THE DIGESTIVE TRACT OF CEPHALOPODS: AT THE INTERFACE BETWEEN PHYSIOLOGY AND ECOLOGY

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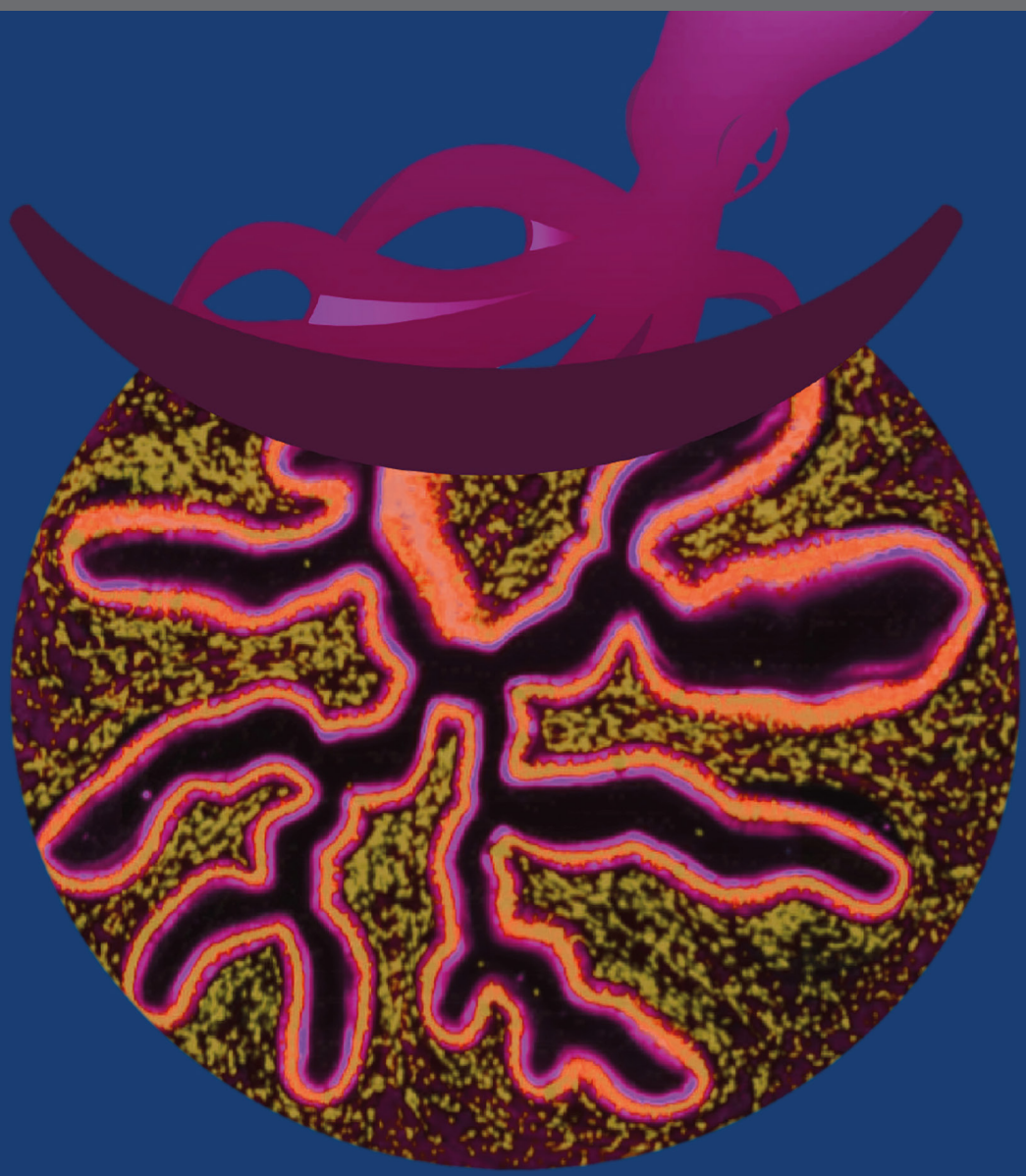

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THE DIGESTIVE TRACT OF CEPHALOPODS: AT THE INTERFACE BETWEEN PHYSIOLOGY AND ECOLOGY

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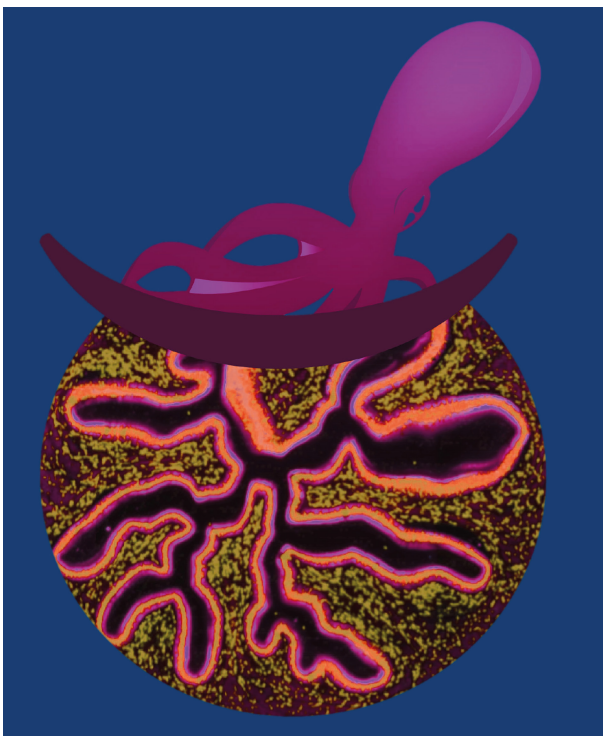

Image: The centrality of the cephalopod digestive system in relation to physiology and welfare is exemplified by a transverse-section of oesophagus of the common octopus and its outline exemplifying a marine predator. The drawing is modified from the logo used for the COST Action FA1301 Workshop on 'The Digestive tract of cephalopods: the interface between ecology and physiology' where the concept for this eBook originated. Drawing by G. Ponte

Aristotle in the Historia animalium, (Book IV) gives one of the earliest descriptions of the anatomy of the cephalopod digestive tract, comparing it to that of other molluscs. From dissections of cuttlefish several key features of the cephalopod digestive tract were described: the beak ("teeth") and radula ("tongue"), the passage of the oesophagus through the brain en route to the "stomach". The stomach is

described as having spiral convolutions like a trumpet snail shell suggesting that the structure described is actually the caecum. The gut then turns anteriorly so that the anal opening is near the funnel leading a modern author to comment that they "defaecate on their heads" (Leroi, 2014).

In the intervening two millennia research on the cephalopod digestive tract has been sporadic with much of the current knowledge arising from a series of studies in the 1950s to the 1970s by A.M. Bidder, E. Boucaud -Camou, R. Boucher-Rodoni and K. Mangold which established the basic mechanisms of digestion and absorption (e.g., Bidder, 1950; Boucaud-Camou et al., 1976). The last 10 years has seen a resurgence of research on the digestive tract stimulated by interest cephalopods (particularly *Octopus vulgaris* and *Sepia officinalis*) as candidate species for aquaculture and the potential impact of climate change on cephalopod ecology. Additionally, the inclusion of cephalopods in the European Union legislation regulating scientific research has necessitated improved understanding of dietary requirements and metabolism as well as the development of methods to monitor digestive tract function to ensure optimal care and welfare in the laboratory. Prompted by this resurgence of interest in the cephalopod digestive tract and an international workshop on the topic held in November 2015 we have collected a series of papers reflecting the current state-of-the art.

The seventeen papers in this book combine original research publications and reviews covering a diversity of topics that are grouped under four main themes reflecting key topics in the physiology and ecology of the cephalopod digestive tract; feeding strategies, early life stages and aquaculture, anatomy and digestive physiology, care and welfare. This book provides a timely synthesis of ongoing research into the cephalopod digestive tract which we hope will stimulate further studies into this relatively neglected aspect of cephalopod biology.

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Table of Contents

ANATOMY AND DIGESTIVE PHYSIOLOGY

CARE AND WELFARE

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EDITORIAL
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Editorial: The Digestive Tract of Cephalopods: At the Interface Between Physiology and Ecology

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Editorial on the Research Topic

The Digestive Tract of Cephalopods: At the Interface Between Physiology and Ecology

The collection of papers included in this Research Topic represents the outcome of some of the activities of the COST Action FA1301, Cephs*In*Action. It emerged from a series of presentations delivered during a workshop in Cascais (Portugal; November 24th, 2015), and from the research activities carried out during Short Term Scientific Missions supported through the COST Action FA1301. The overall aim is to fill some lacunae in knowledge of the digestive tract of cephalopod molluscs. In contrast to other areas of cephalopod biology such as the central nervous system and behavior (e.g., Marini et al., 2017; Nakajima et al.; O'Brien et al.; Shigeno et al.) and the visual system (see Hanke and Osorio), relatively little research has been done on this topic during the last 30 years.

Cephalopods are active marine predators counting more than 800 species. Understanding the physiological adaptations of these fascinating and complex molluscs poses important challenges for several disciplines. Knowledge of the normal functioning (i.e., appetitive drive, signaling satiety, storage and coordinated oro-anal movement of ingested food and digesta, extra- and intra-cellular digestion, epithelial and intra-cellular transport, metabolism, and incorporation of nutrients in the tissues) of the digestive system has wide ranging implications for fisheries, aquaculture, and for the care and welfare of cephalopods in the laboratory and in public displays. Alterations in digestive tract functionality are also a sensitive indicator of gastrointestinal and systemic infections, disease, and external stressors in the broadest sense. Most of the available knowledge on the cephalopod "gut" and physiology of digestion is based on assumptions by analogy with the vertebrate digestive system.

This Research Topic includes 17 papers from more than 70 authors representing a contribution to the outcomes of COST FA1301. The papers present original data and/or reviews on: nutritional requirements and challenges offered by early-life stages, predatory behavior, anatomy and physiology of the cephalopod digestive system, and possible implications with animal care and welfare.

Among other species, the common octopus, *Octopus vulgaris,* is a prime species for cephalopod aquaculture but its potential is limited by poor survival during the paralarval stage. Limited knowledge of feeding habits and the digestive tract physiology are considered major barriers to progress and these areas are reflected by the nine paralarvae papers included here.

Nande et al. studied the predatory behavior and related movements of the digestive tract in 3-days post hatching (dph) *O. vulgaris* paralarvae hatched in the laboratory and fed on eighteen different types of wild caught prey. Capture and ingestion of decapod prey was less efficient (60%)

than cladocerans or copepods (100%). Overall, paralarvae spent only ∼5 min in contact with prey. The temporal sequence of digestive tract motility changes (e.g., crop and stomach filling, intestinal peristalsis) following food ingestion was quantified and pigmented food particles appeared in the digestive gland ∼5 min after the crop had reached maximum volume.

Fernández-Gago et al. provide a 3D reconstruction of the digestive tract during the first 35 days of life, identifying four developmental periods (embryonic, early and late-post-hatching, and juvenile-adult), suggesting that the radula and digestive gland may take longer to mature than other regions. Despite the limitations of a morphological study, this paper provides background information against which the more functional studies can be considered.

A comparison by Estefanell et al. of wild caught with captive bred hatchlings highlights the potential utility of measurement of fatty acids such as $n-3$ highly unsaturated fatty acids from neutral and polar lipids in elucidating the nutritional requirements of *O. vulgaris* paralarvae. Lourenço et al. analyzed the lipid class content and fatty acid profiles of wild paralarvae and their potential prey and proposed that monounsaturated fatty acids (particularly C18:1n7) and the DHA:EPA ratio are trophic markers of the diet of paralarvae. The search for nutritional imbalance biomarkers is explored further by Morales et al. who measured changes in anaerobic and aerobic metabolism, fatty acid oxidation, and gluconeogenesis (from glycerol and amino acids) in *O. vulgaris* paralarvae during an extended part of this life-stage. Authors' findings suggest that phospholipid and n-3 HUFA-enriched *Artemia* reduced mortality and increased paralarval growth, thus contributing to the understanding of the ontogeny of metabolic pathways, an essential requirement for optimizing the diet of paralarvae in culture. A similar dietary enrichment was used by García-Fernández et al. to investigate the epigenetic regulation by diet and age of octopus paralarvae. An age-related demethylation was observed during the first 28 days of life and was accelerated by dietary n-3 HUFA enrichment. A proteomic approach allowed authors to identify specificity in the diet (*Artemia* enriched with microalgae vs. crustacean zoeae), and allowed comparison of fed and food deprived paralarvae suggesting that arginine kinase, NAD+ specific isocitrate dehydrogenase and S-crystallin 3 may be useful as biomarkers of nutritional stress (Varó et al.). Metagenomics provided a different approach to assessing diet in wild paralarvae, by analysis of DNA from the dissected digestive gland (Olmos-Pérez et al.) to identify Molecular Taxonomic Units recognizing decapods, copepods, euphausiids, amphipods, echinoderms, molluscs, and hydroids as part of the natural diet. Some paralarvae showed a preference for cladocerans (see also Nande et al.) and ophiuroids and overall seasonal variability was shown in the presence of copepods and ophiuroids in the diet.

Roura et al. investigated the paralarval microflora (microbiome). Both wild caught paralarvae and those newly hatched in captivity had similar microbial communities which the authors termed the "Core Gut Microflora," the presence of which they considered indicative of healthy *O. vulgaris* paralarvae. A finding of particular relevance to aquaculture was that after 5 dph, in comparison to newly hatched paralarvae the number of bacterial species was reduced by ∼50% with two families (Mycoplasmataceae and Vibrionacea) dominating. The importance of the microbial diversity provided by zooplankton in the wild in contrast to the typically used *Artemia* diet in captivity is discussed by the Authors.

A short overview of cephalopod predatory habits is also included in this Research Topic (Villanueva et al.) including an account of the relative roles of photo-, mechano-, and chemo-reception in the detection of diverse prey types in relation to living habits (e.g., photic zone, primarily vision; deep sea, primarily mechanoreception). A variety of hunting strategies (ambushing, luring, pursuit, stalking, pouncing, cooperation, and scavenging) are employed by different cephalopod species and the authors make an interesting comparison with marine and terrestrial vertebrates. Attention is drawn to the neglected area of the ontogeny of predation by reference to the feeding behavior of both hatchlings and senescent cephalopods.

A contribution to the anatomy and physiology of the digestive system of cephalopods is given by five papers. Ponte and Modica review the largely overlooked topic of the evolution of the salivary glands in molluscs by comparing gastropod and cephalopod molluscs, including a detailed tabulated comparison of the saliva constituents, and the relative role of the secretions from the submandibular gland, the anterior and posterior salivary glands in prey immobilization (by neurotoxins such as cephalotoxin and tetrodotoxin) and digestion (by enzymes). The subsequent steps in digestion are described in detail in *Octopus maya* and *Octopus mimus* by Gallardo et al. by highlighting novel data on the temporal pattern of absorption and assimilation, and providing preliminary evidence that lipid mobilization is dependent upon habitat water temperature.

The digestive gland in cephalopods is the main organ of metabolism and is analogous to the vertebrate liver. It secretes a range of digestive enzymes into the lumen of the digestive tract, receives digested nutrients from the caecum which it assimilates and subsequently transfers to the haemolymph (glucose and lipids). The digestive gland (DG) is also the main site of detoxification and storage of ingested marine pollutants as reviewed by Rodrigo and Costa. High concentrations of both essential (e.g., Cu and Zn) and non-essential (e.g., Ag, Cd, and Pb) metals with metal homeostasis involving *spherulae* formation, chelation and metallothionins characterize the DG. The authors also discuss the involvement of the DG in the storage and metabolism of organic toxicants including amnesic shellfish toxins (e.g., domoic acid), polycyclic aromatic hydrocarbons and polychlorinated biphenyls, and comparisons made with the mechanisms operating in the vertebrate liver including biotransformation, conjugation and elimination with a focus on the cytochrome P450 system.

Understanding the metabolic adaptations of cephalopods to environmental changes is of growing importance because of predicting the effects of climate change and the consequences of coastal eutrophication and assessing the impact of intensive aquaculture. Capaz et al. reported that exposure of adult cuttlefish to sea water with a 50% decreased oxygen for 1 h markedly increased breathing frequency (85%) and reduced oxygen consumption (37%), but there was only a small

increase in mantle muscle octopine levels indicative of anaerobic metabolism. Complementary *in vitro* studies of protein turnover and Na^+/K^+ATPase activity (responsible for ionic gradient maintenance) enabled the authors to hypothesize that the reduced oxygen consumption in hypoxic animals was primarily due to reduced protein synthesis and Na^+/K^+ATPase activity.

In *O. vulgaris* Baldascino et al. utilized RT-PCR to reveal the neurochemical complexity of the gastric ganglion with evidence for putative peptide and non-peptide neurotransmitters (e.g., cephalotocin, FMRFamide, and 5-hydroxytryptamine) and/or their receptors (e.g., $cholecystokinin_{A,B}$ and $orexin_2$). A comparison of gene expression in the gastric ganglion of animals with relatively high or low levels of infection with the common digestive tract parasite *Aggregata octopiana* showed differential gene expression (e.g., increased NFκB, toll-like 3 receptor and decrease superoxide dismutase and glutathione peroxidase).

The regulation in European Union states of scientific research utilizing cephalopods has necessitated the development of guidelines for their care and welfare in the laboratory (Fiorito et al., 2015). Monitoring the functionality of the digestive tract is an important aspect of objective assessment of the overall welfare of captive cephalopods in general and particularly following an experimental procedure. A contribution to care and welfare as identified through the functioning of the digestive system is provided by two papers.

Ponte et al. survey non-invasive methodology for monitoring the physiology of the digestive tract in cephalopods *in vivo*. Measuring the predatory responses, measuring food intake or body weight by non-invasive approaches or measurement of oro-anal transit time (imaging and markers) are challenges that can possibly be overcome by using tools such as ultrasound to monitor movements of the digestive tract or fecal analysis as a "reporter" of digestive tract function.

A wide-ranging overview of the relevance of understanding digestive tract functionality to the welfare of cephalopods in the laboratory and aquaculture is given by Sykes et al. Authors discuss the challenges of feeding cephalopods in captivity and particularly issues around: live food and prepared diets, feeding frequency and quantity, the impact of a range of experimental interventions (e.g., surgery) on the digestive tract, and a discussion of the impact of food deprivation on the overall health and welfare of the animal.

AUTHOR CONTRIBUTIONS

All authors have made a substantial, direct and intellectual contribution to the editorial.

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Cephalopods as Predators: A Short Journey among Behavioral Flexibilities, Adaptions, and Feeding Habits

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The diversity of cephalopod species and the differences in morphology and the habitats in which they live, illustrates the ability of this class of molluscs to adapt to all marine environments, demonstrating a wide spectrum of patterns to search, detect, select, capture, handle, and kill prey. Photo-, mechano-, and chemoreceptors provide tools for the acquisition of information about their potential preys. The use of vision to detect prey and high attack speed seem to be a predominant pattern in cephalopod species distributed in the photic zone, whereas in the deep-sea, the development of mechanoreceptor structures and the presence of long and filamentous arms are more abundant. Ambushing, luring, stalking and pursuit, speculative hunting and hunting in disguise, among others are known modes of hunting in cephalopods. Cannibalism and scavenger behavior is also known for some species and the development of current culture techniques offer evidence of their ability to feed on inert and artificial foods. Feeding requirements and prey choice change throughout development and in some species, strong ontogenetic changes in body form seem associated with changes in their diet and feeding strategies, although this is poorly understood in planktonic and larval stages. Feeding behavior is altered during senescence and particularly in brooding octopus females. Cephalopods are able to feed from a variety of food sources, from detritus to birds. Their particular requirements of lipids and copper may help to explain why marine crustaceans, rich in these components, are common prey in all cephalopod diets. The expected variation in climate change and ocean acidification and their effects on chemoreception and prey detection capacities in cephalopods are unknown and needs future research.

Keywords: predation, feeding behavior, prey capture

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INTRODUCTION

The physiology, behavior, and sensory world of cephalopods have been succesfully adapted from the luminous shallow waters to the dark and cold deep-sea, where they look for the diverse prey that meet their energy requirements. Thus, a variety of feeding behaviors have been recorded both in the wild and laboratory, in association with diverse feeding strategies (see between others, the

reviews of Nixon, 1987; Hanlon and Messenger, 1996; Rodhouse and Nigmatullin, 1996). Despite being limited in number, with 845 cephalopod species described to date (Hoving et al., 2014) when compared with the very populous phylum Mollusca to which they belong, nautiluses and coleoid cephalopods (cuttlefish, squid, octopus) are an astonishing example of diversity of form and function well equipped to deal with the various marine habitats they occupy (Clarke, 1988). This is an example of how evolution can drive potential limitations in design, based to their molluscan clade, to extreme complexities (e.g., Young, 1977; Budelmann, 1995; Godfrey-Smith, 2013; Albertin et al., 2015; Allcock et al., 2015; Shigeno, 2017). Cephalopod coastal species have received more research attention because of their ease of accessibility in the field and their ability to be maintained under laboratory conditions. Most shallow water species are active visual predators with vigorous metabolic activity and sophisticated behaviors (see between others Hanlon et al., 2008; Ebisawa et al., 2011; Benoit-Bird and Gilly, 2012; Vidal et al., 2014). On the other hand, mesopelagic and deep-sea cephalopod species have been less well-studied and their feeding strategies and behaviors are not well known. Cephalopods show a significant negative relationship between metabolism and minimum habitat depth (Seibel et al., 1997; Seibel and Childress, 2000) however, in addition to buoyancy and body mass, phylogenetic position also has an influence on the metabolic rates of each individual species (Seibel and Carlini, 2001). As showed by Seibel et al. (1997), cephalopods of the family Cranchiidae as *Cranchia* and *Liocranchia* have low metabolic rates. These cephalopods live both in epipelagic waters (as subadults) and deep-sea (when adults) and do not follow the negative relationship between minimum depth and metabolic rate showed for most cephalopod species studied. The example illustrate that phylogeny is also an important factor when considering metabolic rates of individual species (Seibel and Carlini, 2001).

The following text seeks to briefly review recent advances on cephalopod predation and identify the main gaps in knowledge on this aspect of cephalopod biology and behavior. Here, we aim to briefly account for the wide spectrum of morphological, behavioral, and physiological features that cephalopods use to meet their energetic needs through predation and food intake. Along this journey we will identify possible gaps in knowledge, thus providing a short guide for future studies.

DETECTING PREYS

The physiology and sensory processing capabilities of cephalopods are adapted to all marine environments. Animals looking for diverse prey needed to meet energetic requirements; metabolic energetic needs that change dramatically according to the ontogenetic state, the habitat they live in and life cycle stage. A variety of feeding behaviors have been recorded in association with diverse feeding strategies (for review see Hanlon and Messenger, 1996; but see also Rodhouse and Nigmatullin, 1996), and such richness is accompanied by a sophisticated set of sensory systems (review in: Budelmann, 1994; Wells, 1994; Budelmann et al., 1997; **Table 1**). This developed sensory system allows them to achieve sophisticated behaviors to detect food, avoid predators and communicate between congeners in a way comparable to vertebrates. Photo-, mechano-, and chemoreceptors provide support for the collection of information about their potential prey.

Probably one of the most striking features of cephalopods is their developed eye, superficially resembling that of teleost fish. It has a single nearly spherical lens with a graded refractive index, the ability to accommodate the len and a similar capacity for eye movement, showing an example of convergent evolution (Packard, 1972). The use of an adjustable pupil to control the amount of light entering the eye distinguishes the cephalopods' eye from their fish counterpart and the light-evoked pupillary constriction in cephalopods is among the fastest in the animal kingdom (Douglas et al., 2005). Among the few exceptions is the deep-sea cirrate octopod *Cirrothauma murrayi,* whose eye lacks lenses and the optic lobes are simply organized (Aldred et al., 1983), however, it is probably able to detect bioluminescence (Warrant and Locket, 2004). Most cephalopods studied have a single type of rhodopsin as a visual pigment, suggesting they are blind to color (Messenger et al., 1973; Marshall and Messenger, 1996; Mäthger et al., 2006). They can achieve spectral and color discrimination by exploiting chromatic aberration and pupil shape (Stubbs and Stubbs, 2016), but this system could work for only a narrow range of visual tasks (Gagnon et al., 2016). The giant (*Architeuthis*) and the colossal (*Mesonychoteuthis*) squids have the largest eyes in the animal kingdom, however their characteristics suggest they are mainly used for detecting and identifying bioluminescent waves generated by sperm whales during their dive into the deep, thus protecting them from potential predation, rather than detecting prey at long distances (Nilsson et al., 2012). The importance of the visual system to locate prey is also reflected in the ability for aerial capture, such as, when *Sepia officinalis* is able to attack and capture prey shown above the water surface by an experimenter (Boletzky, 1972). The complexity of the visual system of cephalopods is also achieved through extra-ocular light perception capabilities, providing an intricate network of sensory devices on their skin (see also Kingston et al., 2015; Ramirez and Oakley, 2015; Kelley and Davies, 2016). In addition, cephalopods are sensitive to polarized light and polarization vision serves to enhance the detection and recognition of prey. Squid hatchlings attack planktonic prey under polarized illumination at a 70% greater distance than under depolarized illumination (Shashar et al., 1998) and the polarization vision helps cuttlefish to see further into turbid water and to better detect prey (Cartron et al., 2013).

Sensory capabilities are not limited to vision. Cephalopods have sensory receptors that form the lateral line system, which detects gentle water currents and vibrations. Ciliated primary sensory hair cells, sensitive to local water movements, are arranged in epidermal lines located on the arms, head, anterior part of dorsal mantle and funnel (e.g., Sundermann, 1983; Budelmann and Bleckmann, 1988; Budelmann, 1994; Lenz et al., 1995) and are known to provide sensory capabilities in detecting prey (Komak et al., 2005). In fact, cuttlefish are able to catch small shrimp in the darkness and behavioral experiments showed

TABLE 1 | Biological and behavioral adaptations utilized by cephalopods for the sake of their predatory behavior.

Predatory adaptations		Activities
Senses	Eyes and vision; Epidermal hair cells; Equilibrium receptor organs for linear and angular accelerations; Epidermal tactile receptors; Contact and distance chemoreceptors; Vibration receptors and hearing	Searching for prey
Respiratory, circulatory and nervous systems	Efficient branchial ventilation; Closed circulatory system; Central nervous system; Giant fiber system	Catching prey
Physical features	Arms and tentacle; Suckers; Beaks; Jet propulsion; Skin color change	Catching and handling prey
Cognitive capabilities	Learning and memory abilities	Searching, recognition and catching prey
Hunting strategies[a]	Ambushing, Luring, Pursuit, Stalking, Speculative and Cooperative Hunting	Catching prey

Morphological, physiological, sensory, neural and behavioral adaptations and corresponding behavioral outcomes (Activities) are listed here as deduced from several reviews (Packard, 1972; Young, 1977; Hanlon, 1988; Hanlon and Messenger, 1996; Borrelli et al., 2006; Borrelli and Fiorito, 2008).
[a]*See also* ***Table 2****.*

they use the epidermal lines to detect prey (Budelmann et al., 1991).

Distant chemoreceptor organs such as, olfactory organs and rhinophores, further provide additional sensory capabilities. Olfactory organs are paired, oval shaped organs situated on either side of the head, ventrally behind the eye and near the mantle edge. Their possible role in prey detection is poorly understood. Water containing food odor (shrimp) is detected by *S. officinalis* (Boal and Golden, 1999) and embryos exposed to the odors of prey later influences prey choice in the same species (Guibé et al., 2010). Increased ventilation rates in response to prey chemicals was described for *Eledone cirrhosa* (Boyle, 1986); and positive chemotaxis for *Octopus maya* during Y-maze experiments, with amino acids (alanine, proline), nucleotids (ATP), and crab extract functioned as excitants, while betaine and taurine functioned as arrestants (Lee, 1992). The rhinophores of *Nautilus* are paired organs located below each eye and open to the exterior by a narrow pore. They are similar to the olfactory organs but are significantly larger (Basil et al., 2005).

In addition, cephalopods have contact receptors in the tentacles, sucker rims, and lips; known to allow sensing of a broad spectrum of chemical and mechanical signals. Sucker receptors are more elaborated in octopus. There are about 10,000 chemoreceptor cells in a single sucker of an octopod, but only about 100 are present in the sucker of a cuttlefish (Budelmann, 1996). The food searching habit of benthic octopods (see below Speculative pounce), that make extensive use of the arms and suckers exploring rocks and crevices, may justify this marked difference. In contrast, cuttlefish use their arms mostly for manipulating their prey (Chichery and Chichery, 1988). Contact receptors located in lips of octopus and cuttlefish are more advanced in structure and organization than those of squid. As cuttlefish and octopus are more sedentary and benthic than pelagic squid, they may rely more on tactile and chemical stimuli (Emery, 1975). Chemical receptors in cephalopods help them to locate prey and also to avoid unwanted prey. Cuttlefish were able to learn that a prey is not acceptable food, to recognize and to avoid it and, as a result, to choose a usually non-preferred prey when necessary (Darmaillacq et al., 2004).

ONTOGENY OF PREDATION: THE YOUNG AND THE SENESCENT

Hatchling cephalopods are of relatively large size, ranging from 0.6 (*Argonauta hians*) to 28 (*Graneledone boreopacifica*) mm mantle length (Villanueva et al., 2016), allowing the animal to start an active mode of food searching marked by the coexistence of two nutritive systems: (a) an embryonic energy in the form of yolk, and (b) a post-hatching energy provided by captured food (Boletzky, 2003). Preference for prey at hatchling when previously exposed during the latest embryonic stages (Darmaillacq et al., 2006) and visual imprinting during a short sensitive period during the first day of life (Darmaillacq et al., 2008) showed some of the available tools employed by the young cuttlefish, *S. officinalis,* to successfully capture prey and survive during the first days of life as a predator. In this species, the development of learning and predatory behavior is observed during late embryonic and early juvenile development. This occurs simultaneously with the maturation of the vertical–subvertical lobe tracts of the brain, allowing the animals to maintain a prey in the frontal field during predatory pursuit (Dickel et al., 1997). Then, during the first 3 months of life, feeding hierarchy has been reported for the same species (Mather, 1986; Warnke, 1994). A comprehensive review on this behavioral development is provided by O'Brien et al. (2016). On the other hand, in the juvenile holobenthic octopuses *O. maya*, preference to attack a prey is not obtained through previous life experience. Juvenile octopuses selected crabs as prey when individuals had previously been fed shrimp earlier in life. This could be the result of innate biological processes (Portela et al., 2014).

In squids, brain developmental differences can be found when observing the relatively large Loliginid *Sepioteuthis lessoniana* hatchlings, with a subvertical lobe of especially complicated domain structure, which may reflect an active predatory behavior (Shigeno and Yamamoto, 2005). In comparison, the minor development of higher motor centers of the small ommastrephid *Todarodes pacificus* hatchlings, suggests these animals are not active predators at this time but perhaps suspension feeders after hatching (Shigeno et al., 2001a,b). The first food and feeding strategy of the ommastrephid paralarvae before they start to actively feed on zooplankton is an unresolved question that

merits further research (O'Dor et al., 1985; Vidal and Haimovici, 1998).

Diet of planktonic cephalopods in the wild is poorly understood (Passarella and Hopkins, 1991; Roura et al., 2012, 2016; Olmos-Pérez et al., 2017). Roura et al. (2016) found that *Octopus vulgaris* hatchlings targeted low abundance prey like decapod crustacean larvae independently of the zooplankton community they inhabit, thus showing a selective behavior in these patchy environments. Stable isotope ratios allowed discrimination of specific feeding strategies during ontogenesis and accumulations of metals as cadmium and mercury also reflected the ontogenetic stage in five species of cephalopods (Chouvelon et al., 2011). Externally, strong morphological changes during early life are recognized in some cephalopod groups, particularly in oegopsid squids and merobenthic octopods, associated with different habitats and feeding modes during early life. Ontogeny of prey capture develops progressively, from a simple type after hatching to an adult-like capture behavior involving structures such as, tentacles and hooks, which are absent or poorly developed in larval forms (Sweeney et al., 1992). In young ommastrephid squids, the fused tentacles forms the proboscis and its functionality, supposedly related to food capture, remain an open question that again needs future research (Uchikawa et al., 2009).

In loliginid squids, ontogeny of prey capture develops progressively, from a simple type after hatching to an adult-like capture behavior involving tentacles after 1 month of age in *Doryteuthis opalescens* raised with copepods (Chen et al., 1996). In merobenthic octopods, a positive allometric arm growth takes place during planktonic life, probably helping the animal to capture benthic prey after settlement. At the same time animals lose the oral denticles of the beaks, of which the trophic function remains unclear (Villanueva and Norman, 2008). However, observations on the external digestion and initial ingestion process in the pymy squid *Idiosepius paradoxus*, suggest that oral denticles may be used to detach the semidigested flesh from the exoskeleton of the crustacean prey (Kasugai et al., 2004). The early development of the muscular, protein-rich arm crown in merobenthic octopods is related to the decrease in lipid content of the animal, due to the relative decrease of the visceral mass, where lipids are abundant. During planktonic life, the octopus feeding behavior is that of a visual predator. The presence of prey increases the turning rate and reduces the swimming speed in *O. vulgaris* paralarvae, possibly improving the exploitation of patchy food environments in the wild (Villanueva et al., 1997).

At the other end of early life is senescence, a period coincident with the end of the single reproductive period characteristic of this group of semelparous molluscs. Chichery and Chichery (1992) found in aging *S. officinalis* signs of degeneration of the anterior basal lobe, a structure that plays an important role in the control of the predatory behavior, as indicated by previous studies by the same authors (Chichery and Chichery, 1987). In addition, they suggested that visual capacities were also affected during the aging process by reducing the attention mechanisms and also the maintenance of the predator's visual tracking behavior, concluding that the low interest in the prey shown by senescent cuttlefish may be related to the deterioration of the basal lobe and the decreasing visual input. The progressive loss of appetite in both senescent male and female octopuses is fairly well documented (see review by Anderson et al., 2002). In the brooding *O. vulgaris,* female food intake decreases about 90% and the method of predation and handling over the scarce prey changes and becomes irregular (Wodinsky, 1978). Interestingly, in the brooding female *Octopus filosus*, Wodinsky (1977) found that removal of optic glands made them cease brooding, start feeding again, and live longer than normal. This surprising behavior after removal of these glands has not been studied in other cephalopod species.

CEPHALOPOD FEEDING REQUIREMENTS AND PREY PREFERENCES

Crustaceans are present in nearly all the cephalopod diets studied to date. Teleost fish and molluscs complement their energetic needs in different proportions, depending on the species, habitat, and ontogenetic stage (see reviews of Nixon, 1987; Rodhouse and Nigmatullin, 1996). Why crustaceans seem to be an indispensable prey in the diet to sustain suitable growth for cephalopods under culture conditions, and particularly for their young stages, is a subject of current debate (Iglesias et al., 2014). Large protein and amino acid content in the diet are required to maintain positive growth, at least in shallow water cephalopod species characterized by vigorous protein metabolism and showing a relatively low quantity of lipids in their body composition. However, phospholipids, cholesterol, and long-chain polyunsaturated fatty acids (PUFA), all of them abundant in marine crustaceans, seem to play an important role. Particularly, the n-3 PUFA, due to their high demand for cell membrane synthesis where they are incorporated, due to the inability of cephalopods to synthesize them (Monroig et al., 2013; Reis et al., 2014), These findings suggest that PUFA, play an essential role in cephalopod nutrition, at least for shallow water, fast growing cephalopod species (Navarro et al., 2014). In addition, the elemental composition of natural food strongly suggests that cephalopod paralarvae and juveniles must require a food rich in copper (Villanueva and Bustamante, 2006). This fact is probably related to the haemocyanin requirements for oxygen transport, as copper is the dioxygen carrier of haemocyanin typical of crustaceans and molluscs. Again, marine crustaceans seem to play a pivotal role in the diet of cephalopods, also considering that diet of a species can change from different locations depending on the prey availability and abundance (Leite et al., 2016). The general tendency of cephalopods to prey mainly on crustaceans, fish, molluscs, and other invertebrates, such as, polychaetes, echinoderms, hydroids (Olmos-Pérez et al., 2017), and also on gelatinous fauna (Hoving and Haddock, 2017) is not followed by the deep-sea species, *Vampyroteuthis infernalis*. This species is able to fuel its low metabolism mainly on detritus (Hoving and Robison, 2012). On the other hand, as an extreme comparative example, the Giant Pacific octopus (*Enteroctopus dofleini*) and *Octopus* cf *insularis* occasionally feeds on large marine birds (Anderson and Shimek, 2014), and attacks and bite damage to the skipjack tuna (*Katsuwonus pelamis*) and yellowfin tuna (*Thunnus*

albacares) inside purse seine nets have also been described for the jumbo squid *Dosidicus gigas* (Olson et al., 2006). These species are extreme examples showing the adaptive capacity of cephalopod species to obtain energy from the different marine habitats in which they live. In addition, when resources are scarce or when the density of congeners is high, cephalopods can choose cannibalism as a feeding behavior. Cannibalistic behavior has been reported from video recordings in the wild for both squids (Hoving and Robison, 2016) and octopods (Hernández-Urcera et al., 2014) independently of fishing operations, which may induce unnatural feeding behaviors. Cannibalism is common in most cephalopod species whose diet has been studied, an uncommon characteristic in the animal kingdom which may be related to their high metabolic demands. Factors influencing this unusual feeding behavior are environmental variations, population density, food availability, body size, and sexual dimorphism (Ibáñez and Keyl, 2010). In addition to visual stomach content analysis, recent tools are being used as trophic indicators and tracers in food chain pathways including stable isotope (Lorrain et al., 2011; Ohkouchi et al., 2013; Guerreiro et al., 2015), heavy metal (Bustamante et al., 1998), and fatty acid signature analysis (Pethybridge et al., 2013; Rosa et al., 2013), as well as molecular techniques (Deagle et al., 2005; Braley et al., 2010; Roura et al., 2012; Olmos-Pérez et al., 2017) and food web models (Hunsicker et al., 2010; Coll et al., 2013).

PREDATORY BEHAVIORAL STRATEGIES AND PREY CAPTURE

Until food satiation is obtained, cephalopods explore their environment looking for food. Known modes of hunting in cephalopods include ambushing, luring, stalking and pursuit, speculative hunting and hunting in disguise, among others (**Table 2**), described in detail by Hanlon and Messenger (1996). Behavioral observations on foraging cephalopods in their natural habitat usually come from shallow-water environments, mostly on cuttlefishes and octopuses using scuba diving. A variety of behaviors have been recorded and mimicry has been observed during octopus foraging (Forsythe and Hanlon, 1997; Hanlon et al., 2008; Krajewski et al., 2009; Caldwell et al., 2015). The sequences of foraging behavior in shallow water octopuses usually showed characteristics of a tactile saltatory searching predator, as well as a visual opportunist (Leite et al., 2009). Using acoustic techniques, coordinated school behavior during foraging was recorded at night in shallow water for jumbo squid *D. gigas*. They were observed using ascending, spiral-like swimming paths to emerge from extremely dense aggregations (Benoit-Bird and Gilly, 2012).

Behavioral studies of predation in the laboratory are more detailed and abundant. The predatory strategy is part of a series of body and locomotory patterns. The visual attack is executed with great accuracy leading to a final strike, a sequence described in cuttlefishes (Messenger, 1968) and identified in different species (*Lolliguncula brevis*, Jastrebsky et al., 2017) revealing similar behavioral performances. During the attack, raised arms and dynamic skin patterns are part of these sophisticated behavioral sequences utilized presumably to deceive the potential prey and facilitate capture. Raised arms are expressed during predation when the cuttlefish has located its prey and is approaching it to reach a position suitable for attack. Arms I appear extended vertically upwards (Messenger, 1968, p. 345) and often separated in a V, each forming an S-shaped curve (review in Borrelli et al., 2006). In some cases, arms II may also be similarly raised. Raised arms are generally dark and may sway to and fro. Messenger (1968) suggests that this peculiar posture and swaying movement of the arms may act as lures, directing the prey's attention away from the tentacles. Chromatic pulses and rhythmic passing waves as been described as dynamic skin chromatic patterns of cephalopods during hunting displays. Chromatic pulses are known in cuttlefishes and also in squids and octopuses and consist of a single band of color contrast sweeping across part of the predator in a particular direction. Rhythmic passing waves are known in cuttlefishes and octopuses, involving the movement of rhythmic bands across the predator in a constant direction (see How et al., 2017 for review).

The most accurate description of full attack response of octopuses (e.g., *O. vulgaris*) is provided by Andrew Packard: "In full attack... an octopus launches itself directly toward the crab... swimming by the propulsion of water from its funnel (siphon) and without touching the bottom" (Packard, 1963, p. 39). The chromatic, postural, and locomotor components (i.e., body patterning), making up the behavior, include: (i) head and eyes raised, with the latter "wide open"; (ii) the body is or darkens to "a deep reddish-brown hue" (Packard, 1963, p. 39); (iii) the arms are outstretched or loose "with the suckers facing downwards" (Packard, 1963, p. 39); (iv) the octopus orients the siphon posteriorly, away from its target. In *O. vulgaris*, as in squid and cuttlefish, the full attack response is elicited by the initial visual recognition of an edible "object" followed by the final outcome of the attack (i.e., obtainment of food; for review see also Borrelli et al., 2006). The full attack is only one example of the variety of predatory behaviors. A full gradient of locomotor patterns appear to be exhibited. As reviewed by Borrelli et al. (2006) during crawling an octopus moves relatively slowly in contact with the ground, and may also be aided by brief swimming sequences. The animal moves along the substrate aided by the suckers of the central half of the arm, while the arms push or pull, depending on their position, to facilitate the direction of movement; this crawling may imply several arms (Finn et al., 2009) or just the posterior pair as in bipedal locomotion, also referred as walking or tiptoeing (see also Huffard et al., 2005; Borrelli et al., 2006). Crawling is adopted by octopuses to explore their surroundings and approach sites that they eventually explore for prey capture.

On the other hand, speculative hunting (or speculative pounce) is characteristic of several octopus species (see for e.g., Borrelli et al., 2006; Leite et al., 2009). While searching for prey, "the octopus moves across the bottom in a combination of swimming and crawling actions. Every 1–2 m it makes a speculative pounce, covering a rock, a clump of algae, or a small area of the bottom with its web. Pausing for a few seconds to feel under the web the octopus continues its trip" (Yarnall, 1969, p. 749).

TABLE 2 | Comparison between different hunting strategies adopted by some species of cephalopods and vertebrates (not an exhaustive list).

	Hunting strategies						
CEPHALOPODS	**Ambushing**	**Luring**	**Pursuit**	**Stalking**	**Pouncing**	**Cooperative**	**Scavenger**
Sepia officinalis	•	•			•		
Euprymna scolopes	•						
Loligo vulgaris	•						
Sepioteuthis lessoniana	•	•	•	•			
Sepioteuthis sepioidea	•	•	•	•	•		
Dosidicus gigas	•	•			•	•	
Architeuthis dux	•		•				•
Mesonychoteuthis hamiltoni	•	•					
Vampyroteuthis infernalis	?	•					•
Octopus vulgaris	•	•	•		•		
VERTEBRATES							
Great white shark *(Carcharodon carcharias)*	•		•	•		•	•
Nile crocodile *(Crocodylus niloticus)*	•		•	•		•	•
Eastern green mamba *(Dendroaspis angusticeps)*	•			•	•		
Golden eagle *(Aquila chrysaetos)*	•		•	•	•		•
Killer whale *(Orcinus orca)*	•		•	•		•	•
Bottlenose dolphin *(Tursiops truncatus)*	•		•			•	
Leopard *(Panthera pardus)*	•		•	•	•		•

Hunting strategies are indicated following Curio (1976). Information included here is deduced from a series of sources including for cephalopods: Moynihan and Rodaniche (1982); Hanlon and Messenger (1996); Robison et al. (2003); Cole and Adamo (2005); Kubodera and Mori (2005); Rosa and Seibel (2010); Sugimoto and Ikeda (2013). Data from Vertebrates are presented here to attempt a possible comparison and are not exhaustive (Guggisberg, 1972; Angilletta, 1994; Martin et al., 2005; Hayward et al., 2006; Watson, 2010; Ferguson et al., 2012). "•": hunting strategy recorded. "?": hunting strategy probable, not recorded.

In addition, cephalopods use different tools to enhance prey capture. For example, disguise strategies using ink during predation, has been reported recently by Sato et al. (2016) for *I. paradoxus*. These pygmy squid use ink during prey attacks in two modes: releasing ink between themselves and the prey and then attack through the ink cloud, and also releasing ink away from the prey and attacking the prey from another position. Another tool used in the darkness is the dinoflagelate bioluminescence, employed by *Euprymna scolopes* and *S. officinalis* to locate non-luminous crustaceans and fish prey (Fleisher and Case, 1995). During foraging under culture conditions, it is remarkable that cuttlefish (*Sepia pharaonis*) are able to identify the amount of prey available, discriminate prey numbers, and the following prey selection, all depending on their satiation state (Yang and Chiao, 2016). When cuttlefish detect a prey, they perform a well-known three-stage visual attack sequence of attention, positioning, and seizure (Hanlon and Messenger, 1996). Observing conspecifics during prey capture, these events do not seem to improve their predation techniques (Boal et al., 2000).

Venom is used by cuttlefishes and octopods to kill the prey and for muscle relaxation. Octopuses bored holes in the carapace, the eye or the arthrodial membrane of crustaceans (Grisley et al., 1996; Pech-Puch et al., 2016). The selection of the preferred area to inject the cephalotoxin in the crab seems to be a combination of factors related to prey and octopus size. For example, large octopuses use eye puncture less frequently than small individuals (Grisley et al., 1999). Prey handling in octopus eating bivalves showed different combinations of pulling and drilling feeding behaviors. The injection of the cephalotoxin into the bivalve and gastropod prey is associated with drilling. Drilling occurs by the combined action of radula and salivary papilla (Nixon, 1980). A combination of drilling and pulling behaviors has been reported for preying on bivalve and gastropod prey (Runham et al., 1997; Fiorito and Gherardi, 1999; Steer and Semmens, 2003; Ebisawa et al., 2011). Octopuses hold the prey within the proximal part of the arms so, they cannot use vision during most prey handling period, probably choosing the most energetic, cost effective feeding behavior based on previous experience (Anderson and Mather, 2007). In the field, other factors may influence the bivalve selection and feeding mode. McQuaid (1994) showed that mussel size selected by small octopuses (<500 g) was related to octopus weight, with small octopuses eating on small mussels because they are unable to remove large mussels attached with the byssus threads from the rocks. In addition to pulling and drilling, shell crushing has been reported as a feeding behavior for the deep-sea octopod *Graneledone* preying on gastropods, a behavior that may be favored due to their relatively larger beaks in comparison with those of shallow water octopods (Voight, 2000). It is remarkable that the elevated diversity of cephalopod hunting behaviors, almost matches the strategies adopted by vertebrate predators (**Table 2**). Both taxa are so diverse and remote in their phylogenetic traits, but clearly there are cases of functional (and behavioral) convergence during evolution.

LOOKING FOR FOOD IN THE COLD DARKNESS

In neritic and epipelagic cephalopods, vision is probably the main sense utilized for prey detection and capture. As light intensity decreases in deep-sea environments, low temperature reduces the metabolic demands and predator-prey distance changes (Seibel et al., 2000). In this environment, the mechanoreceptor structures in the arms, tentacles, and filaments increase in number and complexity. These metabolic and morphological changes considered to be closely related with the prey selected by deep-sea cephalopods result in feeding strategies that are more diverse in the deep-sea that previously believed. The cirrate octopods are characterized by the possession of paired filamentous cirri along the arms, of diverse length according to families, which are interspersed between a single row of suckers, and are thought to have a sensory function involved in prey detection and capture. Cirrates feed mainly on small-sized organisms with low swimming speeds including amphipods and polychaetes (Collins and Villanueva, 2006). In the cirrate *Stauroteuthis syrtensis*, blue-green bioluminescence is emitted by modified suckers without adhesive function; this has been suggested to act as a light lure to attract prey and/or mates (Johnsen et al., 1999). For *S. syrtensis* of 60 g fresh weight, a daily ration of only 1–30 calanoid copepods day^{-1} has been estimated (Jacoby et al., 2009), showing the low metabolic rate of this group of deep-sea cephalopods. In a similar way, the colossal squid (*Mesonychoteuthis hamiltoni*), the world largest invertebrate, reaching 500 kg of total weight, seems to be an ambush or sit-and-float predator that uses the hooks on its arms and tentacles to capture prey and reach a projected daily energy consumption of 45 kcal day^{-1}, equivalent to only 30 g of fish day^{-1} (Rosa and Seibel, 2010). The knowledge of the diet of deep-sea squids needs further research. A comprehensive review of the main prey found in stomachs of deep-sea squids has been provided by Hoving et al. (2014).

As suggested by Young et al. (1998), the great variation in squid tentacle morphologies may reflect variation in target prey and the handling of captured food. The deep-sea squid *Grimalditeuthis bonplandi* is an extreme example: its tentacles have a very thin and fragile elastic stalk, whereas the clubs bear no suckers, hooks, or photophores. It is unknown how these tentacles are used to capture and handle their prey, as they consist on cephalopods and crustaceans (Hoving et al., 2013). Very long dorsolateral arms with photophores are present in *Lycoteuthis lorigera* males (Villanueva and Sánchez, 1993) and extremely large and filamentous arms and tentacles approximately equal in thickness and length are key characters of the genus *Magnapinna*: these reach 15–20 times the mantle length of the animal, reaching to 7 m in total length (Vecchione et al., 2001; Guerra et al., 2002). *V. infernalis* uses its two thin and retractile filaments, which may be up to nine times the body length for food capture, i.e., remains of gelatinous zooplankton, discarded larvacean houses, crustacean remains, diatoms, and fecal pellets (Hoving and Robison, 2012), thus indicating that the use of luring as a mode of hunting is probably common in deep-sea cephalopods. Also the mesopelagic *Spirula spirula* feeds mainly on detritus and zooplankton (Ohkouchi et al., 2013).

FEEDING ON INERT PREY

As mentioned above, cephalopods do not necessarily predate exclusively on live prey. Some cephalopod species are collected in large numbers from the wild using baited traps such as, *Nautilus* (Dunstan et al., 2011) and *O. vulgaris* (Guerra, 1997) showing that scavenger behavior exists in nature. Recent development of the cephalopod culture techniques (review in Iglesias et al., 2014) allowed the use of frozen prey and/or artificial food in supporting growth during part of the life cycle in a number of species including *Nautilus*, cuttlefish (*S. officinalis, S. pharaonis, Sepiella inermis, Sepiella japonica*), squid (*Loligo vulgaris, S. lessoniana*), and octopus (*Amphioctopus aegina, O. maya, Octopus mimus, Octopus minor, O. vulgaris*). The first feeding period usually requires live crustacean prey, particularly for the delicate planktonic stages, although planktonic octopuses are able to detect, capture and ingest inert particles from the water surface (Marliave, 1981) or descending in the water column (Villanueva et al., 2002; Iglesias et al., 2007). A successful semi-humid squid paste-bound gelatine has been developed to feed *O. maya* benthic hatchlings from first feeding, showing that this species can live and reach normal growth with artificial food during the whole life cycle under laboratory conditions (Rosas et al., 2014). In other species, after a variable acclimation period, inert food is readily accepted by advanced juvenile, subadult, and/or adult stages of cephalopods under culture conditions (Vidal et al., 2014).

The training phase from feeding on live prey to inert food shows the behavioral adaptions and learning capacities of these animals under laboratory conditions. As an example noted by Nabhitabhata and Ikeda (2014), *S. lessoniana* aged 20 days can be fed sliced fish meat of two or three times the mantle length of the squid, that seize the food in the water column: when squid feed on live prey, the prey is seized by the tentacles, when the squid are fed dead feed, they change their prey capture behavior, using only their arms to seize the food and do not perform the positioning phase typical of the squid attack (Messenger, 1968). The same behavioral adaptions and prey capture modes are observed in *S. pharaonis* (Nabhitabhata, 2014a) and *S. inermis* (Nabhitabhata, 2014b) when changing from live to inert prey.

FUTURE CHALLENGES ON CEPHALOPOD PREDATION

In this review, we surfed through a number of important topics that require further research and possibly a dedicated effort. Research on cephalopod predatory strategies is needed in a variety of fields, from behavior to ecology. Studies of feeding behavior, nutrition, and feeding requirements are critical in order to develop the nascent cephalopod aquaculture of key species, particularly from early young stages. Studies on nutritional requirements are only at the beginning. The role of lipids on the early growth and survival of shallow water species seems

more important than previously supposed and research is also needed in that field (Navarro et al., 2014). Hatchlings of 13% of the cephalopod species described to date has been obtained under laboratory conditions, most of them belonging to shallow water octopods (Villanueva et al., 2016). As this number increases in the future, new larval and juvenile predatory behavioral strategies will mostly likely be described. Similarly, the future study of deep-sea and oceanic cephalopod forms will provide further instances of novel, undescribed receptors, organs, behaviors, and modes of prey detection and capture in cephalopods (Hoving et al., 2014). In addition, whether our knowledge on diet richness of a given cephalopod species in the wild is affected or not by research effort remains to be explored; data we presented above may represent only a starting-point. The variability of conformation of cephalopod beaks and their functional relation with possible prey-items is another possible challenging avenue of research (Franco-Santos and Vidal, 2014; Franco-Santos et al., 2014). The use of modern techniques as genomics (Olmos-Pérez et al., 2017) and proteomics technologies (Varó et al., 2017), microbiota associated with different diets (Roura et al., 2017), or venom structure (Whitelaw et al., 2016) may further extend our knowledge on cephalopod diets. Some aspects, such as, the hormonal control over feeding in cephalopods are practically unknown (Wodinsky, 1977). Interactions with other species such as, intraguild predation (when species compete simultaneously for resources and interact as prey and predator), is another aspect that may need further attention in cephalopod science. The interaction between shallow water octopus and juvenile lobster is potentially an example of intraguild predation involving interference competition for refuge (Butler and Lear, 2009) but cannibalism (see above) may also be seen under this framework.

Interaction with other species, as well as competition for spatial and feeding resources will probably be modified with global change. A representative example is the case of the jumbo squid *D. gigas*. During the daytime, jumbo squids dive to the depth, suppressing metabolism in the oxygen minimum zone, an energy saving strategy in hours of prey limitation in shallow waters (Rosa and Seibel, 2008). The expected climate change expansion of deep-water hypoxia and the warming and acidification of surface waters will concentrate both prey and predators, with unknown effects on *D. gigas* predatory dynamics (Seibel, 2015). The expected variation in climate change and ocean acidification has been shown to induce complex changes in chemoreception and prey detection, including altered cue detection behaviors in some marine organisms. Changes in CO_2 may have effects on predator handling time, satiation, and search time in coastal molluscs (Kroeker et al., 2014) as shown by increased activity in the pigmy squid *Idiosepius pygmaeus* (Spady et al., 2014). Nonetheless, the possible effects of elevated CO_2 on chemoreception in cephalopods are unknown. The ability of carnivorous fish and sharks to detect chemical cues produced by their prey appears reduced and, as a consequence, the activity levels spent on food searching increases upon exposure to elevated CO_2 (Cripps et al., 2011). This leads to a considerable reduction in the growth rates of sharks (Pistevos et al., 2015). Being carnivorous predators, similar effects may be expected in cephalopods, a research subject that merits future exploration.

AUTHOR CONTRIBUTIONS

RV and GF are responsible for the conceptual design. RV, GF, and VP contributed to the writing of the manuscript.

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frontiers in Physiology

ORIGINAL RESEARCH
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Prey Capture, Ingestion, and Digestion Dynamics of *Octopus vulgaris* Paralarvae Fed Live Zooplankton

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Octopus vulgaris is a species of great interest in research areas such as neurobiology, ethology, and ecology but also a candidate species for aquaculture as a food resource and for alleviating the fishing pressure on its wild populations. This study aimed to characterize the predatory behavior of *O. vulgaris* paralarvae and to quantify their digestive activity. Those processes were affordable using the video-recording analysis of 3 days post-hatching (dph), mantle-transparent paralarvae feeding on 18 types of live zooplanktonic prey. We show for the first time in a live cephalopod that octopus paralarvae attack, immobilize, drill, and ingest live cladocerans and copepods with 100% efficiency, which decreases dramatically to 60% on decapod prey (*Pisidia longicornis*). The majority (85%) of successful attacks targeted the prey cephalothorax while unsuccessful attacks either targeted the dorsal cephalothorax or involved prey defensive strategies (e.g., juvenile crab megalopae) or prey protected by thick carapaces (e.g., gammaridae amphipods). After immobilization, the beak, the buccal mass and the radula were involved in exoskeleton penetration and content ingestion. Ingestion time of prey content was rapid for copepods and cladocerans (73.13 ± 23.34 s) but much slower for decapod zoeae and euphausiids (152.49 ± 29.40 s). Total contact time with prey was always <5 min. Contrary to the conventional view of crop filling dynamics observed in adult *O. vulgaris*, food accumulated first in the stomach of paralarvae and the crop filled after the stomach volume plateaued. Peristaltic crop contractions (∼18/min) moved food into the stomach (contractions ∼30/min) from where it passed to the caecum. Pigmented food particles were seen to enter the digestive gland, 312 ± 32 s after the crop reached its maximum volume. Digestive tract contents passed into the terminal intestine by peristalsis (contraction frequency ∼50/min) and defaecation was accompanied by an increased frequency of mantle contractions. Current results provide novel insights into both, *O. vulgaris* paralarvae—live prey capture strategies and the physiological mechanisms following ingestion, providing key information required to develop an effective rearing protocol for *O. vulgaris* paralarvae.

Keywords: digestion dynamics, digestive tract motility, nutrition physiology, *Octopus vulgaris* paralarvae, predatory behavior, video analysis, zooplankton

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INTRODUCTION

Octopus vulgaris is the best known octopod species among Octopodidae (Norman et al., 2014) and one of the most intensively studied species in various animal research areas such as development and growth (e.g., Villanueva and Norman, 2008; Iglesias and Fuentes, 2014), behavior (e.g., Hanlon and Messenger, 1996; Fiorito and Gherardi, 1999), and neuroscience (for a review see Fiorito et al., 2014). Particularly interesting is the well-developed central nervous system in *O. vulgaris* which makes it a suitable model organism in neurophysiology, ethology, and ecology (for reviews see Wells, 1978; Hanlon and Messenger, 1996; Hochner et al., 2006; Hochner, 2012; Fiorito et al., 2014). Additionally, *O. vulgaris* has been a candidate for aquaculture since Classical Antiquity (Iglesias et al., 2007; Lotze et al., 2011) and such interest continues stimulated by concerns about its sustainability despite the large size of the commercial cephalopod fishery (Iglesias et al., 2007; Vidal et al., 2014; Doubleday et al., 2016).

Massive mortality during the paralarval stage is one of the major bottlenecks to the successful rearing of the common octopus. Such mortality is believed to be caused by our deficient knowledge of early nutritional requirements (Iglesias and Fuentes, 2014; Navarro et al., 2014; Vidal et al., 2014). For instance, prey attack strategies, types of live zooplanktonic prey preferred and the physiology of digestion, are essentials to ensure survival during early developmental stages in the hatchery. The zootechnical advances in paralarvae growth will also facilitate the provision of captive bred animals for a variety of research studies. The latter may become important as European Union Directive 2010/63/EU (European Parliament Council of the European Union, 2010) prohibits the use of animals taken from the wild unless this can be scientifically justified (*Article* 9).

Wild octopus paralarvae are believed to feed on a large number of zooplankton species, some of which have been identified with molecular tools (Roura et al., 2012). Different types of crab zoeae (Villanueva, 1995; Iglesias et al., 2004), copepod prey (Iglesias et al., 2007), and wild zooplankton (Estévez et al., 2009) have been assayed in nutritional trials of common octopus paralarvae and have improved its early growth and survival. Also, adapted live prey diets based on gammarid amphipods have improved growth and survival of benthic octopuses such as *Octopus joubini* and *Octopus maya* (Forsythe and Hanlon, 1980; Baeza-Rojano et al., 2013).

Understanding the nutritional gain achievable using live prey requires the design of parallel studies to properly dissect the different phases of the octopus attack strategy and ingestion dynamics. Feeding strategies have been described in wild adults as well as in captive animals (for review see Wells, 1978; Hanlon and Messenger, 1996).

The buccal mass is the most anterior part of the cephalopod digestive tract and all its components are already present at hatching (Villanueva and Norman, 2008). The buccal mass comprises two chitinous beaks, the radula and the associated musculature (**Figures 1A,B**; Altman and Nixon, 1970; Boucher-Rodoni, 1973; Boyle et al., 1979a,b; Guerra and Nixon, 1987) and can be rotated by the buccal musculature under neural control (Altman and Nixon, 1970; Boyle et al., 1979a,b). Adults use their beak to bite or to drill the prey exoskeleton thus creating an access to inject digestive enzymes from the posterior salivary glands into the prey via the salivary papilla (Wells, 1978). The radula in adults is equipped with an erect part with small teeth which are used to rasp food into the mouth (Nixon, 1968). Such food ingestion has also been reported in paralarvae which fully ingested crab zoeae fluids leaving an empty exoskeleton (Hernández-García et al., 2000). In adults, food passes through the esophagus to the crop, proceeds into the stomach and caecum, and transits to the digestive gland for nutrient absorption (Boucaud-Camou et al., 1976; Boucaud-Camou and Boucher-Rodoni, 1983; O'dor et al., 1984; Linares et al., 2015). The contractile activity of esophagus, crop, stomach, caecum, and intestine, progressively moves the food along the digestive tract (**Figures 1A,B**), i.e., a complex physiological activity believed to be coordinated by the gastric ganglion, although additional hormonal control cannot be excluded (Andrews and Tansey, 1983).

There is a paucity of knowledge of feeding strategies and digestive tract physiology of paralarvae as compared to adult octopus. To date, only indirect assessments of prey consumption have been possible as inferred from molecular analysis of paralarvae (Roura et al., 2012, 2016). In fact, direct assessment of feeding in recently hatched octopus paralarvae is elusive and requires the study of prey-predator relationships (e.g., hunting, defense, and escape) as well as of the subsequent prey capture, ingestion and post-ingestion. One way to access such knowledge is to quantify behavioral and physiological data from high-resolution video recording (e.g., Fiorito and Scotto, 1992). Video recording allows the simultaneous quantification of multiple physiological processes on the same animal. Moreover, the transparency of the thin mantle muscles of cephalopod paralarvae (except for sparse chromatophores) allows real time video monitoring and the quantification of the digestive process in a live, non-invasive manner and therefore, to undertake a comprehensive investigation of octopus paralarvae feeding in captivity (Hernández-García et al., 2000).

The general aim of this study is to understand the feeding strategies employed by common octopus paralarvae on different prey types and the physiological mechanisms operating during their digestion. The specific objectives were the *in vivo* quantification of (a) the attack strategy and related behavior exhibited by octopus paralarvae fed wild zooplankton, spider crab zoeae and edible crab zoeae hatched from broodstock, (b) the dynamics of exoskeleton penetration ("drilling") and content ingestion of different prey types, and (c) the dynamics of food distribution in the crop and the stomach comprising the motility changes occurring throughout the digestive tract until defaecation.

MATERIALS AND METHODS

Biological Material

Octopus Broodstock

In January and February, from 2013 to 2016, adult female and male *O. vulgaris*, Cuvier 1797, were captured in the Ría de Vigo (NW Spain) using artisanal fishing gear. Fourteen individuals were transported to the aquaculture facilities of *Centro Oceanográfico de Vigo* (COV-IEO) using portable—100

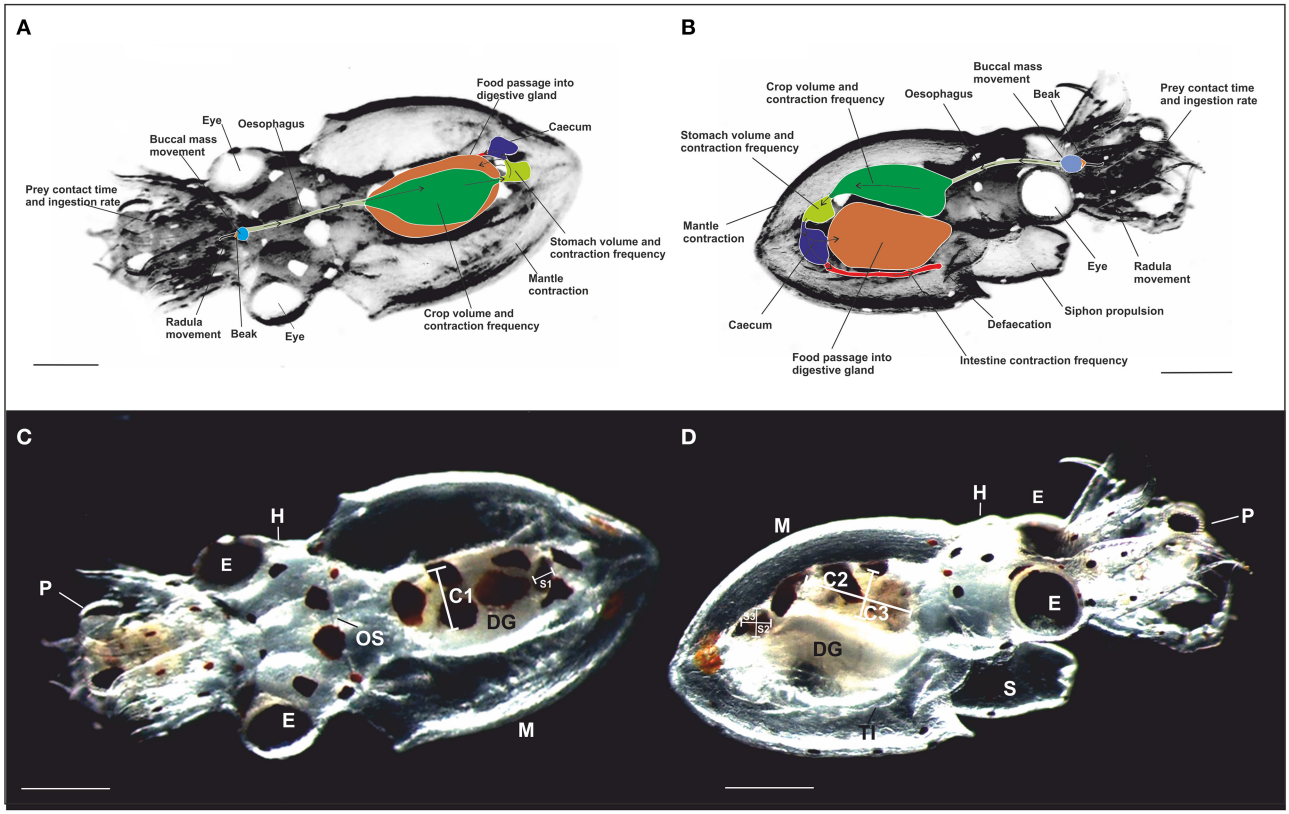

FIGURE 1 | Schematic representation of the dorsal **(A)** and the ventral **(B)** view of a paralarva in contact with prey (crab zoea) showing the location of the main digestive tract structures measured. Arrows indicate the direction of the food passage. **(C,D)** photography of the dorsal view **(A)** and the ventral view **(B)** taken from video recordings of a 3 dph free-swimming paralarvae of *O. vulgaris* with crab zoea prey captured. Note the transparency of the paralarva enabling direct observation of the digestive tract. The diameter of the crop **(C)** and the stomach (S) was measured along three axes: C1 and S1 (medio-lateral width), C2 and S2 (rostro-caudal length), and C3 and S3 (dorso-ventral thickness). OS, Oesophagus; DG, digestive gland; TI, terminal part of the intestine; E, eye; H, head; P, prey; M, mantle; and S, siphon. Scale bar = 0.5 mm.

L tanks at 14°C and O_2 saturation. Transport lasted 20 min and the broodstock was maintained in a flow-through concrete tank (4.60 × 2.10 m) filled with seawater (1.0 m in depth) at 14–18°C and 35 psu (practical salinity unit) as measured weekly using a refractometer ATC (ATAGO©; Iglesias et al., 2016). Several sections of a plastic pipe (0.2 m in diameter and 0.5 m in length) were immersed in the tanks as dens providing shelter for spawning females. The female to male ratio was 3:1 and the broodstock was fed frozen mussels (*Mytilus galloprovincialis*), frozen fish (*Merluccius merluccius* and *Sardina pilchardus*), and frozen crustaceans (*Polybius* spp.) three times a week. The food rations were calculated as 20% of the broodstock biomass introduced into each tank. The spawning females were removed from the broodstock tank and housed individually in smaller tanks (1.0 × 1.0 m) filled with seawater, 1.0 m depth at the ambient temperature of Ría de Vigo (14–18°C). Ammonia, nitrites and nitrates were measured daily and kept close to zero (Nutrafin©). Dissolved oxygen was measured twice a day (early morning and late afternoon) using an oximeter (OxyGuard-10XHM053, Polaris©, UK) and always maintained above 90%. Females spawned for 8–9 days between March and June each year (2013–2016) and laid their egg batches on the upper side of the pipe, so egg clusters remained suspended allowing their constant cleaning and oxygenation by gentle water jets from the female's siphon.

Hatching Paralarvae

The embryonic development of *O. vulgaris* paralarvae lasted 45–65 days at a seawater temperature of 14–18°C (Nande et al., 2017). Paralarvae remained in the hatching tank until day 2 post hatching (dph) and were transferred thereafter to 5 L buckets with filtered seawater (0.1 μm) at 35 psu salinity and 18°C for 24 h before carrying out the experiments. Thirty-five, 3 dph paralarvae per bucket (already devoid of inner yolk to prevent interference with their feeding behavior) were used in the experiments (Nande et al., 2017). Paralarvae were anesthetized at the end of the study by immersing them in a 0.5% $MgCl_2$ seawater solution (Magnesium chloride hexahydrate, Barcelonesa©, Global Chemical Solutions, Barcelona, Spain) at room temperature (18–20°C) for 10 min and was increased to 3.5% for 30 min. All paralarvae were killed by destruction of

the brain with a needle and aided by binocular microscope (LEICA MZ8®). Although, current experiments do not fall under Directive 2010/63/EU (European Parliament Council of the European Union, 2010) the authors followed its principles in terms of minimizing the number of animals used (Fiorito et al., 2014) and by using an appropriate killing method (Andrews et al., 2013; Fiorito et al., 2015). All the experimental procedures were supervised by an ethics committee (Octowelf project, see below, #CEIBA 2014-0108).

Zooplankton Samples

Six marine surveys were conducted between 2013 and 2016 aboard the Oceanographic vessel "José María Navaz" at different sampling points of Ría de Vigo. Three trawls were performed per survey using a planktonic net of 2 m in diameter with a 500 μm mesh placed in the collector tip and dragged to an average depth of 10 m for 10 min. Zooplankton samples were filtered twice through a 2 mm sieve and maintained in 100 L tanks containing seawater and equipped with gentle aeration. Each zooplanktonic sample was transferred and maintained in a specific 500 L tank equipped with gentle aeration and constant temperature (18°C) until completion of each feeding experiment (2 days).

Crustacean Broodstock

Several broodstocks of the crabs *Cancer pagurus* and *Maja brachydactila* were reared between 2012 and 2016 at the COV-IEO facilities to obtain live zoeae for nutritional assays on *O. vulgaris* paralarvae. Female crabs were acclimatized and maintained at the ambient temperature of Ría de Vigo (14–18°C) and at low light intensity (<100 lx) in 1.0 × 1.0 m tanks of 0.75 m in depth, supplied with filtered seawater in a flow through system. Crabs were fed frozen mussels (*M. galloprovincialis*) three times a week at a ratio of 10% in weight of the crab broodstock biomass. Spontaneous hatching events of zoeae were collected in spring and transferred to 100 L tanks using a 500 μm sieve. The water temperature was maintained at 18°C in a recirculating closed circuit.

Experimental Design

Effective Attacks vs. Ineffective Attacks

O. vulgaris paralarvae previously acclimatized in 5 L buckets ($n = 35$ paralarvae/bucket) filled with 0.1 μm filtered seawater of 35 psu at 18°C and kept in low light intensity (100–300 lx) were used in the experiments. Prey density in each bucket was 0.2 individuals/mL and a gentle air-flow was used to mix the water and facilitate prey-paralarvae encounters. This procedure was repeated so that in total three buckets per sampling, each with 35 paralarvae were studied. Only the 18 types of prey captured by paralarvae were identified under a binocular microscope (LEICA MZ8®) as helped by taxonomic identification guides (Rose, 1933; Trégouboff and Rose, 1957). Taxonomic identification reached either the species or the family level (**Table 1**). After 10 min of paralarvae-prey co-habitation in buckets, 15 paralarva-prey combinations (PPC) were carefully collected per replicate using a Pasteur pipette and examined under a binocular microscope (LEICA MZ8®). The number of paralarvae–prey combinations (PPC), the number of "effective attacks"(EA) and the number of "ineffective attacks" (IA) were defined as follows,

- Paralarvae-prey combination (PPC): a sustained predator-prey association, i.e., the prey is immobilized within the paralarva's arms.
- Effective attacks (EA): the paralarva grasps the prey, pierces its exoskeleton, and ingests its body content.
- Ineffective attacks (IA): the paralarva grasps the prey but neither pierces it nor ingests its content.

Dynamics of Food Ingestion and Digestive Passage

Paralarvae-prey combinations (PPC) were photographed and filmed in a Petri dish filled with seawater, using a high-resolution camera (Leica IC80 HD®, 3.1 Mpx) mounted on a binocular microscope (LEICA MZ8®). Recording of food passage through the digestive tract of paralarvae following EA was possible due to the transparency of the mantle musculature. The duration of recordings varied depending on the paralarva—prey association time and the orientation of the paralarva, so the observation time of digestive activities in crop, stomach, and terminal intestine differed between specimens. For those reasons, most parameterization is given as number (No.) of events/10 s rather than as No. events/min. Maximum stomach dimensions (diameter and radius) were estimated using image analysis software (LEICA Application Suite V4®) once the meal stopped moving into the stomach and began accumulating in the crop. Crop dimensions were taken once paralarvae released the prey carcass. Those dimensions were used to calculate the crop volume and the stomach volume, respectively.

Volume of the crop (Cv) and volume of the stomach (Sv) were calculated according to the formula,

$$Cv \text{ or } Sv = \frac{4}{3\pi} \cdot r1 \cdot r2 \cdot r3$$

Diameters of the stomach and the crop were measured from videotape recordings along three axes: as X (rostro-caudal length), Y (medio-lateral width), and Z (dorso-ventral thickness). Diameters of the stomach and the crop were used to calculate their radius per axis (r1, r2, r3) and were applied in the above formula to calculate the volume at specific time points during EA (**Figures 1C,D**).

After some preliminary observations for calibration, the association between paralarvae and prey was split into three phases as follows,

1. Initial phase (IP): the time since paralarvae captured the prey until ingestion began.
2. Middle phase (MP): the time since the food stopped entering the stomach until it began to accumulate in the crop.
3. Late phase (LP): the time since either the crop was full or since paralarvae released the prey.

Videos of PPC involving 18 prey types as well as single paralarvae were reanalyzed to measure the following parameters over 10 s intervals during the three defined phases (IP, MP, LP),

a) Mantle contractions (MC) during PPC.

TABLE 1 | Classification of wild zooplankton and laboratory bred species (*) captured by octopus paralarvae.

Type of prey	*N* Total	EA (%)	IA (%)	L (mm)	W (mm)
Acartia clausii	13.67 ± 1.53	100	0	1.25 ± 0.11	0.25 ± 0.01
Temora longicornis	15.00 ± 2.64	100	0	1.57 ± 0.12	0.61 ± 0.01
Centropages sp.	15.00 ± 1.73	100	0	1.98 ± 0.13	0.57 ± 0.01
Podon intermedius	15.34 ± 1.53	100	0	0.90 ± 0.01	0.45 ± 0.01
Carcinus maenas zoeae	12.67 ± 3.06	80.00 ± 8.66	20.00 ± 8.66	1.73 ± 0.15	0.58 ± 0.01
Maja brachydactyla zoeae*	17.00 ± 2.65	86.39 ± 2.27	13.61 ± 1.27	2.14 ± 0.05	0.69 ± 0.01
Cancer pagurus zoeae*	14.00 ± 3.00	83.30 ± 2.11	16.70 ± 2.11	2.45 ± 0.07	0.71 ± 0.02
Pisidia longicornis	09.00 ± 2.00	62.48 ± 4.87	37.52 ± 4.87	1.47 ± 0.02	0.57 ± 0.01
Paguridae[a]	10.34 ± 3.21	72.95 ± 9.06	27.05 ± 9.06	2.19 ± 0.15	0.86 ± 0.02
Processidae[a]	13.00 ± 2.00	68.73 ± 4.87	31.27 ± 4.87	4.34 ± 0.24	0.65 ± 0.01
Hippolytidae[a]	11.34 ± 4.51	63.77 ± 7.59	36.23 ± 7.59	2.35 ± 0.13	0.75 ± 0.04
Palaemonidae[a]	12.00 ± 2.00	74.52 ± 4.31	25.48 ± 4.31	3.34 ± 0.21	0.73 ± 0.03
Nyctiphanies couchii	13.00 ± 1.00	71.49 ± 6.27	28.51 ± 6.27	5.46 ± 0.34	0.83 ± 0.03
Crangonidae[a]	07.00 ± 1.00	0	100	4.88 ± 0.23	0.88 ± 0.03
Gastropods[a]	03.67 ± 0.58	0	100	0.57 ± 0.01	0.50 ± 0.01
Gammaridae[a]	06.34 ± 1.15	0	100	2.46 ± 0.14	1.45 ± 0.06
Hyperiid amphipod[a]	05.34 ± 1.15	0	100	1.99 ± 0.10	1.15 ± 0.09
Brachyura megalopae[a]	03.67 ± 0.58	0	100	1.68 ± 0.01	2.34 ± 0.11

[a]*A finer taxonomic classification of early stages could not be achieved in this taxon.*

N Total is the mean and standard deviation of the No. of prey captured by paralarvae from 2013 to 2016. EA and IA are the mean percentages (±sd) of effective attacks and ineffective attacks per prey type, respectively. The length (L) and the width (W) of prey are given in millimeters.

b) Siphon propulsions (SP), measured as sudden movements provoked by siphon jetting during the fight between prey and paralarvae (PPC).
c) Buccal mass movement (BM) during different phases (IP, MP, and LP).
d) Radula movement (RM) during prey penetration and ingestion.
e) Stomach contraction frequency (SC), volume (v), and nature of the stomach contents from each prey type.
f) Peristaltic crop movements (PC) propelling food toward the stomach. The crop volume was measured during LP.
g) Ingestion rate (IR), estimated as,

$$\mathrm{IR} = \frac{TF}{Ti}$$

Where *TF* is the total volume of food ingested (crop volume + stomach volume when the paralarvae released the prey) and *Ti* is the total ingestion time employed by paralarvae.

h) Contractions of the terminal intestine (TIC) measured in all phases of the ingestion (IP, MP, and LP).
i) Total food intake (TF) was the sum of the stomach volume (Sv) and the crop volume (Cv) as defined above.
j) Ingestion time (IT) was the time from the start of ingestion (food passing through the esophagus) until the prey is released.
k) Total contact time (TCT), is defined as the time from prey capture until release.
l) Contraction frequency of the mantle during post-ingestion.
m) Peristaltic crop movement and periods of crop inactivity (CI) during post-ingestion. CI measures the time from when the peristaltic activity (PC) ceases until the beginning of the next PC episode in the crop.
n) Description of food passage thought the intestine tract, caecum, digestive gland, excretion, and terminal intestine contraction (TIC) during post-ingestion.

Likewise, three phases were defined for ineffective attacks (IA) and used in video analyses,

1) Initial phase (IP): the period prior to prey capture.
2) Middle phase (MP): the period between prey capture and its immobilization (fight initiation).
3) Late phase (LP): the period from prey immobilization to prey release.

Statistics

Predator-prey data measured from video recordings were tested for homoscedasticity using the Levene's test (Zar, 1999). Data distributions were checked for normality using the one-sample Kolmogorov–Smirnoff test (Zar, 1999). Kruskal–Wallis analysis was used to evaluate the significance of nonparametric data from EA and IA relative to the different prey types. Differences in the mean of all parameters (Mantle contraction, MC; buccal mass movement, BM; radula movement, RM; intestine contraction, TIC; crop volume, Cv; stomach volume, Sv; total time of ingestion, TIT; and ingestion rate, IR) among replicates and among, prey type, were compared with one-way ANOVA using the program STATISTICA 10.0©. Global significant tests led to comparison of pairwise means using the Tukey test. Intra-individual analyses of the same variable taken at different digestion phases (IP, MP, and LP) over time were performed

with a *t*-test and a general linear model analysis of variance (ANOVA-RM) for repeated measures. Data are presented in the text and illustrations as mean ± *SD* and significant differences were assumed below the nominal probability threshold $p = 0.05$.

RESULTS

Paralarvae Attack and Prey Capture

Paralarvae exposed to wild zooplankton captured diverse prey including cladocerans (*Podon intermedius*), copepods (*Acartia clausii*, *Temora longicornis,* and *Centropages* sp.), zoeae of *Carcinus maenas* and *Pisidia longicornis* as well as zoeae of the decapod families Crangonidae, Hippolytidae, Paguridae, Palaemonidae, and Processidae (**Table 1**). Paralarvae also captured larval stages of the euphausiids *Nyctiphanes couchii*, amphipods (e.g., gammarids and hyperiids) and megalopae stages of gastropods and crabs. Prey capture was independent of prey size or prey mobility in the water column (**Figure 2**). The defensive strategy of prey encountering paralarvae varied substantially between species. Copepods showed continuous swimming and sudden direction changes; zoeae (*C. maenas*, *C. pagurus,* and *Maja brachydactyla*) showed rhythmic swimming, increased frequency of abdominal flexion and high speed spinning (Video, from start to second 17; Supplementary Material). Megalopae used their chelipeds as a defensive tool (Video, from second 17 to second 33; Supplementary Material). The sharp spines of zoeae occasionally damaged or even killed paralarvae (Video, from second 33 to second 43; Supplementary Material).

Prey Capture Frequency, Prey Target Site, and Attack Effectiveness

The dorsal-cephalothorax was the site where 100% of effective attacks (EA) occurred on small prey (copepods and cladocerans), crab zoeae, and krill (**Figure 2**; **Table 1**). The frequency of efficient attacks (EA) on *M. brachydactyla* averaged 86.4 ± 2.3% but was less on Hippolytidae and *P. longicornis* (≈60%, **Table 1**). In laboratory-bred strains and in wild decapod species, EA always

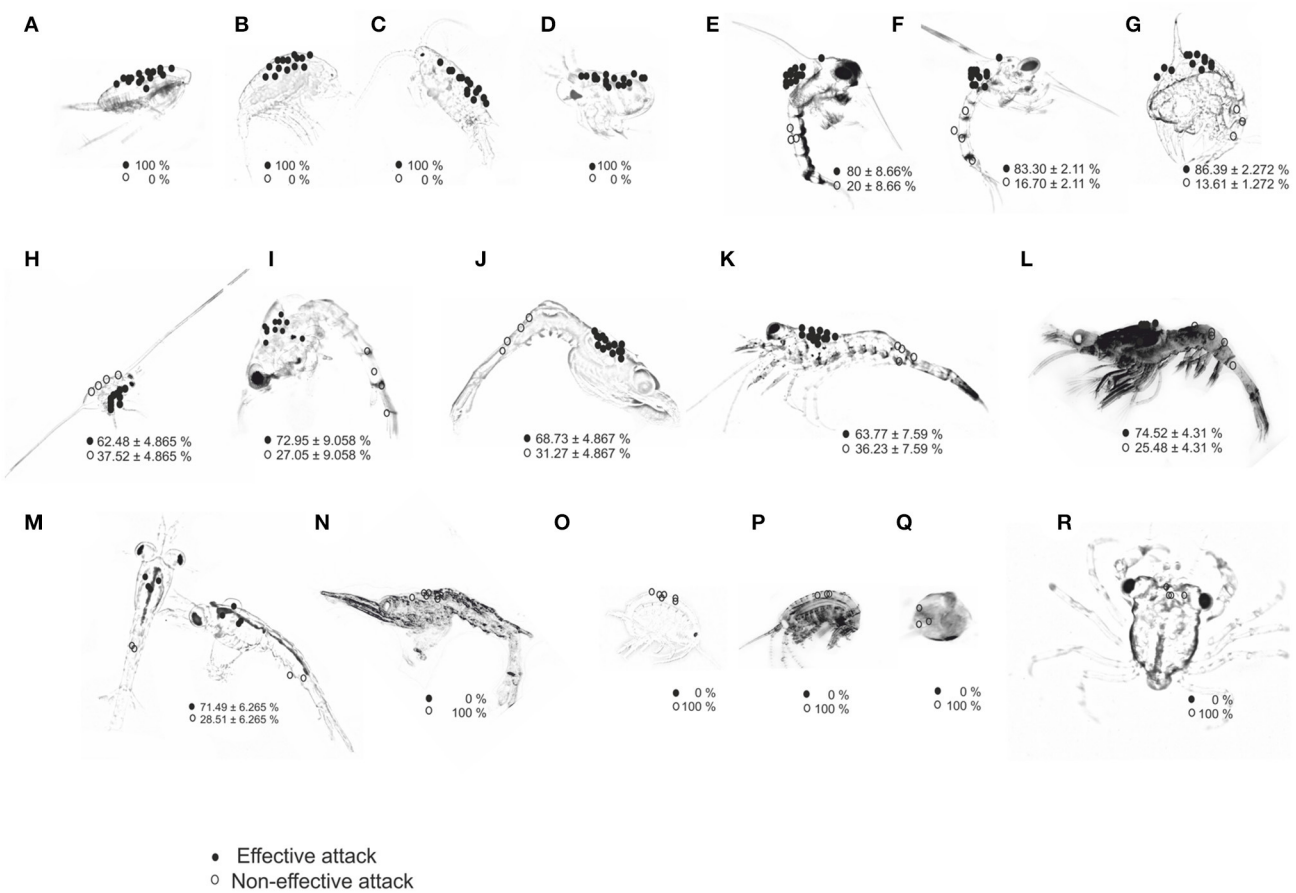

FIGURE 2 | Location of attack sites (circles) by paralarvae of *O. vulgaris* on different prey types: **(A)** *Acartia clausii*; **(B)** *Temora longicornis*; **(C)** *Centropages* sp.; **(D)** *Podon intermedius*; **(E)** *Carcinus maenas*; **(F)** *Cancer pagurus*; **(G)** *Maja brachydactyla*; **(H)** *Pisidia longicornis*; **(I)** Paguridae; **(J)** Processidae; **(K)** Hippolytidae; **(L)** Palaemonidae; **(M)** *Nyctiphanes couchii*; **(N)** Crangonidae; **(O)** Gammarid amphipod; **(P)** Hyperiid amphipod; **(Q)** Gastropods; **(R)** Brachyura megalopae. Prey images are not to scale to identify all preys in the same panel; actual prey size is given in **Table 1**. Percentage of EA (effective attacks, filled circles) and IA (ineffective attacks, open circles) are given per prey type.

targeted the cephalothoracic area, occurring 85.0 ± 5.0% on its dorsal area and 10.0 ± 5.0% on its lateral or ventral areas (**Figure 2**; Video, from second 44 to second 60, Supplementary Material). All ineffective attacks (IA) on laboratory bred decapod zoeae and on wild zooplankton taxa (*C. maenas, P. longicornis,* Paguridae, Processidae, Hippolytidae, Palaemonidae, and the krill *N. couchii*) occurred on the abdominal or on the telson areas (**Figure 2**; **Table 1**).

Significant differences in the frequency of EA were observed between prey types grouped by categories: (a) copepods and cladocerans (100% EA), (b) decapod zoeae and krill (60–90% EA), and (c) Crangonidae, amphipods, gastropods, and crab megalopae (0% EA) [Kruskal–Wallis test, H (18 prey types, $n = 57$) = 51.02; $p = 0.0001$]. All captures of decapod zoeae from families Crangonidae, amphipods, gastropods, and crab megalopae occurred on the dorsal cephalothorax and resulted in ineffective attacks (IA) (paralarvae were unable to drill their exoskeleton). All attacks on crab megalopa occurred on the dorso-anterior region and prey employed their chelipeds to successfully defend from paralarvae.

Prey Killing and Ingestion

Effective Attacks and Initial Digestive Tract Activity

During the initial phase (IP) of the predator-prey combination (PPC), paralarvae fought with the prey to immobilize it (Video, from second 44 to second 60, Supplementary Material).

The frequency of MC (mantle contractions) in the IP, calculated as an average from all EA was 17.08 ± 1.44/10 s ($n = 66$). The MC frequency was significantly less in small prey (*A. clausii*, *T. longicornis,* and *P. intermedius*) than in large prey (Paguridae, Processidae, Hippolytidae, Palaemonidae, and the krill *N. couchii*) in all phases, i.e., IP (ANOVA-RM, $F = 43.34$, $p = 0.0001$), MP (ANOVA-RM, $F = 189.23$, $p = 0.00001$), and LP (ANOVA-RM, $F = 176.23$ $p = 0.0001$; **Figure 3A**; **Table 2**). Mantle contractions were accompanied by siphon propulsions averaging SP = 3.03 ± 0.54/10 s (Table 1 in Supplementary Material), as well as by an average of 12 movements/min of repetitive lateral movements of head and arms. The initiation of the buccal mass activity was coincident with movements of siphon, head and arms (Video, from second 44 to second 60, Supplementary Material). The MC frequency during phase MP (MC = 13.67 ± 1.43/10 s) and phase LP (MC = 12.73 ± 1.76/10 s) were significantly less than the MC frequency during phase IP (MC = 17.08 ± 1.44/10 s) (ANOVA-RM, $n = 66$, $F = 201.46$, $p = 0.0001$; **Figure 3A**).

Once the prey was immobilized, paralarvae prepared to access its internal tissues using the beak and buccal mass movements (BM). In IA (ineffective attacks), the frequency of BM was 6.16 ± 2.64/10 s for copepods and cladocerans as compared to 9.24 ± 1.29/10 s for the rest of the species. BM movement was significantly higher on larger prey than on smaller prey (e.g., copepods and cladocerans) (one-way ANOVA, $F = 29.064$, $p = 0.00001$; **Figure 3B**). Significant differences in BM were also observed between phases IP and LP (e.g., BM_IP = 9.08 ± 2.58/10 s vs. BM_LP = 7.24 ± 2.05/10 s; ANOVA-RM, $F = 112.23$, $p = 0.0001$).

Following exoskeleton penetration (after IP phase), paralarvae inserted the radula into the prey and initiated the ingestion of its internal content (Video, from second 61 to second 69, Supplementary Material). RM (radula movement) frequency in the middle phase (MP_ RM = 6.59 ± 1.7/10 s) did not differ from that in the LP (RM_LP = 6.21 ± 1.8/10 s) (ANOVA-RM, $F = 6.65$, $p = 0.278$). However, the RM frequency differed significantly in copepods and cladocerans ($n = 45$) (RM_{MP} = 4.27 ± 1.03/10 s; RM_{LP} = 4.07 ± 1.16/10 s, respectively) as compared to the rest of the species ($n = 153$) (RM_{MP} = 7.27 ± 1.11/10 s; RM_{LP} = 6.84 ± 1.05/10 s) (one-way ANOVA, F_{MP} = 4.72, $p = 0.0001$; $F_{LP} = 7.02$, $p = 0.0001$; **Figure 3C**). Food ingestion was assisted by buccal mass movements, the beak and the radula. Food passed through the esophagus into the upper digestive tract, bypassed the crop and entered the stomach where it accumulated as feeding proceeded (Video, from second 61 to second 103, Supplementary Material). Before its complete filling (defined as the volume plateauing) the stomach contracted at a frequency of 5.24 ± 0.78/10 s (Table 2 in Supplementary Material) independent of prey type (one-way ANOVA, $F = 3.67$, $p = 0.12$). Stomach contractions stopped after it had filled but the paralarvae continued feeding and accumulating food in the crop. The stomach volume at its maximum filling was Sv = 0.016 ± 0.008 mm^3 ($n = 56$) and was prey-independent (ANOVA-RM, $F = 16.44$, $p = 0.084$; **Figure 4**, Table 3 in Supplementary Material).

The crop exhibited rhythmic peristaltic contractions (PC = 18 ± 6/min; $n = 56$; Video, from second 103 to second 130, Supplementary Material) to deliver food toward the stomach from where it passed into the caecum. It was not possible to define a fixed time at which food began to move from the stomach to the caecum. In some paralarvae, food transfer to the caecum began once the crop was full but in other paralarvae it began either at the end of the ingestion phase or once the paralarvae released the prey.

Crop volume at the end of the ingestion phase varied significantly between small prey such as copepods and cladocerans (Cv = 0.005 ± 0.003 mm^3; $n = 15$) and large prey such as decapods and krill (Cv = 0.09 ± 0.04 mm^3) (one-way ANOVA, $F = 17.805$, $p = 0.0001$; **Figure 5A**). The ingestion rate (IR) differed significantly between all copepod prey (cladocerans, *P. longicornis*, *C. maenas,* and *C. pagurus zoeae*; IR = 0.009 ± 0.002 mm^3/min; $n = 28$) and the rest of prey, particularly the large ones (IR = 0.045 ± 0.007 mm^3/min; $n = 33$; one-way ANOVA, $F = 9.027$, $p = 0.0001$; **Figure 5B**). Rhythmic contractile activity was observed throughout the digestive tract from the initiation of prey drilling (Video, from second 113 to second 130, Supplementary Material). Such contractions were prominent, with a high frequency (7.29 ± 0.41/10 s) in the terminal part of the intestine throughout the intake process and did not depend on prey type (one-way ANOVA, $F = 17.805$, $p = 0.0001$). Total food intake was significantly less in copepods and cladocerans (TF = 0.011 ± 0.005 mm^3) than in larger species (TF = 0.113 ± 0.05 mm^3) (one-way ANOVA, $F = 41.91$, $p = 0.0001$; **Figure 5C**). The ingestion time was significantly shorter in copepods and cladocerans (IT = 73.13 ± 23.34 s; $n = 15$) than in decapod

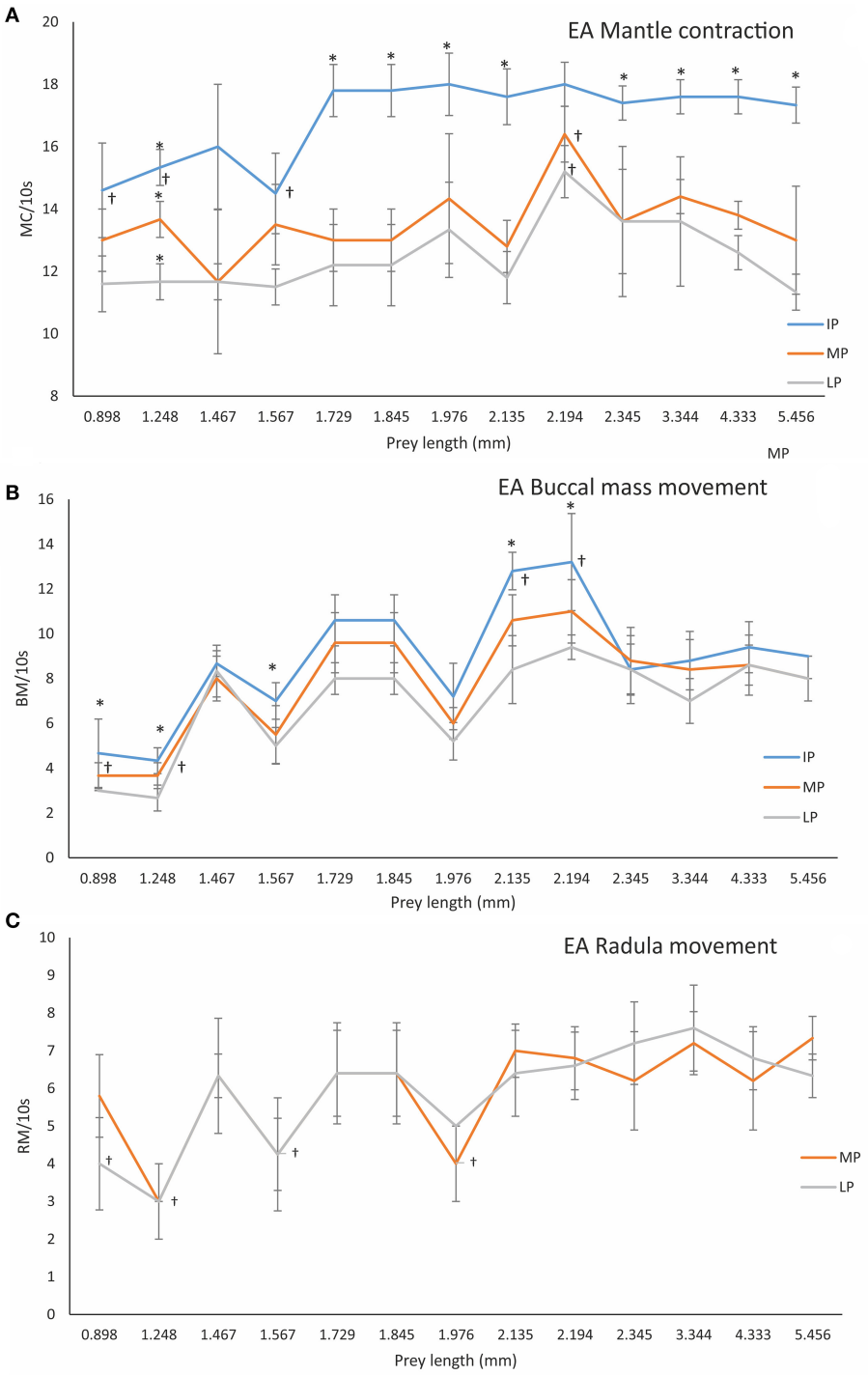

FIGURE 3 | Frequency of MC (Mantle contractions, **A**), BC (Buccal mass movements, **B**) and RM (Radula movements, **C**), in different PPC (predator-prey combination phases), i.e., IP (initial phase), MP (middle phase), and LP (late phase) of EA (effective attacks) by 3 dph *O. vulgaris* paralarvae in relation to prey length. Data are plotted as mean ± *SD*. Symbols (†) indicate significant differences between prey-lengths in the same phase (one-way ANOVA, $p < 0.05$). The asterisk (*) indicates significant differences within prey-type between PPC phases (ANOVA-RM, $p < 0.05$; see Section Materials and Methods for details).

zoeae and euphausiids (IT = 152.49 ± 29.40 s; $n = 41$) (one-way ANOVA, $F = 15.37$, $p = 0.0001$; **Figure 5D**). Total contact time was significantly shorter in copepods and cladocerans (TCT = 84.73 ± 21.86 s) than in the remaining prey (TCT = 220.17 ± 25.44 s) (one-way ANOVA, $F = 31.18$, $p = 0.0001$).

TABLE 2 | Mean frequency and standard deviation of mantle contractions, crop peristaltic contractions, intervals between episodes of crop activity, and terminal intestine contractions during the post-ingestion, i.e., after paralarvae released prey.

Type of prey	No. paralarvae	No. Mantle Contractions /10 s	No. Crop contractions/10 s	Interval between episodes of crop peristaltic movements/10 s	No. terminal intestine contractions/10 s
Copepods (*A. clausii, T. longicornis, Centropages* sp.)	3	5.667 ± 0.577	3.333 ± 0.254	6.667 ± 0.334	8.200 ± 0.545
Cladocera (*Podon intermedius*)	4	6.334 ± 0.577	2.330 ± 0.667	6.167 ± 0.687	8.333 ± 1.334
Zoeae *M. brachydactyla*, *C. pagurus*	6	6.334 ± 1.154	3.600 ± 0.667	6.334 ± 0.236	9.600 ± 0.547
Zoeae *C. maenas, P. longicornis*	6	6.000 ± 1.000	3.200 ± 0.767	6.867 ± 0.381	9.200 ± 0.667
Paguridae	3	6.333 ± 1.155	2.800 ± 0.476	7.000 ± 1.000	8.600 ± 0.845
Processidae, Hippolytidae, Palaemonidae	8	6.667 ± 0.577	3.333 ± 0.564	6.670 ± 0.334	9.333 ± 0.577
Euphausiidae	4	5 ± 1.732	2.800 ± 0.776	5.600 ± 0.658	8.800 ± 0.955

Data are grouped according to the criterion of prey group (see text for definitions and details).

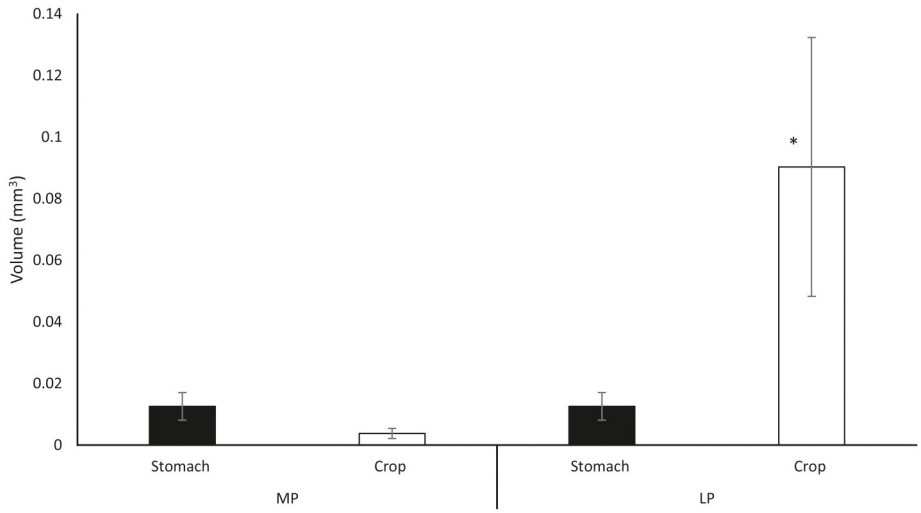

FIGURE 4 | Mean crop volume (open bars) and stomach volume (filled bars) as combining data from all ingested prey types. MP measurements taken at maximum stomach volume during middle phase; LP volume measurements taken in the late ingestion phase at maximum crop volume (Note that there is no change in the gastric volume). Data plotted as mean ± *SD* ($n = 55$). The asterisk (*) indicates significant differences between ingestion phases (MP vs. LP) within organ (ANOVA-RM, $p < 0.05$).

Post-Ingestion Activity in the Digestive Tract

Paralarvae detached from prey in the post-ingestion phase (Video, from second 139 to second 148, Supplementary Material; ≈10 min recording time). Mantle contraction frequency decreased significantly between the ingestion phase (MC = 13.37 ± 0.13/10 s; $n = 56$) and the post ingestion phase (MC = 6.07 ± 0.67/10 s) (ANOVA-RM, $F = 989.33$, $p = 0.00001$). During this latter phase, the crop was fully distended and showed intermittent peristaltic activity characterized by episodes of contractile activity lasting 2.0 ± 1.0 s at a frequency 3.0 ± 1.0 /10 s and at intervals of 6.58 ± 1.85 s ($n = 34$) between episodes (Video, from second 150 to second 176, Supplementary Material). The caecum contained prey pigments (reddish from *T. longicornis* and blackish for *C. maenas*) which colored the digestive gland as food entered the hepatopancreatic duct. Food began entering the digestive gland *via* the hepatopancreatic duct 312 ± 32 s after the crop was full (Video, from second 176 to second 210, Supplementary Material). Contents from the digestive tract not entering the digestive gland proceeded along the intestine and were expelled through rectum and anus. During the latter activity, the terminal part of the intestine contracted at frequency of 8.87 ± 1.46/10 s which did not differ from that observed during prey ingestion (ANOVA-RM, $F = 37.34$, $p = 0.001$). Feces were gray in color and string-like in appearance (Video, from second 210 to second 250, Supplementary Material). During defaecation (observed in 12% of specimens) the MC frequency increased to 14.23 ± 0.44/10 s ($n = 4$), a value comparable to that observed during the ingestion phase (13.37 ± 0.13/10 s; Video, from second 210 to second 250, Supplementary Material).

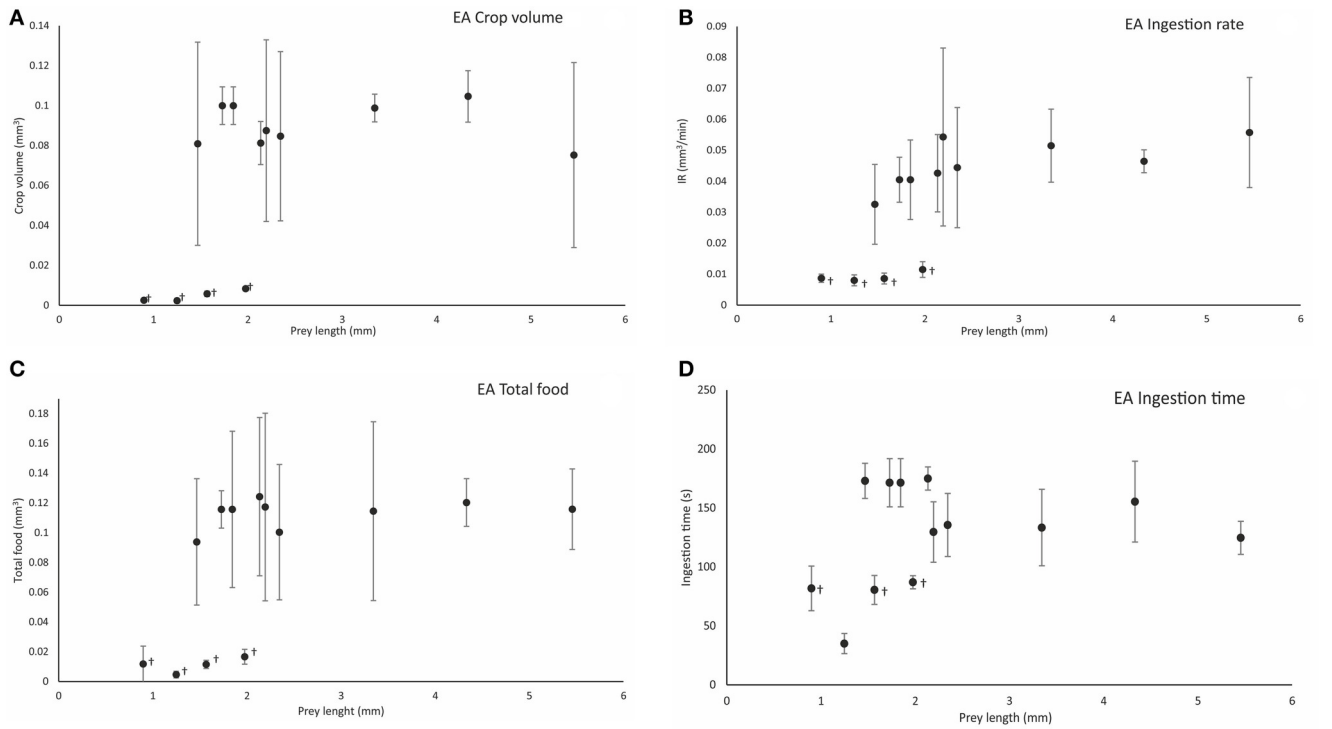

FIGURE 5 | Calculated crop volume (Cv mm^3) **(A)**, ingestion rate (IR, mm^3/min) **(B)**, total food volume (TF, mm^3) **(C)**, and ingestion time (IT, s) **(D)**, in relation to prey length (mm) following effective attacks (EA) on different prey types. Symbol (†) indicates significant differences (one-way ANOVA, $p < 0.05$) between prey types at the end of ingestion. Vertical bars represent the standard deviation of the mean of the variable (see the main text for definitions and details).

Digestive Tract Activity during Ineffective Attacks

The frequency of mantle contractions during ineffective attacks did not differ between PPC phases ($MC_{IP} = 17.37 \pm 1.44/10$ s; $MC_{MP} = 16.86 \pm 1.80$; and $MC_{LP} = 17.4 \pm 1.83/10$ s) (ANOVA-RM, $F = 3.44$, $p = 0.33$), and averaged $17.75 \pm 1.01/10$ s ($n = 105$) overall (**Figure 6A**). The frequency of MC did not differ among prey types (one-way ANOVA, $F = 1.177$, $p = 0.319$) or between the initial phase of effective attacks ($MC_{IP-EA} = 17.08 \pm 1.44/10$ s, $n = 66$; see above) vs. ineffective attacks ($MC_{IP-IA} = 17.75 \pm 1.01/10$ s) (one-way ANOVA, $F = 0.28$, $p = 0.60$). However, MC frequency was significantly lower during the middle-phase of ineffective attacks as compared to effective attacks ($MC_{MP-IA} = 13.67 \pm 1.43/10$ s; $n = 66$; one-way ANOVA, $F = 198.05$, $p = 0.0001$).

Following attack and prey holding (i.e., once PPC was established), paralarvae grasped the prey accompanied by lateral shaking of head and arms and siphon jetting propulsions (SP $= 3.0 \pm 1.0/10$ s; $n = 105$), and attempted to pierce the exoskeleton with the beak. The buccal mass movements were at a frequency MC $= 11.14 \pm 2.37/10$ s, MC $= 10.89 \pm 2.85/10$ s, and MC $= 11.11 \pm 2.75/10$ s in phases IP, MP, and LP respectively, (Table 4 in Supplementary Material). BM frequency did not differ among ingestion phases (one-way ANOVA, $F = 0.098$, $p = 0.910$) and averaged BM $= 11.05 \pm 2.64/10$ s ($n = 105$). BM frequency differed between Crangonidae, megalopae and Gammaridae amphipods (BM $= 13 \pm 1.41$ /10 s) and *P. longicornis* (BM $= 9.89 \pm 1.58/10$ s) (one-way ANOVA, $F = 16.903$, $p = 0.00001$; **Figure 6B**). BM frequency differed significantly between EA (BM $= 11.14 \pm 1.37/10$ s) and IA (BM $= 9.075 \pm 1.57/10$ s) in the initial phase of ingestion (one-way ANOVA, $F = 5.82$, $p = 0.0001$). Although, paralarvae were unable to pierce the prey in ineffective attacks, rhythmic contractile activity was observed in their intestine. The frequency of rhythmic contractions did not differ between prey types (one-way ANOVA, $F_{IP} = 0.96$, $p = 0.49$; $F_{MP} = 1.91$, $p = 0.11$; $F_{LP} = 1.6$, $p = 0.18$) at any phase (ANOVA-RM, $F = 4.33$, $p = 0.07$) and averaged $5.24 \pm 1.23/10$ s ($n = 87$; **Figure 6C**). Significant differences were observed between the higher terminal intestine contraction frequency recorded during EA ($7.34 \pm 0.92/10$ s) and that observed during IA ($5.24 \pm 1.23/10$ s; one-way ANOVA, $F = 342.03$, $p = 0.0001$). Total contact time between paralarvae and prey (TCT) varied significantly with prey type (one-way ANOVA, $F = 3.73$, $p = 0.004$; $n = 35$). The shortest TCT (35.33 ± 8.14 s) was observed in gastropods and the longest TCT (153 ± 19.78 s) was observed in gammarids (**Figure 6D**). TCT was significantly higher in EA attacks as compared to IA attacks (one-way ANOVA, $F = 81.84$, $p = 0.00001$). For instance, TCT $= 230.6 \pm 17.89$ s during EA on *M. brachydactyla* zoeae and TCT $= 69.34 \pm 21.78$ s when an ineffective attack occurred on the same prey (Video, from second 250 to second 303, Supplementary Material).

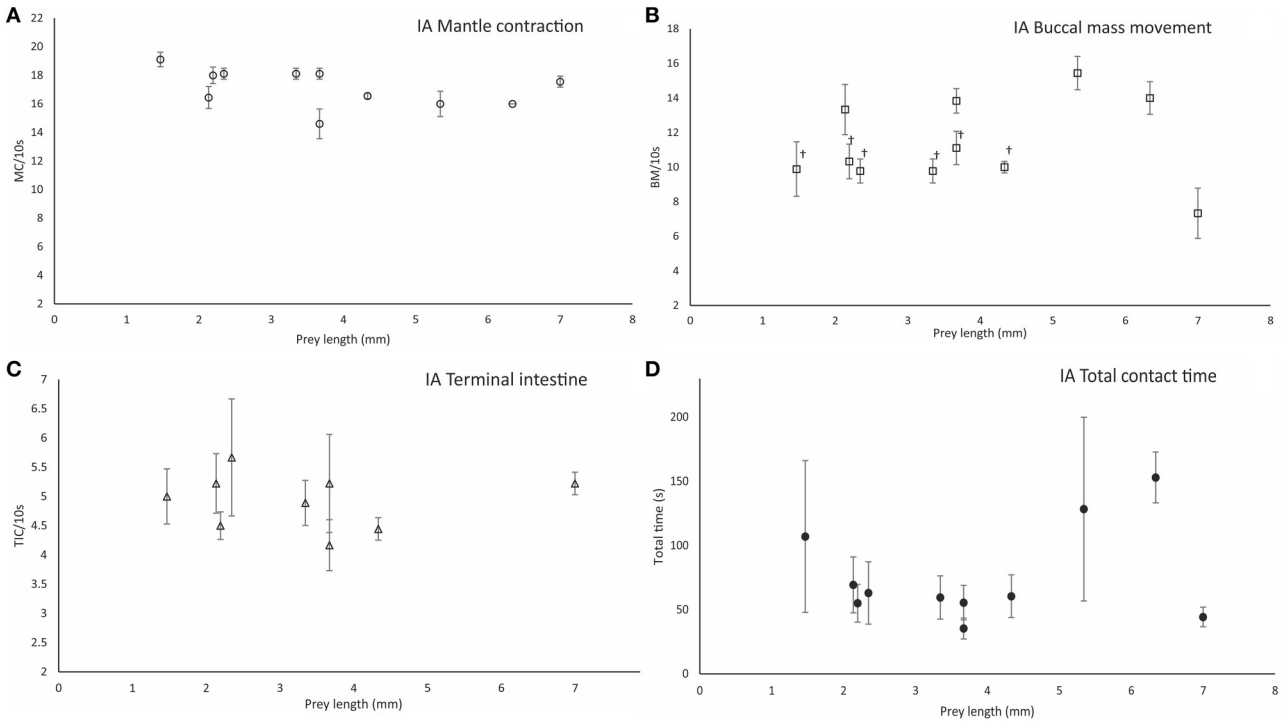

FIGURE 6 | Parameters measured during ineffective attacks (IA). Mantle contraction frequency (MC/10 s) **(A)**, buccal mass movement frequency (BM/10 s) **(B)**, terminal intestine contraction frequency (TIC/10 s) **(C)**, and total contact time between paralarvae and prey (TCT/10s) **(D)**. Prey types are grouped by prey size in length (mm). Symbols (†) indicate significant differences (one-way ANOVA, $p < 0.05$) between prey types. Vertical bars represent the standard deviation of the mean for each variable.

DISCUSSION

The general aim of this study was to understand the feeding mechanisms employed by paralarvae of the common octopus fed different prey types and the physiological dynamics operating during digestion. Specific results for each of the objectives outlined in the Introduction are discussed in relation to knowledge of digestive tract physiology from adult cephalopods.

Paralarvae Attack and Prey Defensive Strategies

The first specific objective of this study was the *in vivo* quantification of the attack strategy and the related behavior exhibited by octopus paralarvae on wild zooplankton and zoeae of spider crab and edible crab hatched from the broodstock. *O. vulgaris* paralarvae coexist in the wild with diverse zooplankton, i.e., copepods, cladocerans, zoeae of different decapods, mysidacea, euphausiids, gammarids and hyperiids amphipods, salpids, gastropods, cirripedia nauplii (Roura et al., 2013), the smallest among them captured and ingested by paralarvae, i.e., cladocerans (*P. intermedius*, length 0.90 ± 0.01 mm) and copepods (*A. clausii* and *T. longicornis,* length 1.25 ± 0.11 and 1.57 ± 0.12 mm, respectively). The ability of octopus paralarvae to capture copepods (Nande, 2016) and cladocerans is confirmed herein. Nevertheless, occasional failed attacks on copepods was due to their escape reaction upon detection the predator movements (Yen et al., 1992; Fields and Yen, 1997; Paffenhöfer, 1998) as has also been observed in paralarvae of the squid, *Loligo opalescens* (Chen et al., 1996).

Notably, current results also indicate that 3 dph paralarvae are able to effectively feed on both, small and large live prey and not only on prey of length >2 mm (Iglesias et al., 2006). The highest percentage of EA among decapods zoeae took place on zoeae of *C. maenas*, *M. brachydactyla,* and *C. pagurus* (80, 86, and 83%, respectively). Consistent with those data, the best growth and survival rates reported in recent years from rearing experiments of *O. vulgaris* paralarvae were obtained using zoeae of *M. brachydactyla* (Iglesias et al., 2004; Carrasco et al., 2006), *Grapsus grapsus* and *Plagusia depressa* (Iglesias et al., 2007), *Liocarcinus depurator* and *Pagurus prideaux* (Villanueva, 1994, 1995). Also, diverse analyses of stomach content from wild paralarvae contained species of decapods zoeae such as *Necora puber*, *Polybius henslowii*, *Pirimela denticulata*, among others (Roura et al., 2012). Other prey types such as zoeae of families Paguridae, Processidae, Hippolytidae, and Palaemonidae were also captured by octopus paralarvae with a lower efficiency of attacks (60–75%) as compared to zoeae of the preferred species.

The cephalothorax area was the site targeted in all effective attacks (irrespective of prey type) but ineffective attacks occurred on the abdomen (e.g., on krill species). Supportive evidence for the hypothesis of a deliberate targeting of the prey cephalothorax is the number of effective attacks on that body area. A

less plausible but untested hypothesis comes from nutritional analyses of krill (*Meganyctiphanes norvegica*) and states that the cephalothorax area contains a higher proportion of food per volume and a different lipid profile as compared to the abdomen (Albessard et al., 2001). Although, paralarvae directed their attacks toward the cephalothorax, about 25% of them were ineffective because of the escape ability of the prey after their initial abdominal contact. For instance, zoeae of *C. maenas*, *C. pagurus,* and *M. brachydactyla* exposed to paralarvae exhibited a defensive behavior consisting on rapid swimming movements and fast spinning, in agreement with previous observations (Hernández-García et al., 2000). Two alternatives to escaping consisted of facing the predator using sharp spines which occasionally caused mortal injuries to the paralarvae (Villanueva and Norman, 2008) and the use of chelipeds as defense tools (*Brachiura megalopae*) as observed in current and previous studies (Bertini and Fransozo, 1999).

A 100% frequency of ineffective attacks was observed in all species of families Crangonidae, gastropods, Gammaridae and Hyperiidae amphipods and Brachiura megalopae, irrespective of the body area targeted. Those IA were due to the inability of the paralarvae to pierce the exoskeleton of those prey (see **Figure 2**). Although, some Cragnonidae prey were previously reported from stomach contents of wild paralarvae (Roura et al., 2012), current observations show that a 3 dph beak is relatively smaller than that of older paralarvae (Perales-Raya et al., 2014) and therefore it is practically ineffective on mineralized exoskeletons.

Mantle contractions are related to swimming and breathing (Villanueva, 1994; Villanueva and Norman, 2008). During the initial phase of PPC, the mantle contraction frequency was ∼100/min and progressively decreased during ingestion and post-ingestion to ∼75/min. Studies of *O. vulgaris* (at 15°C) ranging in weight from 2.5 g to 8 Kg showed a progressive reduction in respiration rate from 51/min to 12/min (Polimanti, 1913, cited in Wells, 1978, Table 3.1, p26). That range is consistent with baseline values for larger octopuses in the later literature (∼150 g to ∼2 Kg, ∼16 bpm to ∼30 b/min; Andrews and Tansey, 1981; Wells and Wells, 1985; Valverde and García, 2005). The higher mantle contraction frequency of paralarvae as compared to specimens weighing >2.5 g is not odd since the metabolic rate is expected to be higher in rapidly developing paralarvae as compared to adults (Iglesias et al., 2004; Semmens et al., 2004). Current data suggest that the lower frequency of mantle contractions relates to a precise prey manipulation, as observed in older octopus paralarvae (Villanueva et al., 1996). Indeed, paralarvae successfully positioned themselves as well as the prey using combined contractions of mantle and siphon, in addition to movements of head and arms. Those actions need coordination between the central nervous system and the visual system (see **Figure 2**) and the mechanisms by which this is achieved require further research.

Prey Drilling and Ingestion Dynamics

The second specific objective of this study was the *in vivo* quantification of the dynamics of both, exoskeleton penetration ("drilling") and ingestion of different prey types. Monitoring those processes was feasible thanks to the transparency of paralarvae (Nande et al., 2017) which allowed the use of high resolution video-recording to characterize and quantify the mechanisms for the first time in a live cephalopod. Current results show that during the initial phase, when paralarvae attempted to drill the prey, it used buccal mass movements (Villanueva and Norman, 2008) which were prey-dependent. The BM frequency was significantly lower when feeding on copepods and cladocerans bearing a less chitinized exoskeleton than on prey with a thicker, more mineralized exoskeleton (e.g., 6.16 ± 2.64/10 s vs. 9.24 ± 1.29/10 s). Consequently, BM frequency was higher (11.05 ± 2.64/10 s) during the initial phase of ineffective attacks on mineralized exoskeletons (amphipods, Crangonidae, Brachiura megalopae, and Gammaridae).

In effective attacks, radula movements (RM) began after prey drilling and proceeded at constant frequency (6.59 ± 1.7/10 s). However, RM frequency was higher on large prey than on small prey, particularly in the later phases. We were unable to disentangle the relative roles that the beak, the radula and the buccal mass played during ingestion, but it is assumed that food particles moved into the esophagus by the coordinated activity of the above three structures. In this regard, Altman and Nixon (1970) reported that ingestion of crab in adult octopus was possible in radula-less specimens by using the lateral buccal palps. Nevertheless, those authors also reported that radula-less specimens were unable to perform the "*more delicate parts of the cleaning process*" (Altman and Nixon, 1970, p. 35). Therefore, it is likely that the radula is important during ingestion requiring the coordination of beak, radula, and buccal mass muscles by the inferior buccal and subradular ganglia (Boyle et al., 1979a,b). Lesion studies have shown that eating also requires connection of those ganglia with the superior buccal ganglia (Young, 1965) and current observations suggest that the inferior buccal, subradular and superior buccal ganglia are sufficiently mature in 3 dph octopus paralarvae to coordinate food ingestion. However, the possibility exists that in such an immature state, ingestion may be regulated solely by the peripheral ganglia as triggered by buccal contact with prey.

Food passed from the buccal cavity into the relatively narrow esophagus where peristaltic contractions conveyed it to crop and stomach (Andrews and Tansey, 1981). The total contact time between prey and paralarvae was short (<5 min) so ingestion was relatively rapid. Therefore, the rapid food ingestion and storage of paralarvae is particularly adaptive because of the high vulnerability of feeding paralarvae to predation. Since attack effectiveness and subsequent ingestion were prey-dependent, paralarvae exhibited an optimized strategy for prey capture, drilling, and ingestion time upon potential food energy intake, density and digestibility. Current data provides a basis for considering how octopus paralarvae feeding fits with published models of feeding (Schoener, 1971), particularly for invertebrate larvae (e.g., Crustacea, Le Vay et al., 2001).

Post Ingestion Digestive Tract Motility

The third specific objective of this study was the *in vivo* quantification of the distribution of food into the crop, the stomach, and the digestive gland as well as the characterization

of the motility patterns. The crop is an elongated sack-like structure located between the esophagus and the stomach of Octopoda (e.g., *O. vulgaris*, Andrews and Tansey, 1983; *O. maya*, Linares et al., 2015; *Octopus cyanea*, Boucher-Rodoni, 1973) and Nautiloids (e.g., *Nautilus pompilius*, Owen, 1832; Westermann and Schipp, 1998; Westermann et al., 2002) which is absent from Sepidae and Teuthoidea where the ingested food passes directly to the stomach (for reviews see: Bidder, 1966; Boucaud-Camou and Boucher-Rodoni, 1983). Studies on adult *O. vulgaris* (Andrews and Tansey, 1983) and *N. pompilius* (Westermann et al., 2002) provided evidence that the crop is adapted for food storage. Such storage function in *O. vulgaris* is facilitated by the thin muscular wall of the crop and by suppression of the contractile activity proposed to be cholinergic (Andrews and Tansey, 1983). *Post mortem* analysis of *O. vulgaris* led to the suggestion that the initial food storage (accommodation) during feeding occurred in the crop (Bidder, 1957; Young, 1960; Wells, 1978; Andrews and Tansey, 1983), then gradually moving toward the stomach where it would be triturated by the reciprocal movement of two thick apposed muscular blocks (Andrews and Tansey, 1983). Current observations on crop and stomach filling in paralarvae challenges the above classical view of the crop-stomach relationship. In the early ingestion phase of paralarvae (MP) the stomach had a higher volume than the crop (**Figure 4**) and its volume plateaued as ingestion proceeded (MP) while the crop volume continued to increase. At the end of feeding (LP, late phase) the crop volume was ∼10x its volume as measured at the end of the MP (when the stomach volume had already plateaued). These results show that although the crop is the location where the majority of ingested food is stored in paralarvae, the first food ingested proceeds straight to the stomach. Interestingly, by the time the stomach volume plateaued (MP, **Figure 4**) and the crop began to fill (phase transition MP to LP), the contractions of the stomach ceased, suggesting a coordinated activity between the crop and the stomach. The gastric ganglion is most likely responsible for such coordination as stimulation of the ganglion in adult octopus can simultaneously inhibit gastric contractions and enhance crop activity (Andrews and Tansey, 1983). Also, *in vitro* studies provide evidence that the contractile activity of crop and stomach of cephalopods is mediated by catecholamines (Bacq, 1934; Wood, 1969; Andrews and Tansey, 1983).

Since the time between prey contact and the end of ingestion did not differ within prey among individuals, a putative satiety signal could exist. One possibility is a sensitive buccal signal upon the full removal of prey content. However, a more plausible explanation is the distension of the crop as the triggering signal for feeding termination as suggested by Nixon (1966) in adult octopus. While digestive tract mechano- and chemo- receptive afferents signaling from the digestive tract to the brain are well established in vertebrates (e.g., Andrews, 1986; Olsson, 2011; Brookes et al., 2013), evidence of afferents from the crop and other regions of the digestive tract projecting to the brain is very limited in cephalopods (Young, 1965, 1971). The possibility that food ingestion could release gut hormones to act on the brain to terminate feeding, as occurs in vertebrates (e.g., Dockray, 2014; Volkoff, 2016) should not be overlooked.

There is no evidence of a sphincter placed between the crop and the stomach in adult *O. vulgaris*, so ingested food (fish or crab) can move in either direction depending on the digestive tract contractile activity (Andrews and Tansey, 1983). It has been proposed that crop and stomach should be regarded as a functional unit coordinated by the gastric ganglion. In such a model, the crop accommodates food and delivers it to the stomach where it is triturated by the muscle blocks and mixed with digestive secretions. Food can then be returned to the crop or delivered to the distal digestive tract, depending on its degree of digestion (Andrews and Tansey, 1983). The nature of the ingested food in paralarvae as compared to adult octopus could explain why such repeated cycling between crop and stomach proposed in adults may not apply in paralarvae, i.e., the higher food digestibility and the low amount (if any) of indigestible residuals.

The peristaltic contractions of the crop moved food to the stomach with periods of contractile activity interspersed with quiescence. The frequency of crop contractions was ∼18/min in paralarvae what is similar to the range of 10–20/min for small amplitude contractions recorded *in vitro* from longitudinal muscle in adult *O. vulgaris* (Andrews and Tansey, 1981). That *in vitro* study also recorded sustained (10–20 s) large amplitude contractions which were not obvious in the present study. Such lack of correspondence between studies regarding duration of contractions can be due to the shorter recording time of the current study. However, the faster passage of food throughout the digestive tract of paralarvae as compared to adults cannot be overlooked. Further recording of crop activity using food marked with fluorescent microspheres might facilitate understanding the relationship between the external appearance of the crop and the movement of its content. The stimulus for initiation of crop contractions is not known but *in vitro* studies show that distension would stimulate contractions in the crop (Andrews and Tansey, 1983). However, there must be coordination between the crop and the stomach which most likely operates via the innervation of the various gut regions from the gastric ganglion (Young, 1967), which is able to modulate both, stomach and crop motility (Andrews and Tansey, 1983).

The contraction frequency of the full stomach was ∼30/min when full, i.e., when food began moving from the stomach to the caecum. Such a frequency is approximately twice the one recorded *in vitro* in adult octopus (Andrews and Tansey, 1983). Subsequently, pigmented food material was observed to move from the caecum to the digestive gland via the hepatopancreatic duct. Although, we were unable to track specific food particles during transit from the crop to the stomach, pigmented particles were seen to enter the digestive gland ∼5 min after the crop filled. Therefore, the total time between food ingestion until it enters the digestive gland in paralarvae is in the range of minutes as opposed to hours in adult octopus (Bidder, 1957, 1966; Boucaud-Camou et al., 1976; Linares et al., 2015).

The terminal part of the intestine showed rhythmic contractile activity during all phases (even during ineffective attacks). An intestine contraction frequency of ∼50/min is high for a tissue assumed to be composed of smooth muscle (no data available in paralarvae) and is well above the range of ∼12

to ∼18/min recorded *in vitro* in the rectum and intestine of adults (Andrews and Tansey, 1983). Defaecation was a rare event, since it was only recorded in 4 animals out of 34 (∼12%) but the string-like appearance of feces was similar to that reported in adult *O. vulgaris* (Bidder, 1957). Defaecation in adult *O. vulgaris* is accompanied by changes in respiration (Wells, 1978) and was accompanied by an increase in the frequency of mantle contractions in paralarvae. Such activity suggests the involvement of central nervous system coordination between the distal digestive tract (regulated by the atriorectal nerve from the palliovisceral brain lobe (Young, 1967, 1971) and the somatomotor system of the mantle muscle (Wells, 1978).

CONCLUSIONS

This paper originally provides a detailed quantification of the attack and ingestion of a range of live prey by *O. vulgaris* paralarvae at a very early post-hatching stage. The first two goals show that effective attacks targeted vulnerable regions of the prey and that dynamics of buccal mass, radula movements, ingestion time and mantle contraction, suggest that paralarvae receive feedback from the prey exoskeleton and its inner content. The third goal indicates that the process of ingestion and transfer to the digestive gland for assimilation is faster than in adults. These results establish the utility of high-resolution video recording of paralarvae as a real-time method for studying the motility of the digestive tract (although limited by relatively short recording times and paralarvae orientation) and suggest that the dogma about crop-stomach relationships in adults may need reconsideration. As paralarvae remain transparent until settlement, this method should enable tracking the full maturation of digestive tract function.

This study provides a new perspective on feeding strategies that could be adopted in octopus aquaculture, where octopus paralarvae survival remains an issue. Therefore, three dph paralarvae would need to consume tenfold more copepods as compared to zoeae in order to obtain a food equivalent. It is critical that paralarvae are fed the adequate live prey, which they can drill and ingest better than *a priori* appealing Crangonidae or Gammaridae species equipped with inaccessible exoskeletons. The characterization of digestive tract function described here (e.g., ingestion rate, motility, time for food entry into the digestive gland, and defaecation) permits the assessment of the digestibility of different prey types to improve paralarvae growth and survival.

AUTHOR CONTRIBUTIONS

MN, MP, and PP worked out the conception, experimental design and execution of this study. All the authors (MN, PP, AR, PA, and MP) contributed significantly to achieve this publication, e.g., discussed the results and implications, commented on the manuscript at all stages and finally approved its submission for publication in Frontiers of Physiology in the Research Topic "The Digestive Tract of Cephalopods: at the Interface Between Physiology and Ecology."

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00573/full#supplementary-material

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3D Reconstruction of the Digestive System in *Octopus vulgaris* Cuvier, 1797 Embryos and Paralarvae during the First Month of Life

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Octopus vulgaris aquaculture is limited due to poor biological knowledge of the paralarval stages (e.g., digestive system functionality), their nutritional requirements (e.g., adequate live diet) and standardization of rearing techniques. These factors are important in explaining the high mortality rate observed in this developmental stage under culture conditions. For a better understanding of nutrition biology of this species, we investigated the 3D microanatomy of the digestive tract of the embryo and paralarvae during the first month of life. *O. vulgaris* paralarvae digestive system is similar to that in the adult. The "descending branch" has a dorsal position and is formed by the buccal mass, oesophagus and crop. Ventrally, the "ascending branch" is formed by the intestine and the anus. The digestive gland, the posterior salivary glands and the inner yolk sac (in the case of the embryo and hatched paralarvae) are located between the "ascending" and "descending" branches. In the curve of the U-shaped digestive tract, a caecum and the stomach can be found. The reconstructions reveal that anatomically the digestive system is already complete when the paralarvae hatch. The reconstruction of the buccal mass at different post-hatching days has demonstrated that all the necessary structures for food intake are present. However, the radula surface in contact with the pharynx is very small on the first day of life. Although the digestive system has all the structures to feed, the digestive gland and radula take longer to reach full functionality. We have established four development periods: embryonic, early post-hatching, late post-hatching and juvenile-adult. The differentiation between these periods was done by type of feeding (endogenous or exogenous), the state of maturation and hence functionality of the digestive gland, type of growth (linear, no net, or exponential), and measurement of the arm lengths with respect to the mantle length. 3D reconstruction represents a new tool to study the morphology and functionality of the cephalopod digestive system during the first days of life.

Keywords: ***Octopus vulgaris*, paralarvae, ontogeny, digestive system, 3D**

INTRODUCTION

Octopus vulgaris aquaculture is limited due to a number of factors including: poor physiological and biological knowledge of the paralarval stages, their nutritional requirements, a live diet with adequate composition, and the standardization of rearing techniques (Moxica et al., 2002; Iglesias et al., 2007; Iglesias and Fuentes, 2014). Despite determined research efforts (Itami et al., 1963; Iglesias et al., 2004; Iglesias and Fuentes, 2014) octopus aquaculture continues to have high mortality during the first month of life. Recent studies have focused on understanding the nutritional requirements in the paralarval stage (Villanueva et al., 2002; Iglesias et al., 2006; Seixas et al., 2008; Reis et al., 2015). However, little research has been undertaken to standardize aquaculture techniques (Moxica et al., 2002; Reis et al., 2015; Iglesias et al., 2004, 2007; Domingues et al., 2010; Fuentes et al., 2011) or to learn about the biology of this developmental stage (Roura, 2013).

Major changes in shape and morphology of the organs occur in the larval stage of development. In teleost ontogeny, these changes in the alimentary tract occur in both morphology and function (Dabrowsky, 1986). This can be observed using methods that detect both, morphological and functional changes. Most larvae have a simple or still undifferentiated digestive tract in the first days of life (Govoni et al., 1986). Changes can include the opening of the mouth, increase in the relative length of the intestine and oesophagus, the formation of the stomach, development of intestinal mucosal foldings and changes in protein digestion or enzymatic activity in general (Porter and Theilacker, 1999; Cahu et al., 2004; Mangetti, 2006). Comparative studies of digestive system development have been performed in a number of fish species including *Solea solea* (Boulhic and Gabaudan, 1992), *Dicentrarchus labrax* (Beccaria et al., 1991), *Dentex dentex* (Crespo et al., 1992), *Pagrus pagrus*, and *Diplodus sargus* (Darias, 2005).

Previous studies of *O. vulgaris* embryonic structures show the organogenesis of the gonad, hepatopancreas and circulatory systems (Naef, 1928; Boletzky, 1967, 1968; Marthy, 1968). Also, general and comparative aspects of the embryonic development have been reported by Boletzky (1969, 1971). Several authors (cited by Boletzky, 1978) have also studied the embryonic digestive system. However, these previous studies focused on the origin and formation of the embryonic structures but do not show the changes occurring between embryonic and paralarval stages. In addition, the digestive system functionality of cephalopods in paralarval stages is poorly known. There are only a few studies concerning the development of the digestive system in cephalopod hatchlings (Moguel et al., 2010; Martinez et al., 2011; López-Peraza et al., 2014). The nutritional requirements in the larval stages are critical in aquaculture (Mangetti, 2006). A comparative study of the embryonic and paralarval developmental stages provides new insights into digestive system morphology that also may facilitate physiological studies of these stages. If the digestive system is underdeveloped or does not possess all the structures capable of digesting the food, its absorption capacity will be limited. This will allow us to better identify the food requirements at the most critical stage of the culture to reduce mortality and to find a specific diet and nutritional protocol for this phase.

The present study describes the anatomical changes in the digestive system of *O. vulgaris* embryos and paralarvae at different phases of its ontogenetic development during the first month of life. This study represents the first 3D reconstruction of octopus paralarvae. In addition, this study describes a new tool to study the functional morphology of cephalopod organs during their first days of life. These data provide an explanation for the adaptation to the use of exogenous food and provide useful information to facilitate aquaculture of this species.

MATERIALS AND METHODS

Paralarvae Rearing

Octopus paralarvae were obtained from the Ría de Vigo on 14th and 22nd August 2013, through the collection of egg strings. These were transported to the ECIMAT (REGA ES360570181401) in tanks with seawater and then placed in 150L tanks with running seawater in the dark until hatching occured. The hatching paralarvae were observed in the laboratory to exclude the selection of premature paralarvae. Subsequent paralarvae rearing was conducted in two cylindrical tanks (150 L) with dark walls. The temperature of the culture was 21°C (Hamasaki and Morioka, 2002), the period of illumination was 12 h, with one water renovation per day and moderate aeration. A total of 1350 paralarvae were reared in each tank. The diet was supplied *ad libitum* each 2 days. This consisted of *Artemia* sp. enriched with *T-iso* and *Rhodomonas lens* over 3 days before they were used. All procedures involving in this study were carried out under Directive 2010/63/EU, in accordance with the recommendations of Bioethics Committee of Santiago de Compostela University (RD. 53/2013 12th of February) and Ethics Committee of Animal Experimentation of Vigo University (Protocol number 10/2013 2nd December).

Photography and Morphometry

We took samples of the eggs in the stages X, XIII, XIX (Naef, 1928) and paralarvae at 5, 7, 9, 12, 15, 20, 29, and 35 post-hatching days. The paralarvae were anesthetized with ethanol prior to photographing to prevent stress suffering during the processing method (Rocha et al., 2015). Each paralarva was extracted individually by siphoning and then it was deposited in a Petri dish with 10 ml of seawater. Subsequently, 96% ethanol drops were added to the seawater to reach a concentration of 1%. No ethanol was added directly on the paralarvae. The signs to consider the individual anesthetized were a cessation of swimming, decreased activity of chromatophores, lack of arm movement, decreased cardiac activity, and relaxation of the musculature of arms and mantle (Gleadall, 2013). Each specimen was maintained anesthetized 5 min to take photos and measures. No toxic effects or indicators of stress were observed during anesthesia, such as ink release, skin and eye irritation, abnormal changes in color and texture of the skin or contraction in body musculature (Andrews et al., 2013; Gleadall, 2013). Image acquisition was performed using a binocular loupe Nikon SMZ 1500. Pre-fixation death was achieved by slowly increasing the

ethanol concentration to 10% when the heartbeat ceased. Total length (TL), dorsal mantle length (DML), arm length (AL), and the number of suckers were measured for paralarval stages.

Semithin Section Series

For the 3D reconstructions, we selected a single specimen of the embryonic stages X, XIII and for 0, 5, 10, 20, and 35 post-hatching days each. The samples were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer for several days. Post-fixation was made in osmium tetroxide (1% in cacodylate buffer) and after dehydration, in a graded acetone series, the samples were embedded in epoxy resin following standard protocols. Semithin section series (1.5 μm) were made with an ultramicrotome (RMC MT 7000) with a diamond knife according to the protocol established by Ruthensteiner (2008). The sections were mounted on glass slides and stained with Richardson blue (**Figure 1A**). Photographs of the complete microscope slides were obtained with an automated Olympus BX61VS light microscope (20x objective) equipped with an Olympus IX2-FCB digital camera and dotSlide system. Using Olympus OlyVIA software (Leadtools, USA) a snapshot of every fourth slice was taken for further digital imaging.

Computer Reconstruction and Volumetry

The images were pre-processed in Photoshop 6.0 (San Jose, USA) to change the color to gray-scale and to enhance contrast followed by unsharp masking. The 3D processing was performed with Amira software (FEI, Germany) following the steps described by Ruthensteiner (2008). In brief: for the selection of the different organs and structures, we used the *Segmentation Editor* (**Figure 1B**). Selections, i.e. tracing the cutting profiles of each histological structure considered, were made manually in every image plane. We used the *SurfaceGen* module to generate smooth 3D surface models (**Figure 1C**) of these structures by its implemented triangulation algorithm. Images of the 3D models in preferred perspectives and organ reconstructions were obtained using the *Snapshot* tool (see also Laforsch et al., 2012). Volume measurements of organs were made with the *Measurements* module (counting all voxels of each structure and multiply the value by the single voxel volume). We calculated the relative volume of each structure of interest in relation to the "total volume," i.e., the volume of the animal body and external yolk for embryo and from the dorsal tip of the end of the external lips for paralarvae.

Statistical Analysis

All results are presented as mean ± SD using Microsoft Excel (Microsoft, USA). The total length, the relative and total volume of the digestive system structures curves at different ages were interpolated between the measured values using the graph function in Microsoft Excel (Microsoft, USA). As point trend adjustment measures have been used for the coefficient of correlation, values close to 1 indicate a reliable trend of data.

RESULTS

The morphometric changes of *O. vulgaris* paralarvae show an exponential growth curve from the fifth post-hatching day (**Figure 2**). The total length (**Table 1**) at the fifth post-hatching day was 2.99 ± 0.23 mm, the dorsal mantle length was 2.08 ± 0.15 mm and the arms present a mean length of 0.625 ± 0.066 mm with 3 suckers. At the end of the rearing phase (35 days post hatching), the mean total length of the paralarvae was 5.58 ± 0.67 mm, the mean dorsal mantle length was 3.56 ± 0.37 mm and the arm length was 1.44 ± 0.18 mm with 7 functional suckers and 3 primordial suckers.

The **embryo** reconstructions (**Figures 3A,B**) show different yolk absorption periods. Stage X of development (**Figure 3A**) shows outer and inner yolk: while the outer yolk represents 89% of the total animal volume, the inner yolk contributes only 5%. The connection between these two structures is wide, the only difference in these structures is their position inside

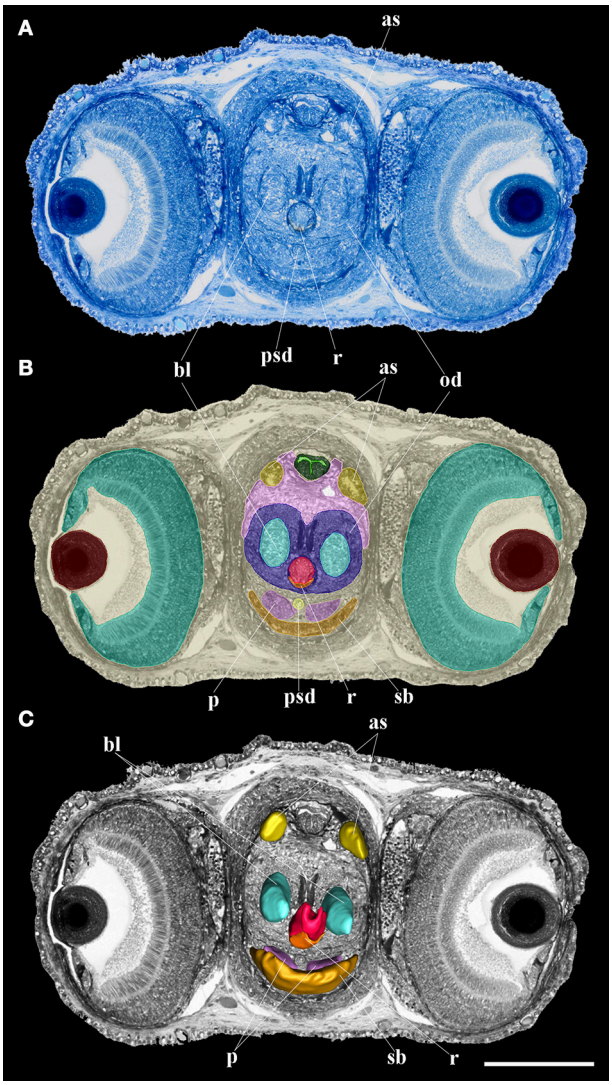

FIGURE 1 | Oral bulb: from histology to reconstruction. **(A)** Semithin section stained in Richardson blue; **(B)** Segmentation of the different oral bulb organs and structures; **(C)** 3D reconstruction of the different oral bulb organs and structures. as, anterior salivary glands; bl, bolster; od, odontophore; p, salivary papilla; psd, posterior salivary duct; r, radula; sb, submandibular gland. Scale bar 200 μm.

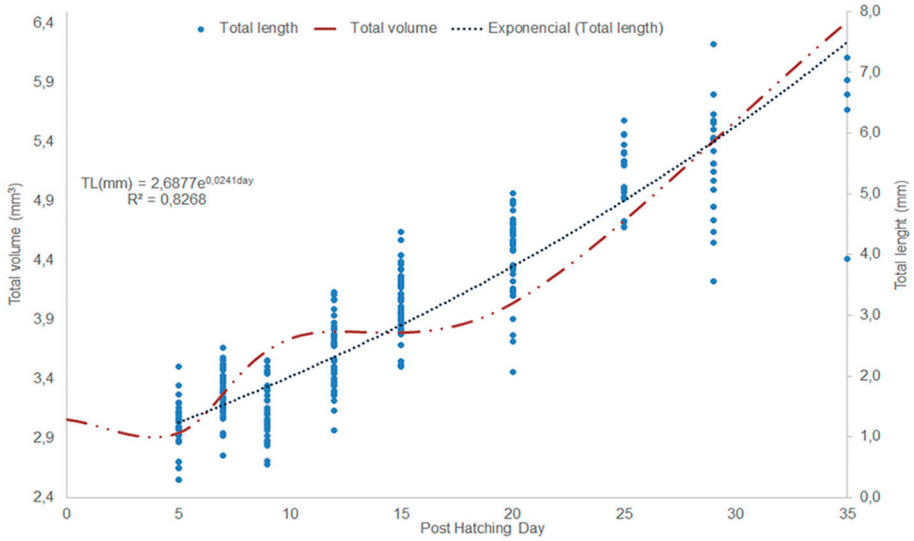

FIGURE 2 | Changes in *Octopus vulgaris* paralarvae total length (mm) and total volume during the first 35 days of life. The dots represent an animal total length measurement.

TABLE 1 | The growth of *O. vulgaris* paralarvae from 5–35 post hatching days.

Stage (age, sample size)	Total length (mm)	Dorsal mantle length (mm)	Mean arm length (mm)	Relation DML/AL	Suckers
(5 day, $N = 30$)	2.99 ± 0.23	2.08 ± 0.15	0.625 ± 0.066	3.328	3
(7 day, $N = 40$)	3.29 ± 0.19	2.21 ± 0.14	0.657 ± 0.083	3.364	3
(9 day, $N = 40$)	3.12 ± 0.24	2.22 ± 0.19	0.523 ± 0.098	4.245	3
(12 day, $N = 40$)	3.60 ± 0.29	2.59 ± 0.19	0.706 ± 0.101	3.669	4
(15 day, $N = 40$)	4.03 ± 0.21	2.78 ± 0.21	0.789 ± 0.104	3.523	5
(20 day, $N = 40$)	4.43 ± 0.22	3.04 ± 0.22	0.912 ± 0.135	3.333	5
(25 day, $N = 20$)	5.10 ± 0.28	3.41 ± 0.22	1.129 ± 0.163	3.020	6
(29 day, $N = 20$)	5.22 ± 0.47	3.40 ± 0.25	1.278 ± 0.213	2.660	7
(35 day, $N = 5$)	5.58 ± 0.67	3.56 ± 0.37	1.44 ± 0.180	2.472	7

or outside the embryo. The inner yolk is not bilobulated. The reconstructions of this stage show the second absorption period according to Portmann and Bidder (1929). In this stage, it is possible to see the brain primordium which is surrounded by the anterior part of inner yolk. The XIII Naef's stage reconstruction (**Figure 3A**) represents the third yolk absorption period. The yolk is represented by inner (1% of total body volume) and outer yolk (82%). The connection between them is made by a narrow neck and the inner yolk has a bilobulated shape. This structure is not in connection with any part of digestive primordium. In the anterior region, it is limited by the brain, eyes and statocysts. At the posterior region, it is limited by the still undifferentiated digestive tract. At this stage, the onset of oral bulb differentiation is observed (**Figures 3B**, **4a**). This consists of the following structures: the outer lips, the primordia of the beak, salivary papilla, and odontophore. The reconstruction of embryo stage XIX (not shown) represents the last stage of the third period of yolk absorption: In this stage, the yolk is only represented by the inner yolk. The digestive system is similar to day 0 (i.e., just hatched) paralarvae (**Figures 5A,B**).

The digestive system of *O. vulgaris* **paralarvae at hatching** already has a structure like that of the adult (**Figures 5A,B**). The "descending branch" has a dorsal position and it goes from the anterior to the posterior region of the paralarva. It is formed by the buccal mass, oesophagus, and crop. The "ascending branch," which is situated ventrally and runs from the posterior to the anterior region of the paralarva, is formed by the intestine and the anus. Between both branches, the digestive gland, the posterior salivary glands and the inner yolk are located. The latter structure is not connected with the digestive gland. In the curve of the U, the caecum and the stomach can be found.

The oral bulb or buccal mass of this stage already has all the structures necessary for food intake (**Figures 4b,e**), although some of them are not yet fully developed. The mouth is completely open to the outside, showing no membrane that would block the entry of food. The center is occupied by the salivary papilla and the radula. All these structures are

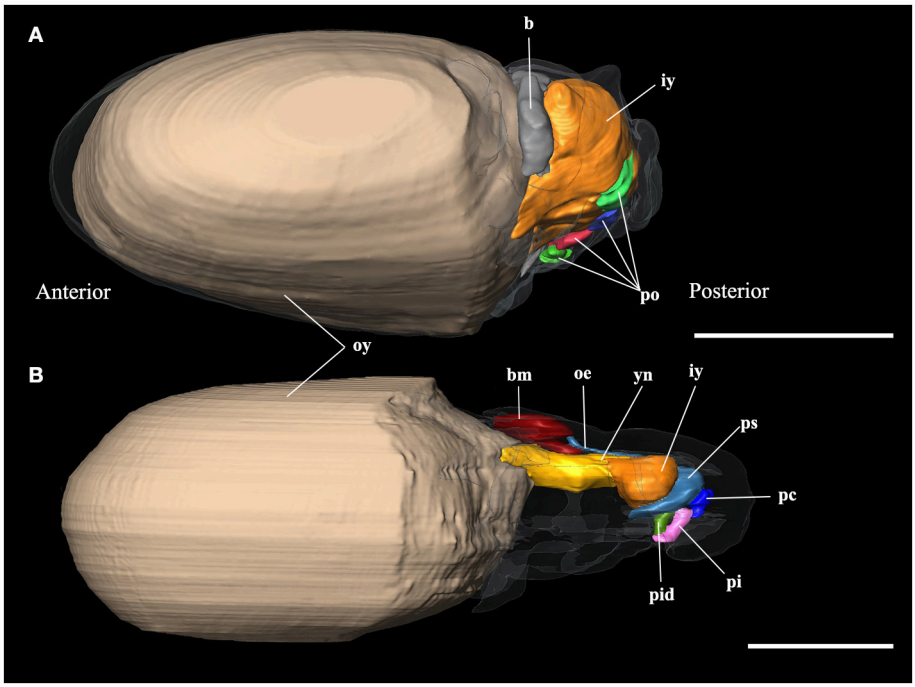

FIGURE 3 | 3D reconstructions of the digestive system of embryo stages of *Octopus vulgaris*. **(A)** Stage X of Naef (1928); **(B)** Stage XIII of Naef (1928); b, brain; bm, primordium of the buccal mass; iy, inner yolk; oe, oesophagus; oy, outer yolk; pc, primordium of the caecum; pi, primordium of the intestine; pid, ink duct and ink sac primordium; po, promordia of different organs; ps; posterior salivary glands; yn, yolk neck. Scale bars 500 μm.

surrounded by the beak. The radula surface in contact with the pharynx is very small on the first day of life (see **Table 3**). The salivary glands, anterior and posterior, are connected to the buccal mass. The latter structure is connected with the stomach by the oesophagus. It is a narrow tube which, at the level of the posterior salivary glands, gets wider posteriorly and finally opens into the crop (**Figure 5A**). The stomach, caecum and the intestine are connected through the vestibule. The intestine is a narrow uncoiled tube that starts in the vestibule, and at the end of it, two papillae and the opening of the ink duct are present (**Figure 5B**). This structure begins in the dorsal region of the animal and ends in the ventral region after turning back on itself. From the first day, all the major glands are present. The digestive gland is the largest gland of the digestive system with a volume of 4.4% of total body volume. This gland has two defined regions: the glandular region and the digestive appendages in the posterior region. It connects to the caecum by two ducts which unify before entering it (**Figure 5B**). The posterior salivary glands are the next smallest glands with a volume fraction of 0.67%, located dorsally in the digestive gland. These glands are connected to the oral bulb through the salivary ducts. These ducts merge and have a common opening near the entrance of the mouth (**Figure 4B**). The other two glands present in the digestive system are the anterior salivary glands and submandibular gland (**Figures 4b, 5A**), while the first pair is externally to the buccal mass the submandibular gland is embedded in the buccal mass.

Paralarvae at 5 and 35 post-hatching days have an even more elongated body shape (**Figures 5C,D**) and display an increasing complexity of inner organs (**Table 2**). However, the internal yolk is no longer present at the 5 post-hatching days. Oral bulb reconstruction at different ontogenetic phases (**Figure 4**) demonstrates that at hatching day the radula (**Figures 4b,e**) is present but represented by a small undeveloped structure. The volume, contact surface with pharynx and the proliferation of teeth increase with age. This has a considerable growth increment from the first day of life until the 5th day (**Table 2**). The 3D reconstruction allows us to see the differentiation of this structure with age (**Figures 4c,d,f,g**). The intestine remains uncoiled in the examined developmental stages at least until posthatching day 35. At day 35 the radula structure is similar to that of the adult.

The creation of 3D models of the digestive system at different stages of paralarvae development (**Figure 4**) allowed us to measure total volumes (**Table 2**) and to observe significant differences in the relative volumes of the internal structures (**Table 3**). All considered structures have a positive allometry (**Figure 6**). The structures that have a higher growth in the first month of life are the digestive gland, posterior salivary glands (**Figure 6A**) and the radula surface, which is in contact with the pharynx (**Figures 4e–g**). The major increment in the volume of the glands occurs from 10 to 35 post-hatching days. The relative volumes of these structures show the same trend (**Figure 6B**). However, the total volume of the organs (**Figure 6C**) show two different growth periods. The greater increments occur from 5 to 10 days and from 20 to 35 post hatchings days. The relative volumes of these structures show that the development of these structures does not have the same trend. In the case of the

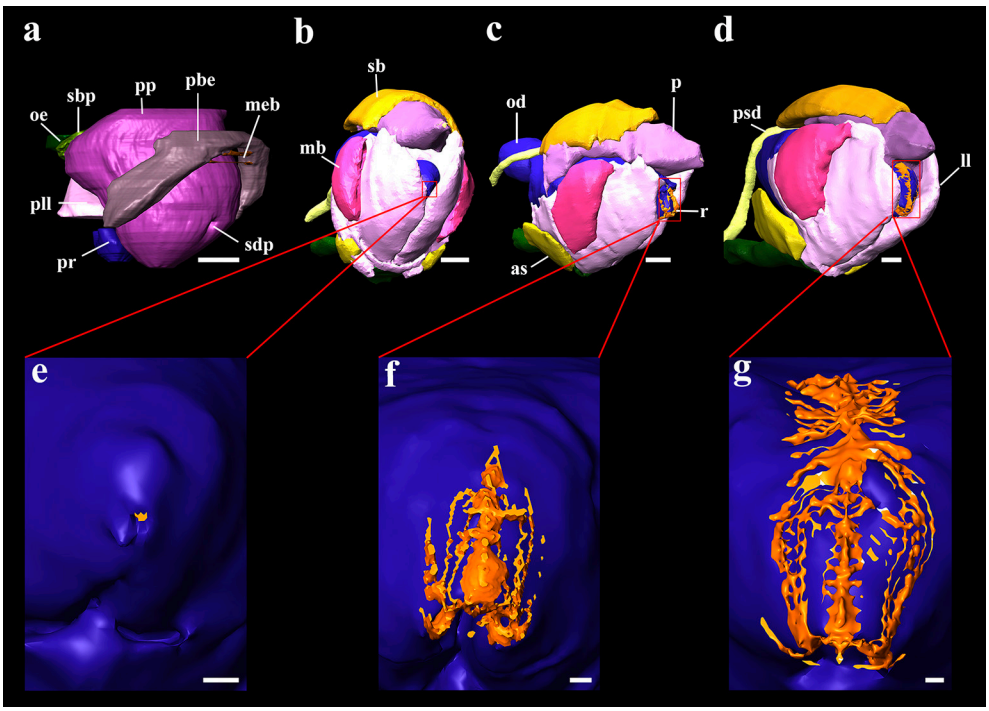

FIGURE 4 | 3D reconstructions of the buccal mass and odontophores of *Octopus vulgaris* embryos and paralarvae at different development stages. **(a)** Primordium of buccal mass at stage XIII of Naef (1928); **(b)** buccal mass of the hatching paralarva; **(c)** buccal mass of 5 days post hatching paralarva; **(d)** buccal mass of 20 days post hatching paralarva; **(e)** odontophore and surface of the radula in contact to pharynx in the hatching paralarva; **(f)** odontophore and surface of the radula in contact to pharynx in 5 days post hatching paralarva; **(g)** odontophore and surface of the radula in contact to pharynx in 20 days post hatching paralarva. as, anterior salivary glands; ll, lateral lobes; mb, muscles of beaks; meb, membrane of beaks; od, odontophore; oe, oesophagus; p, salivary papilla; pbe, primordium of beaks; pll, primordium of lateral lobes; pp, salivary papilla primordium; pr, odontophore primordium; psd, posterior salivary duct; r, radula; sb, submandibular gland; sbp, submandibular gland primordium; sdp, salivary duct primordium. Scale bars (**a–d**) 50 μm; Scale bar (e-g) 10 μm.

oesophagus and intestine, the volumes increase from the first day of life until the end of the rearing period (35 days) in the present study. However, the caecum and stomach volume do not show t a clear trend (**Figure 6D**). The paralarvae total volume (**Figure 2**) shows a no net growth phase (Vidal et al., 2002) from 0 to 5 post-hatching days. After this time, the total volume of paralarvae shows two different growth periods. The first from 5 to 12 post-hatching days, and the second from day 15 to the end of the rearing.

DISCUSSION

Octopus vulgaris completes embryonic development as a planktonic stage called paralarva (Young and Harman, 1988). During this post-hatching stage, the transition between endogenous and the exogenous feeding occurs, which is considered as a critical period characterized by high mortality (Iglesias and Fuentes, 2014). This study provides the first visualization of the anatomical ontogeny of the digestive system from embryonic stage X to the 35 days post hatching paralarvae. This was performed using 3D reconstruction of the structures that make up the digestive system at different stages.

Boucaud-Camou and Boucher-Rodoni (1983) established three developmental phases in cephalopods called: embryonic (1), post-hatching (2) and juvenile-adult (3). The differentiation between these 3 phases lies in the type of feeding (endogenous or exogenous) and the state of maturation and enzymatic activity of the digestive gland. Moguel et al. (2010) have added more features to differentiate these phases including the growth pattern, the length of the arms relative to the length of the mantle and the response to prey. The embryonic stage (1) is characterized by an intracellular digestion of the yolk, linear growth, and weak activity of digestive gland. The post-hatching phase (2) starts with exogenous feeding, in which the size of the digestive gland increases and its enzymatic activity is initiated (Villanueva et al., 2002). Despite the beginning of enzymatic activity, this gland is still relatively immature and erratic function of enzymes occurs (Villanueva et al., 2002; Moguel et al., 2010), with lapses of enzyme synthesis in the digestive gland cells. The inner yolk is still present; this implies both exogenous and endogenous feeding occur at the same time. This phase has a no net growth (Vidal et al., 2002) and the arms are shorter than the mantle thus limiting the ability to capture prey. In the last phase, juvenile-adult (3), the digestive gland appears fully developed with stable enzyme activity (Villanueva et al., 2002). In this phase, the growth is exponential and arms get longer than the mantle. These phases have been identified for cephalopods species with direct development, in which the embryo hatches

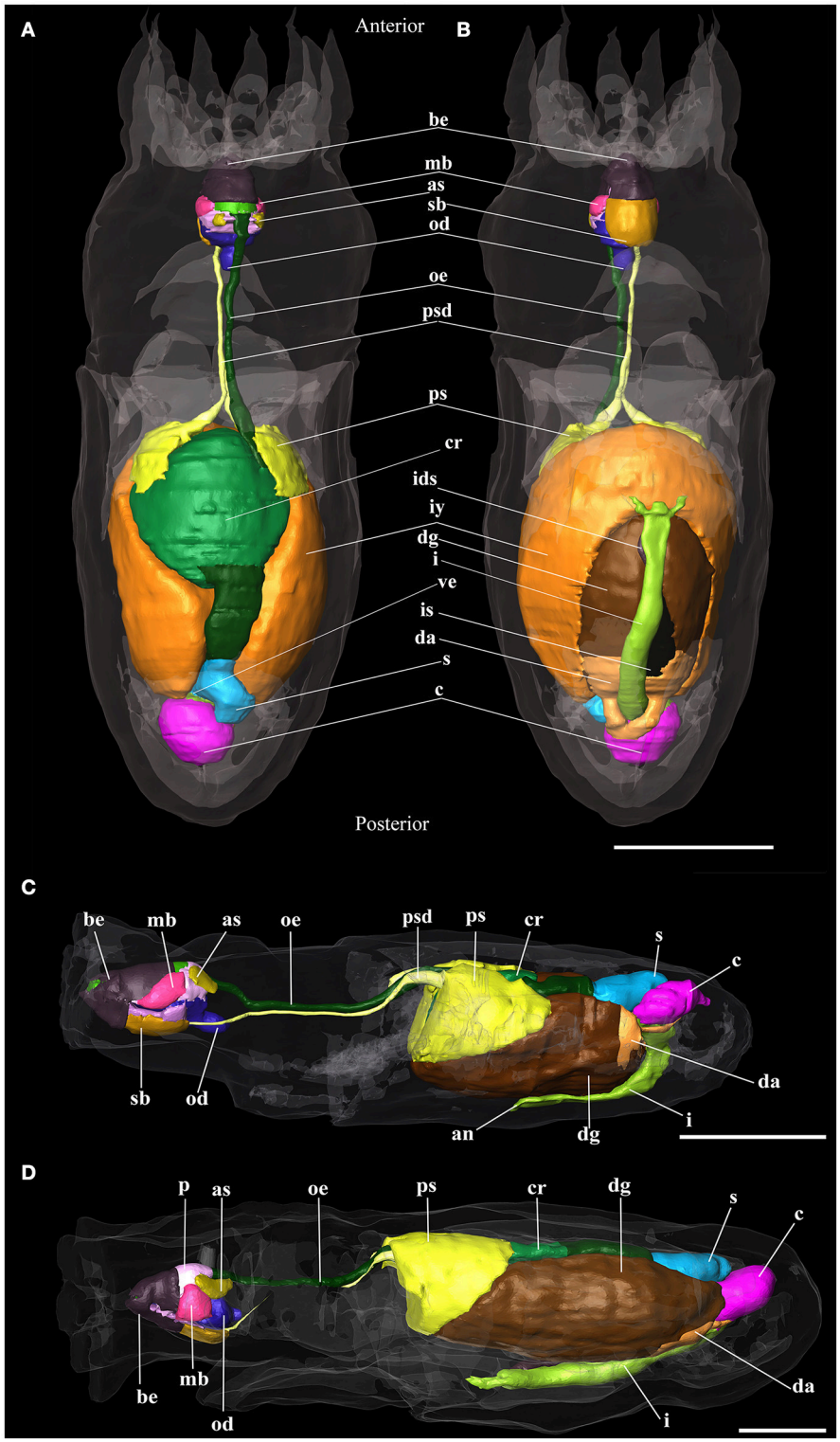

FIGURE 5 | 3D reconstructions of paralarva digestive system of *Octopus vulgaris*. **(A)** Dorsal view and **(B)** Ventral view of hatching paralarvae; **(C)** 5 days old paralarva reconstruction; **(D)** 30 days old paralarva reconstruction. an, anus; as, anterior salivary glands; be, beaks; c, caecum; cr, crop; da, digestive appendages; dg, digestive gland; i, intestine; ids, ink sac duct; is, ink sac; iy, internal yolk; mb, muscles of beaks; od, odontophore; oe, oesophagus; p, salivary papilla; ps, posterior salivary glands; psd, posterior salivary gland duct; s, stomach; sb, submandibular gland; ve, vestibule. Scale bars **(A,B)** 100 μm; Scale bars **(C,D)** 500 μm.

TABLE 2 | Absolute volume (mm^3) of the digestive system structures and organs and total body volume from the end of the mantle until the end of the external lips.

Structures \ DPH	0	5	10	20	35
Anterior salivary glands	0.0001	0.0006	0.0010	0.0017	0.0043
Posterior salivary glands	0.0089	0.0255	0.0532	0.0645	0.1333
Submandibular gland	0.0007	0.0013	0.0020	0.0036	0.0069
Digestive gland	0.0567	0.1140	0.2846	0.4390	0.7469
Oesophagus	0.0037	0.0030	0.0090	0.0158	0.0285
Stomach	0.0033	0.0051	0.0088	0.0141	0.0400
Caecum	0.0032	0.0034	0.0107	0.0100	0.0279
Crop	0.0160	0.0039	0.0076	0.0103	0.0504
Intestine	0.0033	0.0038	0.0107	0.0160	0.0436
Odontophore	0.0045	0.0068	0.0131	0.0156	0.0327
Radula	0.0001	0.0001	0.0002	0.0002	Broke
Total volume	1.2805	1.0574	2.6110	3.1997	7.8395

DPH, days post hatching.

TABLE 3 | Relative volume (%) of the digestive system structures and organs.

Structures \ DPH	0	5	10	20	35
Anterior salivary glands	0.012	0.059	0.039	0.054	0.056
Posterior salivary glands	0.695	2.413	2.038	2.015	1.700
Submandibular gland	0.055	0.120	0.075	0.112	0.087
Digestive gland	4.427	10.778	10.920	13.720	9.527
Oesophagus	0.288	0.288	0.346	0.495	0.364
Stomach	0.256	0.483	0.338	0.442	0.511
Caecum	0.248	0.319	0.410	0.312	0.356
Crop	1.248	0.369	0.290	0.323	0.642
Intestine	0.256	0.361	0.410	0.500	0.556
Odontophore	0.355	0.640	0.504	0.487	0.418
Radula	0.008	0.014	0.008	0.007	Broke
Radula in contact with pharynx	0.728	34.651	42.86	35.804	Broke

DPH, days post hatching. The relative volume is calculated considering the total volume from the end of the mantle until the end of the external lips. The "radula in contact with the pharynx" relative to the absolute radula volume.

with characteristics very similar to the adults, as *O. maya* (Moguel et al., 2010).

Anatomical and Morphological Changes

The embryonic development stages described here coincide with those described previously by Naef (1928) for *O. vulgaris*, based on the visible anatomical development of structures through the chorion. However, the development of the 3D technique gives much more information about the development of this species. These types of 3D model allow simultaneous analysis of both external structures and internal structures. This allows inferences to be made about development and physiology.

Our results confirm that in *O. vulgaris* the embryonic phase finishes when the animal hatches for nutritive reasons (represented here by the reconstruction of post-hatching day 0 paralarvae). At day 0 paralarvae have the capacity to start exogenous feeding although the paralarvae carry out very few attacks maybe due to the fact that they are not physiologically ready to start their exogenous feeding (Iglesias et al., 2006). Yet, in the first days of life, the paralarvae no longer depend on the yolk resources only, rather they progressively change to a mixed feeding depending on both, the yolk and exogenous sources. In this phase, we can see the inner yolk, an open mouth, small radula (radula surface in contact with the pharynx only 0.73% of the total radula surface), and small digestive gland. Other important structures for prey capture are the arms. At hatching day, they are shorter than the mantle and have only 3 suckers. Although food is available, the animal may have a limited ability to capture it because the arms are relatively undeveloped. Overall, these features could reduce the capacity of the paralarvae to capture and ingest prey.

The duration of this phase is directly related to the incubation and rearing temperature of the paralarvae. Vidal et al. (2005) showed in squid that higher temperatures increase metabolic rates. In our case (21°C) this early post-hatching phase extends from the first day of life to the fifth rearing day. At that day, the inner yolk is depleted, hence its volume is zero, and the paralarvae can only exploit the resource of the exogenous food. Besides, the combination of total length and the total volume of the paralarvae allows us to differentiate the no net growth phase. For all these characteristics, we refer to the "early post-hatching phase" as the period from hatching to 5 days post hatching at 21°C.

We conclude that a late post-hatching phase occurs. In this phase, paralarvae have characteristics intermediate between the early post-hatching and juvenile-adult phases. This is characterized by exogenous feeding, an exponential growth, arms still shorter than mantle length, and increase in the digestive gland size. In this phase, the organism becomes better prepared for effective autonomous feeding (catching success, prey size). The capacity of paralarvae to feed exclusively on exogenous food depends on the ability to find and capture prey (Moxica et al., 2002). The reconstruction of the buccal mass shows that all the necessary structures to actively feed are present. At the fifth day post hatching the radula surface, in contact with the pharynx, increased considerably (34.62%). This enables the animal to more efficiently consume prey. The activity of proteolytic enzymes, however, may be still somewhat limited (see below). In order to define the end of this phase and the starting point of the juvenile phase, it will be necessary to carry out a more detailed histological and histochemical study of the digestive gland to establish when it is completely developed. However, better exogenous food adaptation is demonstrated by the positive allometry of the different structures and total length.

In conclusion, the present study shows that the first 20 days of life in *O. vulgaris* are a transition period until juvenile life starts. In this time of transition, the animal will adapt to the new environmental conditions in which it will live, and we propose four developmental phases in *O. vulgaris*: embryonic, early post-hatching, late post-hatching and juvenile-adult phase. A critical period is represented by the transition between the early and late post-hatching phase.

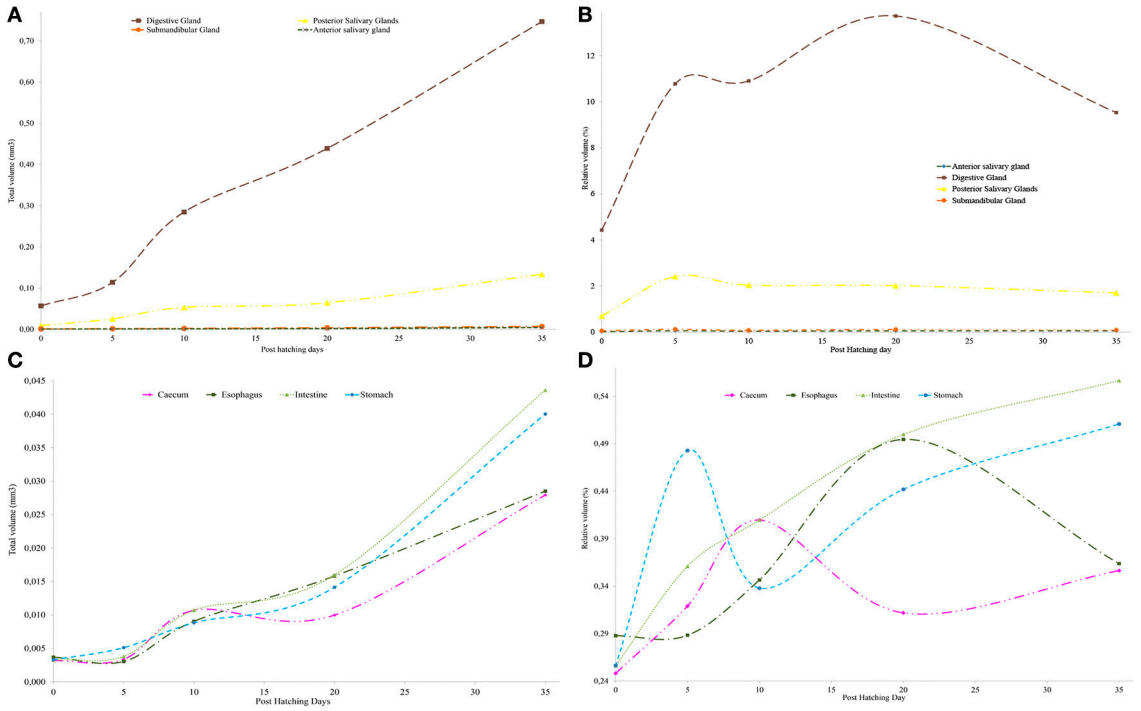

FIGURE 6 | Changes in relative (%) and total volume (mm^3) of the different digestive system organs. **(A)** Changes in glands absolute volume; **(B)** changes in glands relative volume **(C)** changes in the absolute volume of organs. **(D)** Changes in the relative volume of organs.

Potential Physiological Implications

The morphological findings described and discussed above, e.g., changes in the absolute size and relative volumes of all parts of the digestive system of *O. vulgaris* paralarvae in combination with the changing possibilities to use endogenous and/or exogenous food forebode changes in, or maturation of, physiological processes during development. These physiological processes, of course, have to be proven in future physiological experiments.

Early Post-hatching Phase

Although the internal yolk is in contact with the digestive gland in just hatched *O. vulgaris* paralarvae, no specialized structure connects these two organs. Portmann and Bidder (1929) established that in the hatching *Loligo* sp. paralarvae, called period III, the yolk nutrients passage is directly to the digestive gland, however, Boletzky (1975) stated that the passage of nutrients from the yolk syncytium directly into the digestive gland is inconceivable and the nutrient absorption is through the posterior sinus. The hatched paralarvae 3D reconstruction presented here cannot confirm Boletzky (1975) or Portmann and Bidder (1929) theory about the yolk absorption. Anyway, in a future study, it has to be clarified when exactly (between D0 and D5) the internal yolk is depleted and how the anatomical relation to the posterior sinus changes.

Late Post-hatching Phase

In this phase, the fast growth of the digestive gland points to the still immature activity of this gland (Villanueva et al., 2002). The transition between this phase and the juvenile phase should be investigated to look for a mature tubule structure and the presence of boules in the cells (Bidder, 1966). These digestive gland structures could indicate maturation of digestive gland function. In other cephalopods paralarvae as *O. maya* (Lopez-Ripoll, 2010) and *S. officinalis* (Boucaud-Camou and Yim, 1980) is observed that the 30D post hatching digestive gland has a more complex tubular structure than 5D post hatching paralarvae.

The juvenile phase starts when the digestive system is matured morphologically and physiologically. Our study demonstrates that after 20 rearing days the rate of increase in the size of this gland slows down. This is in accordance with previous studies on the digestive enzymes in *O. vulgaris* paralarvae where Villanueva et al. (2002) found that at 20 rearing days total proteolytic activity was stabilized, suggesting that by 20 rearing days at 21°C the digestive gland is fully developed and functional.

Considering all that has been said about the development of the digestive system and the use of the yolk in *O. vulgaris*, the early post-hatching phase must be considered critical. This, because it represents the transition from endogenous to exogenous feeding, at a time when some structures of the digestive system are not fully developed. The cephalopod paralarvae are active predators from the time of hatching (Iglesias et al., 2006). Thus, choice of the correct prey as food during the first days of cultivation is critical. In this sense, probably an initial diet based on larvae of decapod

crustaceans (*zoeas*) might be more adequate than one based on *Artemia* (Iglesias et al., 2007; Iglesias and Fuentes, 2014).

AUTHOR CONTRIBUTIONS

RF: Conception and design of study. RF and HG: Acquisition of data. RF and MH: Analysis and/or interpretation of data. RF and MH: Drafting the manuscript. RF, MH, and FR: revising the manuscript critically for important intellectual content. RF, MH, HG, and FR: Approval of the version of the manuscript to be published.

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Fatty Acid Profile of Neutral and Polar Lipid Fraction of Wild Eggs and Hatchlings from Wild and Captive Reared Broodstock of *Octopus vulgaris*

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The culture of *Octopus vulgaris* is constrained by unsolved problems in paralarvae rearing, mainly associated to the unknown nutritional requirements of this species in early stages. In this article we studied the fatty acid profile (total, neutral, and polar lipid fractions) in wild eggs and wild hatchlings, collected in Gran Canaria (SW) (Spain) with artificial dens, in comparison to hatchlings obtained in captivity from broodstock fed on trash fish species. Total lipids were 11.5–13.5% dw, with the polar fraction representing a 70.6–75.5% of total lipid, with lower values in wild hatchling in comparison with captive ones. Docosahexaenoic acid (DHA) was the main component in neutral and polar fatty acid profile in all samples, underlying its importance in this species. Decreasing levels of saturates and arachidonic acid (ARA) from wild eggs to hatchlings, mainly associated to the polar fraction, suggest their use during embryonic development. In hatchlings, increasing levels of oleic acid in the neutral fraction and eicosapentaenoic acid (EPA) in the polar fraction, suggests their importance in hatchlings quality. Wild hatchlings showed in the polar fraction higher oleic acid and ARA, and lower DHA/ARA and EPA/ARA ratios in comparison with captive hatchlings, suggesting a difference in paralarvae nutritional status. These results suggest the importance of n-3 highly unsaturated fatty acids (HUFA), oleic acid, and ARA, presented in the adequate lipid fraction, in the diet of broodstock and paralarvae of *O. vulgaris*.

Keywords: fatty acids, neutral and polar lipids, *Octopus vulgaris*, hatchlings, eggs, artificial dens, wild and captive reared

INTRODUCTION

The common octopus *Octopus vulgaris* is a promising candidate to diversify marine farming for its wide market demand and high growth rates (Vaz-Pires et al., 2004; García García and Cerezo Valverde, 2006; Estefanell et al., 2012a). However, the low survival of the paralarvae after the planktonic phase still constrains the industrial rearing of this species (Iglesias et al., 2007; Iglesias and Fuentes, 2014). To date, best paralarvae growth and survival were obtained when crab zoeas were added as a complement to *Artemia* (Villanueva, 1994, 1995; Iglesias et al., 2004; Carrasco et al., 2006; Fuentes et al., 2011; Reis et al., 2015; Garrido et al., 2016a; Roo et al., 2017), which suggests that

nutrition is the main factor affecting the biological performance of early life stages in this species (Navarro et al., 2014). In order to estimate its nutritional requirements, biochemical analysis of wild hatchlings, wild paralarvae (6–8 days old), wild juveniles, and potential natural preys have been performed. In general, these wild individuals showed high phospholipids and high n-3 HUFA (EPA, DHA) and n-6 HUFA (ARA) content (Navarro and Villanueva, 2000, 2003; Estefanell et al., 2013; Garrido et al., 2016b; Roo et al., 2017), abundance in certain amino acids (lysine, leucine, arginine, glutamate, aspartate) (Villanueva et al., 2004) and high levels in some microelements (copper, calcium, strontium, sulfur) (Villanueva and Bustamante, 2006). However, in comparison with these estimated nutritional requirements of paralarvae, the enriched *Artemia* successfully used as live prey in marine fish larval rearing, shows low polar lipid content and an imbalance in the n-3 and n-6 HUFA fatty acid profile (Navarro and Villanueva, 2000; Estefanell et al., 2013; Reis et al., 2015; Garrido et al., 2016b; Roo et al., 2017). Even though *Artemia* enrichment in DHA and polar lipids was attained using marine lecitine (Guinot et al., 2013a), the rapid bioconversion of DHA from the polar to the neutral fraction (Guinot et al., 2013b) suggests the inadequacy of *Artemia* as live prey for *O. vulgaris* paralarvae. These findings also underline the importance of the fraction in which the fatty acids are supplied for the successful rearing of this species. For instance, reared paralarvae of *O. vulgaris* showed most n-6 and n-3 HUFA in the polar fraction and most monoenes in the neutral fraction after 10–30 days of feeding (Navarro and Villanueva, 2003; Viciano et al., 2011). However, no data is available regarding the fatty acid profile of the neutral and polar lipid fractions in eggs and hatchlings, which represents useful information to estimate the nutritional requirements of early stages, contributing to the improvement of enrichment protocols for *Artemia* and specific compound microdiets.

In recent years, new data has been published on the ecology of *O. vulgaris* paralarvae from the NW Atlantic cost of the Iberian peninsula. For instance, decapod crab zoeas were identified as main natural preys by molecular methods (Roura et al., 2012), an oceanic life strategy far from the shelf in paralarvae was observed (Roura et al., 2016) and a preference for spawning areas with hard bottom substrate and moderate depth (<20 m) was detected (Guerra et al., 2015). However, data regarding the initial biochemical profile of wild paralarvae and eggs of *O. vulgaris* is still scarce, which could provide useful information regarding the nutritional requirement in early stages. In particular, one wild egg mass was analyzed from the Mediterranean sea (Navarro and Villanueva, 2003). To our knowledge, only the fatty acid profile from total lipids were obtained in 10 wild paralarvae of 6–8 days old in NW Spain (Garrido et al., 2016b) and in two samples of wild hatchlings and egg masses at the Canary Islands (Estefanell et al., 2013). Generally, the egg of marine species contains all the nutrients that the larvae require during the lecithotrophic phase, prior to exogenous feeding, and is related to the broodstock diet (Mourente and Vazquez, 1996). In *O. vulgaris*, an effect of the broodstock diet was observed on the biochemical profile of gonads (ovary and testis) (Estefanell et al., 2015), eggs and hatchlings (Quintana et al., 2015). Also, in a recent rearing trial with paralarvae of *O. vulgaris*, stress and nutritional condition biomarkers showed significant variability associated to geographical origin, despite applying the same feeding protocol and diet (Garrido et al., 2017). These authors concluded that further research must be carried out in order to understand the physiology of *O. vulgaris* associated to different geographical origins. Indeed, differences in the fatty acid profile were observed in the ovary of wild *O. vulgaris* collected from the natural environment in distant areas (Rosa et al., 2004; Sieiro et al., 2006; Lourenço et al., 2014; Estefanell et al., 2015), probably related to differences in the natural diet (Hanlon and Messenger, 1996). For these reasons, samples of wild eggs and wild hatchlings from different areas must be collected and analyzed in order to obtain information on the nutritional requirements of this species, and search for potential regional differences.

In this study we used indirect methods to obtain information about the neutral and polar fatty acid nutritional requirements in early stages in *O. vulgaris*. For this, we collected wild egg masses in Gran Canaria (Canary Islands, Spain) from the natural environment and obtained wild hatchlings at the lab. Also, we obtained hatchlings from captive broodstock fed on trash fish species commonly used during the grow out phase (Estefanell et al., 2012b).

MATERIALS AND METHODS

Ethics in Animal Research

The protocols for handling and rearing of broodstock of *O. vulgaris*, as well as the protocol for paralarvae euthanasia were approved by the Committee of Ethics in Animal Welfare of the University of Las Palmas de Gran Canaria in compliance with Directive 2010/63/EU.

Wild Eggs and Hatchlings

To obtain wild eggs and wild hatchlings, artificial dens were specifically designed to capture females caring eggs. For this, a black "T" shaped PVC 160 mm diameter pipe, with two ends closed with a PVC lid, was attached to a concrete base of 60 × 40 × 15 cm, weighing ∼15 kg. Several dens were placed at 10–20 m depth in rocky areas (with abundant crevices and holes) in the SW coasts of Gran Canaria (Las Palmas, Canary Islands). In November, several artificial dens were spotted with eggs. In total, three artificial dens with the female and the egg mass were carefully placed in a 250 L tank to be transported, by boat to the nearest harbor and by car to the ULPGC aquaculture facilities. In total, transport took ∼1 h.

Upon arrival to the facility, each den with the female and the eggs was placed individually in 500 L circular tanks, using 5 μm filtered natural seawater (37 ppt) in an open flow through system adjusted to a renovation of 50%/h. Natural photoperiod (November–December) were used during embryonic development. Each tank was covered with a shadowing net and the females were not fed during this period (∼1 month). Once the paralarvae started hatching the renovation was reduced to 50 L/h, and the newly hatched paralarvae were retained by a filter (net mesh of 375 μm) in a nearby 100 L tank connected to the 500 L tank. Hatchlings were daily collected

(8:00 a.m.). The water temperature was ranged 20–22°C and the oxygen levels were above the 90% saturation.

Captivity Hatchlings

Wild specimens of *O. vulgaris* were provided by professional fishermen and transported to ULPGC aquaculture facilities (Telde, Las Palmas, Canary Islands) in the conditions described by Estefanell et al. (2012b). Subadults of *O. vulgaris*, males:female sex ratio 1:1 ($N = 6$, initial weight: 975 ± 128 g) were kept under social conditions in 1.5 m^3 rectangular tanks under natural photoperiod (September–October), using 5 μm filtered natural seawater (37 ppt) in an open flow through system adjusted to a renovation of 100%/h. The tank was provided with 12 dens (PVC tubes of 160 mm diameter and 50 cm length) and covered with a shadowing net. During the rearing period the specimens were fed *ad libitum* once a day (six times/week) with fresh bogue *Boops boops* (Estefanell et al., 2012b). The males were removed after 2 weeks. The remaining females naturally spawned after ∼2 months. Same methodology as described above was used to collect hatchlings.

Paralarvae Euthanasia Protocol

Hatchlings were anesthetized by immersion in seawater with a 1.0% ethanol (96%) for 5 min, prior to being sacrificed by immersion on iced seawater. The same protocol was applied for eggs.

Dry Weight Determinations

The hatching period lasted 2–3 weeks. For each female, dry weight (dw) of hatchlings was determined four times during the hatching period. For each time, 30 paralarvae were randomly selected and separated in 3 pools of 10 paralarvae. After being sacrificed, the hatchlings were rinsed with distilled water, prior to being carefully placed on a crystal slide. The dry weight was determined by drying them at 105°C until constant weight.

Biochemical Samples

The following samples were taken: a sample of eggs (3 strings) from each artificial den was taken upon arrival to the aquaculture facility ("wild eggs," $N = 3$, from different females), hatchlings from the natural environment ("wild hatchlings," $N = 3$, hatched from eggs from the same females) and hatchlings from broodstock fed on trash fish species under common aquaculture conditions ("captive hatchlings," $N = 3$, from different females). For each female, ∼1,000 hatchlings were sacrificed four times during the hatchling period, and mixed to obtain an homogeneous pool sample (∼4 g wet weight). After being sacrificed, the eggs and the hatchlings were rinsed with distilled water to remove ethanol traces, dried on absorbent paper and immediately frozen at −80°C.

Biochemical Analysis

Proximate composition of eggs and hatchlings were analyzed following standard procedures (AOAC, 1997). Moisture was determined after drying the sample in an oven at 105°C to constant weight; ash by combustion in a muffle furnace at 600°C for 12 h; protein content (N × 6.25) was determined by Kjeldahl method and crude lipid was extracted following the method described by Folch et al. (1957). Neutral and polar fractions of total lipids were separated by adsorption chromatography on silica cartridges (Sep-pak; Waters S.A., Massachussets, USA) using 30 mL chloroform and 20 mL chloroform/methanol (49: 1, v/v) as solvent for neutral lipid, followed by a 30 mL methanol wash to obtain the polar fractions according to Juaneda and Rocquelin (1985). Fatty acids methyl esters from total, neutral, and polar lipids were extracted by transmethylation as described by Christie (1982) and separated by gas chromatography under the conditions described by Izquierdo et al. (1992). All analyses were conducted in triplicates.

Statistical Analysis

All data, presented as mean ± standard deviation, were tested for normality (Kolmogorov Smirnov) and homogeneity of variances (Levene's test). When necessary, an arcsin transformation of the data was carried out, particularly when data was presented as % (Fowler et al., 1998). The dry weight of wild and captive hatchlings was compared using a Student "t" model. The proximate composition, neutral, and polar lipid proportions, as well as the % of fatty acid from total, neutral, and polar lipids of wild eggs, wild hatchlings and captive hatchlings were submitted to a one way ANOVA test. In addition, differences among groups were determined with a Tukey *post-hoc* test. When normality or homogeneity of variances was not achieved, non-parametric tests were used (Kruskal Wallis, Games-Howell). In all this manuscript significant differences were considered when $P < 0.05$. For the different analysis, the statistical computer package SPSS v15 (SPSS, Chicago, IL, USA) was used.

RESULTS

Proximate Composition and Fatty Acid Profile from Total Lipids

A lower lipid content (% dw) and a higher ash content (%) were observed in eggs (wild) in comparison with hatchlings ($P < 0.05$) (**Table 1**). Similar lipid and protein content was observed in wild and captive hatchlings, while ash content was higher in wild than in captive hatchlings ($P < 0.05$) (**Table 1**).

Regarding the fatty acids from total lipids, a decrease in saturates (tetradecanoic acid, 14:0; palmitic acid, 16:0) and n-6 (ARA, 20:4n-6), and an increase in monoenes (oleic acid, 18:1n-9) and n-3 HUFA (ETE, 20:3n3; EPA, 20:5n-3) were observed in hatchlings regardless of origin, in comparison with eggs (wild) ($P < 0.05$) (**Table 1**). Also, a higher ARA and lower DHA content were observed in wild hatchlings in comparison with those obtained from captive broodstock fed on trash fish species ($P < 0.05$) (**Table 1**).

Neutral and Polar Lipid Proportion from Total Lipids and Fatty Acid Profile from Each Fraction

In general, a higher polar lipid proportion was observed in comparison with the neutral fraction regardless of sample ($P < 0.05$). In particular, the neutral and polar fraction represented a 25.6 ± 0.5 and a 74.4 ± 0.5% in eggs (wild), a 29.4 ± 1.6

TABLE 1 | Dry weight of hatchlings (mg), proximate composition (lipids, proteins, moisture, ash) (%), and main fatty acids profile (% of total fatty acids) from total lipids in wild eggs ($N = 3$), wild hatchlings ($N = 3$), and hatchlings obtained in the lab from captive broodstock fed on trash fish species ($N = 3$).

	Eggs (wild)	Hatchlings (wild)	Hatchlings (captivity)
Dry weight (mg)	–	0.20 ± 0.02	0.22 ± 0.01
Lipids (%dw)	11.5 ± 0.8^{a}	13.3 ± 0.2^{b}	13.5 ± 0.5^{b}
Proteins (%dw)	72.7 ± 3.2	71.6 ± 2.3	72.0 ± 4.2
Moisture (%)	70.4 ± 2.8^{a}	85.4 ± 0.2^{b}	86.3 ± 5.2^{b}
Ash (%)	1.6 ± 0.1^{c}	1.3 ± 0.1^{b}	1.0 ± 0.1^{a}
14:0 (%)	2.8 ± 0.7^{b}	1.1 ± 0.0^{a}	0.9 ± 0.1^{a}
16:0 (%)	21.6 ± 0.2^{b}	17.0 ± 0.1^{a}	17.3 ± 1.3^{a}
16:1n-7 (%)	0.6 ± 0.1^{c}	0.3 ± 0.0^{b}	0.2 ± 0.0^{a}
18:0 (%)	3.8 ± 0.4	4.3 ± 0.1	3.8 ± 0.1
18:1n-9 (%)	5.8 ± 0.2^{a}	9.5 ± 0.1^{b}	10.4 ± 0.3^{c}
18:1n-7 (%)	3.6 ± 0.4^{b}	2.6 ± 0.0^{a}	2.4 ± 0.3^{a}
18:1n-5 (%)	1.9 ± 0.1^{c}	1.5 ± 0.1^{b}	0.7 ± 0.1^{a}
18:2n-6 (%)	0.2 ± 0.1^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
20:1n-9 (%)	3.6 ± 0.4	3.7 ± 0.1	3.7 ± 0.1
20:1n-7 (%)	0.3 ± 0.1^{a}	0.6 ± 0.0^{b}	0.5 ± 0.0^{b}
20:2n-6 (%)	1.3 ± 1.1	0.8 ± 0.0	0.9 ± 0.2
20:4n-6 (%)	13.0 ± 1.2^{c}	8.5 ± 0.3^{b}	4.5 ± 0.5^{a}
20:3n-3 (%)	0.1 ± 0.0^{a}	2.0 ± 0.1^{b}	2.1 ± 0.1^{b}
20:4n-3 (%)	0.1 ± 0.0^{a}	0.7 ± 0.2^{b}	0.5 ± 0.1^{b}
20:5n-3 (%)	9.6 ± 0.8^{a}	14.0 ± 0.5^{b}	15.6 ± 0.4^{c}
22:1n-9 (%)	0.2 ± 0.0^{a}	0.6 ± 0.0^{b}	0.5 ± 0.0^{b}
22:4n-6 (%)	1.4 ± 0.2^{b}	1.1 ± 0.1^{b}	0.3 ± 0.0^{a}
22:5n-6 (%)	1.3 ± 0.2^{b}	1.1 ± 0.1^{b}	0.6 ± 0.1^{a}
22:5n-3 (%)	1.5 ± 0.3^{b}	2.2 ± 0.1^{c}	1.0 ± 0.0^{a}
22:6n-3 (%)	24.7 ± 1.0^{a}	25.0 ± 0.1^{a}	31.9 ± 1.4^{b}
∑ Saturates (%)	28.7 ± 0.8^{b}	22.8 ± 0.2^{a}	22.2 ± 1.5^{a}
∑ Monoenes (%)	16.4 ± 0.8^{a}	19.3 ± 0.2^{b}	18.6 ± 0.2^{b}
∑ n-3 (%)	36.6 ± 1.3^{a}	44.4 ± 0.9^{b}	51.5 ± 1.6^{c}
∑ n-6 (%)	17.3 ± 1.9^{c}	11.5 ± 0.4^{b}	6.4 ± 0.4^{a}
∑ n-9 (%)	6.4 ± 0.1^{a}	11.5 ± 0.1^{b}	11.9 ± 0.2^{c}
∑ n-3 HUFA (%)	36.1 ± 1.4^{a}	43.8 ± 0.8^{b}	51.1 ± 1.6^{c}
DHA/EPA	2.6 ± 0.2^{c}	1.8 ± 0.1^{a}	2.1 ± 0.1^{b}
DHA/ARA	1.9 ± 0.2^{a}	3.0 ± 0.1^{b}	7.1 ± 0.3^{c}
EPA/ARA	0.8 ± 0.1^{a}	1.6 ± 0.1^{b}	3.4 ± 0.1^{c}

All variables are shown as mean ± SD. No significant difference were found in dry weight in hatchlings regardless of origin ($P < 0.05$). Different superscript letter within a row denotes significant difference among samples ($P < 0.05$). The ∑ included all detected fatty acids. Selected FA represented 95–98% of total FA.

TABLE 2 | Neutral lipids from total lipids (%) and main fatty acids in the neutral fraction in wild eggs ($N = 3$), wild hatchlings ($N = 3$), and hatchlings obtained in the lab from captive broodstock fed on trash fish species ($N = 3$).

	Eggs (wild)	Hatchlings (wild)	Hatchlings (captivity)
Neutral Lipids/ Total lipids (%)	25.6 ± 0.5^{ab}	29.4 ± 1.6^{b}	24.8 ± 2.5^{a}
14:0 (%)	5.5 ± 1.3^{b}	2.0 ± 0.4^{a}	2.1 ± 0.7^{a}
16:0 (%)	16.7 ± 1.0	17.3 ± 1.8	15.3 ± 2.1
16:1n-7 (%)	0.9 ± 0.2	0.8 ± 0.3	0.9 ± 0.2
18:0 (%)	4.1 ± 0.3^{a}	7.9 ± 0.9^{b}	8.8 ± 0.2^{b}
18:1n-9 (%)	9.4 ± 0.8^{a}	20.7 ± 2.5^{b}	16.8 ± 0.4^{b}
18:1n-7 (%)	2.5 ± 0.3^{b}	2.1 ± 0.2^{b}	1.2 ± 0.0^{a}
18:1n-5 (%)	0.5 ± 0.0	1.4 ± 1.2	0.2 ± 0.0
18:2n-6 (%)	0.8 ± 0.1^{b}	0.1 ± 0.1^{a}	0.2 ± 0.3^{ab}
20:1n-9 (%)	7.2 ± 0.5^{b}	2.5 ± 0.6^{a}	3.1 ± 0.4^{a}
20:1n-7 (%)	1.5 ± 0.1^{b}	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}
20:2n-6 (%)	0.9 ± 0.1	0.4 ± 0.5	1.4 ± 0.4
20:4n-6 (%)	7.9 ± 1.0^{b}	4.4 ± 1.1^{a}	4.1 ± 0.1^{a}
20: 3n-3 (%)	0.6 ± 0.1^{a}	2.0 ± 0.8^{b}	1.4 ± 0.3^{b}
20:4n-3 (%)	0.2 ± 0.0^{a}	0.3 ± 0.2^{a}	4.0 ± 0.8^{b}
20:5n-3 (%)	8.7 ± 0.5	8.5 ± 0.9	7.2 ± 1.3
22:1n-9 (%)	1.0 ± 0.1	1.0 ± 0.7	1.5 ± 0.4
22:4n-6 (%)	0.8 ± 0.8	1.1 ± 0.7	0.4 ± 0.2
22:5n-6 (%)	1.1 ± 0.3^{b}	0.9 ± 0.6^{b}	0.1 ± 0.2^{a}
22:5n-3 (%)	2.2 ± 0.5^{b}	1.5 ± 0.8^{ab}	0.6 ± 0.2^{a}
22:6n-3 (%)	21.6 ± 2.5^{b}	16.9 ± 2.3^{a}	21.7 ± 1.4^{ab}
∑ Saturates (%)	27.4 ± 2.2	28.7 ± 1.4	27.3 ± 2.1
∑ Monoenes (%)	24.9 ± 2.1^{a}	30.6 ± 1.7^{b}	25.8 ± 0.9^{a}
∑ n-3 (%)	34.1 ± 2.8^{ab}	30.6 ± 2.8^{a}	36.5 ± 1.7^{b}
∑ n-6 (%)	11.8 ± 1.8^{b}	7.7 ± 0.2^{a}	7.2 ± 0.9^{a}
∑ n-9 (%)	12.3 ± 0.9^{a}	22.9 ± 2.4^{c}	18.8 ± 0.6^{b}
∑ n-3 HUFA (%)	33.4 ± 2.9^{ab}	29.1 ± 2.3^{a}	35.1 ± 1.2^{b}
DHA/EPA	2.5 ± 0.2^{ab}	2.0 ± 0.5^{a}	3.1 ± 0.6^{b}
DHA/ARA	2.8 ± 0.5^{a}	4.0 ± 1.2^{ab}	5.3 ± 0.3^{b}
EPA/ARA	1.1 ± 0.2^{a}	2.0 ± 0.3^{b}	1.8 ± 0.3^{b}

All variables are shown as mean ± SD. Different superscript letter within a row denotes significant difference among samples ($P < 0.05$). The ∑ included all detected fatty acids. Selected FA represented 92–95% of total FA.

and a 70.6 ± 1.6% in hatchlings (wild), and a 24.8 ± 2.5 and a 75.2 ± 2.5% in hatchlings (captivity), respectively (**Tables 2**, **3**). A higher neutral lipid proportion and a lower polar lipid proportion were observed in wild hatchlings in comparison with hatchlings obtained from captive broodstock ($P < 0.05$) (**Tables 2**, **3**).

Regarding the fatty acids from the neutral fraction, the highest monoenes and n-9 were observed in wild hatchlings in comparison with the other samples ($P < 0.05$) (**Table 2**). In contrast, 20:1n-9 (Eicosenoic acid) and 20:1n-7 (Paullinic acid) showed higher values in eggs in comparison with hatchlings ($P < 0.05$). A decrease in ARA was observed in hatchlings regardless of origin, while EPA showed similar levels in the different samples in the neutral fraction ($P < 0.05$) (**Table 2**).

Regarding the fatty acids from the polar fraction, the lowest levels of saturates (particularly 18:0, estearic acid) and the highest levels of monoenes and n-9 (mainly associated to oleic acid) were observed in wild hatchlings in comparison with the other samples ($P < 0.05$) (**Table 3**). A reduction in ARA was observed from wild eggs to wild hatchlings, with hatchling obtained from captive broodstock showing the lowest levels ($P < 0.05$) (**Table 3**). Increments in EPA, DHA/ARA, and EPA/ARA ratios were observed from wild eggs to wild hatchlings, with hatchling obtained from captive broodstock showing the highest levels ($P < 0.05$) (**Table 3**). Higher levels of n-3 HUFA (associated to 20:3n3, ETE and EPA) were detected in hatchlings, regardless of origin, in

TABLE 3 | Polar lipids from total lipids (%) and main fatty acids in the polar fraction in wild eggs ($N = 3$), wild hatchlings ($N = 3$), and hatchlings obtained in the lab from captive broodstock fed on trash fish species ($N = 3$).

	Eggs (wild)	Hatchlings (wild)	Hatchlings (captivity)
Polar Lipids/ Total lipids (%)	74.4 ± 0.5^{ab}	70.6 ± 1.6^{b}	75.2 ± 2.5^{a}
14:0 (%)	2.8 ± 0.9^{b}	1.1 ± 0.1^{a}	0.9 ± 0.0^{a}
16:0 (%)	25.2 ± 1.5^{b}	18.1 ± 1.0^{a}	20.2 ± 0.3^{a}
16:1 n-7 (%)	0.6 ± 0.1^{b}	0.1 ± 0.0^{a}	0.1 ± 0.1^{a}
18:0 (%)	6.6 ± 0.4^{b}	4.1 ± 0.4^{a}	11.5 ± 0.2^{c}
18:1n-9 (%)	3.5 ± 0.6^{a}	9.7 ± 0.6^{b}	2.6 ± 0.0^{a}
18:1n-7 (%)	1.8 ± 0.1^{b}	2.5 ± 0.2^{c}	0.8 ± 0.0^{a}
18:1n-5 (%)	0.4 ± 0.0^{b}	1.4 ± 0.0^{c}	0.3 ± 0.0^{a}
18:2n-6 (%)	0.2 ± 0.0^{a}	0.6 ± 0.0^{b}	0.6 ± 0.0^{b}
20:1n-9 (%)	3.0 ± 0.3^{a}	3.4 ± 0.2^{ab}	3.8 ± 0.2^{b}
20:1n-7 (%)	0.2 ± 0.1^{a}	0.6 ± 0.0^{b}	0.6 ± 0.1^{b}
20:2n-6 (%)	0.7 ± 0.3	0.7 ± 0.1	0.9 ± 0.1
20:4n-6 (%)	13.3 ± 1.0^{c}	7.7 ± 0.5^{b}	4.5 ± 0.2^{a}
20: 3n-3 (%)	0.1 ± 0.0^{a}	1.8 ± 0.2^{b}	2.1 ± 0.1^{b}
20:4n-3 (%)	0.3 ± 0.4	0.2 ± 0.1	0.2 ± 0.0
20:5n-3 (%)	9.3 ± 0.8^{a}	12.5 ± 1.3^{b}	15.7 ± 1.0^{c}
22:1n-9 (%)	0.3 ± 0.1^{a}	0.5 ± 0.0^{b}	0.6 ± 0.1^{b}
22:4n-6 (%)	0.5 ± 0.6	1.4 ± 0.4	0.1 ± 0.2
22:5n-6 (%)	1.2 ± 0.2^{ab}	1.6 ± 0.6^{b}	0.6 ± 0.1^{a}
22:5n-3 (%)	1.2 ± 0.2^{a}	3.0 ± 0.8^{b}	1.0 ± 0.1^{a}
22:6n-3 (%)	22.2 ± 1.2^{a}	26.3 ± 2.4^{ab}	27.9 ± 1.7^{b}
$\sum$ Saturates (%)	35.0 ± 2.2^{b}	23.6 ± 1.5^{a}	32.9 ± 0.0^{b}
$\sum$ Monoenes (%)	10.3 ± 0.8^{a}	18.4 ± 1.0^{b}	9.0 ± 0.5^{a}
$\sum$ n-3 (%)	33.6 ± 1.8^{a}	44.1 ± 2.2^{b}	47.2 ± 0.3^{b}
$\sum$ n-6 (%)	16.3 ± 1.4^{c}	12.3 ± 0.4^{b}	6.9 ± 0.2^{a}
$\sum$ n-9 (%)	4.0 ± 0.5^{a}	10.9 ± 0.7^{b}	3.9 ± 0.2^{a}
$\sum$ n-3 HUFA (%)	33.2 ± 1.8^{a}	43.8 ± 2.2^{b}	46.9 ± 0.4^{b}
DHA/EPA	2.4 ± 0.2	2.1 ± 0.4	1.8 ± 0.2
DHA/ARA	1.7 ± 0.2^{a}	3.4 ± 0.5^{b}	6.2 ± 0.7^{c}
EPA/ARA	0.7 ± 0.1^{a}	1.6 ± 0.1^{b}	3.5 ± 0.0^{c}

All variables are shown as mean ± SD. Different superscript letter within a row denotes significant difference among samples ($P < 0.05$). The $\sum$ included all detected fatty acids. Selected FA represented 92–98% of total FA.

comparison with wild eggs ($P < 0.05$) (**Table 3**). Wild hatchlings showed higher levels of monoenes (series 18:1n) in comparison with hatchlings from captive broostock ($P < 0.05$) (**Table 3**).

DISCUSSION

In this study, *O. vulgaris* hatchlings showed lower dry weights in comparison to data from different regions (0.30–0.48 mg dw; Navarro and Villanueva, 2000; Carrasco et al., 2006; Seixas et al., 2010; Fuentes et al., 2011; Domingues et al., 2013; Iglesias et al., 2014), but similar to previous studies in the Canary Islands (0.17–0.25 mg dw; Reis et al., 2015; Garrido et al., 2017; Roo et al., 2017). This could be related to the higher seawater temperature in Canarian latitudes. Indeed, the incubating temperature is inversely related to hatching size in *O. vulgaris* (Repolho et al., 2014) and in other cephalopod species (*Sepia officinalis, Loligo opalescens*, *Loligo vulgaris*) (Bouchaud, 1991; Villanueva, 2000; Domingues et al., 2002; Vidal et al., 2002).

Lipid content observed in hatchlings (13.3–13.5% dw) was similar to previous data (Navarro and Villanueva, 2000; Seixas et al., 2010; Iglesias et al., 2014; Roo et al., 2017) and higher than those reported in juveniles and adults of *O. vulgaris* (Navarro and Villanueva, 2003; García García and Cerezo Valverde, 2006; Estefanell et al., 2012b), underlying the importance of the lipid fraction in early stages. This is probably associated to the higher relative size of the digestive gland and especially the nervous and the visual system in hatchlings in comparison with adults. Indeed, lipids in cephalopods are abundant in the digestive gland (García Garrido et al., 2010; Lourenço et al., 2014; Estefanell et al., 2015) and are probably main components of the nervous and visual system, as in several marine fish larvae (Navarro et al., 1995; Benítez-Santana et al., 2007). Regarding the ash content, higher levels were detected in wild eggs and wild hatchlings in comparison with those obtained from captive broodstock, which may have important physiological implications (Davis and Gatlin, 1996). Minerals have several essential functions in cephalopods, such as regulation of acid-base equilibrium and as component of hormones, enzymes and structural proteins, and are affected by fasting conditions (Villanueva and Bustamante, 2006). The analysis of mineral content in wild and reared paralarvae may provide useful information to improve paralarvae survival.

In this study, the proportion of the polar lipid fraction (70–75%) was slightly higher than in previous reports in hatchlings (60–65%) (Navarro and Villanueva, 2000; Quintana et al., 2015; Reis et al., 2015), underlying the importance of the polar fraction in early stages in *O. vulgaris* (Navarro et al., 2014). The importance of the dietary polar lipid fraction has been shown in subadults of this species by its very high digestibility regardless of total dietary lipid content, while the digestibility of the neutral fraction was generally low and inversely related with total dietary lipids (Morillo Velarde et al., 2015). Whether the paralarvae show this selective lipid digestion is unknown. However, low lipid content in crab zoeas (5–10%dw) with high relative levels of phospholipids (Andrés et al., 2010) were suggested to be responsible for the positive effect of these live prey on paralarvae rearing (Iglesias et al., 2014; Reis et al., 2015). Also, the addition of crab zoeas in low quantities to an *Artemia* diet induced a better histological nutritional status of the digestive gland in comparison with paralarvae fed on single *Artemia* (Roo et al., 2017). In contrast, enriched *Artemia* is abundant in lipids (18–28%dw) (Viciano et al., 2011; Iglesias et al., 2014; Roo et al., 2017) and shows a rapid turnover of polar to neutral lipid fraction (Guinot et al., 2013b), inducing negative effects on growth and survival on paralarvae rearing when supplied as a single live prey (Reis et al., 2015; Roo et al., 2017). For these reasons, the supply of the lipids and fatty acids in the adequate fraction appears to be essential for paralarvae rearing success in *O. vulgaris*.

In general, all samples in this study showed high levels of palmitic acid, estearic acid, oleic acid, ARA, EPA, and DHA, in agreement with previous findings (Navarro and Villanueva,

2000, 2003; Quintana et al., 2015; Reis et al., 2015; Roo et al., 2017). The essentiality of ARA, EPA, and DHA in *O. vulgaris* has been suggested by the very low activity of their biosynthesis pathways from n-3 to n-6 substrates (Monroig et al., 2013; Reis et al., 2014). In this study, deviations in the fatty acid profile from total lipids between eggs and hatchlings suggests the use of saturates and ARA during embryonic development, whereas other monoenes and n-3 HUFA are retained or show increasing values in hatchlings. Similar findings were observed in eggs and hatchlings of *O. vulgaris* (Navarro and Villanueva, 2000, 2003). The increase in n-3 HUFA (ETE and EPA) in the polar fraction from wild eggs to wild hatchlings suggest their importance as phospholipids components in paralarvae. The decrease observed in monoenes of the 20:1n series, ARA and DHA in the neutral fraction from wild eggs to wild hatchlings suggest their use as energy substrates during embryonic development. In contrast, increasing values of oleic acid in wild hatchlings, both in the polar and neutral fraction, suggests its importance as energy substrate during the transition from endogenous to exogenous feeding and also as component of phospholipids in paralarvae tissues. Also, the decrease in ARA in the polar fraction in hatchlings is probably associated to a change in phospholipid class, as observed from eggs to hatchlings of *O. vulgaris* fed on different diets (Quintana et al., 2015). In our study, hatchlings obtained from captive broodstock showed a significantly different proportion of polar and neutral lipids and deviations in the fatty acid profile in comparison with wild ones, probably related to the broodstock diet (Quintana et al., 2015). The bogue *Boops boops* used as food shows high DHA and linoleic acid and low ARA content (Estefanell et al., 2012b), mainly provided as triglycerides (neutral lipids) (Cerezo Valverde et al., 2012). In captive hatchlings, the neutral fraction fatty acid profile was relatively similar to wild hatchlings. In contrast, important deviations were observed in the polar fraction, with captive hatchlings showing increasing levels of EPA and the lowest levels of monoenes (18:1n series) and ARA in comparison with wild ones. These variations probably affected the fatty acid profile of the phospholipid classes in hatchlings obtained from captive broodstock (Bell et al., 1995), with potential negative effects on the paralarvae nutritional status. Indeed, different fresh broodstock diets induced differences in spawn quality, related to a change in the fatty acid and the phospholipid class profile in hatchlings (Quintana et al., 2015). In a previous study, important deviations were also observed in the fatty acid profile in gonads (ovary and testis) between wild and reared *O. vulgaris*, especially in the EPA/ARA ratios from total lipids, associated to dietary input (Estefanell et al., 2015). Indeed, difference in the natural diet is probably responsible for the different relation observed in this study among DHA, EPA, and ARA in wild eggs in comparison to previous reports (Navarro and Villanueva, 2003; Estefanell et al., 2013), since cephalopods normally feed on the most readily available prey (Hanlon and Messenger, 1996). Seasonal changes in natural preys and specific oceanographic conditions may explain the important effect of the geographical region on paralarvae rearing success, recently noted in *O. vulgaris* (Garrido et al., 2017).

In the present study, different ratios among ARA, EPA, and DHA in total and polar lipids were observed between wild and captive hatchlings, with several well-known physiological implications in marine species. DHA is especially important in the neural tissue, retina, and the optic nerve which develop during early larval stages in marine fish (Benítez-Santana et al., 2007). In marine fish, ARA and EPA compete with each other for the enzymes that regulate the synthesis of eicosanoids, hormone-like compounds involved in blood clotting, immune and inflammatory response, renal and neural function, cardiovascular tone and reproduction (Tocher, 2003). Also, the deficiency or imbalance of DHA, EPA, and ARA in broodstock diets reported negative effects on reproduction in several marine fish species, affecting egg and sperm quality, and decreasing fecundity, and reducing egg vitality, hatching rate and larval survival (Izquierdo et al., 2001; Furuita et al., 2003; Mazorra et al., 2003; Fernández-Palacios et al., 2011).

In conclusion, our results underline the importance of the polar lipid fraction in paralarvae lipid profile, in particular oleic acid, ARA, EPA, and DHA. The highest oleic acid content in wild paralarvae in neutral lipids also suggest the importance of this fatty acid as energy reserve, probably related to a better nutritional status in comparison with hatchlings obtained from captive broodstock. The authors would like to emphasize that the analysis of fatty acid from neutral and polar lipids provides useful information to elucidate the nutritional requirements of this species. More research must be carried out in order to understand the physiological mechanisms involved in paralarvae quality and feeding during early stages in *O. vulgaris*.

AUTHOR CONTRIBUTIONS

JE, JS, and AM designed the experiment. BR and AL built the artificial dens and did the samplings at sea, in colaboration with JE and AM. JE did the biochemical analysis. The paper was writen by JE and AM, and revised by JS and CH, who also helped discussing the results. MI provided funding and helped discussing the results.

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Feeding Relationship between *Octopus vulgaris* (Cuvier, 1797) Early Life-Cycle Stages and Their Prey in the Western Iberian Upwelling System: Correlation of Reciprocal Lipid and Fatty Acid Contents

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Under the influence of the Western Iberian upwelling system, the Iberian Atlantic coast holds important hatcheries and recruitment areas for *Octopus vulgaris.* Recently identified as an octopus hatchery, the Ría de Vigo harbors an important mesozooplankton community that supports *O. vulgaris* paralarvae during the first days of their planktonic stage. This study represents a preliminary approach to determine the nutritional link between wild *O. vulgaris* hatchlings, paralarvae and their zooplankton prey in the Ría de Vigo, by analyzing their lipid class content and fatty acid profiles. The results show that octopus hatchlings are richer in structural lipids as phospholipids and cholesterol, while the zooplankton is richer in reserve lipids like triacylglycerol and waxes. Zooplankton samples are also particularly rich in C18:1n9 and 22:6n3 (DHA), that seem to be successfully incorporated by *O. vulgaris* paralarvae thus resulting in a distinct fatty acid profile to that of the hatchlings. On the other hand, content in C20:4n6 (ARA) is maintained high through development, even though the zooplankton is apparently poorer in this essential fatty acid, confirming its importance for the development of *O. vulgaris* paralarvae. The content in monounsaturated fatty acids, particularly C18:1n7, and the DHA: EPA ratio are suggested as trophic markers of the diet of *O. vulgaris* paralarvae.

Keywords: ***Octopus vulgaris,*** **paralarvae, fatty acids, lipid content, zooplankton, prey-predator relationship**

INTRODUCTION

The common octopus (*Octopus vulgaris* Cuvier, 1797) is the most important commercially harvested octopus worldwide. With global landing estimates of 42,457 ton/year (FAO, 2016), it is consumed in many countries of Asia, Latin-America and Mediterranean. The high market demand and value, along with the biological characteristics like the short life span, high growth rates and

high food conversion, makes *O. vulgaris* a desirable species for aquaculture production (Vaz-Pires et al., 2004). However, after decades of research the low paralarvae survival remains an important constraint for industrial farming (Iglesias et al., 2007; Garrido et al., 2016b).

The Iberian Atlantic coast is an important hatchery and recruitment area for *O. vulgaris* (Moreno et al., 2014; Guerra et al., 2015). Here, *O. vulgaris* paralarvae find the optimal environmental conditions to grow favored by the strong summer upwelling (Moreno et al., 2009; Roura et al., 2016). In the first days of life, the paralarvae combine endogenous (yolk) with exogenous feeding, preying mainly upon larval stages of crustaceans of the families Crangonidae, Alpheidae, Brachyura, Paguridae, Thalassinidae, Porcellanidae, Cladocera, Copepoda, and Euphausiidae, but also fish larvae and Cnidaria (Roura et al., 2012; Olmos-Pérez et al., 2017). In fact, during summer, all these potential prey are naturally "enriched" with essential fatty acids (EFA) by the seasonal coastal upwelling where the frequent diatom and dinoflagelate blooms are responsible for the production of polyunsaturated fatty acids (PUFA) as 20:5n3 (eicosapentaenoic acid, EPA) and 22:6n3 (docosahexaenoic acid, DHA). The phytoplankton fatty acid (FA) composition and, particularly, the ratios PUFA (n-3)/(n-6) and EPA/DHA will influence the FA composition of the linked trophic levels like meso- and microzooplankton and planktivorous fishes (Dalsgaard et al., 2003) like *Sardina pilchardus* (Garrido et al., 2008) and to certain extent tissues and eggs of higher trophic levels species as *O. vulgaris* (Lourenço et al., 2014). In fact, the FA composition of muscle and eggs in marine organisms reflects to certain level the biochemical and ecological conditions of ecosystems and can be used to identify food web interactions (Bergé and Barnathan, 2005) being used as qualitative markers, or biomarkers, to trace or confirm predator-prey relationships (Dalsgaard et al., 2003; Budge et al., 2006).

From the metabolic perspective, marine lipids have key roles in the physiology and reproductive processes of heterotrophic organisms. The neutral lipids triacylglycerols and wax esters, are energy reserves that produce free fatty acids through oxidation, which will be incorporated into phospholipids and again in fat reserves (Budge et al., 2006). Phospholipids are the building blocks for the membrane lipid bilayer. The lipids facilitate the absorption of fat-soluble vitamins (e.g., Vitamins A, D, E, and K), and play an important role in the production and regulation of eicosanoids (Bergé and Barnathan, 2005). Cholesterol is the predominant sterol in cephalopod's lipid reserves (Sieiro et al., 2006) and it is precursor of steroid hormones including cortisol, corticosterone, and cortisone. From these, cortisol has an important role in stress responses and is involved in the regulation of the carbohydrates and protein metabolism (Tocher and Glencross, 2015). Despite the low content of lipids in cephalopod body composition (6% dw in muscle, 24% in digestive gland of adults, Sieiro et al., 2006) and 12% dw of the paralarvae (Navarro and Villanueva, 2003), lipids have critical roles in cephalopod metabolism and development (Navarro and Villanueva, 2000; Okumura et al., 2005; Miliou et al., 2006; Seixas et al., 2010; Monroig et al., 2013; Reis et al., 2015). The lipid-rich nervous system of hatchlings represents approximately one quarter of the animal's fresh weight (Navarro et al., 2014) and the long-chain PUFA, namely EPA, DHA, and C20:4n6 (arachidonic acid, ARA) are identified as EFA for cephalopods, particularly in early life-cycle stages (Monroig et al., 2012; Reis et al., 2014). In fact, several studies have suggested that *O. vulgaris* paralarvae require prey of low lipid content, rich in polar lipids, long-chain PUFA, and cholesterol content (Navarro and Villanueva, 2000, 2003; Okumura et al., 2005; Seixas et al., 2008).

Despite the extended knowledge about the environmental physical factors that drive the distribution, abundance, and recruitment success of *O. vulgaris* paralarvae (González et al., 2005; Otero et al., 2008, 2009; Moreno et al., 2009; Roura et al., 2013, 2016), there are few studies regarding the nutritional profile and requirements of wild *O. vulgaris* paralarvae and their natural prey. In recent years, major efforts have been conducted to understand the nutritional needs for paralarvae in captivity (Garrido et al., 2016b) and their fatty acid profile in the wild (Estefanell et al., 2013; Garrido et al., 2016a), however the nutritional link between them and their prey in natural conditions is still largely unknown.

To fulfill this gap, this study aimed to identify the lipid class content of wild *O. vulgaris* hatchlings and paralarvae and that of their potential preys—i.e., the mesozooplankton community—in the Ría de Vigo (NW Spain). The contents in phospholipids, cholesterol, triacylglycerol, free fatty acids, and wax esters were determined in the mezooplankton samples and *O. vulgaris* hatchlings samples. The FA profile was evaluated in the mesozooplankton, hatchlings and paralarvae samples in terms of individual FA, saturated FA (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), n-6 highly unsaturated FA (n-6), and n-3 highly unsaturated FA (n-3). Based in significant dissimilarities analyses, trophic markers were selected and compared between the zooplankton, hatchlings and paralarvae, aiming to understand which FA were incorporated into planktonic *O. vulgaris* paralarvae through their diet.

MATERIALS AND METHODS

Zooplankton Sampling

A total of 12 mesozooplankton samples were collected at 5 m depth of the Ría de Vigo (NW Spain, **Figure 1**) in three surveys conducted under the LARECO project (CTM2011-25929) in autumn 2012, September 17th (d1); October 1st (d2); and October 5th (d3) in the outer part of the Ría de Vigo. Samples were collected with a multitrawl (MultiNet®) sampler (0.71 × 0.71 m opening frame, 200 μm mesh), East (inn samples) and West of Cies Islands (outer samples) and visually examined on board, looking for *Octopus vulgaris* paralarvae, which were manually sorted. Six zooplankton samples ($n = 6$) were washed with sea water and filtered with a 1,000 μm sieve and frozen at −80°C, freeze dried during 48 h and stored again at −80°C for further analytical methods (see below). The zooplankton size selection was supported by the evidence that *O. vulgaris* paralarvae feed preferentially upon prey >1 mm (Passarella and Hopkins, 1991; Villanueva, 1994; Villanueva et al., 1996; Iglesias et al., 2006; Roura et al., 2010). The remaining samples were fixed in 70% ethanol and then used to identify the

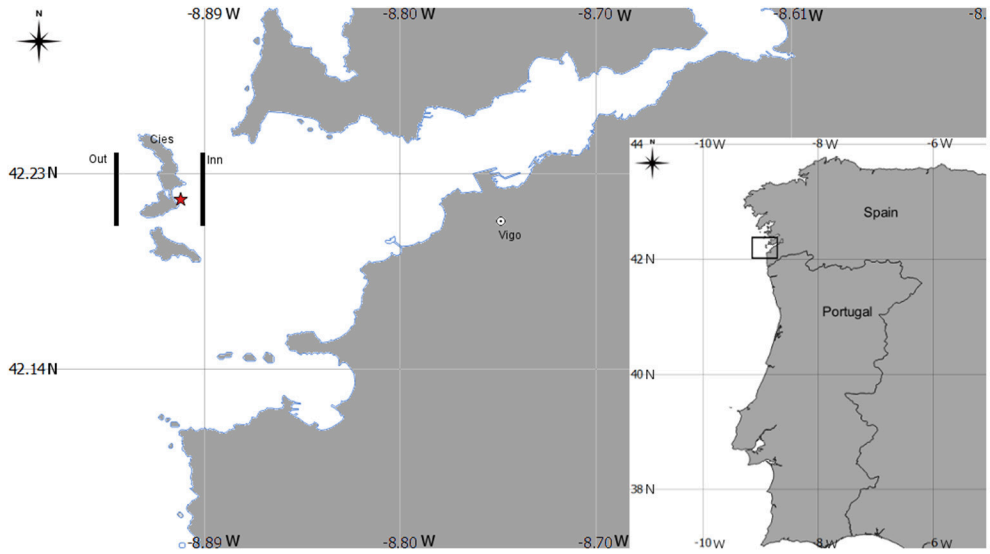

FIGURE 1 | Geographical position of the sampling area in the Ría de Vigo, Spain Northwest Atlantic coast. Legend: lines indicate the sampling transepts at east (inner) and west (out) of Cies Islands. The symbol * indicates the sampling site for the *Octopus vulgaris* paralarvae collected during diving.

mesozooplankton community cohabiting with the paralarvae. Organisms were identified under a binocular (Nikon SMZ800) or inverted microscope (Nikon Eclipse TS100) to the lowest possible taxonomic level. The community (holoplankton/meroplankton) ratio was determined based in the number of species identified and classified as holoplankton or meroplankton accordingly to Roura et al. (2013).

The *O. vulgaris* paralarvae collected ($n = 44$) were pooled in a single sample and stored at −20°C in a Methanol: Dichloromethane (2:1) solution to avoid long-term degradation of lipids.

To determine the basal biochemical profile of *O. vulgaris* paralarvae before external feeding, newly hatched paralarvae (hereafter called hatchlings) were obtained from ripe eggs from a single female batch collected by scuba diving in October 9th 2012 off the Ría de Vigo (site coordinates: 42°14′N, 8° 54′W, **Figure 1**). The hatchlings were pooled and analyzed in duplicate.

Biochemical Methods

Lipids were first extracted from each zooplankton samples and from the single hatchlings with chloroform: methanol (1:2) and after centrifugation, the precipitate was re-extracted with chloroform: methanol (2:1). Both supernatants were subsequently washed with chloroform: methanol: water (8:4:3) as described by Fernández-Reiriz et al. (1989). Total lipids were quantified following the method described by Marsh and Weinstein (1966) with a tripalmitine standard (Sigma Aldrich Inc., Buchs, Switzerland). Wax esters (WAXES), triglycerides (TAG), free fatty acids (FFA), cholesterol (CHL), and phospholipids (PL) content were determined by thin-layer chromatography (TLC)/densitometry. Silica gel 60 W plates (Merck 16486), with a size of 20 × 20 cm and a layer thickness of 0.25 mm, were used. Samples were applied by automatic TLC sampler (Camag 27220). The chromatographic staining was conducted accordingly to Freeman and West (1966). The plates were stained with a 10% CuSO4 solution in 0.85% H_3PO_4 by heating to 180°C (Bitman and Wood, 1982). Standards employed for the quantitative analysis of the WAXES, TAG, FFA, and CHOL were oleyl oleate, triolein, oleic acid, and cod liver oil (CHOL, Sigma), respectively. A standard obtained from *Mytilus galloprovincialis* was used for PL. The plates were scanned with a Shimadzu CS9000 densitometer, using a monochromatic 370 nm beam of 0.4 × 0.4 mm working in the zigzag mode, reading the whole spot, and with automatic autozero for baseline correction. All solvents, reagents and fatty acid standards used in this work were of analytic grade (Merck, Darmstadt, and Sigma). FA content of total lipids fraction of zooplankton, *O. vulgaris* hatchlings and paralarvae was determined converting total lipids into FA methyl esters (FAME), accordingly to the method described by Lepage and Roy (1984). Fatty acids methyl esters (FAME) were analyzed by gas chromatography. Peaks corresponding to FAME were identified by comparison of their retention times with standard mixtures and the concentration of each fatty acid or fatty acid group was expressed as % FAME.

Statistical Analysis

Zooplankton samples were identified according to the correspondent transect (out or inn) and sampling day (d1, d2, and d3) resulting in the following sampling code: Out_d1, out_d2, out_d3, inn_d1, inn_d2, and inn_d3. The zooplankton sample composition, lipid classes and FA content were analyzed using metric multidimensional techniques aiming to identify dissimilarities between groups. Prior to analysis, zooplankton abundance data was transformed log (x+1) and screened to select the taxa that appeared at least in 10% of the samples. Zooplankton dissimilarity matrix was calculated using the

Bray-Curtis dissimilarity index and analyzed with principal coordinate analysis (PCO). The species with highest correlation with the first and second coordinate axes were identified as the potential prey group for the lipid analysis (species highlighted in **Table 1**). The lipid class content and FA with mean concentration higher than 1% FAME were normalized, the similarity matrix was determined using Euclidean distance and analyzed with principal component analysis (PCA) (Zuur et al., 2007). The dimension (axes) eigenvalues and FA scores in each dimension obtained were used to select the FA that explained most of the variance (FA in bold in **Table 2**). The zooplankton species, lipid class and FA groups identified were tested for differences related with sampling area and species composition by non-parametric permutational ANOVA (PERMANOVA) considering type I errors. A constrained canonical analysis (CCA) was applied to the set of zooplankton prey using FA as explanatory variables to identify significant correlations between these FA and the zooplankton species.

Following, the lipid class and FA content were compared between zooplankton, *O. vulgaris* hatchlings and paralarvae applying PCA to determine which lipid classes and FA could differentiate between the groups. The groups identified were tested with PERMANOVA for sample type and sample site to test the significance among groups. CCA was applied to the set of most influential FA, using selected trophic markers as explanatory variables to identify significant differences between the FA profile of prey, hatchlings and paralarvae and the zooplankton species selected. The trophic markers selected were ΣSFA (SFA), ΣMUFA (MUFA), ΣPUFA (PUFA), Σn-6HUFA (n-6), Σn-3HUFA (n-3), SFA/PUFA; n-3/n-6; DHA/ARA; DHA/EPA). The metric multidimensional analysis was conducted applying the "envfit" function of VEGAN package in R (Oksanen et al., 2013).

RESULTS

The species composition of the zooplankton in the Ría de Vigo (**Table 1**) showed a dominance of holoplankton both in the inner zone (67.65%) and the outer zone (83.61%) with the copepods *Paracalanus parvus, Acartia clausii,* and the euphausid *Nyctiphanes couchii* being the most frequent species. The meroplankton species contributed with 32.35% in the inner zone and 16.39% in the outer zone, with the most frequent larvae being bivalves and gastropod larvae and cirripeds, mainly in the inner zone stations. The holo/meroplankton ratio of the zooplanktonic community ranged between 1.77 in the outer zone and 3.14 in the inner zone indicating that the two sampling groups belonged to the same coastal community. The inner and outer zone zooplankton communities presented similar total lipids and lipid class content, only differing in the concentration of a single FA. The FA C18:1n7 is particularly high in the zooplankton community of the inner zone (blue arrow in the **Figure 2**). The correlation results showed that C18:1n7 was highly correlated with zoaea of different crustaceans and cnidarians.

The lipid class composition of *Octopus vulgaris* hatchlings was significantly different of that of the zooplankton community (**Figure 3**). The *O. vulgaris* hatchlings were richer in PL, followed by CHOL and low content in FFA and no TAG and WAXES were detected. In general, hatchlings are richer in FA than zooplankton, in detail, the FA profile of both zooplankton and hatchlings (**Table 2**) showed that, while the two groups had similar content of ΣSFA and ΣPUFA, the zooplankton had higher content of ΣMUFA, particularly in C16:1n7, C18:1n7, and C18:1n9. Despite the similarity in the ΣPUFA, zooplankton samples were richer in EPA, while hatchlings and paralarvae had higher content in ARA and DHA.

PCA showed that the lipid class content allowed to separate zooplankton samples from *O. vulgaris* hatchlings, explaining 95% of the model variation (**Figure 4A**) supported by PERMANOVA, $F = 6.67$, p-value $= 0.025$, 999 perm). The *O. vulgaris* hatchlings were correlated with higher content of CHOL, while the zooplankton samples were correlated with higher content in TAG, FFA, and WAXES (particularly the sample out_d2). Comparing the FA profile of *O. vulgaris* hatchlings with the zooplankton samples, most FA showed different concentrations with exception of C24:0, C22:1n9, C24:1n9, and C18:2n6. Some FA were only identified in *O. vulgaris* hatchlings as the C18:1n9 and C20:2n6, while others were only identified within the zooplankton samples, like C18:3n3 and C20:4n3. The overall FA profile is significantly different when comparing the zooplankton and the *O. vulgaris* hatchlings (PERMANOVA, $F = 139.29$, p-value $= 0.01$, 999 perm). The biplot in **Figure 4B** shows that the first axis explained about 96% of the variation observed and the zooplankton samples were correlated with higher content of short-chain C14:0, C16:1n7, and the family of C18:0. However C18:0 was positively correlated with *O. vulgaris* hatchlings, as well as the long-chain FA C20:1n9, C22:5n3, and ARA, and the MUFA C17:1.

By comparing the zooplankton samples with *O. vulgaris* hatchlings, some differences arose. The trophic markers selected to compare zooplankton with *O. vulgaris* hatchlings showed significant differences between the two groups (blue arrows in **Figure 4B**), particularly MUFA, n-3/n-6, DHA/ EPA, and DHA/ARA. For instance, n-3/n-6 is two times higher in the zooplankton prey (12.74 ± 0.99) than in *O. vulgaris* hatchlings (7.27 ± 2.33), which influences in the same degree the DHA/ARA (zooplankton 9.88 ± 2.36; hatchlings 4.87 ± 2.00).

The FA profile identified in the sample of 40 paralarvae collected in the wild showed that some of the minority FA (<1% FAME) identified in hatchlings were not identified in this older stage (**Table 2**). It is noteworthy that C16:1n7 and C18:1n9 contents were particularly high in the paralarvae in comparison with that of the hatchlings. The ARA content was identical in both, hatchlings and paralarvae, and higher than the zooplankton samples. The DHA content of paralarvae was identical to the zooplankton and lower to that of the hatchlings. EPA content in wild paralarvae was particularly low in comparison with the other groups. Overall, planktonic *O. vulgaris* showed higher concentrations of ΣMUFAs and lower concentrations of ΣPUFAs when compared with hatchlings. The PCA reflected those differences separating the planktonic paralarvae from hatchlings and zooplankton samples, mainly based in the differences in the content of

TABLE 1 | Mesozooplankton community abundance (n/1,000 m^3) and % (in parenthesis).

	species code	out_d1	out_d2	out_d3	inn_d1	inn_d2	inn_d3
HOLOPLANKTON							
Acartia clausi	cop_ac	186 (6.93)	90 (6.52)	288 (10.87)	162 (5.25)	549 (24.05)	468 (18.27)
Calanoides carinatus	**cop_cca**	**72 (2.68)**	**24 (1.74)**	**6 (0.23)**	**24 (0.78)**	**18 (0.79)**	**3 (0.12)**
Calanus helgolandicus	**cop_ch**	**6 (0.22)**	**12 (0.87)**	**12 (0.45)**	**12 (0.39)**	**3 (0.13)**	
Centropages chierchiae	cop_cch	3 (0.11)				3 (0.13)	
***Clausocalanus* spp.**	**cop_cla**	**48 (1.80)**	**97 (0.65)**	**3 (0.11)**	**21 (0.68)**	**9 (0.39)**	
***Corycaeus* spp.**	**cop_cor**		**18 (1.30)**	**3 (0.11)**		**24 (1.05)**	**36 (1.41)**
Paracalanus parvus	cop_pp	624 (23.30)	327 (23.70)	390 (14.72)	183 (5.93)	225 (9.86)	372 (14.52)
Paraeuchaeta hebes	**cop_peh**	**54 (2.01)**	**3 (0.22)**				
Paraeuchaeta sp.	cop_pe	3 (0.11)		6 (0.23)			3 (0.12)
Ctenocalanus vanus	**cop_cv**	**30 (1.12)**	**6 (0.43)**	**24 (0.91)**		**24 (1.05)**	
Pseudocalanus elongatus	cop_pce	6 (0.22)	6 (0.43)	12 (0.45)	24 (0.78)	30 (1.31)	3 (0.12)
Subeucalanus crassus	cop_sec	3 (0.11)		3 (0.11)		9 (0.39)	3 (0.12)
Temora longicornis	**cop_tl**	**21 (0.78)**	**3 (0.22)**	**93 (3.51)**	**57 (1.85)**	**9 (0.39)**	**3 (0.12)**
Candacia armata							3 (0.12)
Oithona plumifera		3 (0.11)	3 (0.22)	12 (0.45)	12 (0.39)	12 (0.52)	6 (0.23)
Oncaea media		21 (0.78)		291 (10.99)	99 (3.21)	6 (0.267)	
Harpacticoida					3 (0.10)	6 (0.26)	3 (0.12)
Copepodid stages	cop	72 (2.68)	42 (3.04)		78 (2.53)	45 (1.97)	6 (0.23)
Evadne nordmanni		306 (11.41)	63 (4.57)	9 (0.34)	831 (26.92)	156 (6.83)	54 (2.11)
Podon intermedius			15 (1.09)		21 (0.68)	42 (1.84)	33 (1.29)
Nyctiphanes couchii calyptopa		51 (1.90)	102 (7.39)	600 (22.65)	144 (4.66)	48 (2.10)	24 (0.94)
Nyctiphanes couchii furcilia	nyc_cou	300 (11.18)	129 (9.35)	99 (3.74)	69 (2.24)	69 (3.02)	222 (8.67)
Mysidacea	mys	3 (0.11)		3 (0.11)			
Cnidaria	**cnid**			**27 (1.02)**	**15 (0.49)**		**81 (3.16)**
Chaetognatha	chaet	108 (4.03)	21 (1.52)	72 (2.72)	66 (2.14)	84 (3.68)	90 (3.51)
Syphonophora	**syph**	**33 (1.23)**		**30 (1.13)**	**96 (3.11)**		
Tunicata	salp	81 (2.93)	12 (0.86)	30 (1.14)	117 (3.65)	63 (2.68)	156 (5.74)
Platyhelminthes							12 (0.47)
MEROPLANKTON							
Amphioxus		3 (0.11)					
Gammaridea					6 (0.19)	3 (0.13)	3 (0.12)
Cirripida cipris	cirripid	57 (2.12)	3 (0.22)		27 (0.87)	15 (0.66)	24 (0.94)
Polichaeta larvae	polich		9 (0.65)	12 (0.45)	6 (0.19)	12 (0.53)	3 (0.12)
Bivalvia larvae		429 (16.00)	381 (27.61)	312 (11.78)	699 (22.64)	582 (25.49)	642 (25.06)
Gastropoda		57 (2.13)	69 (5.00)	117 (4.41)	84 (2.72)	138 (6.04)	243 (9.48)
Ophiuridea larvae		45 (1.68)	3 (0.22)	3 (0.11)	159 (5.15)	6 (0.26)	
Equinoidea larvae					9 (0.29)		
Cirripida nauplius		18 (0.67)	9 (0.65)	171 (6.46)	51 (1.65)	27 (1.18)	21 (0.82)
Brachyura zoeae	brach_zoea	30 (1.13)	6 (0.43)	15 (0.57)	6 (0.19)	24 (1.05)	18 (0.70)
Crangonidae zoeae	crang_zoea					12 (0.53)	
***Paguridae* zoeae**	**pag_zoea**				**3 (0.10)**		**6 (0.23)**
***Palaemonidae* zoeae**	**palam_zoea**			**3 (0.11)**		**6 (0.26)**	**3 (0.12)**
Bryozan larvae		6 (0.22)	12 (0.87)			15 (0.66)	9 (0.35)
***Pisidia longicornis* zoeae**	**p_long_zoea**					**3 (0.13)**	**3 (0.12)**
Porcellana platycheles zoeae	p_platy_zoea	3 (0.11)	3 (0.22)				6 (0.23)
Processidae zoeae	process_zoea				3 (0.10)	6 (0.26)	
Fish eggs				3 (0.11)			
Fish larvae						3 (0.13)	
Holoplankton/Meroplankton		1.91	1.77	1.83	2.56	2.11	3.14

Out_d1, out_d2, out_d3, inn_d1, inn_d2, and inn_d3 represent the zooplankton samples. Zooplankton species with individuals bigger than 1 mm were selected as prey for Octopus vulgaris paralarvae and analyzed for their nutritional profile (identified with a species code). The species in bold were selected for the constrained canonical analysis (CCA).

TABLE 2 | Fatty acid concentration (mean ± SD, % FA) of zooplankton community and *Octopus vulgaris* hatchlings in the Ría de Vigo.

	Mesozooplankton	*O. vulgaris*	
		Hatchlings	Paralarvae
Saturated fatty acids (SFA)			
C14:0	5.46 ± 0.84[a]	2.47 ± 0.17[b]	2.33
C15:0	0.63 ± 0.10[a]	0.33 ± 0.03[b]	0.65
C16:0	18.88 ± 1.21[a]	19.35 ± 1.46[b]	21.97
C17:0	1.35 ± 1.18[a]	1.42 ± 0.11[b]	1.49
C18:0	4.57 ± 0.41[a]	9.96 ± 0.75[b]	10.58
C24:0	0.77 ± 0.11[a]	0.65 ± 0.06[a]	
ΣSFA	31.69 ± 6.97[a]	34.19 ± 2.59[a]	37.02
Monounsaturated fatty acids (MUFA)			
C15:1		0.37 ± 0.04[b]	0.46
C16:1n7	7.18 ± 1.10[a]	0.54 ± 0.05[b]	1.31
C17:1	1.21 ± 0.20[a]	3.29 ± 0.26[b]	3.03
C18:1n7	3.15 ± 0.50[a]	1.67 ± 0.19[b]	1.94
C18:1n9	4.61 ± 0.51[a]	2.82 ± 0.25[b]	7.65
C20:1n9	0.73 ± 0.41[a]	4.09 ± 0.29[b]	4.61
C22:1n9	0.62 ± 0.66[a]	0.90 ± 0.14[a]	
C24:1n9	0.65 ± 0.09[a]	0.52 ± 0.03[a]	0.63
Σ MUFA	19.40 ± 2.41[a]	15.66 ± 1.38[b]	21.10
Poli-unsaturated fatty acids (PUFA)			
C18:2n6	1.78 ± 0.04[a]	0.37 ± 0.02[a]	0.67
C18:4n3	2.61 ± 0.28[a]	0.39 ± 0.03[b]	
C18:3n3	1.41 ± 0.12		
C20:2n6		0.76 ± 0.07	
C20:4n6[1] (ARA)	1.79 ± 0.19[a]	5.32 ± 2.40[b]	5.06
C20:4n3	0.81 ± 0.12		
C20:5n3 (EPA)	22.23 ± 1.51[a]	18.43 ± 1.44[b]	15.40
C22:5n3	0.90 ± 0.05[a]	1.63 ± 0.13[b]	1.78
C22:6n3 (DHA)	17.34 ± 2.69[a]	23.25 ± 1.78[b]	18.97
Σ PUFA	50.17 ± 8.17[a]	50.15 ± 5.86[a]	41.88
Σn-6	3.57 ± 0.16	6.37 ± 1.63	5.73
n-3/n-6	12.74 ± 0.16	7.27 ± 2.33	3.62
DHA/EPA	0.79 ± 0.16	1.26 ± 0.00	1.23
EPA/ARA	12.47 ± 0.76	3.85 ± 1.56	3.04
DHA/ARA	9.88 ± 2.36	4.87 ± 2.00	3.75

[1] *The FA C20:4n6 and FA C20:3n3 have the same retention time, and the concentration of FA C20:4n6 is dominant in marine products, the concentration presented here is representative of C20:4n6.*

Different superscripts indicate significant statistical differences ($p < 0.05$) between mesozooplankton, O. vulgaris hatchlings and paralarvae.

C18:1n9 (**Figure 4C**). PERMANOVA results showed that the FA profile is different between these three groups in terms of both FA identified and FA content ($F = 85.08$, p-value $= 0.004$, 999 perm). The trophic markers (blue arrows in the **Figure 4C** biplot) were highly correlated with axis 1 (95% explained variation), indicating that differences found in these trophic markers ratios were more significant between *O. vulgaris* samples and zooplankton samples than between hatchlings and paralarvae.

DISCUSSION

This study represents the first attempt to analyse the FA contents of *O. vulgaris* paralarvae and that of the zooplankton community where they fed on during the first days of their planktonic life. Moreover, a detailed description of the lipid class composition of wild *O. vulgaris* hatchlings and zooplankton was carried out to understand how they differ. Being aware of the seasonal, regional, and sampling limitations, this study still represents an important snapshot on the nutritional support provided by the zooplanktonic community to the *O. vulgaris* paralarvae. *Octopus vulgaris* paralarvae are lecithotrophic and in the first days of life, their survival depends of the embryonic yolk which nutritional composition is directly influenced by female's diet (Quintana et al., 2015). After some hours or a few days in the water column the paralarvae start to feed, and with 7 days-old (Garrido et al., 2016a) they are able to feed in a large variety of prey from decapod zoaea, krill, fish larvae, cladocerans, copepods, siphonophores, and jellyfish (Roura et al., 2012; Olmos-Pérez et al., 2017). Here, we observed that the FA profiles of wild *O. vulgaris* hatchlings and paralarvae are different from those of the zooplankton. Given that the zooplankton samples analyzed were constituted by numerous phyla, with different FA and lipid class compositions (Dalsgaard et al., 2003), this difference may be the result of the trophic selection displayed by *O. vulgaris* paralarvae (Roura et al., 2016).

The zooplanktonic samples analyzed during this study included a heterogeneous assemblage of organisms dominated by two copepods *Paracalanus parvus* and *Acartia clausi,* the euphausiid *Nyctiphanes couchii,* chaetognaths and small Tunicata. This assemblage was particularly rich in FFA, TAG, and WAXES. The zooplankton accumulates TAG and WAXES, important energy reserves produced by the microalgae during the frequent upwelling events (Lee et al., 2006). The higher content in TAG in these samples is probably related with the presence of meroplankton species in some samples, particularly cirripeds and brachyuran larvae that are known to storage TAG in large lipid globules (Lee et al., 2006), in opposition to the copepod dominated samples richer in WAXES (Lee et al., 1970, 1971).

This zooplankton community was rich in SFA and PUFA because of the dominance of calanoid copepod species. The higher availability of bacteria, detritus, and green algae during autumn may account for the increase of the content in SFA (∼30%) and PUFA (∼49%) (Falk-Petersen et al., 2002; Gonçalves et al., 2012). Moreover, MUFA, particularly C18:1n7 had an important role in the nutritional characterization of the zooplankton (see **Figures 2**, **4B**). Despite the relatively low content in comparison with other FA like C18:1n9, increasing concentrations of C18:1n7 might be related with higher abundance of the meroplankton fraction in the zooplankton samples, which is characteristic of coastal communities (Roura et al., 2013). We suggest that this FA can be used as a trophic marker evaluating the contribution of holoplankton and meroplankton to the *O. vulgaris* paralarvae diet.

Several authors have previously shown that newly hatched paralarvae have low lipid content with relatively high PL and CHOL and very low TAG contents (Navarro and Villanueva, 2000; Reis et al., 2015). In comparison with the zooplankton

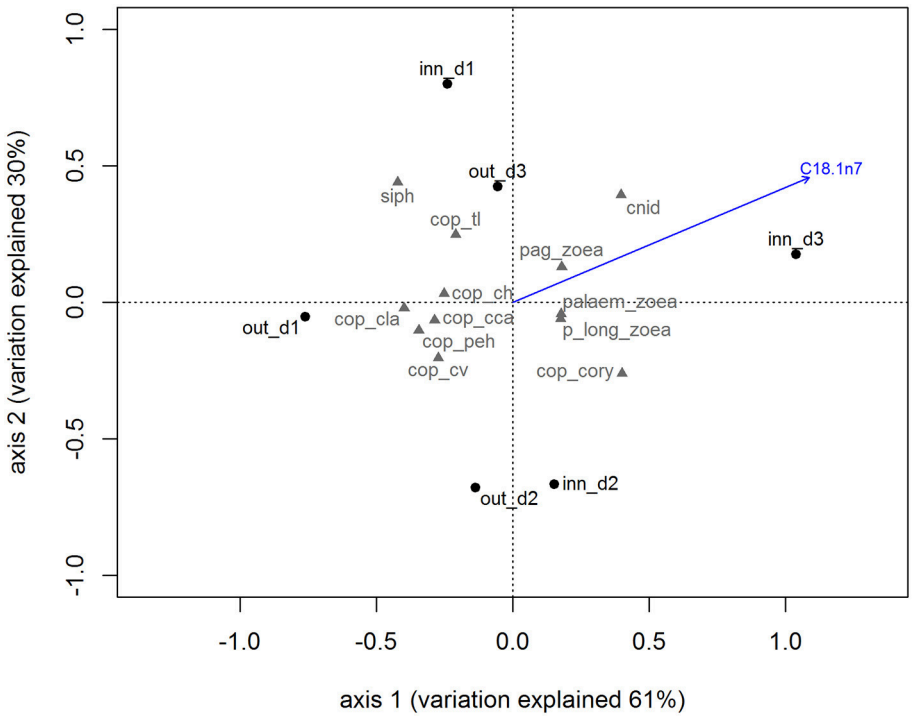

FIGURE 2 | Biplot for principal component analysis of zooplankton community accordingly with sampling site. The blue vector represents the most correlated variable obtained by canonical constrained analysis. Out_d1, out_d2, out_d3, inn_d1, inn_d2, and inn_d3 represent the zooplankton samples scores and the gray codes represent the zooplankton species scores (see **Table 1** for species names).

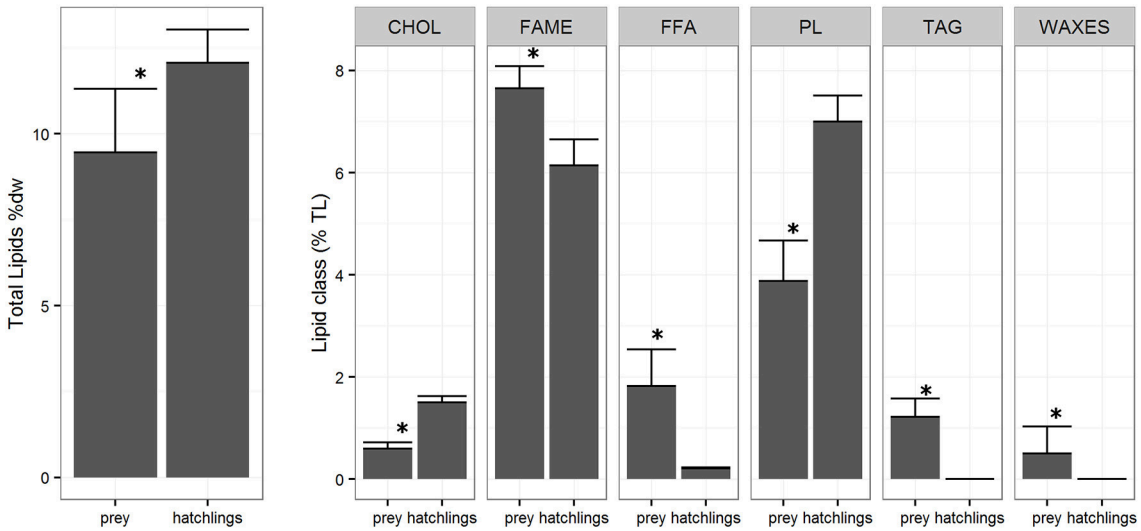

FIGURE 3 | Zooplankton and *Octopus vulgaris* hatchlings total lipids (TL %) (± SD) and lipid classes (% TL) (± SD). *Indicates a significance level of $p < 0.05$ between groups.

samples, the hatchlings sample presented higher content of PL and CHOL, and lower content in FFA. The CHOL and PL, important components of cell membranes, have origin in the maternal reserves (Quintana et al., 2015) explaining their relative high content in the hatchlings. On the other hand, we couldn't detect WAX and TAG in hatchling samples, suggesting a very low content as observed in the work of Navarro and Villanueva (2000). These results show that besides the total lipid contents,

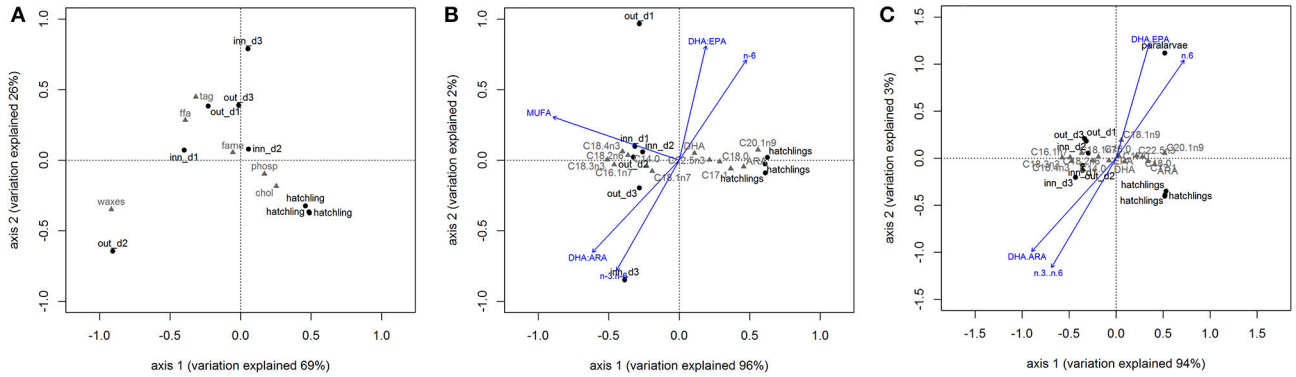

FIGURE 4 | Biplots of principal component analysis of correlation between zooplankton and *Octopus vulgaris* hatchlings based in lipid classes content **(A)** and fatty acid profile **(B)**. Biplot C represents the principal component model comparing the fatty acid profile of zooplankton samples with *Octopus vulgaris* hatchlings and paralarvae. The vectors represent the most correlated trophic markers from canonical constrained analysis. Legend: out_d1, out_d2, out_d3, inn_d1, inn_d2, and inn_d3 represent site score for zooplankton samples, the gray codes represent lipid classes scores **(A)** and fatty acid scores **(B,C)**.

is the proportion and content of some lipids classes that have high relevance for the paralarvae (Navarro et al., 2014; Reis et al., 2015). In this transitional phase, the digestive gland is still developing (Moguel et al., 2010) and is not able to store and digest the neutral lipids as TAG and WAX until 12 days after hatching (Martínez et al., 2011), explaining why, despite being highly energetic nutrients, these lipid classes appear in a very low concentration in the paralarvae. In fact, previous studies on paralarvae nutritional requirements that used *Artemia* as live feed, seem to have produced paralarvae with important shifts from the natural nutritional profile of the paralarvae (including high TAG content), resulting in high paralarvae mortality probably due to the poor essential lipid composition of the *Artemia* (Navarro et al., 2014).

Capturing *O. vulgaris* paralarvae in zooplankton samples is challenging, as it occurs with many other cephalopod paralarvae with pelagic stages (Moreno et al., 2009; Roura et al., 2016). *Octopus vulgaris* paralarvae are among the less abundant meroplanktonic organisms in the zooplanktonic community (Roura et al., 2013; Zaragoza et al., 2015) and it is very difficult to collect high numbers of individuals to conduct biochemical analyses. To overcome this problem, the approach adopted in the present study was to pool all the paralarvae collected in a unique sample, losing individual information. Alternatively, Garrido et al. (2016b) using the same collection method (the multinet sampler) analyzed 10 *O. vulgaris* paralarvae individually, resulting in high variability between individuals. Both approaches are valid, however, some differences arise, particularly in the content of C18:1n9, and DHA with obvious reflection in the content in Σ MUFA and Σ PUFA. To decrease the uncertainty associated to the FA profiles obtained from *O. vulgaris* paralarvae from nature, the sampling approach could be improved by conducting triplicate field samples of pooled paralarvae collected under the same environmental conditions. However, this approach would only be viable by means of increasing the chance of collecting paralarvae. This could be achieve by filtering more water using bongo nets (González et al., 2005; Roura et al., 2016) or by using light traps, which probed to be quite effective in capturing octopod paralarvae off the NW coast of Australia (Jackson et al., 2008).

DHA and C18:1n9 and are essential for *O. vulgaris* paralarvae (Monroig et al., 2013; Reis et al., 2015) and the difference found between this and the study conducted by Garrido et al. (2016b) might be associated with the high variability in mesozooplankton community composition (Roura et al., 2013), together with the variety of prey hunt by the paralarvae (Roura et al., 2012; Olmos-Pérez et al., 2017). High C18:1n9 is common in neutral lipids (e.g. TAG, Viciano et al., 2011) accumulated by decapod zoaea (see **Figure 3**; Letessier et al., 2012), one of the preferential prey of *O. vulgaris*, while DHA is associated with dinoflagellate blooms (Dalsgaard et al., 2003) common during autumn (Crespo et al., 2008) and probably dominated in the plankton community during our sampling season.

In marine larvae, SFA and MUFA are the main substrates to incorporate neutral lipids as TAG to satisfy energy demands, while long chain PUFA are preferentially esterified in structural lipids as the phospholipids in cell membranes (Reis et al., 2015). In this study, the paralarvae had higher content of C16:0, C18:0, C16:1n7, and C18:1n9 than hatchlings. This accumulation in SFA and MUFA was probably related with the diet rich in decapod zoaea and other omnivorous and carnivorous holo and meroplankton rich in TAG, consequently in TAG and MUFA (Dalsgaard et al., 2003; Lee et al., 2006). Moreover, ARA content of the paralarvae was similar to that observed in the hatchlings and significantly higher to the prey. The high ARA content was already observed in the mature ovary of females (Rosa et al., 2004; Lourenço et al., 2014; Estefanell et al., 2015) and in hatchlings collected off the Gran Canaria Island (Estefanell et al., 2013). Reis et al. (2015) proved that ARA is efficiently incorporated by the paralarvae. In fact, exists a competition mechanism of incorporation of ARA and EPA that are esterified by the same enzymes, and it is this mechanism that is responsible of the high variability in the EPA/ARA obtained for paralarvae in different studies ranging between 0.95 (for paralarave fed with *Grapsus*

adcensionis in Reis et al., 2015), 2.7 in Garrido et al. (2016b), and 3.04 in the present study.

Trophic markers are used to follow the interactions between prey and predators in the marine trophic marine web. In this study, CCA results showed that ratio of essential DHA/ ARA, DHA/ EPA, EPA/ ARA, n-3/ n-6, SFA/ PUFA ratios and the content of ΣMUFA and Σn-6 allowed the discrimination between preys and predators (Budge et al., 2006). In this context, it would be expected to find similar trophic ratios between prey (zooplankton) and predators (paralarvae). In fact, only the paralarvae content in ΣMUFA, Σn-6 and the DHA: EPA ratio seemed to follow the prey composition. As occurs with their prey, *O. vulgaris* paralarvae seem to have a lower content in DHA and higher content in ΣMUFA, presenting the same tendency presented by feeding experiments where *O. vulgaris* hatchlings were fed with known prey (Iglesias et al., 2013; Reis et al., 2014). It is noteworthy, that the MUFA C18:1n7 and C18:1n9 showed an increase in relation to hatchlings following the pattern of their prey.

Even though the low number of samples analyzed, we believe that the lipidic profile and trophic ratios determined for *O. vulgaris* hatchlings, paralarvae and their potential prey, allowed a first approach to understand the impact of the available prey pool in the nutritional profile (in terms of lipids) of *O. vulgaris* paralarvae. The impact of feeding in the FA content, particularly C18:1n7, C18:1n9, and DHA is notable, showing that ΣMUFA, DHA/ EPA, and C18:1n7 can potentially be used as trophic markers of the diet of *O. vulgaris* paralarvae in the wild. Further biochemical and physiological studies targeting the neutral and polar lipid reserves of wild paralarvae and their prey will certainly untangle the nutritional deficiencies obtained under culture conditions for *O. vulgaris* paralarvae.

AUTHOR CONTRIBUTIONS

SL: Contibuted with the conception, sampling design of the work, and with acquisition, analysis, and interpretation of data, manuscript drafting and preparation for submission. ÁR: Contributed to the work with acquisition, analysis, and interpretation of the data, with critical revision of the manuscript, and final approval for the paper submission. MF: Contributed to the present work with the samples biochemical analysis and interpretation, and for the manuscript critical revision in all aspects considering the lipidic analysis. LN: Contributed for the work conception, critical revision and final approval of the version to be published. ÁG: Contributed for the work conception, critical revision, and final approval of the version to be published.

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Time Course of Metabolic Capacities in Paralarvae of the Common Octopus, *Octopus vulgaris,* in the First Stages of Life. Searching Biomarkers of Nutritional Imbalance

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The culture of the common octopus (*Octopus vulgaris*) is promising since the species has a relatively short lifecycle, rapid growth, and high food conversion ratios. However, recent attempts at successful paralarvae culture have failed due to slow growth and high mortality rates. Establishing an optimal nutritional regime for the paralarvae seems to be the impeding step in successful culture methods. Gaining a thorough knowledge of food regulation and assimilation is essential for paralarvae survival and longevity under culture conditions. The aim of this study, then, was to elucidate the characteristic metabolic organization of octopus paralarvae throughout an ontogenic period of 12 days post-hatching, as well as assess the effect of diet enrichment with live prey containing abundant marine phospholipids. Our results showed that throughout the ontogenic period studied, an increase in anaerobic metabolism took place largely due to an increased dependence of paralarvae on exogenous food. Our studies showed that this activity was supported by octopine dehydrogenase activity, with a less significant contribution of lactate dehydrogenase activity. Regarding aerobic metabolism, the use of amino acids was maintained for the duration of the experiment. Our studies also showed a significant increase in the rate of oxidation of fatty acids from 6 days after-hatching. A low, although sustained, capacity for *de novo* synthesis of glucose from amino acids and glycerol was also observed. Regardless of the composition of the food, glycerol kinase activity significantly increased a few days prior to a massive mortality event. This could be related to a metabolic imbalance in the redox state responsible for the high mortality. Thus, glycerol kinase might be used as an effective nutritional and welfare biomarker. The studies in this report also revealed the important finding that feeding larvae with phospholipid-enriched *Artemia* improved animal viability and welfare, significantly increasing the rate of survival and growth of paralarvae.

Keywords: ***Octopus vulgaris*, paralarvae, metabolic organization, nutritional imbalance, biomarkers**

INTRODUCTION

Commercial octopus fishing has been exploited for several decades leading to strict regulations on fishing practices. As a result, attempts have been made to culture *Octopus vulgaris* in captivity. These practices remain promising since the species has a relatively short lifecycle, fast growth, and high food conversion ratios. However, under culture conditions, paralarvae of this cephalopod species show slow growth and high mortality rates, reflecting the main obstacle to successful culture. Nutritional deficiencies and food source imbalances are considered the primary causes for low paralarvae survival and growth, although other factors related to zootechnical conditions (such as, tank volume, culture density, and light) cannot be ruled out (Iglesias and Fuentes, 2014).

In terms of nutrition, Lee (1994) highlighted the importance of closely balanced amino acid levels in larval food needed as substrates for metabolism and protein synthesis. On the other hand, Navarro et al. (2014) reported that a nutritional imbalance in both content and profile of the fatty acid in artificial food sources may be responsible for high mortalities since reared paralarvae differ from recently hatched individuals especially in these aspects. Establishing an optimal nutritional regime is of paramount importance for successful thriving of paralarvae and efficient aquaculture. Thus, it is essential to have a thorough knowledge of the physiological processes regulating food assimilation and metabolism.

Regarding metabolic capabilities of cephalopods, studies have shown predominance in protein catabolism, regardless of individual body mass (Boucher-Rodoni and Mangold, 1985; Lee, 1994; Katsanevakis et al., 2005; Petza et al., 2006). This implies a high requirement of protein in available food sources. Additionally, a low capacity to use lipids as metabolic fuel has been reported throughout the cephalopod literature (Ballantyne et al., 1981; Storey and Storey, 1983; O'Dor and Webber, 1986; Lee, 1994; Hochachka, 1995).

After the larval yolk reserves are exhausted, paralarvae depend solely on exogenous food. Octopuses in this stage survive on live prey, which are actively captured using bursts of anaerobic swimming (Baldwin, 1982). In vertebrates, energy needed for early stage activity is supported by the creatine phosphate/creatine kinase ATP regeneration system, followed by the pyruvate fermentation by lactate dehydrogenase (LDH). Interestingly, in mollusks, anaerobic conditions, in addition to the LDH activity, have been shown to produce energy by the arginine phosphate/arginine kinase system in which octopine dehydrogenase (ODH) is involved (Lyzlova and Stefanov, 1991). In many species of mollusks only ODH activity is present; when both ODH and LDH are operating, ODH exhibits the higher activity (Regnouf and van Thoai, 1970; Gäde, 1980; Speers-Roesch et al., 2016). Regarding the advantage of ODH over LDH, Fields and Quinn (1981) reported that ODH maintains a lower cytosolic redox ratio ($NADH/NAD^+$) than LDH during anoxia, where the glycolytic pathway is prevalent.

Although metabolic capacities in adult cephalopods have been assessed in several studies (O'Dor and Wells, 1987; Lee, 1994), such studies have not been reported for early stage larvae. Thus, this report seeks to elucidate the metabolic organization in common octopus paralarvae throughout ontogenic development and to assess the capacity of adaptations to changes in food composition.

MATERIALS AND METHODS

All experimental work was performed according to Spanish law (RD 53/2013) based on the European Union's directive on animal welfare for the protection of animals used for scientific purposes (Directive 2010/63/EU). Guidelines for the care and welfare of cephalopods proposed by Fiorito et al. (2015) were followed in this study. The present study was also approved (register document CEIBA2014-0108) by the Ethics Committee for Animal Research and Welfare (Comité de Ética de la Investigación y Bienestar Animal, CEIBA) from the University of La Laguna (Spain).

Paralarvae Rearing Conditions

Two experiments were carried out to characterize the metabolic profile in common octopus paralarvae. The first experiment analyzed the time course of metabolic capacities during the first 12 days of life. The second experiment analyzed the influence of food composition (with a diet rich in highly unsaturated fatty acids and phospholipids) on metabolic capacities and survival.

To carry out both experiments, a total of 20 adult *Octopus vulgaris* were captured by local fishermen using artisanal octopus traps in Tenerife coastal waters (Canary Islands, Spain) and maintained in the facilities of the Oceanographic Centre of the Canary Islands (Spanish Institute of Oceanography). Adult specimens were kept in 1,000 L tanks (with a maximum density of 10 kg/tank) with water renovation (5 L/min), under oxygen saturation conditions and low light intensity. Two batches of paralarvae obtained from this broodstock were used in the experiment described below.

Experiment 1. Time Course of Metabolic Capacities During the First 12 Days of Life

A total of 15,000 paralarvae, (5,000 paralarvae/tank; 5 paralarvae /L), were reared in triplicate during 12 days in 1,000 L black fiberglass cylinder-conical tanks with a flow-through seawater system at 60 mL/s from 18:00 to 8:00 (over 2.5 renewals/day). The renovation flow allowed the unfed *Artemia* to go through a 500 μm outflow mesh located in the middle of the tank. Moderated flux aeration stones were placed on the edge of the tanks. Green water (1×10^6 cell/mL *Nannochloropsis* sp supplied by Phytobloom Green Formula®, Olhão, Portugal) was added at 8:00. The natural photoperiod was attained at a maximum intensity (around mid-day) of 300 lx. Temperature and oxygen were measured daily, and nitrite, ammonium, and salinity once a week (see **Table 1**). Paralarvae were fed with *Artemia* (Sep-Art BF INVE Aquaculture, Dendermonde, Belgium) enriched for 20 h after hatching with freeze dried *Isochrysis galbana* (supplied by easy algae®, Cádiz, Spain; 10 metanauplii/mL, $1{\cdot}10^7$ cell/mL). *Artemia* was supplied at 0.3 *Artemia*/mL divided in three times a day (at 10:00, 13:00, and 16:00). In order to avoid enrichment lost,

TABLE 1 | Physicochemical parameters for paralarvae reared.

	Experiment 1	Experiment 2
T (° C)	22.3 ± 0.5	22.57 ± 0.1
0_2 (%)	91.8 ± 0.8	94.50 ± 1.0
Salinity (psu)	36.8 ± 0.1	36.8 ± 0.1
pH	8.1 ± 0.0	8.1 ± 0.0
$N0_2^-$ (mg/L)	<0.3	<0.3
NH_4^+ (mg/L)	0.0	0.0

Data are shown as mean ± standard deviation.

Artemia cultures were kept in the dark at 4°C with soft aeration until the moment of feeding.

Experiment 2. Influence of Food Composition on Metabolic Capacities and Survival

A total of 30,000 paralarvae, (5,000 paralarvae per tank; 10 paralarvae/L) were reared over 28 days in 500 L black fiberglass cylinder-conical tanks. Two fluorescent lights (OSRAM Dulux superstar 36W/840) were placed above each tank to attain 700 lx focused in the middle of the tank surface with a 12L:12D photoperiod (8:00–20:00). A flow-through seawater system equipped with 20, 5, and 1 μm filter cartridges and UV lamps was used. A water flow per tank of 1 L/min (over 1.5 renewals/day) was applied from 18:00 to 8:00. In similar protocol to the first experiment, the renovation flow allowed the unfed *Artemia* to go through a 500 μm outflow mesh located in the middle of the tanks. Two moderated flux aeration stones were placed in front each other in the edges of the tanks. Green-water system using 5×10^5 cell/mL of *Nannochloropsis sp* (Phytobloom Green Formula®, Olhão, Portugal) was added to the tanks before illumination. Temperature and oxygen were measured daily, and nitrite, ammonium and salinity once a week (see **Table 1**).

Paralarvae were fed with the same *Artemia* used in the first experiment, but in this case, the control group (C) was enriched with microalgae (freezer dried *Isochrysis galbana*, and *Nannochloropsis sp*) or with an experimental diet of Marine Lecithin LC 60® (PhosphoTech Laboratoires, Saint Herblain, France; LC60). Each treatment was carried out in triplicate. To quantitate *Artemia* size over the duration of the experimental period, three prey sizes were used: first, nauplii from day 0 to 3; second, metanauplii from day 4 to 11; and third, 8 day old metanauplii from day 12 to 27. Enrichments and on-growing of *Artemia* were made according to Garrido et al. (2017). *Artemia* was supplied at 0.5 *Artemia*/mL divided in three times a day (at 10:00, 13:00, and 16:00). In order to avoid enrichment loss, *Artemia* cultures were kept in the dark at 4°C with soft aeration until the moment of feeding.

Growth and Survival

No dry weight was determined in Experiment 1. In the second experiment, 15 individuals' dry weight (DW) was determined for each treatment at day 0, 12, and 28. Paralarvae were euthanized in chilled seawater (−2°C), washed in distilled water, oven dried (110°C, 20 h) and weighed. Specific growth rate (SGR, % DW/day) was calculated as (Ln DWf-Ln DWi) 100/(tf-ti), where DWf and DWi are the dry weight at final time (tf) and initial time (ti) respectively. Survival was assessed in both experiments at the termination of the experiment. Survival (S, %) was calculated as S = 100 Xf/(Xi-Xs), where Xf is the number of live individuals at the end of experiment, Xi is the initial number of individuals and Xs is the number of individuals sacrificed during the experiment.

Sample Collection

Due to the usual mortality in this type of culture and the large size of samples that require analyses, sampling could only be extended up to 12 days. Thus, sample collections in experiment 1 were carried out at 0, 3, 6, 9, and 12 days.

Likewise in the second experiment, samples were taken at 12 days from C and LC groups, and also at 28 days in LC groups since this treatment showed greater survival allowing for a suitable sample size.

Pools of 300 paralarvae per tank were taken in each sampling. Paralarvae were euthanized using ice seawater (−2°C), frozen in liquid nitrogen and stored at −80°C until further analysis.

Enzyme Assays

Pooled paralarvae of each sample were homogenized in four volumes of ice-cold 100 mM Tris-HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8. All procedures were performed on ice. Homogenates were centrifuged at 30,000×g for 30 min at 4°C and the resultant supernatants were kept in aliquots and stored at −80°C for further enzyme assays.

All enzyme assays were performed at 25°C using a PowerWaveX microplate scanning spectrophotometer (Bio-Tek Instruments, Inc., USA) and run in duplicate in 96-well microplates (UVStar Greiner Bio-One, Germany). The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme in each tissue were established by preliminary assays. The millimolar extinction coefficients used for NADH/NADPH and DTNB, were 6.22 and 13.6 mM^{-1} cm^{-1}, respectively.

Activities of fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11), glycerol kinase (GyK; EC 2.7.1.30), pyruvate kinase (PK, EC 2.7.1.40), glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), citrate synthase (CS; EC 4.1.3.7), β-hydroxyacyl CoA dehydrogenase (HOAD; EC 1.1.1.35), glutamate pyruvate transaminase (GPT; EC 2.6.1.2), glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1), and glutamate dehydrogenase (GDH; EC 1.4.1.2) were determined as previously described by Hidalgo et al. (2017). Octopine dehydrogenase (ODH; EC 1.5.1.11), and lactate dehydrogenase (LDH, EC 1.1.1.27) were assayed according to the method of Baldwin and England (1980). See Supplementary Material provided for detailed assay conditions in a final volume of 200 microliters.

Soluble protein concentration in homogenates was analyzed using the method of Bradford (1976), with bovine serum albumin used as standard.

Data Analysis and Statistic

Data were checked for normal distribution with one-sample Kolmogorov-Smirnoff test, as well as for homogeneity of variances with the Levene's test (Zar, 1999) and transformed (natural logarithm) when needed (Fowler et al., 1998). Differences between two groups were assessed by Student's *t*-test. Multiple comparisons in experiment 1 were performed by mean of one-way ANOVA test and Tukey's HSD post hoc test. When normal distribution and/or homoscedasticity were not achieved, data were subjected to Kruskall–Wallis non-parametric test, followed by Games-Howell non-parametric multiple comparison test (Zar, 1999). Statistical significance was established at $P < 0.05$. Statistical analyses were performed using the SPSS package version 15.0 (SPSS Inc., Chicago, USA).

RESULTS

Table 2 shows the time course of metabolic capacities during the first 12 days of life: newly hatched (0), 3, 6, 9, and 12 day old paralarvae (Experiment 1).

The PK (glycolysis), G6PDH (NADPH provision), CS (oxidative metabolism), and GPT (amino acid catabolism) activities did not show significant changes during the period analyzed. Nevertheless, FBPase activity decrease at day 3, to subsequently increase and remain unchanged until the end of the experimental period.

Activity of enzymes involved in anaerobic metabolism revealed that LDH reached maximum activity on day 6, after decreasing to values similar to the 0 day. ODH activity increased progressively from day 3 of life, reaching a significant maximum at 12 days old.

Activity of β-oxidation of fatty acids (HOAD) showed a significant increase from day 6 onwards. Enzymes involved in protein metabolism, specifically, the activity of GOT, showed significant increase from day 3 post-hatching, remaining significantly higher until day 9. However, at day 12 the activity was similar to that of day 0. In turn, GDH activity increased gradually, reaching statistical significance at day 9 post-hatching. Again on day 12, activity significantly decreased.

The HOAD/CS ratio increased since the sixth day forward, and the GPT/CS ratio was higher in newly hatched paralarvae and decreased from the third day onwards (**Figure 1**).

In experiment 2, the effect of feeding with *Artemia* enriched with marine phospholipids on metabolic enzyme activity of hatchlings, 12 day and 28 day old paralarvae was studied. **Table 3** shows the results of dry weight (DW), specific growth rate (SGR), and survival (S) at the hatchling stage, 12 day, and 28 day-old paralarvae fed with *Artemia* enriched with phytoplankton (Control Diet) or *Artemia* enriched with Marine Lecithin (LC diet). The results showed that DW was significantly higher in paralarvae from the LC group compared to the control group at 12 days old ($P < 0.05$). Also, survival in 28 day old paralarvae was significantly higher in the LC group compared to the control group ($P < 0.05$).

Table 4 shows the results of metabolic enzyme activities in newly hatched paralarvae, 12 day old paralarvae fed on microalgae-enriched (12C) and on LC60-enriched (12LC) *Artemia*, and in 28 day old paralarvae fed on LC60-enriched *Artemia* (28LC). Of these activities, ODH, CS, HOAD, and GOT increased significantly in 12 day old paralarvae. The only difference among 12C and 12LC paralarvae was the significantly higher GyK activity in 12C. The 28 day old paralarvae fed on LC60-enriched *Artemia* (28LC) showed significant increases in GyK, PK, G6PDH, LDH, GOT, and GDH activities with respect to 12LC.

DISCUSSION

Time Course of Metabolic Capacities during the First 12 Days of Life

Common octopus paralarvae have a planktonic lifestyle that lasts until the first 30–60 days of life. Remaining yolk reserves provide energy substrates during the first few days post-hatching, however paralarvae immediately begin to actively capture live prey (Iglesias et al., 2007). This simultaneous use of endogenous reserves and exogenous food in cephalopods may last up to several days or weeks (Boletzky and Villanueva, 2014). Specifically, this type of feeding pattern has been shown in the common octopus to last approximately 5 days (Nande et al., 2017). Once the remaining yolk reserves are exhausted, paralarvae depend on active foraging involving episodes of burst swimming under anaerobic conditions (Baldwin, 1982). Our results indicated that both LDH and ODH provide energy to support such episodes of burst swimming during the first days of life. However, by day 9 ODH adopts a predominant role while LDH activity decreases. Therefore, the relative role of both systems in providing energy would be opposite to what has been observed in vertebrates (Lyzlova and Stefanov, 1991). This

TABLE 2 | Ontogenic changes in the activity of key enzymes of intermediary metabolism in *Octopus vulgaris* palarvae.

Day	FBPase	GyK	PK	LDH	ODH	G6PDH	CS	HOAD	GPT	GOT	GDH
0	0.8 ± 0.0^{ab}	49.3 ± 5.0^{b}	126.1 ± 13.2	30.4 ± 0.6^{ab}	46.7 ± 6.9^{a}	8.2 ± 2.6	59.6 ± 7.1	5.0 ± 0.6^{a}	10.5 ± 0.7	201.2 ± 29.9^{a}	17.1 ± 1.2^{a}
3	0.6 ± 0.0^{a}	37.4 ± 8.1^{ab}	108.6 ± 5.8	40.4 ± 1.6^{bc}	41.5 ± 5.0^{a}	11.4 ± 0.9	81.2 ± 1.2	6.5 ± 0.8^{a}	8.3 ± 1.1	272.3 ± 5.8^{b}	21.4 ± 2.0^{a}
6	1.2 ± 0.1^{b}	29.9 ± 1.0^{a}	131.0 ± 8.8	45.0 ± 3.7^{c}	51.6 ± 8.0^{a}	13.2 ± 1.1	74.1 ± 7.2	16.2 ± 4.3^{b}	8.5 ± 0.2	262.1 ± 0.8^{b}	29.9 ± 2.6^{a}
9	1.2 ± 0.2^{b}	37.9 ± 2.2^{ab}	113.0 ± 7.7	34.8 ± 2.1^{abc}	73.9 ± 8.0^{ab}	12.3 ± 1.2	72.3 ± 2.1	13.5 ± 1.5^{b}	10.2 ± 0.4	261.8 ± 12.5^{b}	44.0 ± 3.7^{b}
12	1.1 ± 0.2^{b}	36.8 ± 3.8^{ab}	106.0 ± 4.9	27.2 ± 3.9^{a}	95.2 ± 7.4^{b}	12.4 ± 0.8	67.2 ± 4.5	12.6 ± 0.9^{b}	7.5 ± 0.4	223.1 ± 12.6^{ab}	27.7 ± 3.7^{a}

Values are mean ± S.E. (n = 3). Enzymatic activities are expressed as mU mg protein^{-1}. Different letters in the same column indicate significant differences (P < 0.05).

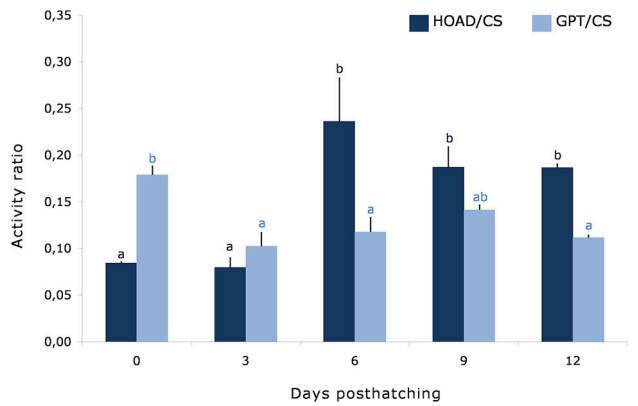

FIGURE 1 | Time course of the ratio between glutamate pyruvate transaminase and citrate synthase (GPT/CS), and β-hydroxyacyl CoA dehydrogenase and citrate synthase (HOAD/CS) activities in octopus paralarvae from Experiment 1. Different superscripts indicate significant differences between sampling points for each parameter ($n = 3$).

TABLE 3 | Dry weight (DW), specific growth rate (SGR), and survival (S) in common octopus paralarvae at hatching and reared for 12 and 28 days with control diet (C, *Artemia* enriched with phytoplankton) or LC diet (*Artemia* enriched with Marine Lecithin).

	Hatchlings	12 days		28 days	
		12C	12LC	28C	28LC
DW (mg)	0.23 ± 0.03	0.30 ± 0.04	0.36 ± 0.06 *	0.50 ± 0.10	0.57 ± 0.16
SGR (%)		2.08	3.59	2.77	3.24
S (%)				1.50 ± 1.28	11.70 ± 3.41*

Data are presented as mean ± SD. n=15 for SGR, n = 3 for S. (*) Indicate significant differences between C and LC groups (P < 0.05).

TABLE 4 | Activity of key enzymes of intermediary metabolism in *Octopus vulgaris* palarvae at hatching (0) and after 12 and 28 days of feeding with control diet (C, *Artemia* enriched with phytoplankton) or LC60 diet (*Artemia* enriched with Marine Lecithin).

	0	C Diet	LC 60 Diet	
		12C	12LC	28LC
FBPase	0.9 ± 0.2	0.6 ± 0.1	0.9 ± 0.1	1.2 ± 0.1
GyK	44.2 ± 1.8	58.5 ± 2.4[#]	40.9 ± 3.5	88.9 ± 5.6[a]
PK	123.9 ± 6.7	133.1 ± 9.3	155.6 ± 7.8	175.9 ± 12.7[a]
LDH	24.7 ± 1.2	23.1 ± 1.3	23.2 ± 1.6	33.1 ± 2.5[a]
ODH	37.2 ± 1.1*	80.9 ± 8.2	78.9 ± 7.7	62.6 ± 8.8
G6PDH	12.2 ± 0.6	12.8 ± 0.1	11.2 ± 1.7	17.8 ± 0.7[a]
CS	53.0 ± 3.3*	87.2 ± 7.5	80.6 ± 5.8	87.4 ± 3.5
HOAD	6.2 ± 0.2*	10.7 ± 1.9	12.0 ± 1.5	14.0 ± 1.0
GPT	8.0 ± 0.4	7.5 ± 0.4	6.7 ± 1.3	10.1 ± 0.9
GOT	232.2 ± 14.4*	292.9 ± 22.4	277.0 ± 1.5	295.9 ± 5.9[a]
GDH	19.6 ± 4.5	19.8 ± 0.6	16.3 ± 2.4	59.8 ± 2.5[a]

Values are mean ± S.E. (n = 3). Enzymatic activities are expressed as mU mg protein^{-1} (*) P < 0.05 vs. 12C and 12LC; (#) P < 0.05 vs. 12LC; (a) P < 0.05 vs. 12LC.

increase in anaerobic activity from day 9 could be due to the depletion of yolk reserves that would force paralarvae to increase their swimming activity for prey capture. Most mollusks only express ODH activity, and in the few cases where LDH is also present, ODH exhibits a higher level of activity (Regnouf and van Thoai, 1970; Gäde, 1980). Additionally, in a recent study on the enzymatic capacities of juvenile cuttlefish, no LDH activity was reported (Speers-Roesch et al., 2016). The coexistence of both pathways indicates that glycogen and arginine phosphate deposits in mantle muscle are used during burst swimming. Sustained PK activity would ensure the provision of pyruvate necessary for both reactions. Regarding the role of ODH, octopine and arginine are among the predominant free amino acids in cephalopod tissue (Lee, 1994). It has been reported that production of octopine might be advantageous in maintaining a low cytosolic redox ratio and, thus, glycolysis (Fields and Quinn, 1981) as well as intracellular osmotic pressure (Fields, 1983). Arginine has been reported to be the most abundant free amino acid in common octopus paralarvae (Villanueva et al., 2004), ensuring adequate levels of arginine phosphate and substrate for ODH. The importance of glycogen deposits in cephalopod muscle have been noted in the literature (O'Dor et al., 1984; Lee, 1994), and confirmed by LDH activity in the present study.

Once episodes of burst swimming ends, it is necessary for paralarvae to restore glycogen and arginine phosphate pools. However, data showing the importance of gluconeogenesis in cephalopods are scarce and contradictory. For example, where some studies indicate that gluconeogenesis occurs in several cephalopod tissues (Ballantyne et al., 1981; Fields and Hochachka, 1982; Hochachka and Fields, 1982), a recent study by Speers-Roesch et al. (2016) reported that *de novo* glucose synthesis is restricted to the digestive gland of juvenile cuttlefish, although the carbon source was unclear. In the present study, after an initial decrease at day 3, a significant increase occurred from day 6 post-hatching. Such increased activity, in parallel to that reported for anaerobic activity, might indicate an increased glycogen use or deposition. Based on the activity of transaminases and GyK in the present study, the substrate for glucose synthesis might be both amino acids and glycerol. It has been amply reported that amino acids are excellent gluconeogenic substrates that are incorporated into glycogen deposits of cephalopod mantle (Hochachka and Fields, 1982). Likewise, transaminases, mainly GOT, are among the enzymes that show higher specific activity in nature. The high GyK activity in hatched paralarvae seems consistent with a high hydrolysis of yolk lipids, resulting in glycerol incorporation into gluconeogenic/glycolytic pathways. Likewise, composition of yolk in cephalopods seems to include approximately 15% lipids (Caamal-Monsreal et al., 2015; Matozzo et al., 2015). Bouchaud and Galois (1990) reported that glyceryl monoesters are abundant in cuttlefish yolk and that probably plays an important role in energy metabolism. The slight decline in GyK activity until the sixth day post-hatching could be due to the depletion of yolk reserves while the subsequent increase may be attributed to greater availability of glycerol derived from catabolism of triacylglycerol present in live prey (Iglesias and Fuentes, 2014). Regarding the use of glycerol as a metabolic

intermediate, overexpression of GAPDH has been reported in 4 day old common octopus paralarvae (Varó et al., 2017), which may be related to a higher rate of incorporation of G3P derived from GyK, to gluconeogenesis/glycolysis. Also, GAPDH would provide the NADH required for ODH and LDH reactions. Therefore, the present results seem to indicate that paralarvae develop metabolic capacities in a short period of time. A poor catabolic capacity would lead to a deficit of energy in the development of burst swimming which is necessary for capturing prey. These results also confirm the necessity of early metabolic capacity to fuel the capture of prey, even when yolk reserves have not been exhausted.

In addition to the energy demands imposed by the capture of prey supported by anaerobic metabolism, paralarvae need a supply of energy for growth, food processing, and maintenance of routine activity. These demands are typically supported by aerobic metabolism. In the present study, CS activity did not show significant changes during the ontogenic period analyzed, although the slight tendency to increase from third day could be related to growth and development processes that impose greater energy needs. GOT activity increased significantly in parallel to the observed changes in CS, indicating a positive correlation between both activities ($r = 0.802$, $P < 0.001$). This would ensure the provision of oxaloacetate necessary for the CS reaction. The data indicate that aspartate (a substrate of GOT) and glutamate, representing almost half of the non-essential amino acids in the body of cephalopods, are relevant (Villanueva et al., 2004). Protein catabolism in cephalopods has been widely accepted as the necessary fuel for aerobic metabolism, regardless of the body mass of the animal (Boucher-Rodoni and Mangold, 1985; Lee, 1994; Katsanevakis et al., 2005; Petza et al., 2006). This would imply a high requirement of this macronutrient in food (Houlihan et al., 1990; Navarro et al., 2014). Sustained GPT and GDH activities would also reflect active protein catabolism during the ontogenic period analyzed. However, the GPT/CS ratio (**Figure 1**) is higher in newly hatched paralarvae, possibly related to the predominance of the catabolism of protein, consisting of the major constituent of cephalopod yolk (Quintana et al., 2015). The reduced GPT/CS ratio from the third day onwards seems to indicate that part of the acetyl CoA incorporated to the CS reaction would come from a source different to amino acids. In this way, both HOAD activity and the HOAD/CS ratio (**Figure 1**) clearly show that since the sixth day forward a significant increase of fatty acid oxidation occurs. It has been widely accepted that cephalopods have a low capacity for lipid metabolism (Ballantyne et al., 1981; Storey and Storey, 1983; O'Dor and Webber, 1986; Lee, 1994; Hochachka, 1995), and as such, the requirement for lipid in food was very low (Seixas, 2009). However, Speers-Roesch et al. (2016) recently reported that cuttlefish efficiently use lipid-based fuels. The present study also shows that, at least during this phase, fatty acids are actively oxidized by common octopus paralarvae. The significant lower HOAD activity detected the first days of life would indicate a predominance of protein catabolism, as described above, probably due to the small proportion of lipid content in yolk and the higher protein content (Quintana et al., 2015).

Influence of Food Composition on Metabolic Capacities and Survival

The main result in this nutritional assay was that the paralarvae that were fed on LC60-enriched *Artemia* showed a 12% survival rate after 28 days, whereas less than 2% of the C group survived before that day. The results for SGR in the present study were similar to those reported previously for *O. vulgaris* paralarvae by Seixas et al. (2010) and Villanueva et al. (2004) using HUFA-enriched *Artemia* as live prey. Conversely, SGR results were lower than those previously found when LC60-enriched *Artemia* was provided (Garrido et al., 2017). However, SGR in both 12 and 28 day old paralarvae were higher in LC groups than in C groups. Thus, these data would indicate that food enrichment provided a beneficial effect on the welfare of paralarvae, although those paralarvae remaining after the 28 day sampling did not survive much longer.

Regarding the possible beneficial effect of food enrichment, in spite of the increased lipid content of LC60-enriched *Artemia*, oxidation rate of fatty acids did not increase in LC group, either at 12 or 28 days. This result may indicate that rather than being used as fuel, the higher amount of lipids could be contributing to the development of the nervous system, essential in such early stages (Nixon and Mangold, 1998). In this sense, the lipid-rich nervous system of *O. vulgaris* paralarvae hatchlings represents approximately one quarter of the animal's fresh weight (Packard and Albergoni, 1970). This may indicate the role of lipids for suitable growth during planktonic life. Increased G6PDH activity observed in 28LC groups would also contribute to such biosynthetic processes. Also, free glycerol derived from triacylglycerol hydrolysis might be used as substrate for glycolysis as indicates by the result of GyK, PK, and LDH at day 28. Increasing lipid levels in food would be not exerting the sparing effect of protein for growth amply reported for fish (Watanabe, 2002). According to Houlihan et al. (1990) and Moltschaniwskyj and Carter (2010), such a sparing effect of protein should give a high rate of protein synthesis and low protein degradation. However, 28 day old paralarvae showed a significant increase in GDH activity. It has been reported that the primary role of GDH in mantle muscle of squid is to regulate the catabolism of amino acids for energy production (Storey et al., 1978). The increased amino acid catabolism, registered in 28LC paralarvae, might be indicative of some kind of nutritional deficiency/imbalance.

In searching for a possible metabolic biomarker, GyK might be considered, since a significant increased activity was observed in both 12C and 28LC groups and a few days later an event of massive mortality took place. Although the role of G3P in cephalopods is controversial (Storey and Hochachka, 1975; Zammit and Newsholme, 1976), this metabolic intermediate can act as a carrier of cytosolic NADH to the mitochondrial electron transport chain. This shuttle activity is thought to coordinate glycolytic and mitochondrial metabolism in highly active tissues and is linked to ROS generation in mammals (Orr et al., 2012). Overproduction of ROS has been reported to exert detrimental effects on aquatic organisms (Abele et al., 2012). Hence, an increased G3P production might be linked to oxidative stress responsible for the low paralarvae survival.

CONCLUSION

In summary, throughout the ontogenic period studied, an increase in anaerobic metabolism takes place, probably related to the increased dependence of paralarvae on exogenous food. Such activity is mainly supported by ODH activity, although LDH activity also contributes to the process. Regarding fuels that support aerobic metabolism, the use of amino acids was maintained and a significant increase in the rate of fatty acid oxidation was observed from the sixth day post-hatching. A low, albeit sustained, capacity for *de novo* synthesis of glucose from amino acids and glycerol was also observed. Feeding of larvae with phospholipid-enriched *Artemia* improved animal welfare, since there was a significant increase in the rate of survival and growth. Regardless of the composition of the food, GyK activity significantly increased a few days prior to a massive mortality event. This data may, in fact, indicate an imbalance in the redox state that could be responsible for the observed high mortality. Thus, GyK might be used as a nutritional and welfare biomarker.

AUTHOR CONTRIBUTIONS

AM Analytical procedures, interpretation of the findings, and design, writing, and revision of the manuscript. GC Analysis, interpretation of the findings, and design, writing, and revision of the manuscript. MH Analysis and interpretation of the findings, and revision of the manuscript. DG and MM octopus paralarvae cultures and execution of the experiments, revision of the manuscript. EA design and execution of experiments, interpretation of results and revision of the manuscript. All authors read and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00427/full#supplementary-material

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Epigenetic DNA Methylation Mediating *Octopus vulgaris* Early Development: Effect of Essential Fatty Acids Enriched Diet

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The common octopus, *Octopus vulgaris*, is a good candidate for aquaculture but a sustainable production is still unviable due to an almost total mortality during the paralarvae stage. DNA methylation regulates gene expression in the eukaryotic genome, and has been shown to exhibit plasticity throughout *O. vulgaris* life cycle, changing profiles from paralarvae to adult stages. This pattern of methylation could be sensitive to small alterations in nutritional and environmental conditions during the species early development, thus impacting on its health, growth and survival. In this sense, a full understanding of the epigenetic mechanisms operating during *O. vulgaris* development would contribute to optimizing the culture conditions for this species. Paralarvae of *O. vulgaris* were cultured over 28 days post-hatching (dph) using two different *Artemia* sp. based diets: control and a long chain polyunsaturated fatty acids (LC-PUFA) enriched diet. The effect of the diets on the paralarvae DNA global methylation was analyzed by Methyl-Sensitive Amplification Polymorphism (MSAP) and global 5-methylcytosine enzyme-linked immunosorbent assay (ELISA) approaches. The analysis of different methylation states over the time revealed a global demethylation phenomena occurring along *O. vulgaris* early development being directly driven by the age of the paralarvae. A gradual decline in methylated loci (hemimethylated, internal cytosine methylated, and hypermethylated) parallel to a progressive gain in non-methylated (NMT) loci toward the later sampling points was verified regardless of the diet provided and demonstrate a pre-established and well-defined demethylation program during its early development, involving a 20% of the MSAP loci. In addition, a differential behavior between diets was also observed at 20 dph, with a LC-PUFA supplementation effect over the methylation profiles. The present results show significant differences on the paralarvae methylation profiles during its development and a diet effect on these changes. It is characterized by a process of demethylation of the genome at the paralarvae stage and the influence of diet to favor this methylation loss.

Keywords: aquaculture, DNA methylation, epigenetic, MSAP, *Octopus vulgaris*, paralarvae

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INTRODUCTION

One of the cephalopod species with a great potential for intensive aquaculture diversification is the common octopus, *Octopus vulgaris*, since it fulfills many of the criteria for this purpose: a short life cycle, fast growth, good food conversion rate, high reproduction performance, fast adaptation to life in captivity, high nutritional value and market price. Unfortunately, while great efforts have been put into raising octopuses in captivity, the sustainable production of this species is still unviable due to the mass mortality during the planktonic paralarvae phase (Vaz-Pires et al., 2004; Iglesias and Fuentes, 2014). Factors, such as: water quality, temperature, light exposure or nutrition; directly influence on growth, health, and ultimately, survival. In addition, octopus paralarvae from distinct geographical origins could have different behavior under similar culturing conditions, suggesting an interaction between paralarvae adaptability to captivity and genotype (Garrido et al., 2017).

Nutrition has been identified as one of the most critical factors influencing octopus paralarvae viability and survival in captivity conditions (Navarro et al., 2014). In contrast to the standard feeding protocols based on live *Artemia* sp. prey, experimental enriched diets supplemented with long chain polyunsaturated fatty acids (LC-PUFAs) and phospholipids have been shown to be beneficial in terms of growth and survival (Guinot et al., 2013a; Garrido et al., 2016a). LC-PUFAs are known to modulate gene expression by provoking local and global effects over DNA methylation in several organisms (see Burdge and Lillycrop, 2014 for review). DNA methylation, the addition of a methyl group to the C-5 position of a cytosine nucleotide by a DNA methyltransferase (Jin et al., 2011), is the most widely studied epigenetic mechanism. Increasing evidence points out DNA methylation as a mechanism with an important role in gene expression regulation in the eukaryotic genome (Wu and Zhang, 2014). Unprogrammed alterations on the methylation profiles triggered by diet and environmental stressors would lead to aberrant gene expression associated with spurious consequences (Faulk and Dolinoy, 2011). This is particularly true during the early development, when DNA methylation is crucial on genomic reprogramming. These early acquired epigenetic landmarks may affect the phenotype, provoke diseases at the adulthood or cause premature mortality (Faulk and Dolinoy, 2011). Moreover, they may persist throughout the entire animal life and even be transmitted to the following generations by genomic imprinting, conditioning their offspring (Feil and Berger, 2007). Considering the direct impact on gene expression and potential heritability, the analysis of methylation profiles should become a valuable tool, if not essential, for biomonitoring the physiological status of cultured specimens in aquaculture (Moghadam et al., 2015).

Although there is wide evidence demonstrating an interaction between epigenetic mechanisms and environment in mammals, research on invertebrates is still ongoing (Sarda et al., 2012). One of the most representative examples of this phenomenon is found in the honeybee *Apis melifera*, with diet-controlled larvae differentiation into either queen or worker casts positively correlating with their brain methylomes (Lyko et al., 2011). Equally, DNA methylation on the crustacean *Daphnia magna* is labile to exposure to toxic pollutants, conditioning fertility and affecting their future offspring by genome imprinting (Vandegehuchte et al., 2009a,b). DNA methylation research in mollusks is scarce and limited to a few species by using methylation-specific restriction enzymes (Petrović et al., 2009; Díaz-Freije et al., 2014; Sun et al., 2014), quantification by LC-MS (Fneich et al., 2013) and ELISA approaches (Rivière et al., 2013) and genome-wide bisulfite sequencing (Gavery and Roberts, 2013). Gavery and Roberts (2010) confirmed the presence of intragenic CpG island methylation in *Crassostrea gigas*, demonstrating a relationship between predicted methylation status and gene expression. Moreover, the availability of *C. gigas* methylome has exemplified the importance of methylation during the molluscan embryo development and in their adaptability to environmental fluctuations (Gavery and Roberts, 2010; Rivière et al., 2013; Rivière, 2014). All these evidences support a conservative role of methylation in invertebrates, presenting a plastic response to environmental changes and allowing the integration of these signals in the genome, as it happens in vertebrates. In fact, previous studies in *O. vulgaris* have highlighted the important role of DNA methylation during the paralarvae period, when major morphological changes take place (Díaz-Freije et al., 2014).

Under the premise that the paralarvae stage should be sensitive to the environment (including rearing conditions and nutritional aspects), monitoring the methylation status of *O. vulgaris* during this life stage will help assessing the impact of the rearing conditions on their development and, ideally, will predict the later outcome of the culture.

In this sense, we focused our attention on DNA methylation in *O. vulgaris* paralarvae fed two different diets commonly used during the rearing of this life stage. First, the global methylation level in paralarvae was examined using an Enzyme-linked immunosorbent assay (ELISA) and then, methylation status changes, associated with early stages of development and diets, were quantified by means of methylation-sensitive amplified polymorphism (MSAP).

MATERIALS AND METHODS

Experimental Design and Diets

Adult octopuses were captured using artisanal traps in Tenerife coastal waters (Canary Islands, Spain) and maintained as a breeding stock in the facilities of the Oceanographic Centre of the Canary Islands (Spanish Institute of Oceanography). Individuals were kept in 1,000 L tanks (with a maximum density of 10 kg/tank) with water renovation (5 L/min), under dissolved oxygen 100% saturation conditions and low light intensity (400 lx on average). Broodstock were fed *ad libitum* with 50% of frozen crab (*Portunus validus*) and 50% of squid (*Loligo opalescens*) every day. PVC shelters were provided as refuges to enrich the environment and induce natural spawning.

Hatchlings were obtained from spontaneous spawning of one adult octopus female (2 kg) kept in captivity. The female was mature at the moment of the capture and after 2 months was paired with only one male (2.4 kg) which was the main contributor to the offspring. A total of 30,000 paralarvae, 6

replicates of 5,000 paralarvae per tank (10 paralarvae/L) were reared during 28 days in 500 L black fiberglass cylinder-conical tanks. Two fluorescent lights (OSRAM Dulux superstar 36 W/840) were placed above each tank to attain 700 lx focused in the middle of the tank surface with a 12L:12D photoperiod (8:00–20:00). A flow-through seawater system equipped with 20, 5, and 1 μm filter cartridges and UV lamps were used. A water flow per tank of 1 L/min (which promoted over 1.5 renewals/day) was applied from 18:00 to 8:00, removing the excess of *Artemia* sp. through a 500 μm outflow mesh located in the middle of the tank. Two moderated flux aeration stones were placed in front each other in the edges of the tanks. The green-water technique was applied, using $5{\cdot}10^5$ cell/mL of *Nannochloropsis* sp. (Phytobloom Green Formula®, Olhão, Portugal) that was added to the tanks before turn on light. Temperature and oxygen were daily checked, while nitrite, ammonium and salinity were verified once a week.

Paralarvae were fed with either *Artemia* sp. (Sep-Art BF, INVE Aquaculture, Dendermonde, Belgium) enriched with microalgae *Isochrysis galbana* (T-Iso) and *Nannochloropsis* sp. (control diet from now onwards) or *Artemia* sp. enriched with Marine Lecithin LC 60® (PhosphoTech Laboratoires, Saint Herblain, France) (enriched diet from now onwards). In order to adapt prey size along the experimental period, three *Artemia* sp. sizes (on-growing at different ages) were used along the experimental period: nauplii from 0 to 3 days post-hatching (dph) paralarvae, 4 days old metanauplii from 4 to 11 dph and 8 days old metanauplii from day 12 to 28 dph. The enrichments and on-growing of the *Artemia* sp. was carried out according to Garrido et al. (2017).

Paralarvae Sampling and DNA Isolation

Paralarvae growth was assessed at 0, 10, 20, and 28 dph. Dry weight (DW) measurements were individually determined as described by Fuentes et al. (2011). Briefly, paralarvae were euthanized in chilled seawater (−1°C), washed in distilled water, oven dried (110°C, 20 h) and weighted. The specific growth rate (SGR, %DW/day) was expressed as: $SGR\left(\frac{\%DW}{day}\right) = \frac{(\ln DW_f - \ln DW_i)\times 100}{(t_f - t_i)}$, where DW_f and DW_i are the DW at final time (t_f) and initial time (t_i), respectively following Garrido et al. (2017) protocol. Dorsal mantle length (DML) measurements were done for each individual with a stereomicroscope (Nikon SMZ-10A. Nikon, Tokyo, Japan) following Villanueva (1995). Survival ($S\%$) was assessed at the end of the experiment as: $S\% = 100 \times \left(\frac{X_f}{(X_i - X_s)}\right)$, where X_f is the number of alive individuals at the end of experiment, X_i is the initial number of individuals and X_s is the number of sacrificed individuals during the experiment. To detect significant changes in terms of these growth parameters an unpaired T-test was performed using the software R.

For DNA methylation analysis, 10 paralarvae were sampled at 0, 10, 20, and 28 dph for each of the two tanks conditions. Larvae were euthanized in chilled seawater (−1°C) and stored in ethanol 100% at −20°C until their analysis. Genomic DNA was individually purified from entire paralarvae using an NZY Tissue gDNA Isolation kit (NZYtech). Subsequently DNA quality and concentration were checked with a Nanodrop-1000 spectrophotometer. DNA extracted samples were adjusted to a final concentration of 100 ng/μL and frozen until use.

All animal experiments were performed in compliance with the Spanish law 65/2013 within the framework of European Union directive on animal welfare (Directive 2010/63/EU) for the protection of animals employed for scientific purposes, following the Guidelines for the care and welfare of cephalopods proposed by Fiorito et al. (2015), and approved by the Ethic Committee of the National Competent Authority.

Global 5-Methylcytosine Levels

A global 5-methylcytosine (ELISA) y (5-mC DNA ELISA Kit, ZYMO) was used as a first attempt to measure in an easy and fast way the patterns of the global DNA methylation levels in octopus paralarvae. DNA from three individuals at two different developmental stages including starting developmental point (0 dph) and also 20 dph for both diets were analyzed. Measurements were tested in duplicates, according to the manufacturer instructions. The optical density at 415 nm was determined after 45 min using an iMark™ Microplate Absorbance Reader, (Bio-Rad). The global DNA methylation levels were expressed in percentages as the mean of the two technical replicates and further analyzed using paired T-tests in the software R.

Methylation Sensitive Amplification Polymorphism (MSAP)

A MSAP protocol, adapted from Reyna-López et al. (1997) was applied to 10 paralarvae per sampling point (0, 10, 20, and 28 dph). Briefly, each DNA sample was digested in parallel reactions with either *Eco*RI/*Hpa*II or *Eco*RI/*Msp*I endonucleases. The obtained DNA fragments were ligated with specific adapters and subjected to two consecutive PCR amplification rounds: a first pre-selective PCR, using an *Hpa*II/*Msp*I+T and *Eco*RI+A primer pair was followed by a second selective PCR with 6-FAM labeled *Hpa*II/*Msp*I+TAG and *Hpa*II/*Msp*I+TCC primers. All reactions were run in a GeneAmp PCR system 9,700 (Applied Biosystems). A detailed protocol of the entire procedure is given in Morán and Pérez-Figueroa (2011). Following AFLP reading on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) restriction profiles were scored using the GeneMapper v.3.7 software (Applied Biosystems).

Methylation Sensitive Amplification Polymorphism (MSAP) profiles were assessed from the resulting absence/presence matrix with the R package MSAP (Pérez-Figueroa, 2013). Loci were categorized as non-methylated (NMT) on specimens amplifying bands for both *Hpa*II and *Msp*I digestions, internal cytosine methylated (ICM), or hemimethylated (HMM) if bands were, respectively present only on either *Msp*I or *Hpa*II, or hypermethylated (HPM) whenever both bands were not present for a given specimen. Loci below a 5% error rate threshold and showing < 2 occurrences of each state were systematically excluded from the analysis. Differences among experimental groups were assessed with a multivariate Principal Coordinates Analysis (PCoA) and Analysis of Molecular Variance (AMOVA).

To further assess whether locus-specific methylation on the paralarvae is dependent on diet and/or age, Fisher's exact tests were used to detect candidate loci among the methyl sensitive

loci (MSL). After statistical adjustment of the resulting *P*-values according to Benjamini and Hochberg false discovery rate (FDR), only loci showing $P < 0.05$ were selected. Estimates of relationships among selected loci were computed by Gower's Coefficient of Similarity and expressed as Euclidean distances. The resulting matrix was clustered by the average linkage method (UPGMA) and visualized as a heatmap matrix with the R "ComplexHeatmap" package (Gu et al., 2016).

RESULTS

Culture Data: Growth and Survival

A total of 30 individual paralarvae were measured at four different sampling points (0, 10, 20, and 28 dph) for each of the two tested diets. **Table 1** shows the DW, DML, SGR, and survival ratio parameters of paralarvae fed with control and enriched diet. The individuals at the starting point (0 dph), showed a DW of 0.23 ± 0.03 mg. This DW increased at 10 dph when the paralarvae were fed with the enriched diet, showing a DW significant higher than the control diet. However, at 28 dph the differences in terms of DW were not significant between the tested feeds. The DML increased from 2.15 ± 0.08 mm at hatching to 2.48 ± 0.35 mm at 28 dph in the control diet group. Concerning the enriched diet group, the DML at 28 dph increased reaching 2.71 ± 0.25 mm. The control diet group showed a survival ratio of 1.47 ± 1.28% at the end of the experiment. For enriched diet the survival ratio increased up to reach 11.7 ± 3.41%.

Global 5 MeC Levels

The 5-mC (ELISA) performed showed that the methylation level of cytosines ranged from 1.21% (minimum values) to 1.24% (maximum value). The methylated cytosine mean level was 1.22 ± 0.07% at the initial stage (0 dph). At 20 dph methylated cytosine mean level was slightly inferior (1.19 ± 0.19%) for larvae fed with the control diet and it slightly increased (1.24 ± 0.25%) for larvae fed with the enriched diet. No differences were found between groups.

MSAP Analyses

The MSAP analyses yielded a total of 297 polymorphic loci when all data from the 70 individuals under study were included (7 groups: 0, 10, 20, and 28 dph for control and enriched diets; 10 individuals per group). Of these, 269 loci were identified as MSL, whereas the remaining 28 ones were non-methyl sensitive (NML). A 100% (28 loci) of the NML were classified as polymorphic whilst the proportion of polymorphic loci reached 70% (188 loci) of the MSL.

The analysis of different methylation states over the time revealed a global demethylation phenomena occurring along *O. vulgaris* early development and directly driven by the age of the paralarvae. A gradual decline in methylated categories (HMM, ICM, HPM) parallel to a progressive gain in NMT loci toward the later sampling points was verified regardless of the diet provided (see **Figure 1**). In fact, the NMT state, representing a 26.12 ± 2.45% of the total at hatching (0 dph), reached maximum values at 28 dph: 37.93 ± 4.08% for the control diet and was even slightly higher, 39.84 ± 3.94%, for the enriched diet group. The AMOVA tests supported a more significant effect of paralarvae development on the methylation profile both in the overall samples ($\Phi_{ST} = 0.1726$, $P < 0.0001$) and separately in the two diets (Control diet: $\Phi_{ST} = 0.1641$, $P < 0.0001$; Enriched diet: $\Phi_{ST} = 0.2155$, $P < 0.0001$). On the other hand, and as expected from the same female and egg spawn paralarvae, AMOVA results on the NML were not statistically significant either when covering all samples ($\Phi_{ST} = 0.01484$, $P = 0.1633$) nor separately for each diet (Control diet: $\Phi_{ST} = 0.02472$, $P = 0.1617$; Enriched diet: $\Phi_{ST} = -0.008439$, $P = 0.8231$), demonstrating the genetic homogeneity among the study samples and thus confirming the validity of the assay.

The PCoA performed with the MSL revealed a grouping of the samples according to this age-driven demethylation process (**Figure 2**). The first principal component, explaining a 19.4% of the observed variation, clearly discriminates between early (0 dph and 10 dph) and late (28 dph) time-points, with samples from the same period clustering together into relatively compact groups. Nevertheless, a differential behavior between diets was observed at 20 dph. At this age, paralarvae subjected to an enriched feeding diet grouped closer to the later samples (28 dph) than to their equivalents for the control diet. A larger intragroup variability was observed at 20 dph in control paralarvae with regards to their LC-PUFA enriched equivalents. This may be interpreted in terms of a slower and/or random loss of epigenetic marks in the absence of enriched diet supply during

TABLE 1 | Data of growth (reported as DW, dry weight; SGR, specific growth rate; DML, dorsal mantle length) and survival ratio (S) of paralarvae reared with control and enriched diet.

	0 dph	10 dph		20 dph		28 dph	
		Enriched diet	Control diet	Enriched diet	Control diet	Enriched diet	Control diet
DW (mg)	0.23 ± 0.03	0.36 ± 0.06*	0.3 ± 0.04*	0.37 ± 0.06	0.33 ± 0.06	0.57 ± 0.16	0.50 ± 0.10
SGR (%)		3.59	2.08	2.26	1.74	3.31	2.82
DML (mm)	2.15 ± 0.08	2.52 ± 0.14	2.3 ± 0.14	2.45 ± 0.19	2.25 ± 0.12	2.71 ± 0.25	2.48 ± 0.35
S (%)						11.7 ± 3.41	1.47 ± 1.28

Differences between control and enriched diet groups at same age were analyzed with an unpaired T-test.
DW, Dry weight; SGR, specific growth rate; DML, dorsal mantle length.
*Data reported with standard deviation. *Indicate significant differences between treatments at the same age (P < 0.05).*

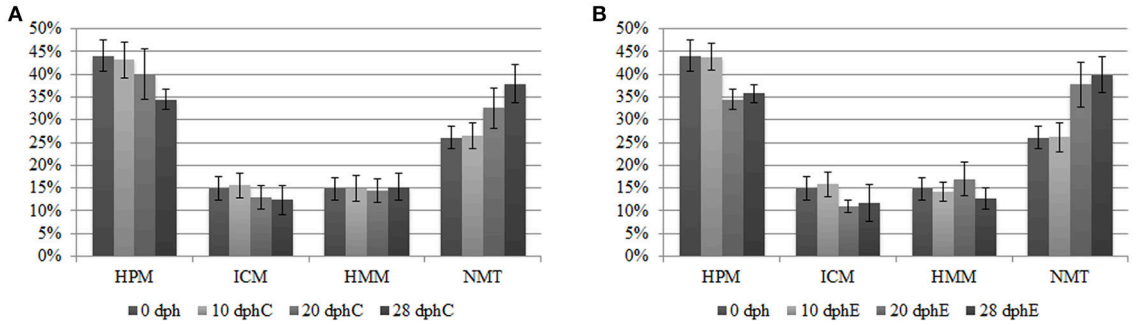

FIGURE 1 | DNA methylation status of methylation-sensitive loci from MSAP (*HpaII/MspI* comparison) in *O. vulgaris* paralarvae (expressed as percentages) measured at 0, 10, 20, and 28 dph. Methylated loci corresponded to categories: ICM, internal cytosine methylated; HMM, hemimethylated; HPM, hypermethylated, and no methylated loci is referred as NMT, non-methylated. Percentages are referred to the total number of polymorphic loci after the error rate filtering. **(A)** Control diet MSAP status. **(B)** Enriched diet MSAP status.

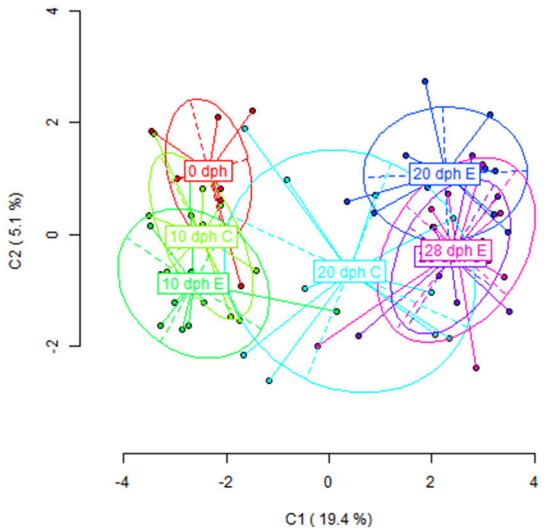

FIGURE 2 | Results from Principal Coordinate Analysis (PCoA) for the *HpaII/MspI* comparison in all age-diet groups of *O. vulgaris* paralarvae (C, control diet; E, enriched diet). The first two axes are shown, indicating the percentage of the global variance explained on the corresponding axis. Points were the representation of the paralarvae individuals and the ellipse delimitates the variance of each group (age + diet).

TABLE 2 | Comparisons of methylation-sensitive loci distribution for both diets and identification of specific loci.

	Φ_{ST} (Pairwise AMOVA)	*P*	#Loci (Fisher test)	#Loci (Fisher test and FDR < 0.05)
CONTROL DIET				
0–10 dph	0.0051	0.3867	2	0
10–20 dph	0.1411	<0.0001***	11	2
20–28 dph	0.0928	0.0022**	10	0
ENRICHED DIET				
0–10 dph	0.1172	0.0005	5	1
10–20 dph	0.3365	<0.0001***	27	15
20–28 dph	0.0637	0.0021**	9	0

Pairwise AMOVA between adjacent ages was analyzing separately for control and enriched diets. Identification of specific loci by Fisher's test and False Discovery Ratio adjust (P < 0.05 and FDR < 0.05).
***Indicate significant differences between treatments at the same age (P < 0.01).*
****Indicate significant differences between treatments at the same age (P < 0.001).*
Φ_{ST} Analysis of molecular variance Φ_{ST} genetic differentiation (Excoffier et al., 1992).
#Number of.

this period. More specifically, pairwise comparisons between adjacent sampling times (**Table 2**) revealed significant differences in DNA methylation profiles only at diet groups the 10–20 dph (Control diet: $\Phi_{ST} = 0.1411$, $P < 0.0001$; Enriched diet: $\Phi_{ST} = 0.3365$, $P < 0.0001$) and 20–28 dph comparisons for both diet groups. Overall, these MSAP results were consistent with a pre-programmed gradual loss of methylation throughout the early development of *O. vulgaris*, having a diet-sensitive period around 20 dph.

The statistical analysis of the different loci by the Fisher Exact test allowed the identification of a total of 51 statistically significant loci (Adjusted $P < 0.05$) among experimental groups. These loci presented a differential distribution of its methylation status mainly between diets at the 10–20 dph comparison (**Table 2**). The result indicates that 15 loci exceeded the FDR cut-off established in the enriched diet compared with the only 2 loci for the same comparison in the control diet. These 2 loci detected in 10–20 dph control diet were also present in the enriched diet group so they were diet independent and correspond to development driven changes. The rest of the loci were identified in the enriched diet group and highlight a specific dietary effect.

The representation of these 51 statistically relevant loci in a heatmap split the samples into two major clusters, discriminating between early (0 and 10 dph) and late (28 dph) developmental stages (**Figure 3**). In a similar way to the previous PCoA on the MSL, samples of 20 dph showed a distinct behavior between diets, with all enriched diet samples grouping with 28 dph paralarvae whilst most control diet samples (9/10) clustered together with 0 and 10 dph samples.

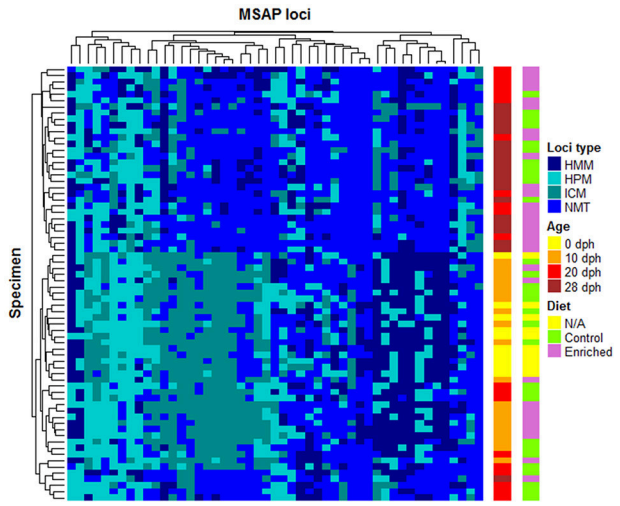

FIGURE 3 | Heatmap of 51 highly differentially methylated MSL of *O. vulgaris* paralarvae. Specimens (rows) and loci (columns) were clustered by the average linkage method. These 51 MSL are the most highly differentially methylated loci identified by Fisher's test ($P < 0.05$). Diet (N/A for 0 dph because have not received diet) and age are shown at right side of the heatmap.

More in detail, the clustering analysis shows that these 51 loci are grouped in 5 clusters (see **Figure 3**). The first four clusters, ordered from left to right, are characterized by losing methylation with the paralarvae development. In the first one, loci showed mainly transitions from HPM to HMM or NMT from the early developmental paralarvae group to the later one. Loci undergoing complete demethylation from ICM in 0 and 10 dph cluster to 28 dph, but also in 20 dph control samples were found on the second cluster, confirming its intermediate status between early and advanced culturing times are found on the second cluster. The third cluster presents a less clear patterning, although it could group those loci losing its HPM status from 0 dph onwards. There was a fourth cluster of loci which change from HMM status at 0 and 10 dph samples to NMT status on later days. Finally, and despite the loss of methylation is associated with development, the fifth and last cluster of the heatmap contained some loci suffering de novo methylation. The latter showed that, even though there is a general pattern of loss of methylation over this developmental period, there is room for certain positions to undergo de novo methylation, although such events can be considered rare.

DISCUSSION

Nowadays the only commercial available prey for *O. vulgaris* paralarvae is *Artemia* sp. However, nutrient enrichments are necessary to improve their nutritional quality. In this regard, LC-PUFA and phospholipid enriched supplements are particularly promising, since they boost the paralarvae viability (Guinot et al., 2013b; Garrido et al., 2016a). Our results show a significant improvement of the survival ratio at 28 dph for the *Artemia* sp. enriched with LC-PUFA and phospholipids respect to control diet. Nonetheless, no significant effect was detected in terms of size and DW at 28 dph between both diets despite the higher values found in the DW of LC-PUFA enriched diet. These results appear to be contrary to the positive effect of LC-PUFA described by Garrido et al. (2016a) in the same species, but it must be considered that the last study was a meta-analysis, which found differences integrating many independent experiments whose results were not often significant. In fact, the variability among studies has been also highlighted by the same authors (Garrido et al., 2017). However, a statistically significant increment of the DW was detected at 10 dph in the enriched diet group (see **Table 1**) suggesting a critical sensitive window where diet could have major effects.

In fish culture, special attention has been taken on lipid and fatty acid requirements due to their essentiality for a correct development and the link between fatty acids and gene expression has already been shown (Tocher, 2010; Xu et al., 2014). However, in *O. vulgaris*, little is known about the molecular basis of the effects of the lipids requirements (Monroig et al., 2013; Reis et al., 2014). For this reason, a better knowledge of octopus genomic regulation mechanisms will be valuable to diet formulation, managing welfare conditions related to culture facilities and to identifying the best conditions to improve breeding programs. To our knowledge, the present research is the first attempt to link diet and methylation in the context of the *O. vulgaris* aquaculture.

The methylation levels herein described for *O. vulgaris* paralarvae using 5-mC (ELISA) (on average 1.2%) are low but in line with those of other mollusks (for example *Biomphalaria glabrate* and *C. gigas*), around 2% (Fneich et al., 2013; Gavery and Roberts, 2013). In contrast to vertebrates, invertebrates present an exceptional variability of how DNA methylation is distributed in their genomes and its function is yet to be completely elucidated. Species of invertebrates like *Caenorhabditis elegans* or dipteran insects like *Drosophila melanogaster* (two model organisms), present an apparent absence of cytosine methylation on their genomes, illustrating that changes in gene expression are independent to DNA methylation (Schübeler, 2015). Nevertheless, DNA methylation in mollusks, such as the oyster *C. gigas*, is likely to vary among life history stages, playing an important role during the embryogenesis of this species and progressively decreasing its levels toward the adult stages, when it no longer exerts a relevant function in the control of gene expression (Rivière, 2014). In fact, previous results on *O. vulgaris* have remarked for an important role of methylation during the earlier development stages (at 1 dph) and having no effect in adults (Díaz-Freije et al., 2014). Our results extend the range of knowledge for this species and demonstrate a pre-established demethylation program during its early development. As methylation levels are low, the 5-mC (ELISA) failed to detect methylation changes in paralarvae either related to age or to diet. However, in this work, the potential of MSAP to analyze methylation patterns in paralarvae have been proved to be highly informative, revealing significant changes in methylation levels, from more methylated DNA of the larvae at hatching to less methylated DNA larvae after 28 days.

Far from being a stochastic phenomenon, our results demonstrate that this loss of methylation follows a well-defined pattern involving a 20% of the MSAP loci, that highlights how some genomic regions are specifically demethylated. Previous research has demonstrated for a pre-defined pattern of active and directed demethylation throughout the early development of vertebrates (Razin and Shemer, 1995; Paranjpe and Veenstra, 2015). The enzymatic machinery behind the methylation process and the changes produced in gene expression are known in detail in vertebrates. Unscheduled alterations provoked by either environmental agents or artificially induced with drugs, such as AZA-5 (5-aza-2′-deoxycytidine) during this period leads to developmental arrest and/or disorders at the adulthood (Rivière et al., 2013). Up to date, octopus DNA methyltransferases have not been described but the presence of cytosine methylation cannot be possible without the enzymatic machinery. In bivalves, these methylation changes correlate with alterations in the expression of *DNMT* orthologue genes, as shown for *C. gigas* and the scallop *Chlamys farreri* (Wang et al., 2014; Lian et al., 2015). In both cases this age-driven demethylation process correlates to low expression levels of the maintenance methyltransferase *DNMT1* and, apparently, undetectable de novo methyltransferases (*DNMT3*). Consequently, this loss of methylation might occur in a passive way, yet preferentially directed toward certain genomic regions.

Although the pattern of methylation loss in octopus paralarvae is mainly age-driven, our results also demonstrate a certain diet influence on the methylation profiles. Indeed, LC-PUFA fed paralarvae showed a premature transition into 28 dph methylation profiles. These results have been demonstrated for a diet-sensitive period in the paralarvae of *O. vulgaris* at around 20 dph and for an effect of LC-PUFA supplementation over the methylation profiles. Despite there are no previous studies correlating this type of dietary supplementation with changes in methylation in invertebrates, the addition of LC-PUFA has shown a global effect over the levels of DNA methylation (Boddicker et al., 2016) and an impact over regulator regions of specific genes (Ma et al., 2016) in vertebrates (well-known in *Mus musculus*, *Rattus norvegicus* and *Sus scrofa*). The implication of fatty acids on the methylome landmark is well-known but the mechanism behind this effect and their targets are still under study (see Burdge and Lillycrop, 2014 for review). Recent studies have started to elucidate this mechanism, correlating the addition of PUFA and changes over the DNA methyltransferase expression patterns (Huang et al., 2016). Thus, in some cases the dietary input of fatty acids finally acts, trough methylation mechanism, over the regulator regions of clue genes in the metabolic homeostasis. The final consequence is a change in the lipid metabolism. The results obtained by (Xu et al., 2014) have special interest since they show how the dietary input of LC-PUFA induce DNA methylation changes which directly affect the main pathway of biosynthesis of these LC-PUFA. These fatty acids are an essential nutritional requirement during *O. vulgaris* early development (Monroig et al., 2013; Reis et al., 2014). In view of the effect of the diet at 20 dph some similar mechanisms may be running in the octopus paralarvae, which could be sensitive to small alterations in nutritional but also environmental conditions during the paralarvae stage, including the digestive tract functionality, and immune system competence, and thus have a great impact on its health, growth and survival.

In vertebrates, the effect over DNA methylation of a diet rich in LC-PUFA and the impact of that over gene expression modulation have been demonstrated. Also, the knowledge of that kind of mechanisms of regulation has promising uses in aquaculture in terms of improve welfare and performance trough diet design. Nevertheless, it remains unknown if this also applies to invertebrates. For other dietary elements and environmental factors, a plastic response during sensitive periods on the early development has been proposed. This mechanism would act as an adaptation mechanism (Saint-Carlier and Rivière, 2015). For instance, in the honeybee, larvae differentiation into either workers or queens is directly dependent on the diet via global methylation changes (Lyko et al., 2011). In the case of *O. vulgaris* paralarvae our results show a dietary effect at 10–20 dph with no changes on loci methylation at 28 dph between control and enrichment diet. Previous studies have shown that differences in lipid composition between cultured paralarvae and their wild equivalents start to be appreciated at around 10 dph (Navarro and Villanueva, 2003; Garrido et al., 2016b). Such differentiation could be due to alterations in the expression of genes regulating lipid metabolism, mediated, among other mechanisms, by DNA methylation as it has been shown for fishes (Xu et al., 2014).

In summary, we have described the existence of an age-driven predetermined demethylation process during the early development of *O. vulgaris*, with a sensitive period to a LC-PUFA supplemented diet at around 20 dph. Enriched diet paralarvae accelerate its transition into latter developmental methylation profiles and show better survival than those verified in the control diet individuals. Nevertheless, more exhaustive studies have to be performed to check the effect of these changes over gene expression patterns with special interest on those genes involved in fatty acid metabolism.

AUTHOR CONTRIBUTIONS

PG performed laboratory experiments, statistical analysis and wrote the manuscript. DG collaborated in laboratory experiments, statistical analysis and manuscript writing. EA performed paralarvae culture and sampling. PM supervised DNA methylation analysis and statistical analysis. CG conceived and designed the study and collaborated in the manuscript writing. All authors helped with the draft of the manuscript and approved the final document.

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Dietary Effect on the Proteome of the Common Octopus (*Octopus vulgaris*) Paralarvae

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Nowadays, the common octopus (*Octopus vulgaris*) culture is hampered by massive mortalities occurring during early life-cycle stages (paralarvae). Despite the causes of the high paralarvae mortality are not yet well-defined and understood, the nutritional stress caused by inadequate diets is pointed out as one of the main factors. In this study, the effects of diet on paralarvae is analyzed through a proteomic approach, to search for novel biomarkers of nutritional stress. A total of 43 proteins showing differential expression in the different conditions studied have been identified. The analysis highlights proteins related with the carbohydrate metabolism: glyceraldehyde-3-phosphate-dedydrogenase (GAPDH), triosephosphate isomerase; other ways of energetic metabolism: $NADP^+$-specific isocitrate dehydrogenase, arginine kinase; detoxification: glutathione-S-transferase (GST); stress: heat shock proteins (HSP70); structural constituent of eye lens: S-crystallin 3; and cytoskeleton: actin, actin-beta/gamma1, beta actin. These results allow defining characteristic proteomes of paralarvae depending on the diet; as well as the use of several of these proteins as novel biomarkers to evaluate their welfare linked to nutritional stress. Notably, the changes of proteins like S-crystallin 3, arginine kinase and NAD^+ specific isocitrate dehydrogenase, may be related to fed vs. starving paralarvae, particularly in the first 4 days of development.

Keywords: ***Octopus vulgaris*, paralarvae, nutritional stress, proteome, novel biomarkers, welfare**

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INTRODUCTION

The common octopus (*Octopus vulgaris*) is one of the most important cephalopod species recommended for European aquaculture diversification due mainly to its rapid growth, elevated food conversion index, and high market demand and price (Navarro and Villanueva, 2000; Vaz-Pires et al., 2004; Iglesias et al., 2007). Nowadays its culture is hampered by massive mortalities (Iglesias and Fuentes, 2014) occurring during early life-cycle stages, (paralarvae), representing the main obstacle for commercial production of this species (Iglesias et al., 2007, 2014; Villanueva and Norman, 2008). In fact, the life cycle of *O. vulgaris* under captivity conditions was completed for the first time in 2001 (Iglesias et al., 2004), but until now, it has not been possible to successfully rear the paralarvae up to juveniles and sub-adults with acceptable survivals for the commercial production

of this species (Rodríguez and Carrasco, 1999; Móxica et al., 2002; Iglesias et al., 2014). The actual causes of such mortality remain unknown, although failure to fulfill the dietary requirements for key nutrients particularly essential lipids appear to be a major factor (Navarro et al., 2014). Thus, substantial efforts have been made to associate dietary lipids to mortality and growth at paralarval stages (Navarro and Villanueva, 2000, 2003; Seixas et al., 2010; Monroig et al., 2012a,b; Navarro et al., 2014), with meta-analysis techniques pointing out clearly at this link (Garrido et al., 2016a). However, the lack of substantial differences in performance between the experimental dietary regimes and control treatments in paralarval cultures fed diets with different essential lipid profiles suggested that other not yet explored factors account for the high mortality mentioned above. Thus, the zootechnical productions may undergo some unspecific stress that adds up to the putative nutritional deficiencies contributing very significantly to reduced paralarval welfare manifested in very low survival and depressed growth.

Like for most early stages of marine fish and crustaceans, feeding is based in the use of live preys. *Artemia* nauplii and metanauplii are extensively used as food for availability reasons, but crustacean zoeae (*Maja*, *Pagurus*, *Grapsus*) seem a more suitable and natural prey from the view point of their nutritional composition, and have been used with some success (Iglesias et al., 2004, 2014; Navarro et al., 2014; Garrido et al., 2016a). Recently, the culture in big volumes (1,000 L tanks) with the use of ongrown *Artemia* biomass using microalgae, like *Nannochloropsis* or *Isochriysis*, as food (Iglesias et al., 2004, 2006; Fuentes et al., 2011) have moderately improved paralarval survival, but still far from industrial scenarios. To the nutritional stress caused by inadequate and/or unbalanced diets (Iglesias et al., 2007; Garrido et al., 2016b), and lack of optimal reproducible culture protocols, it should be added that there is little knowledge on paralarvae physiology and behavior allowing to understand the poor culture performance. In fact, studies on the basic processes involved in nutritional and physiological stress are limited in cephalopod paralarvae. Under such an unmanageable panorama, omics technologies, specifically proteomic approaches come at hand.

Proteins form a major class of macronutrients, because they participate in every cellular process. Therefore, the global profiling of the proteome, defined as the entire protein complement of the genome expressed at a particular time, offers the potential for identification of important biomarkers of nutritional state that respond to alterations in diet. Currently, nutritional research is taking advantage of proteomics technologies to discover biologically active food components, to assess their quality and safety, and to demonstrate their biological efficacy. For example, using proteomics, it was shown that in mice, the consumption of different dietary oils induced either differential expression of long chain acyl-CoA thioester hydrolase protein as an indicator of β-oxidation of fatty acids in the liver, or differential expression of adipophilin protein as an indicator of selective hepatic lipid accumulation and triglyceride secretion (Roos and McArdle, 2008). Proteomics was also applied to explain the mechanisms underlying changes in hepatic lipid metabolism of rats during zinc deficiency (Tom Dieck et al., 2005 cited by Roos and McArdle, 2008). Regarding fish species, zebrafish proteome was altered by calorie restriction (Jury et al., 2008) and rainbow trout proteome was found to reflect dietary manipulations (Martin et al., 2003). Thus, application of proteomics techniques to "map" and read metabolic dysfunctionalities of octopus paralarvae seem a more than promising field.

Up to date, all previous studies of nutritionally-derived stress in paralarvae cultures of the common octopus have been carried out using conventional approaches, testing the effects of a variable on potential (bio)markers of such effects (Garrido et al., 2017). Global protein profiling offers the potential of comparing control vs. treated animals (or the effects of different dietary treatments) beyond the frame of an aprioristic approach, in the search of highlighted differences that can help to establish clues about the metabolic pathways affected. We report here on an experiment analyzing the proteomes of *O. vulgaris* paralarvae, comparing the effect of fasting during the early days of development, as well as the response of two dietary treatments based on either enriched *Artemia* metanauplii or crustacean zoeae as live preys.

MATERIALS AND METHODS

Paralarval Rearing

O. vulgaris paralarvae were obtained from a broodstock kept at the Spanish Institute of Oceanography IEO (Vigo), following the rearing conditions described by Móxica et al. (2002). Paralarvae were raised up in black cylindrical 500 L tanks until 16 days, before massive mortalities start. The initial paralarvae density was 10 individuals L^{-1} (5,000 individuals per tank). A closed water circuit was used during the first 5 days and partly opened (4 h/day) until the end of the experiment. The temperature was kept at 21–23°C, and the salinity at 35 psu. Central aeration and drainage were used for water renovations and surface cleaners based on air pressure were applied. The light intensity in the tank surface was of 800–1,000 lux during 24 h.

Two dietary treatments were tested. *Artemia* group (A) consisted of paralarvae fed *Artemia* nauplii (Sep-Art EG, INVE Aquaculture, Belgium) enriched with the microalgae *Nannochloropsis* sp. and *Isochrysis galbana* at 0.5 individuals mL^{-1} per day. Zoeae group (Z) consisted of paralarvae fed live crustacean zoeae (*Maja brachydactyla*) at 0.01 Maja zoeae mL^{-1} per day in co-feeding with (A). Co-feeding was unavoidable from the evidence that the production of Maja zoeae did not suffice to keep a prey density equivalent to treatment A. Thus, every effort was made to try to keep similar prey densities in both treatments. Also, an unfed paralarvae group, named (I), was kept from hatchling to day 4. *M. brachydactyla* zoeae were produced as described in Iglesias et al. (2014).

Paralarvae dry weight was determined individually after oven drying for 20 h at 110°C as described in Iglesias et al. (2014). Before, animals were sacrificed in chilled seawater (−2°C) and rinsed in distilled water.

Pooled paralarval (5–10) samples were collected from each experimental group at days 0, 4, and 16 for proteomic analysis. The samples were rinsed, frozen in liquid nitrogen and stored

at −80°C until analyzed. The study was exempt from ethics approval, since the zootechnical experiments were performed in 2013 before the Spanish Legislation made it compulsory by established Ethical Committees in the Research Institutions. The experiments were conducted under ethical protocols and recommendations that are nowadays fully compliant with the European directive (2010/63/EU), the Spanish law (RD 53/2013), and the Guidelines for the Care and Welfare of Cephalopods in Research (Fiorito et al., 2015).

2D Differential in Gel Electrophoresis (2D-DIGE): Sample Preparation and Protein Labeling

Proteins from samples were directly extracted in DIGE lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris and 1x complete protease inhibitor EDTA free, Roche) using the 2D grinding kit system (General Electric Healthcare). The solubilized proteins were separated from non-solubilized cellular components by centrifugation (20,000 g × 20 min). Salts and any interfering components were removed using the 2D Clean-up kit (GE Healthcare) and after precipitation, proteins were resolublized in DIGE label buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-pH 8.5). Protein concentration was determined using the Bradford Bio-Rad Protein Assay (RcDc Kit) with bovine serum albumin (BSA) as standard.

Proteins from each experimental group were randomly labeled either with Cy3 or Cy5 following to the manufacturer's instructions (GE Healthcare). Briefly, 50 μg protein of each sample was labeled with 400 pmol CyDye DIGE Fluor minimal Dye by vortexing and incubated on ice in the dark for 60 min. The labeling reaction was stopped with 1 μL of 10 mM lysine followed by incubation on ice for 10 min. An internal standard sample was prepared by pooling 25 μg of protein from each sample, and by labeling by Cy2 as described above. Differentially labeled samples (150 μg total protein) were mixed and 65 mM DTT and 1% ampholytes (pH = 3–10 NL) were added to the mixture before running the first dimension.

Gel Electrophoresis (2D-Dige Gel): Separation and Image Capture

A total of 24 protein samples (6 experimental groups × 4 biological replicates, Supplementary Table 1) were combined in pairs, and analyzed on a total of 12 2D gels following the experimental design given in Supplementary Table 2. 24 cm- IPG strips (pH = 3–11 NL) were rehydrated in 8 M urea, 4% CHAPS, DeStreak (12 μL mL^{-1}), and 1% ampholytes (pH = 3–10 NL) overnight at room temperature. Cy-labeled samples were applied onto IPG rehydrated strips via anodic cup loading, and IEF (first dimension) was performed on a Ettan IPGphor II horizontal electrophoresis system (GE Healthcare) at 20°C using the following IEF protocol: step 1:300 V 4 h, gradient to 1,000 V 6 h, gradient to 8,000 V 3 h; step 2: 8,000 V until reached 32,000 V h.

After IEF, the strips were reduced in equilibration buffer [Tris 50 mM, urea 6 M, and glycerol 30% (v/v), 2% SDS (w/v)] containing 2% DTT (w/v), for 15 min at room temperature; followed by alkylation in equilibration buffer containing 2.5% (w/v) iodoacetamide, for 15 min at room temperature. The proteins were then separated (second dimension) on 12.5% acrylamide SDS-PAGE gels (25 cm × 21 cm × 1 mm) using an Ettan DaltSix Unit (GE Healthcare) electrophoresis system at 2 W per gel for 1 h and 15 W per gel for 6 h.

After electrophoresis, the 2-D gels were scanned with a Typhoon™ 9400 Variable Mode Imager, at 100 μm resolution to visualize the labeled proteins. Excitation/emission wavelengths were chosen specifically for Cy2, Cy3, and Cy5 according to manufacturer's recommendations (GE Healthcare).

Data Analysis

Fluorescence images were analyzed using DeCyder™ 2D software (v.7.0) and the multivariate statistical module EDA (Extended Data Analyses; GE, Healthcare), as described in Varó et al. (2010). First, the intra-gel images were individually processed by DeCyder-DIA (Differential In-gel Analyses) software module to co-detect and differentially quantify the protein spots in the images, taking the internal standard as reference to normalize the data, and with the threshold set to 2 standard deviations. Thereafter, the DeCyder-BVA (Biological Variation Analysis) was applied to inter-gel matching, and differences in average ratios of protein expression were analyzed by the Student's *t*- test and One-Way ANOVA, with $p \leq 0.05$ being considered significant. Finally, EDA software was used for multivariate statistical analysis of data. Principal components analyses (PCA) were carried out using an algorithm included in the EDA software, incorporating only data from proteins present in at least 90% of the spot maps and applying a *t*-test filter ($p \leq 0.05$).

Mass Spectrometry

Protein spots showing significantly altered expression levels between groups were manually excised from analytical silver stained gels and digested with sequencing grade trypsin (Promega) as described elsewhere (Shevchenko et al., 1996). The digestion was stopped with 0.1% TFA (trifluoroacetic acid, Sigma) and the digested peptides were concentrated to 7 μL. BSA plug was analyzed in the same way to control the digestion process.

Digested samples were subjected to PMF-MS/MS (MALDI-TOF-TOF) and/or LC-MS/MS analyses.

PMF-MS/MS (MALDI-TOF-TOF) Analysis

Previously, MALDI plate and the acquisition methods were calibrated with 0.5 μL of the CM5 calibration mixture (ABSciex) in 13 positions. The resulting mixtures were analyzed in a 5800 MALDI- TOF-TOF (ABSciex) in positive reflection mode (3,000 shots in every position). Five of the most intense precursors (according to the threshold criteria: minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap: 200 ppm, maximum fraction gap: 4) were selected for every position for the MS/MS analysis, and data was acquired using the default 1 kV MS/MS method. The MS and MS/MS information was sent to MASCOT via the Protein Pilot (v 4.5 ABSciex) to be identified.

LC-MS/MS Analysis

Protein spots without a positive identification were analyzed by LC-MS/MS. 5 μL of each sample were loaded onto a trap column (NanoLC Colum, 3 μ C18-CL, 350 μm × 0.5 mm; Eksigen) and desalted with 0.1% TFA at 3 μL/min for 5 min. Then, peptides were loaded onto an analytical column (LC Column, 3 μm C18-CL, 75 μm × 12 cm, Nikkyo) and equilibrated in 5% acetonitrile (ACN), 0.1% formic acid (FA). The peptide elution was carried out with a linear gradient of 5–45% B in A for 15 min (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nL/min. Peptides were analyzed in a nanoESI qQTOF mass spectrometer 5600 Triple TOF (ABSciex). The tripleTOF was operated in information-dependent acquisition mode, followed by 0.05-s product ion scan from 100 to 1,500 m/z on the 50 most intensive 2–5 charged ions. LC-MS/MS information was analyzed using Protein Pilot search engine software (v.4.5; ABSciex).

Protein Identification

The PMF search was performed on NCBI databases. Searches were done with tryptic specificity allowing one missed cleavage and a tolerance on the mass measurement of 100 ppm in MS mode and 0.8 Da for MS/MS mode. Carbamidomethylation of Cys was used as a fixed modification and oxidation of Met and deamidation of Asn and Gln as variable modifications.

For LC-MS/MS data Protein Pilot search engine software (v.4.5 ABSciex) was used. Protein Pilot default parameters were used to generate a peak list directly from 5600 TripleTof wiff files. The Paragon algorithm of Protein Pilot was used to search NCBI protein database with the following parameters: trypsin specificity, iodoacetamidecys-alkylation, no taxonomy restriction, and the search effort set to rapid.

For ESTs identifications, the MGF (mascot generic file) generated by Protein Pilot were sent to MASCOT via the Deamon software (Matrix Science). Database search was performed on ESTs database: EST_cephalopoda cephalopoda_140224 (684204 sequences; 143385132 residues). Searches were done with tryptic specificity allowing one missed cleavage and a tolerance on the mass measurement of 50 ppm in MS mode and 0.6 Da in MSMS mode. Carbamidomethylation of Cys was used as a fixed modification and oxidation of Met and deamidation of Asn and Gln as variable modifications.

RESULTS

Dry Weight

The dry weight of paralarvae at hatching was 0.333 ± 0.080 mg. At 4 days unfed paralarvae decreased their weight to 0.177 ± 0.070 mg, whereas values of 0.411 ± 0.014 mg and 0.367 ± 0.033 mg were reached by Z and A groups. At 16 days, these raised again to 0.713 ± 0.062 mg and 0.621 ± 0.051 mg respectively.

DIGE Analyses and Protein Identification

The 12 gel images were subsequently analyzed using the DeCyder-DIA and BVA modules comparing the proteome of the 4 different conditions considered important as function of dietary group and age (Supplementary Table 3). Representative 2D-DIGE gels (gel master) of octopus paralarvae proteins from condition 1, comparing unfed 4 days old paralarvae (I4) with hatchlings (I0), is shown in **Figure 1**. A total of 4,507 proteins were detected in the gel master over the range of pH 3–11 NL and a molecular weight from approximately 10–250 kDa used in this study. BVA analyses revealed in condition 1 (I4 vs. I0), a total of 23 spots of proteins that showed significant changes in expression related with age (t-test, $p \leq 0.05$). Among these protein spots, 11 were up regulated and 12 down regulated in the older unfed paralarvae (I4). From this set of proteins, it was possible to identify 16 proteins using mass spectrometry (Supplementary Tables 4–6). When fed and unfed 4 days old paralarvae (condition 2: A4, Z4 vs. I4) were compared, 24 proteins (22 up regulated and 2 down regulated) presented differential expression in fed paralarvae (one-way ANOVA, $p \leq 0.05$). Of these, 15 protein spots were identified by mass spectrometry. In condition 3 (Z4 vs. A4) and 4 (Z16 vs. A16), where 4 and 16 days old paralarvae of both dietary groups were compared, a total of 10 (8 up regulated and 2 down regulated) and 7 protein spots (3 up regulated and 4 down regulated) respectively showed significant differences in expression (t-test, $p \leq 0.05$) between zoeae and *Artemia* dietary groups. A total of 6 protein spots were identified in each condition by mass spectrometry. The 2D-DIGE analyses (spot no., p-value, fold change) and the results of protein identities differentially expressed in each condition are given in **Table 1**. Principal component analyses (PCA) and hierarchical cluster analyses of data for each condition are shown in **Figures 2–5**. The PCA results revealed a good separation of paralarvae groups from a specific sub-set of significant protein spots as function of diet and age in each condition. The dendogram after hierarchical cluster analyses also show a good separation of the spot maps as function of diet and age in each condition.

A total of 55 spots that showed significant differences in the experimental groups after gel image analyses, and with enough quantity of protein to be manually excided from analytical gels, were selected for protein identification by MS. From these, 43 protein spots were successfully identified in public databases. Different protein spots were identified as the same protein due post-translational modifications. Some of the identified proteins were found to be involved in several metabolic pathways, related with carbohydrate metabolism [glyceraldehyde-3-phosphate-dedydrogenase (GAPDH), triosephosphate isomerase], and other pathways of energetic metabolism (NADP+-specific isocitrate dehydrogenase and arginine kinase). Other identified proteins were related with detoxification [glutathione-S-transferase (GST)], stress [heat shock proteins (HSP70)], structural constituent of eye lens (S-crystallin 3), and cytoskeleton (actin, actin-beta/gamma1, and beta actin). **Table 2** summarizes the main changes in expression of the above-mentioned proteins in the different conditions studied as function of dietary group and age. It is interesting to note the up-regulation of GAPDH, a key protein in carbohydrate metabolism, in all conditions, with the 16 days old zoeae fed group (Z16, condition 4) showed the highest fold change (3.21-fold). In condition 1, unfed paralarvae (I4) presented 2 proteins involved in carbohydrate metabolism,

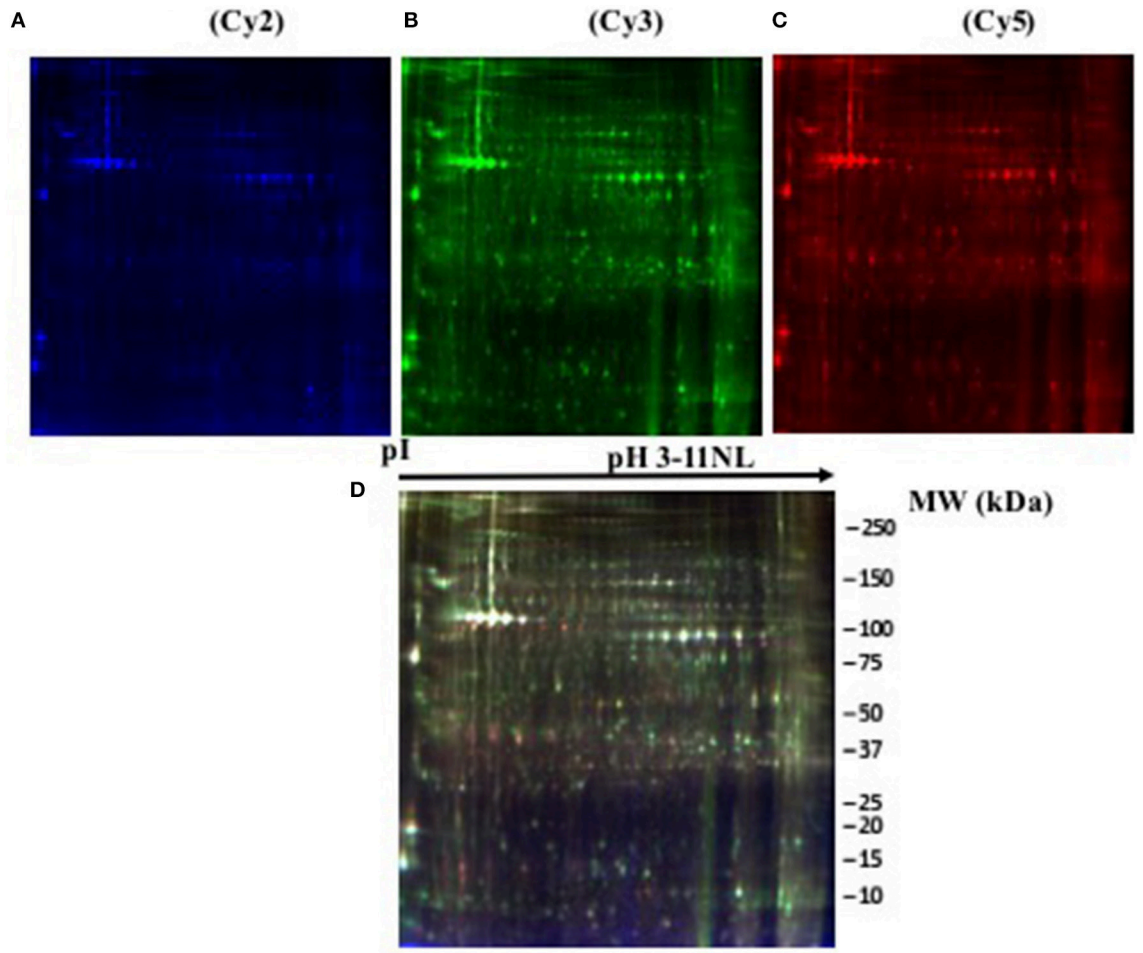

FIGURE 1 | Representative two-dimensional differential in gel electrophoresis (2D-DIGE) of *Octopus vulgaris* paralarvae proteins corresponding to condition 1. Gel was scanned to obtain single images corresponding to: **(A)** (Cy2, blue), **(B)** (Cy3, green), **(C)** (Cy5, red) labelled samples, and **(D)** (overlay gel image of the three dyes). Details in section 2D Differential in Gel Electrophoresis (2D-DIGE): Sample Preparation and Protein Labeling.

one of which was up regulated (GAPDH), whereas another (triosephosphate isomerase) was down regulated. Other 2 up regulated proteins were associated with vision function (S-crystallin 3, by over ∼2-fold) and cytoskeletal structure (beta actin, increased 1.35-fold). Finally, another 2 down regulated (by over ∼2-fold) proteins were related with stress (HSP70) and detoxification (GST). Four days old fed paralarvae (A4 and Z4, condition 2) showed an increase in abundance in all the proteins identified, except for the spot 1,090 (hypothetical protein LOTGIDRAFT_231565), which decreased by ∼1-fold. Also, it is to note that in fed paralarvae groups there was overexpression of proteins involved in energy metabolism (arginine kinase, 19.5-fold) and NADP+-specific isocitrate dehydrogenase, 7.72-fold) and vision (S-crystallin 3, 8.44-fold) compared with unfed paralarvae group (I4). Moreover, an increase in a protein involved in nitrogen metabolism (nitrilase, 4.25-fold), and in two isoforms of actin (actin, 3.76-fold and actin beta/gamma I, 2.25-fold) was identified in fed paralarvae groups. When both dietary groups were compared (condition 3 and 4), the common proteins identified with differential expression, showed also similar changes in abundance. Interestingly, most of the proteins showing significant changes were up regulated in 4 days old paralarvae, while the opposite occurs in 16 days old paralarvae. It is to note that in Z4 group (condition 3), two of the three proteins involved in energy metabolism (GAPHD and arginine kinase) increased, whereas the third (NADP+-specific isocitrate dehydrogenase) decreased. S-crystallin3 decreased only (2.27-fold) in Z16 group (condition 4).

DISCUSSION

Common practice in *O. vulgaris* paralarval rearing is to use spawns from single females. This is practically unavoidable because *O. vulgaris* is a semelparous species, and under the current state of the art, broodstock has to be captured from the wild, each with a different feeding and maturation background. Once captured, each female spawns at different times, and the

TABLE 1 | Protein identities differentially expressed in *Octopus vulgaris* paralarvae in the different conditions studied as function of dietary group and age.

Condition	Spot[a] no.	*P*-value[b]	Fold change[c]	Accession ID[d]	Protein name	Biological process	Species name for which protein was identified
1 (I4 vs. I0)							
Up-regulated	2,535	0.0042	2.06	gi|268376225	–	–	–
	2,619	0.0190	2.01	gi|84447377	–	–	–
	2,668	0.0034	1.94	gi|2495111	S-crystallin 3	Structural constituent of eye lens	*Octopus vulgaris*
	2,711	0.0250	1.83	gi|84417280	–	–	–
	1,802	0.0290	1.71	gi|149688630	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Carbohydrate metabolism	*Octopus vulgaris*
	2,135	0.0074	1.60	gi|380324113	–	–	–
	1,229	0.0360	1.35	gi|9049272	Beta actin	ATP-binding	*Carassius auratus*
Down-regulated	912	0.0064	−1.20	gi|156407978	Predicted protein	–	*Nematostella vectensis*
	2,803	0.0170	−1.23	gi|84425674	–	–	–
	1,601	0.0003	−1.32	gi|342655056	–	–	–
	2,438	0.0110	−1.97	gi|378919614	–	–	–
	596	0.0091	−2.08	gi|158147451	Heat shock protein 70 kDa	Response to stress	*Segonzacia mesatlantica*
	1,645	0.0068	−2.20	gi|342662620	–	–	–
	2,600	0.0030	−2.23	gi|554913552	Triosephosphate isomerase	Carbohydrate metabolism	*Moniliophthora roreri* (MCA 2997)
	2,574	0.0280	−2.28	gi|565322954	Glutathione S-transferase 2	Detoxification	*Ophiophagus hannah*
	950	0.0350	−2.54	gi|342662199	–	–	–
2 (A4, Z4 vs. **I4)**							
Up-regulated	2,742	0.00000021	19.50	gi|340742817	Arginine kinase	Energetic metabolism	*Amphioctopus fangsiao*
	2,592	0.0000056	11.96	gi|378905056	FO174848 whole embryos embryonic stages 16–28. cDNA clone ADY0AAA20YI14	–	*Sepia officinalis*
	2,745	0.00033	8.91	gi|268376271	gb|GT617746.1|GT617746	–	–
	2,571	0.0000099	8.81	gi|84443490	–	–	–
	3,059	0.00002	8.44	gi|2495111	S-crystallin 3	Structural constituent of eye lens	*Octopus vulgaris*
	3,098	0.0000041	7.72	gi|501292852	$NADP^+$-specific isocitrate dehydrogenase	Energetic metabolism	*Riptortus pedestris*
	2,724	0.0005	4.29	gi|342650012	–	–	–
	2,558	0.00014	4.25	gi|518224143	Nitrilase	Nitrogen compound metabolism	*Bacillus endophyticus*
	3,050	0.000019	3.76	gi|508701	Actin	ATP-binding	*Cryptococcus neoformans* var. *grubii*
	1,827	0.00023	3.58	gi|391324898	Predicted: Actin, cytoplasmic 2-like isoform	ATP-binding	*Metaseiulus occidentalis*
	1,897	0.001	2.25	gi|350035483	Actin beta/gamma 1	ATP-binding	*Clonorchis sinensis*
	2,166	0.018	1.56	gi|149688630	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Carbohydrate metabolism	*Octopus vulgaris*
	1,889	0.043	1.30	gi|350035483	Actin beta/gamma 1	ATP-binding	*Clonorchis sinensis*
	2,151	0.019	1.04	gi|149688630	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Carbohydrate metabolism	*Octopus vulgaris*
Down-regulated	1,092	0.00033	−1.10	gi|556108726	hypothetical protein LOTGIDRAFT_231565	–	*Lottia gigantea*
3 (Z4 vs. **A4)**							
Up-regulated	2,742	0.0056	2.66	gi|340742817	Arginine kinase	Energy metabolism	*Amphioctopus fangsiao*
	1,092	0.001	2.25	gi|556108726	hypothetical protein LOTGIDRAFT_231565	–	*Lottia gigantea*
	2,166	0.033	2.06	gi|149688630	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Carbohydrate metabolism	*Octopus vulgaris*

(Continued)

TABLE 1 | Continued

Condition	Spot[a] no.	*P*-value[b]	Fold change[c]	Accession ID[d]	Protein name	Biological process	Species name for which protein was identified
	2,151	0.0066	2.05	gi|149688630	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Carbohydrate metabolism	*Octopus vulgaris*
	1,889	0.023	1.52	gi|350035483	Actin beta/gamma 1	ATP-binding	*Clonorchis sinensis*
Down-regulated	2,558	0.01	−1.73	gi|518224143	Nitrilase	Nitrogen compound metabolism	*Bacillus endophyticus*
4 (Z16 vs. A16)							
Up-regulated	2,151	0.013	3.21	gi|149688630	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Carbohydrate metabolism	*Octopus vulgaris*
Down-regulated	2,724	0.041	−1.15	gi|342650012	–	–	–
	3,059	0.011	−2.27	gi|2495111	S-crystallin 3	Structural constituent of eye lens	*Octopus vulgaris*
	1,827	0.037	−2.41	gi|391324898	Predicted: Actin, cytoplasmic 2-like isoform	ATP-binding	*Metaseiulus occidentalis*
	2,558	0.021	−2.65	gi|518224143	Nitrilase	Nitrogen compound metabolism	*Bacillus endophyticus*
	3,098	0.017	−2.85	gi|501292852	NADP-specific isocitrate dehydrogenase	Energetic metabolism	*Riptortus pedestris*

[a]*Spot numbering.*
[b]*Student t-test P-value.*
[c]*Average fold change ratio in each condition as calculated by DeCyder BVA analysis.*
[d]*Protein accession number from NCBI.*
− Denotes non-identified.

egg masses undergo a long embryonic phase, making almost impossible to synchronize different hatchings. One point to consider then, is whether the molecular behavior of the offspring is linked to the genetic background of the female in particular. It has to be noted, however, that intercalibration approaches carried out among different laboratories (Garrido et al., 2017) and meta-analysis studies (Garrido et al., 2016a) reveal that above of the unavoidable variability inherent to the geographical origin and rearing scenarios, most rearing problems and physiological responses of the paralarvae are repetitive.

As far as we know, this is the first study using a proteomic approach to analyze the effect of diet and to search novel biomarkers of nutritional stress on octopus paralarvae. The proteome of octopus paralarvae according to the multivariate analyses (PCA and hierarchical), showed that changes in specific sub-sets of proteins differentially expressed as function of diet and age, allowed to describe characteristic proteomes for each experimental condition (see **Figures 2–5**). Similar result has been previously reported by Sveinsdóttir and Gudmundsdóttir (2010) for Atlantic cod (*Gadus morhua*) larvae using proteome analysis to study feeding effects. These authors found changes in abundance in a sub-set of 13 protein spots in the proteome of cod larvae fed with saithe (*Pollachius virens*) protein hydrolysate (SPH). Among the proteins identified here, it is noteworthy that most are involved in energy metabolism such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triosephosphate isomerase, NADP+-specific isocitrate dehydrogenase, and arginine kinase. This is not surprising since, early development of many marine organisms undergoing larval phases, and particularly of octopus paralarvae is highly dependent on energy for growth (von Boletzky and Villanueva, 2014). Other proteins identified are involved in several cellular processes related with detoxification (GST), stress [heat shock proteins (HSP70)], vision (S-crystallin 3) and cytoskeleton (actin, actin-beta/gamma1 and beta actin), physiologically crucial during early life stages of development.

The enzyme GAPDH has been known mainly as a "housekeeping" protein (Barber et al., 2005), with a role as key intermediate component of glycolysis and as a source of NADH. In fact, GADPH transforms glyceraldehyde-3-phosphate to glycerate 1,3-bisphospate and mediates the formation of NADH and ATP. The up-regulation of GAPDH in all conditions analyzed in our work could be related with these functions. Paralarval stages show a very active metabolism and fast growth, so obtaining energy and reducing power are essential. Besides, GADPH has demonstrated to be a multifunctional protein at least in vertebrates (Sirover, 1999; Baumgarner et al., 2012), displaying diverse activities including membrane, cytoplasmic and nuclear functions in endocytosis, mRNA regulation, DNA replication and repair, as well as regulation of apoptosis, as has been demonstrated in mammals (Sirover, 1997, 2011; Tristan et al., 2011). GAPDH expression has also been associated with exposure to hypoxia or anoxia and temperature variation in fish (Smith et al., 2009; Baumgarner et al., 2013; Jayasundara et al., 2015), and in mammals with a function of the cell proliferative state (Meyer-Siegler et al., 1992; Mansur et al., 1993; Gong et al., 1996). This last fact could also be the common origin of the over expression of GAPDH found in paralarvae of all conditions, considering the

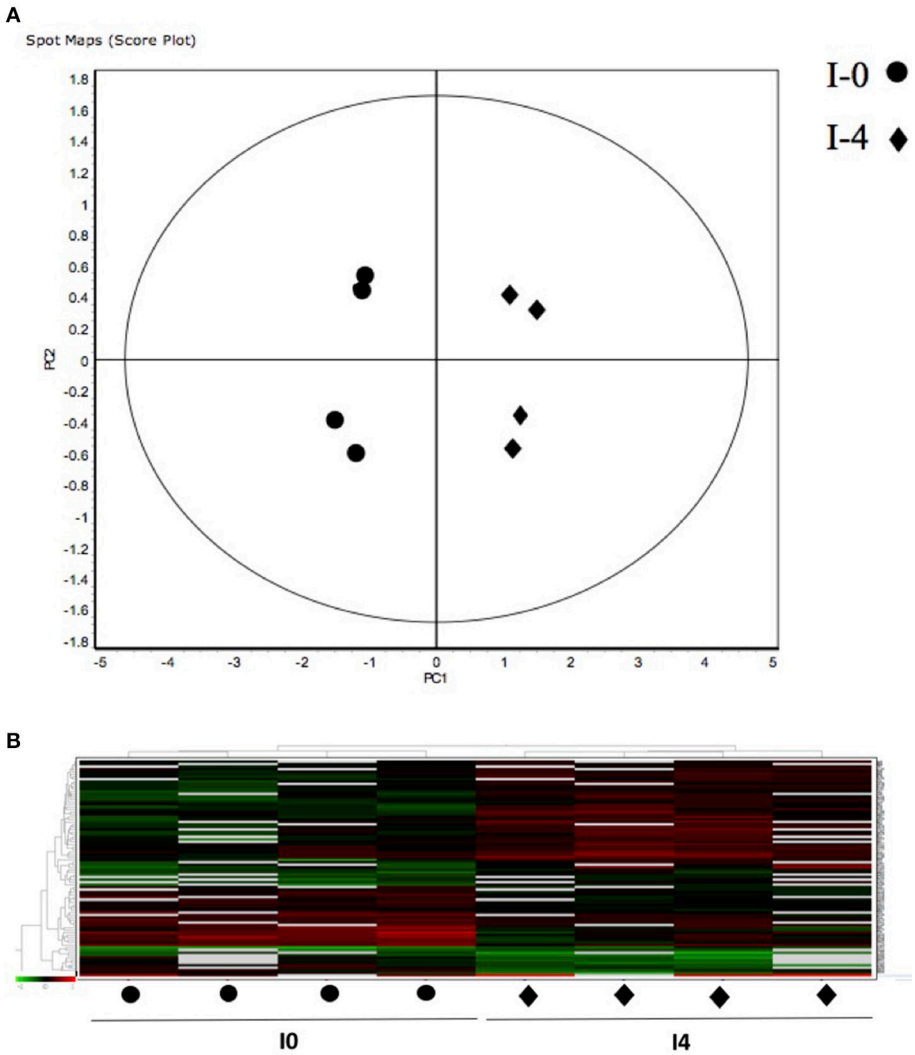

FIGURE 2 | (A) Principal Component Analyses (PCA) and **(B)** hierarchical cluster analyses of the protein spots (sub-sets) differentially expressed of paralarvae of *Octopus vulgaris* corresponding to the condition 1 (I4 vs. I0) considered as function of dietary group and age (details in Supplementary Table 3). % of variance explained: PC1 = 66.6; PC2 = 11.4.

rapid growth undergone in this phase of the biological cycle, with amino acids as the main source of energy (Lopes et al., 2016) and glycolysis playing a secondary role in paralarvae development (Lee, 1994; Navarro et al., 2014). It is important to note, however, that in the early days posthatching paralarvae use yolk reserves. These include glycogen and triglycerides (Quintana et al., 2015). In both cases substrate is supplied for the GAPDH, either by glycolysis or by the incorporation of the glycerol from mono or triglycerides into the glycolytic pathway, as evidenced by the glycerol kinase (GyK) activity found in newly hatched paralarvae (Cardenete et al., 2016).

In condition 1, unfed 4 days old paralarvae showed up-regulation of S-crystallin 3 respect to hatchlings. This protein that is a structural constituent of the lenses of the eyes in octopus. S-crystallins are soluble proteins in eye lens that contribute to the transparency and optical clarity (De Jong et al., 1989). The over-expression of this protein involved in vision, shows the importance of eyesight in paralarvae from the very beginning of their development, even under starving conditions. Although paralarvae are provided with chemoreceptors, they are mainly visual hunters (Lee, 1994; von Boletzky and Villanueva, 2014). Hatchlings enter in a planktonic phase, where they need to start feeding from the first day after hatching, and the active search (hunt) for food has been recorded 2 days after hatch at temperatures between 18 and 20°C (Iglesias et al., 2006), even before the yolk reserves are exhausted (Villanueva and Norman, 2008).

Regarding energetic metabolism, in condition 1 it can be observed how triosephosphate isomerase (TPI) is down regulated in 4 days starved paralarvae with respect to hatchlings. This enzyme catalyzes the reversible transformation of dihydroxyacetone phosphate (DHAP) to glyceraldehyde 3-phosphate (GAP) in the glycolysis pathway. DHAP is mainly provided from glucose or glycerol from triglycerides. Thus, this

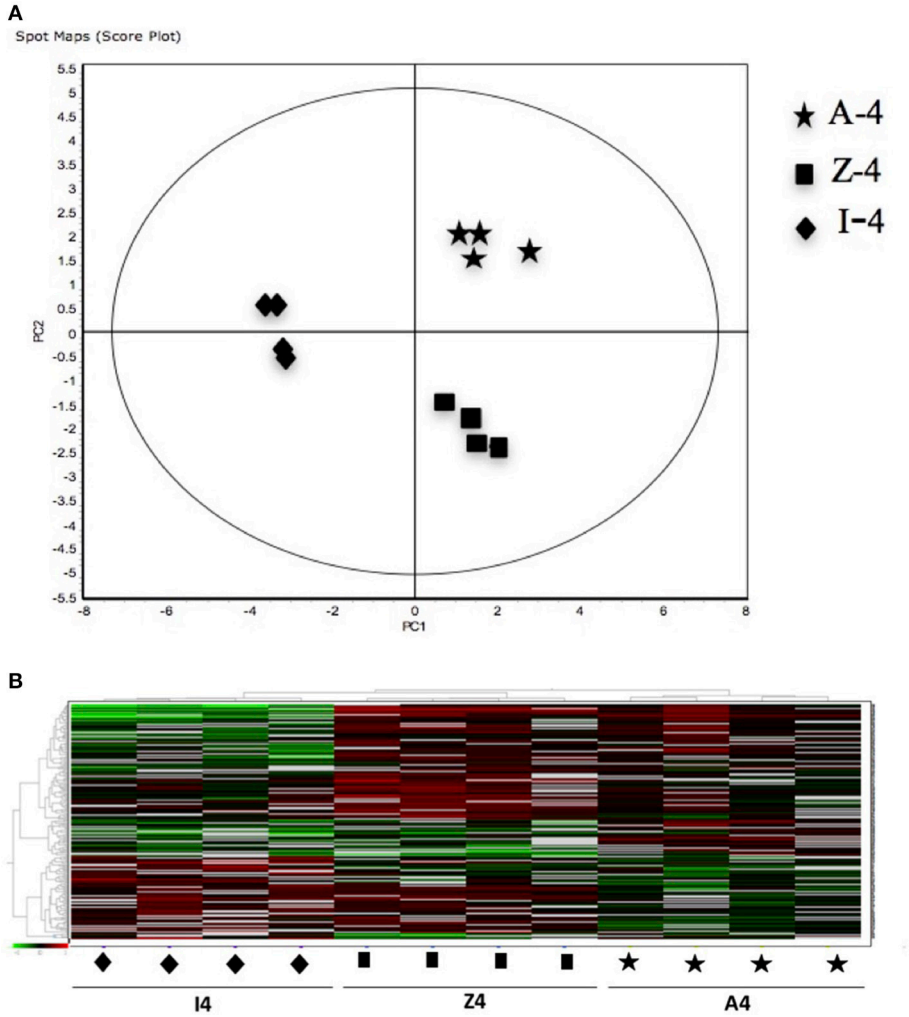

FIGURE 3 | (A) Principal Component Analyses (PCA) and **(B)** hierarchical cluster analyses of the protein spots (sub-sets) differentially expressed of paralarvae of *Octopus vulgaris* corresponding to the condition 2 (A4, Z4 vs. I4) considered as function of dietary group and age (details in Supplementary Table 3). % of variance explained: PC1 = 73.7; PC2 = 9.6.

fact points to a decrease in energy production by glycolysis and/or catabolism of triglycerides, which is in agreement with the fasting situation of paralarvae, and with the foreseeable exhaustion of the yolk reserves which, under normal conditions, are completely depleted around 4–5 days after hatching (Nande et al., 2017)

Usually the enzymatic reaction catalyzed by TPI runs to the formation of GAP, due that this one is rapidly eliminated by the following reaction of the glycolytic pathway that is catalyzed by the GAPDH enzyme (Blacklow et al., 1988; Harris et al., 1998). In this case, GADPH is lightly over regulated and therefore, there is a contrasting situation that should lead to a decrease of the GAP substrate. Obviously, in this case, the over expression of GADHP found, would be more related with the other functions of this enzyme mentioned above (i.e., NADH production) than with the obtention of energy by glycolysis.

Beta actin is also up-regulated in unfed 4 days old paralarvae which is part of the components of the cell cytoskeleton. This protein is highly conserved and is involved in support and different types of cell motility. Cephalopods, especially at paralarvae stages, display high growth rates, and proteins of cytoskeleton may be of paramount importance during their development.

HSP70 was down-regulated in unfed octopus paralarvae. These results are in line with a previous study, where starvation was related with decreased HSP70 levels in 4 days old octopus paralarvae, whereas increased HSP70 levels were detected in fed paralarvae (Varó et al., 2013). Previous studies on early life stages, mainly in fish, indicate variability in HSP70 response related to starvation. In fact, increased (Cara et al., 2005), decreased (Deng et al., 2009), or unchanged (Han et al., 2012) HSP70 levels have been found in fish larvae related with starvation or during food restriction. HSP70 is a chaperone protein that assists in the folding and transport of other proteins, and thus can be critical in periods of rapid growth (Kiang and

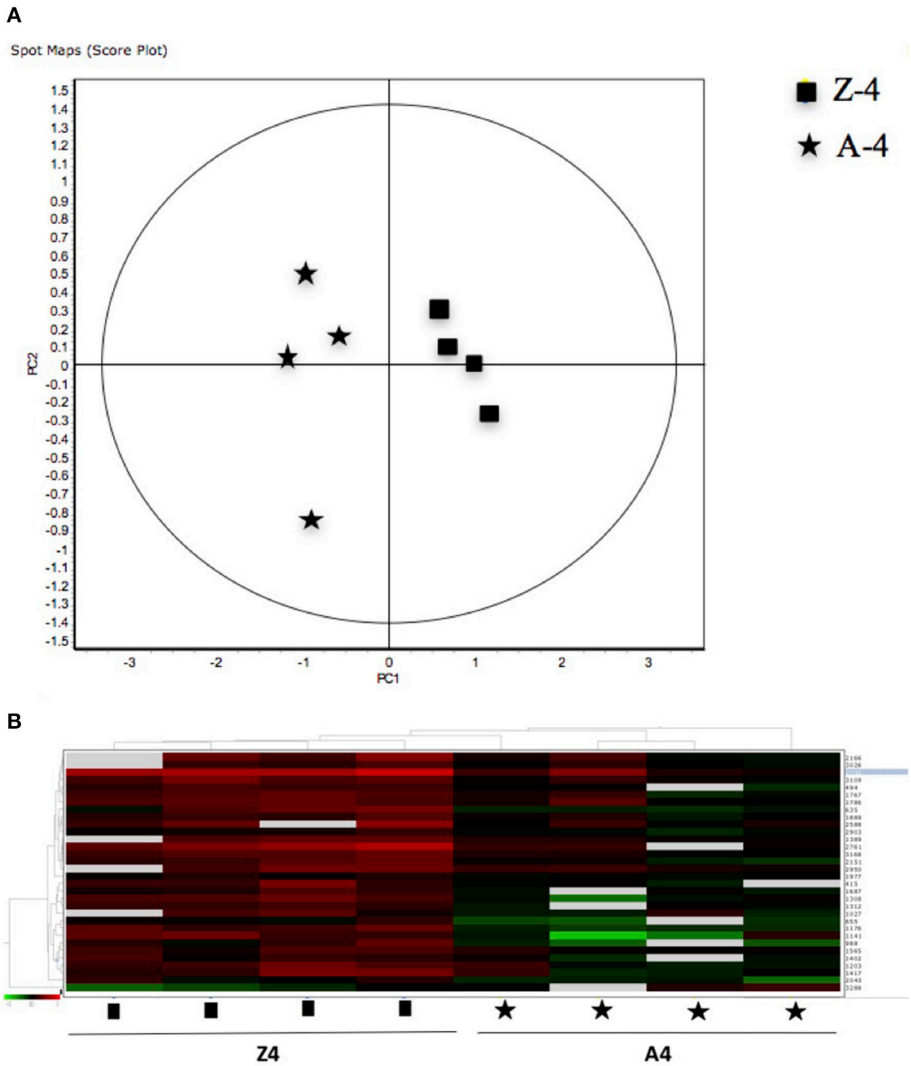

FIGURE 4 | (A) Principal Component Analyses (PCA) and **(B)** hierarchical cluster analyses of the protein spots (sub-sets) differentially expressed of paralarvae of *Octopus vulgaris* corresponding to the condition 3 (Z4 vs. A4) considered as function of dietary group and age (details in Supplementary Table 3). % of variance explained: PC1 = 77.1; PC2 = 8.3.

Tsokos, 1998). Thus, its expression can be altered by several stressors, including nutritional stress, caused by starvation. High growth rates in cephalopods are based in increasing body mass by protein synthesis and accretion, especially at paralarvae stages, that require large amounts of proteins and amino acid in the diet to satisfy the energy demands (Navarro et al., 2014). In larval fish, starvation is associated to amino acid restriction and enhanced proteolysis that affect cellular protein homeostasis, which translates into lower growth and survival (Conceição et al., 1997). For example, in white sturgeon larvae (*Acipenser transmontanus*) starvation reduced the induction of HSPs (HSP70 and HSP90), and decreased the body weight (Han et al., 2012). The growth (dry weigh) of unfed paralarvae group was much lower (0.177 ± 0.070 mg), compared to hatchlings (0.333 ± 0.080 mg) and 4 days old fed paralarvae groups (Z: 0.411 ± 0.014 mg; A: 0.367 ± 0.033 mg), with a loss of their initial weigh around 50% (47.7%). In agreement with former larval fish observations, the down-regulation of HSP70 found in starved paralarvae could be related with lower metabolic rates, linked with restriction of protein and amino acid anabolism, resulting in a lower or poor growth.

GST also showed down-regulation in unfed paralarvae. This enzyme is implicated in the detoxification of reactive electrophilic compounds and xenobiotic substances as antioxidant defense. Although changes of antioxidant defense have been associated with aging in invertebrates, including cephalopods (Zielinski and Pörtner, 2000; Barata et al., 2005), the accumulation of defective macromolecules caused by age has been related with increase of oxidative damage and/or by loss of the ability to repair or degradate these molecules (Stadtman, 1992, in Zielinski and Pörtner, 2000). Our results suggest that the decrease in GST could lead to the accumulation of electrophilic molecules indicating

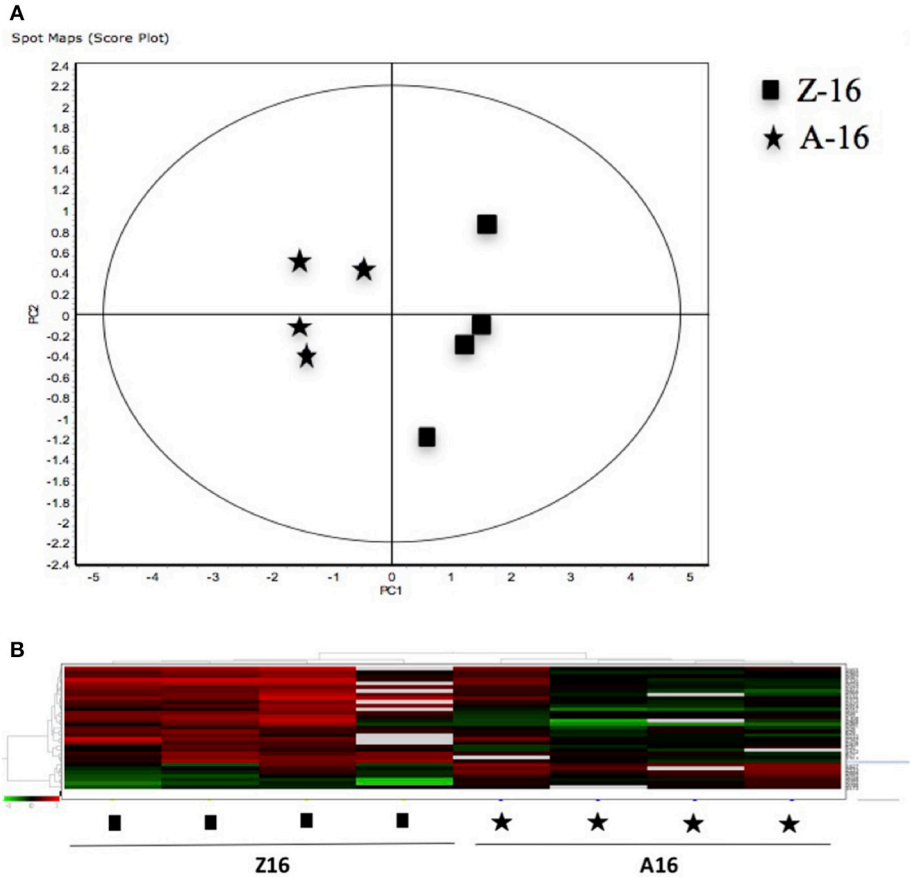

FIGURE 5 | (A) Principal Component Analyses (PCA) and **(B)** hierarchical cluster analyses of the protein spots (sub-sets) differentially expressed of paralarvae of *Octopus vulgaris* corresponding to the condition 4 (Z16 vs. A16) considered as function of dietary group and age (details in Supplementary Table 3). % of variance explained: PC1 = 72.6; PC2 = 8.

TABLE 2 | Summary of the fold changes in expression of protein identities differentially expressed in *O. vulgaris* paralarvae in the different condition studied as function of dietary group and age.

Protein name	Condition 1 I4 vs. I0	Condition 2 A4, Z4 vs. I4	Condition 3 Z4 vs. A4	Condition 4 Z16 vs. A16
S-crystallin 3	↑ (1.94)	↑ (8.44)		↓ (−2.27)
Glyceraldehyde3-phosphate dehydrogenase (GAPDH)	↑ (1.71)	↑ (1.56)	↑ (2.06)	↑ (3.21)
		↑ (1.04)	↑ (2.05)	
Beta actin	↑ (1.35)			
Heat shock protein 70KDa	↓ (−2.08)			
Triosephosphate isomerase	↓ (−2.23)			
Glutahione-S-transferase 2	↓ (−2.28)			
Nitrilase		↑ (4.25)	↓ (−1.73)	↓ (−2.56)
$NADP^+$specific isocitrate dehydrogenase		↑ (7.72)		↓ (−2.85)
Actin		↑ (3.76)		
Actin beta/gamma1		↑ (2.25)	↑ (1.52)	
Arginine kinase		↑ (19.5)	↑ (2.66)	

↑, up-regulated; ↓, down-regulated in each condition studied. ( ), fold change values. I, unfed group; A, Artemia group; Z, zoeae group. Number indicates age (days). vs., versus.

a detrimental effect on starved paralarvae. This, together with the decrease in HSP70, also involved in cellular defense, would constitute a response to an advanced stress scenario in unfed paralarvae. However, the lower metabolic rate probably produced by fasting could also reduce the levels of GST, as pointed out by Morales et al. (2011). In fish the transcriptional response of

GST to starvation varies according to tissue, species and fasting length. Thus, while in cod muscle (*G. morhua*) GST does not show modifications, in liver GST increases after short periods of fast in rock bream (*Oplegnatus faciatus*), whereas in rainbow trout (*Onconhynchus mykiss*) GST decreases in fish starved for 3 or more weeks (Morales et al., 2011). Interestingly, GST is related with S-crystallin in cephalopods. In fact, as pointed out by Tomarev et al. (1991) the use of detoxification stress proteins like GST and aldehyde dehydrogenase as cephalopod crystallins, is indicative of a common strategy for recruitment of enzyme-crystallins during the convergent evolution of vertebrate and invertebrate lenses. A recent study on *O. vulgaris* has revealed that the loss of GST enzyme activity in lens tissue is linked to the enhanced protein stability of S-crystallin via glutathione binding (Tan et al., 2016). In the light of these data, and although protein identification in our study was not carried out at the cellular location level, it is tempting to suggest that the decrease of GST might also be indicative of possible alterations in the lens structure of starved paralarvae.

In 4 days old fed paralarvae (condition 2), all proteins showing differential expression respect to unfed group were over expressed. It is interesting to highlight particularly the high over-expression of S-Crystalin 3 (8.44-fold change) that is in line with the former responses of this protein in 4 days old starving paralarvae, and support the crucial importance of eyes (vision). Particularly, S-Crystalin proteins, for their role in the refractive properties of the eye lens (Tomarev et al., 1991; Tomarev and Piatigorsky, 1996) may be of paramount importance in active visual hunters like octopuses for their adequate feeding and growth, especially during the first days of development, when adequate provision of essential nutrients is necessary for their rapid buildup. Thus, eye structure and function may be modulated by the feeding/nutritional (fed vs. starving) status of paralarvae and "vice versa."

Actin and actin beta/gamma1, cytoskeleton proteins, showed high increases in their expression in 4 days old fed groups respect to unfed ones, paralleling the high growth (dry weight, see above) and pointing at their importance in the development.

Nitrilase is also up-regulated in fed 4 days old paralarvae. This protein is part of a superfamily consisting in thiol enzymes involved in biosynthesis and post-translational modification of natural product in plants, animals, and fungi (Pace and Brenner, 2016). In this study, the sequence producing significant alignment of this protein (NCBI gi| 518224143) corresponds to a nitrilase described in *Bacillus endophyticus*. Bacterial nitrilases are used for biochemical synthesis and for environmental remediation (Cowan et al., 1998). A recent study has shown that *B. endophyticus* compared to different *Bacillus* strains has more genes related with energetic and transport metabolism of carbohydrates and lipids, than those related to cell envelope biogenesis and signal transduction mechanisms (Jia et al., 2015). This could suggest that the up-regulation of nitrilase could be linked to the bacterial metabolism that accompanies the gut microbiome of fed paralarvae, more than with the paralarval metabolism itself.

The high up regulation (7.72-fold change) of an isocitrate dehydrogenase isozyme dependent of $NADP^+$ (NADP-IDH), found in 4 days fed paralarvae (condition 2), point to a high activity of the Krebs cycle (TCA). In eukaryotes, there are three isozymes of IDH, two located in the mitochondrial matrix (NAD and NADP dependent respectively) related with energy production by TCA cycle and another one NADP dependent IDH in cytosol, which provides NADPH. The latter is necessary for the maintenance of the reduced glutathione and peroxiredoxin antioxidant systems by mean of the enzymes glutathione reductase (GR) and thioredoxin reductase (TrxR) respectively (Xu et al., 2004; Tomanek and Zuzow, 2010; Tomanek, 2015). Both systems are required to prevent the deleterious effect of reactive oxygen species (ROS). In condition 2, fed paralarvae seem to have a high aerobic metabolism and an up regulation in the production of NADPH. To account for this production, it is necessary to provide continuously substrate to the IDH catalyzed reaction. In short, sufficient citrate synthesis should be produced by a previous reaction of the Krebs cycle catalyzed by the enzyme citrate synthase. Therefore, a sustained supply of citrate synthase substrates: oxaloacetate and acetyl coenzyme A, are required (Tomanek and Zuzow, 2010). The results of Cardenete et al. (2016) seem to support this. In fact, 3 days old octopus paralarvae (fed *Artemia*) showed a significant increase in glutamate oxaloacetate transaminase activity (GOT, enzyme that produce oxaloacetate) as well as an increase in the activity of β-hydroxyacyl CoA dehydrogenase (HOAD), a key enzyme in beta oxidation of fatty acids, process that produces acetyl CoA. Both activities are also indicative of a high level of aerobic metabolism.

With regard to arginine kinase, the enzyme that shows the higher up regulation (19.5-fold), it catalyzes the first step of the arginine phosphate system that provides anaerobic fuel in several invertebrates including cephalopods (Fields et al., 1976; Gäde, 1980; Ellington, 2001). The system produces ATP in a first step breaking arginine phosphate, stored in mollusk muscles (Regnouf and van Thoai, 1970), by means of the activity of arginine kinase (AK), producing arginine and ATP. In a second step the condensation of the amino acid arginine with pyruvate takes place. This reaction produces octopine and it is catalyzed by the NADH dependent enzyme octopine dehydrogenase (ODH; Fields et al., 1976). In this context, the over expression of GAPDH found may be explained, since it provides the NADH required by AK activity.

The function of this anaerobic way of obtaining energy is related in paralarvae with episodes of physiological hypoxia in muscle when there is a great demand of energy as it happens in fast and intensive swimming (Baldwin, 1982; Lee, 1994). Indeed, it is associated to prey capture by paralarvae, that involves bursts of swimming energetically very expensive (Baldwin, 1982). In agreement with these data, octopine dehydrogenase activity shows a significant increase in the first days of paralarvae life (Cardenete et al., 2016), indicating an increase in the predatory capacity of the paralarvae with their development.

In conditions 3 and 4, when both dietary groups are compared (see **Table 2**), proteins with differential expression showed similar changes in their abundance, suggesting that they were most probably related to development (age) than to diet. The two diets supplied were more different in qualitative than in quantitative composition, with the co-feeding treatment

providing essential lipids in comparison with the diet based solely in *Artemia*. In fact, every effort was made to adjust food so that there was equivalent prey density in both dietary treatments, hence the co-feeding schedule. All these suggestions, however must be contemplated with care since working with live preys is always difficult to handle and control. Suffice to say that octopus paralarvae are active selective predators as has been reported recently (Roura et al., 2012). It must be pointed out however, that until the nutritional requirements of the paralarvae are well established, helping to design and produce adequate inert diets, live food remains the best alternative. Experimental designs focused on unveiling differential expressions of proteins linked to specific aspects of food composition should be foreseen in future works to help to cope with these aspects, but were beyond the scope of the present work.

It is to note that in the zoeae fed group, S-crystallin 3 showed significant change only in 16 days old paralarvae (Z16, condition 4), decreasing by 2.27-fold. In view of all the changes of this protein in the different condition studied (see **Table 2**), one would conclude that starvation seems to affect more to the eye lens structure in 16 days old paralarvae fed with zoeae than with *Artemia*. As mentioned above, alterations in the eye lens structure, lower transparency and optical clarity should produce altered vision and less success in hunting and feeding. This should translate in lower growth. Although Z16 paralarvae showed higher DW (0.713 ± 0.062 mg) and specific growth rate (SGR, % DW day^{-1}: 4.961 ± 0.58) compared with *Artemia* fed group (DW: 0.621 ± 0.051 mg; SGR: 4.043 ± 0.55), as previously discussed by Garrido et al. (2016a), no significant differences in growth were found between both dietary groups. Although it is always delicate to make direct comparisons due to differences in methodologies and feeding schemes (aggravated by the use of live preys), as well as variability in spawn quality (Garrido et al., 2017), it is to note that other trials using zoeae as food have reported higher growth (Iglesias et al., 2004, 2014; Carrasco et al., 2006; Iglesias and Fuentes, 2014). Thus, bearing in mind that adequate prey density at this stage of development is critical (Villanueva et al., 2002), the decrease in S-crystallin 3 in zoeae group could be a response of paralarvae to starvation, more than to the qualitative composition of the diet. This protein would act then as a biomarker of starvation pointing at this condition in the co-feeding treatment as it did in 4 days old starved paralarvae. In fact, co-feeding was used because there was not guarantee of enough provision of zoeae to fulfill the necessary prey density, and paralarvae are selective feeders.

The down-regulation of nitrilases found in the zoeae group at 4 and 16 days (condition 3 and 4) compared to the *Artemia* group suggest, in agreement with the former observations (condition 2) linking these proteins to bacterial nitrilases metabolism present in the gut microbiome of fed paralarvae, that this bacterial metabolism was lower in co-fed paralarvae, perhaps in line with a scarcity of food scenario.

The up-regulation of cytoskeleton proteins, actin and actin beta/gamma1, together with the increase in arginine kinase, involved in energy production (ATP) found only in 4 days old paralarvae fed with zoeae, supports the importance of structural and energetic proteins in the enhanced growth of this group compared with *Artemia* in periods of high energy demand as occurs in the first 4 days of the octopus' development. On the other hand, the down regulation of $NADP^+$ specific isocitrate dehydrogenase suggests a shift toward anaerobic metabolism in 16 days old paralarvae fed with zoeae, as compared with the high up regulation found in 4 days old fed paralarvae.

Overall, the results show that in 4 days old zoeae fed paralarvae, all the proteins identified were up regulated, which could be associated with higher growth (DW) than in the case of those *Artemia* fed. On the contrary, at 16 days the proteins identified were down regulated in the zoeae group, except GAPDH that was always up regulated in all the conditions studied. The consistency of the up-regulation in the rest of the different feeding conditions suggests that the decrease in the zoeae group at 16 days might represent an indication of nutritional stress in paralarvae linked to insufficient availability of food.

CONCLUSION

In summary, the results obtained in the pattern of protein expression allow defining characteristic proteomes of paralarvae of *O. vulgaris* depending on diet and age. This study showed that proteome of *O. vulgaris* paralarvae is affected by fasting during the first 4 day of development, with proteins like S-crystallin, arginine kinase and NAD^+ specific isocitrate dehydrogenase showing the highest over expression in fed respect to unfed paralarvae. When both *Artemia* and *Artemia* plus zoeae dietary groups are compared, the up regulation of the proteins identified in 4 days old zoeae fed paralarvae, followed by the decrease found at 16 days old, might suggest an indication of nutritional stress in this group linked to starvation in a sub-optimal prey concentration scenario, more than with the quality of the diet. Although co-feeding is currently a good strategy to balance the quality of diet for achieving the best growth and survival in on growing octopus paralarvae during the first days of development, later around16 days of development, every effort has to be made to supply adequate amounts of food. Overall, the changes in the abundance of proteins like S-crystallin, arginine kinase and NAD^+ specific isocitrate dehydrogenase may be used as novel biomarkers to assess the nutritional status of octopus paralarvae. Their increase/decrease in abundance may be related to fed vs. starving paralarvae, particularly in the first 4 days of development. Proteomics techniques represent not only an invaluable approach to go beyond the "state of evidence" in the search for the causes of poor performance of paralarval cultures at present, but are also a promising tool for fine tuning welfare once the main problems and constraints are overcome.

AUTHOR CONTRIBUTIONS

IV: Design of proteomic procedures, analysis, and interpretation of the findings, and draft and writing of the manuscript. GC: Interpretation of the findings, writing discussion (metabolism) and revision of the manuscript. OM and FH: Analysis and interpretation of the findings, and general writing of the manuscript. JI and JO: Octopus paralarvae cultures and execution of the experiments. EA: Design experiments and

revision of the manuscript. JN: Interpretation of the findings, writing and revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00309/full#supplementary-material

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Diet Composition and Variability of Wild *Octopus vulgaris* and *Alloteuthis media* (Cephalopoda) Paralarvae: A Metagenomic Approach

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The high mortality of cephalopod early stages is the main bottleneck to grow them from paralarvae to adults in culture conditions, probably because the inadequacy of the diet that results in malnutrition. Since visual analysis of digestive tract contents of paralarvae provides little evidence of diet composition, the use of molecular tools, particularly next generation sequencing (NGS) platforms, offers an alternative to understand prey preferences and nutrient requirements of wild paralarvae. In this work, we aimed to determine the diet of paralarvae of the loliginid squid *Alloteuthis media* and to enhance the knowledge of the diet of recently hatched *Octopus vulgaris* paralarvae collected in different areas and seasons in an upwelling area (NW Spain). DNA from the dissected digestive glands of 32 *A. media* and 64 *O. vulgaris* paralarvae was amplified with universal primers for the mitochondrial gene COI, and specific primers targeting the mitochondrial gene 16S gene of arthropods and the mitochondrial gene 16S of Chordata. Following high-throughput DNA sequencing with the MiSeq run (Illumina), up to 4,124,464 reads were obtained and 234,090 reads of prey were successfully identified in 96.87 and 81.25% of octopus and squid paralarvae, respectively. Overall, we identified 122 Molecular Taxonomic Units (MOTUs) belonging to several taxa of decapods, copepods, euphausiids, amphipods, echinoderms, molluscs, and hydroids. Redundancy analysis (RDA) showed seasonal and spatial variability in the diet of *O. vulgaris* and spatial variability in *A. media* diet. General Additive Models (GAM) of the most frequently detected prey families of *O. vulgaris* revealed seasonal variability of the presence of copepods (family Paracalanidae) and ophiuroids (family Euryalidae), spatial variability in presence of crabs (family Pilumnidae) and preference in small individual octopus paralarvae for cladocerans (family Sididae) and ophiuroids. No statistically significant variation in the occurrences of the most frequently identified families was revealed in *A. media*. Overall, these results provide new clues about dietary preferences of wild cephalopod paralarvae, thus opening up new scenarios for research on trophic ecology and digestive physiology under controlled conditions.

Keywords: trophic ecology, NW Iberian Peninsula, paralarvae culture, NGS diet analysis, Illumina Miseq

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INTRODUCTION

Historically, cephalopods in European waters have always been viewed as a minor fisheries resource (Pierce et al., 2010). However, they can be of considerable local economic importance, especially in southern Europe's artisanal fisheries. Galician waters (NW Spain) support an economically important cephalopod fishery for *Octopus vulgaris* (Otero et al., 2006; Pita et al., 2016) and loliginid squid, mainly *Loligo vulgaris* but also *Alloteuthis media* and *Alloteuthis subulata* (Jereb et al., 2015), species that are not easily distinguished due to the similarity of their external characters (Jereb et al., 2010). Reflecting the short life cycle and rapid individual growth rates, cephalopod populations are sensitive to effects of environmental variation on reproduction and recruitment (Boyle, 1990; Boyle and Rodhouse, 2005; Pierce et al., 2008; Hastie et al., 2009; Rodhouse et al., 2014), resulting in wide year to year fluctuations in captures. From 2000 to 2013, reported cephalopod landings in Europe varied from a minimum of 38,600 tons in 2009 to a maximum of 55,500 tons in 2004 (ICES, 2014).

In recent years, there has been growing interest in the culture of cephalopods, primarily for human consumption, due to their high growth rates, high protein contents, and high ratios of food conversion and short life cycles (Segawa, 1990; Lee, 1995; Villanueva and Bustamante, 2006). Bearing in mind the variability of the wild cephalopod resources, there is a need for a stable and reliable source of cephalopods. Additional impetus for captive rearing arises from the use of cephalopods as model organisms in biomedical science (Bullock, 1948; Hanlon, 1990; Fiorito and Scotto, 1992; Calisti et al., 2011) and for ornamental purposes (Dunstan et al., 2010; Rodhouse et al., 2014). Despite progress in cephalopod culture methods (e.g., Iglesias et al., 2014), cephalopod species with planktonic stages have very low survival rates of paralarvae in captive conditions (Villanueva and Norman, 2008). Therefore, rearing relies on wild captured juveniles, subadults, and adults, preventing commercial viability (Hernández Moresino et al., 2014; Xavier et al., 2015).

Juvenile and sub-adult cephalopods are mainly fed with live prey, including crustaceans, fishes, and mollusks (Domingues et al., 2004; García García and Cerezo Valverde, 2006; Sykes et al., 2013), fisheries discards (Socorro et al., 2005; Estefanell et al., 2011), frozen prey (Ferreira et al., 2010; Sykes et al., 2013), or artificial feed stuffs (Garcia et al., 2011; Estefanell et al., 2013). Paralarvae in captivity have been traditionally fed with enriched *Artemia* (Iglesias et al., 2006) or supplemented with decapod zoeae, copepods, mysids, shrimps, or fish larvae, which increases survival and growth rates (Hernández-García et al., 2000; Iglesias et al., 2004, 2006; Ikeda et al., 2005; Carrasco et al., 2006; Kurihara et al., 2006; Martínez et al., 2014; Farías et al., 2015). Despite all previous attempts, cephalopod paralarval mortality is still close to 100% in captivity.

It has been suggested that the high mortality of paralarvae in captivity is due to a lack of knowledge regarding the physiology and nutrition of paralarvae (Domingues et al., 2003; Villanueva et al., 2009; Garrido et al., 2016a) or the lack of a suitable diet meeting all micronutrient requirements (Iglesias et al., 2007). Several experiments have shown that feeding new born paralarvae with different diet can influence its survival (Villanueva, 1994; Iglesias et al., 2004; Farías et al., 2015). Moreover, physiological changes in digestive gland lipid composition (Garcia et al., 2011) and higher proteolytic activity were observed in paralarvae fed on other zooplankton organisms, rather than Artemia (Pereda et al., 2009).

Moreover, laboratory experiments have shown that hatchlings present restricted swimming capacity and prey hunting skills. They progressively develop the ability to capture different zooplankton prey (Hanlon, 1990; Chen et al., 1996), suggesting the necessity to adapt their diet at their different stages. Thus, increasing the knowledge of dietary preferences of wild cephalopod paralarvae and ontogenetic dietary changes over the course of their early development could help to design a suitable diet for rearing in captivity.

A few investigations have analyzed the diet of wild paralarvae by visual identification of stomach contents, revealing that they mainly fed on copepods (*Illex argentinus*, Vidal and Haimovici, 1998), amphipods (*Ommastrephes bartramii*, Uchikawa et al., 2009), and other crustaceans (*Abralia trigonura* and *Sthenoteuthis oualaniensis*; Vecchione, 1991). However, a high proportion of stomach contents comprises unrecognizable soft material (Roura et al., 2012; Camarillo-Coop et al., 2013) or small pieces of exoskeleton (Passarella and Hopkins, 1991; Vecchione, 1991; Vidal and Haimovici, 1999). Alternative approaches have also been attempted: Specific prey species of *Loligo reynaudii* were detected by applying immunoassays (Venter et al., 1999) and in *O. vulgaris* paralarvae up to 20 different prey were detected cloning PCR products with group-specific primers (Roura et al., 2012). However, these methods are costly and time-consuming, and thus can only be applied to a limited number of samples and clones sequenced.

Zooplankton communities in the Ría de Vigo (NW Iberian Peninsula) are highly dynamic, presenting rapid changes in species composition and abundance according to environmental conditions (Roura et al., 2013; Buttay et al., 2015). Previous research suggests that *O. vulgaris* paralarvae are specialist predators, eating decapods independently of the zooplankton communities they inhabit (Roura et al., 2016). However, a specialist diet focusing on low abundance prey could lead to starvation and death. It is therefore expected that cephalopod paralarvae have certain degree of plasticity in terms of the different prey they can capture, based on their hunting abilities, and that they also eat prey species that have not been yet detected in their diet.

The development of next generation sequencing (NGS) has permitted the elucidation of the diet of a wide variety of animal species including vertebrates and invertebrates (King et al., 2008; Boyer et al., 2013; Leray et al., 2013b). These techniques are more efficient and, in many cases, less costly than traditional diet analysis in terms of time and prey species resolution (Pompanon et al., 2012). Thus, NGS could be applied to reveal previously undetected prey species of cephalopod paralarvae and to extend dietary analysis to a higher number of paralarvae.

Therefore, the aim of this study was to develop a NGS approach to provide a detailed analysis of the diet of the paralarvae of the two cephalopod species most abundant in the

plankton in NW Iberian Peninsula. First of all, we describe for the first time the diet of paralarvae of *Alloteuthis media*, and secondly, we present new information on the dietary preferences of paralarvae of *O. vulgaris* in the coastal environment. Environmental conditions (such as season and feeding area) affecting paralarval prey preferences are also assessed. Diet is thought to be the main factor affecting paralarval survival and determining diet is an essential step toward understanding the physiological status of healthy paralarvae, knowledge, which can then be transferred to increase their survival in captive conditions.

MATERIALS AND METHODS

This study was performed in accordance with existing Spanish guidelines and regulations on animal research (Ley 32/2007, November 7th), and was consequently exempt from an ethics review process.

Sample Collection

Zooplankton samples were collected in the Ría de Vigo (NW Spain) onboard RV "*Mytilus*" in 2012 and 2014. The timing of the sampling was based on previously identified periods of maximum paralarval abundance (Rocha et al., 1999; González et al., 2005) in 2012 and 2014: we carried out ten nocturnal surveys each year, four in summer (July), and six in early autumn (September and October). Additionally, diurnal surveys were conducted in summer and in autumn 2012 (one per season). Sampling surveys were conducted along four transects (**Figure 1A**). For each transect, a Multinet® Hydrobios Mammoth of 250 μm mesh size, fitted with two electronic flow meters, was lowered at 2.5 knots to the sea floor and lifted up gradually to the surface. We defined seven depth layers: from 105 to 85 m, Z7; 85 to 55 m, Z6; 55 to 35 m, Z5; 35 to 20 m, Z4; 20 to 10 m, Z3; 10 to 5 m, Z2; and 5 m to the surface, Z1; see **Figure 1B**). Within each layer, the Multinet® filtered up to 200 m^3 of seawater (approximately from 5–10 min for each layer and hence in total between 20 and 70 min), and collected independent samples. The collected zooplankton was fixed onboard in 96% ethanol and frozen at −20°C until sorting. In the laboratory, all cephalopod paralarvae were separated and preserved individually in 70% ethanol and stored at −20°C.

Identification of Paralarvae and Morphological Measurements

Dorsal mantle length (DML) was measured to the nearest 0.05 mm on the dorsal side of all octopus and squid paralarvae using a Leica M205C stereomicroscope and Leica Application System image analysis software (Leica Microsystems, Germany). All octopus paralarvae ($n = 492$) were identified as *O. vulgaris* based on morphological characters following Sweeney et al. (1992). Due to the difficulty of identifying squid paralarvae using morphological characters, all loliginid paralarvae ($n = 163$) were identified genetically. Molecular identification relies on previous work with adult specimens identified morphological and subsequently genetically.

Briefly, DNA from the mantle of each loliginid paralarva was extracted with a QIAamp DNA Micro Kit (QIAGEN) following manufacturer's instructions, with the exception of two steps: Digestion at 56°C was done overnight and the final elution was done in two steps using 15 μl buffer AE in each elution. The barcoding region of the Cytochrome c Oxidase subunit I (COI) was amplified with the universal primers HCO2198 and LCO1490 (Folmer et al., 1994) and PCR products were sequenced by Sanger sequencing (Stab Vida, Portugal). Each sequence was compared to the following GenBank reference sequences using the BLAST algorithm (Altschul, 2014): *Alloteuthis media*, EU668085 (Anderson et al., 2008); *A. subulata* EU668098 (Anderson et al., 2008), and *L. vulgaris,* KF369142 (Lobo et al., 2013).

Loliginid paralarvae were identified as *A. media* ($n = 93$), *A. subulata* ($n = 35$), and *L. vulgaris* ($n = 22$) (Olmos-Pérez et al., unpublished data). For dietary analysis, a total of 64 *O. vulgaris* paralarvae (summer, $n = 26$; autumn, $n = 38$) and 32 *A. media* (summer, $n = 16$; autumn, $n = 16$) were selected. *Alloteuthis media* was selected for molecular diet analyses because it was the most abundant loliginid present. Samples were chosen to maximize information from different seasons, transects and depth.

Digestive Gland Dissection, DNA Extraction, and Prey Detection

Digestive glands of all 96 paralarvae were dissected out, cleaned with sterile distilled water and placed into DNA-free tubes (Suzuki et al., 2006). DNA was extracted with a QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany) following the modifications in the elution step as stated before. DNA purity and concentration were controlled with NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA).

Many dietary studies recommend the use of restriction enzymes (Blankenship and Yayanos, 2005) or blocking primers (Vestheim and Jarman, 2008; Deagle et al., 2009; Leray et al., 2013a) to avoid amplifying predator DNA and maximize detection of prey DNA. However, the huge number of sequences currently obtained with NGS platforms allows the use of universal primers that facilitate the detection of unexpected prey (Boyer et al., 2013) without the necessity of using restriction enzymes or blocking probes (Piñol et al., 2014). Accordingly, we employed the universal pair of primers HCO2198 (Folmer et al., 1994) and mlCOIintF (Leray et al., 2013b), to amplify 315 base pairs (bp) of the barcoding region of the mitochondrial cytochrome c oxidase subunit I (mt-COI) gene (**Table 1**). Cycling conditions for the touch-down PCR with COI primers were: initial denaturation at 95°C 3 min, 10 initial cycles of denaturation at 95°C for 30 s, annealing for 30 s at 57 (−1°C per cycle), and extension at 72°C for 40 s, followed by 29 cycles of denaturation at 95°C for 30 s, annealing at 47°C for 30 s, extension at 72°C for 40 s, and a final elongation for 4 min at 72°C. PCR amplification was performed in a total volume of 25 μl:1 μl (10 μM) of each forward and reverse primers, 12.5 μl Thermo Scientific™ Phusion™ High-Fidelity PCR Master Mix

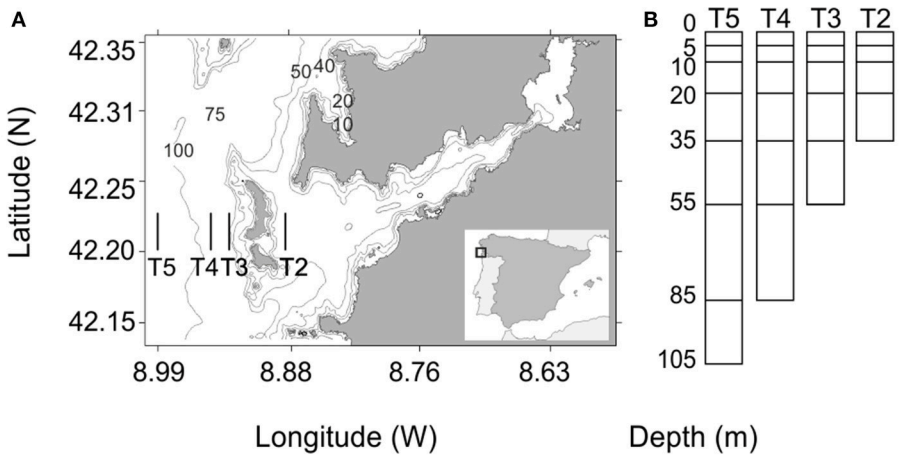

FIGURE 1 | (A) Map of the study area showing the four transects performed in 2012 and 2014. **(B)** Depth layers sampled for each different transects.

TABLE 1 | All of them amplify different regions of mitochondrial (mt) DNA. Product size (bp): Approximate product size of each PCR product (without overhang) expressed as number of base pairs (bp).

Gen	Target	Forward	Reverse	Product size (bp)	References
mt-COI	Universal	ml COlintF[a] GGWACWGGWTGAACWGTWTAYCCYCC	HCO2198[b] TAAACTTCAGGGTGACCAAAAAATCA	315	Folmer et al., 1994[b]; Leray et al., 2013b[a]
mt-16Sa	Malacostraca	16S1F* TGACGATAAGACCCT	16S2R* CGCTGTTATCCCTAAAGTAACT	205	Deagle et al., 2005
mt-16Sb	Chordata	Chord_16S_F_TagA ATGCGAGAAGACCCTRTGGAGCT	Chord_16 s_R_Short CCTNGGTCGCCCCAAC	155	Deagle et al., 2009

**These primers include two nucleotide modifications from the original to amplify specifically the Malacostraca family. All primers were synthesized with the following overhang on the 5′ ends: Forward (5′–3′) TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGACGATAAGACCCT and Reverse (5′–3′) GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGCTGTTATCCCTAAAGTAACT.*

with HF Buffer (Thermo Fisher Scientific Inc., Massachusetts, USA), 1 μl of DNA (20 ng/μl), and 9.5 μl H_2O.

Since decapods, krill and fishes had been previously detected in the digestive tract of *O. vulgaris* paralarvae (Passarella and Hopkins, 1991; Vecchione, 1991; Roura et al., 2012), we also employed two pairs of specific primers to amplify the 16S mitochondrial gene of malacostracan crustaceans (mt-16Sa, **Table 1**) and chordates (mt-16Sb; **Table 1**). Cycling conditions for both 16S pairs of primers were: initial denaturation at 94°C 15 min, 33 cycles of: denaturation at 94°C for 20 s, annealing at 48.7°C for 90 s, extension at 72°C for 45 s, and a final elongation step at 72°C for 2 min. PCR amplification was performed in a total volume of 25 μl:2 μl (10 μM) of each forward and reverse primers, 12.5 μl of Promega GoTaq® Green Master Mix (Promega Corporation, Wisconsin, USA), 1 μl of DNA (20 ng/μl) and 7.5 μl H_2O.

All primers were synthesized following New Zealand Genomics Ltd. (NZGL) recommendations, with an overhang on the 5′ ends to permit the ligation with Illumina multiplexing indices and sequencing adapters (**Table 1**). The optimum annealing temperature with the overhangs was determined with a gradient PCR. For each primer, 2 μl of PCR product were checked on 1.5% agarose gels. Those that presented a clear band of expected size were cleaned up with Agencourt AMPure beads following the manufacturer's protocol (Beckman Coulter Life Science Inc., México). Afterwards, PCR products were quantified using a Qubit™ 3.0 fluorometer (Thermo Fisher Scientific Inc., Massachusetts, USA). Purified PCR products of the same individual with concentration higher than 1.0 μg/ml were pooled together. Library preparation with 96 Nextera Index Primers, quantification, normalization, and pooling were performed by New Zealand Genomics Ltd. (NZGL) in their laboratories. The library was then sequenced with MiSeq Reagent Kit V3 in MiSeq sequencer (Illumina Inc., USA).

Bioinformatic Analysis

MiSeq reporter was used to separate and remove the adapters for the 96 samples. The software Fastq-Multx (Aronesty, 2011) was used to demultiplex amplicons according to the primer sequences. Due to the wildcard characters in the primer sequences, up to 7 base pair mismatches were permitted. Software SolexaQA++ 3.1.4 was used to ensure the reads were still paired (Cox et al., 2010). The paired end reads (read 1 and read 2) were merged using VSEARCH 1.9.5 (Rognes et al., 2016). Paired reads that did not meet the following quality filtering were discarded (Edgar and Flyvbjerg, 2015): (i) reads with quality

score over 3, (ii) reads longer than 140 bp, (iii) reads with less than one expected error in the primer sequence or barcodes. Unique sequences were clustered using a 97% identity threshold and remaining singleton Molecular Taxonomic Units (MOTUs) were discarded. Chimeric sequences were then removed using UCHIME (Edgar et al., 2011). Using the final list of representative sequences, each MOTU was searched against the GenBank database using BLAST 3.2.31 (Camacho et al., 2009).

MOTUs with BLAST query coverage under 60% or BLAST identities lower than 74% were also deleted from the database. Potential contamination and predator MOTUs (i.e., *A. media* and *O. vulgaris*) were removed from the database. Potential prey MOTUs were assigned using the following criteria to taxonomical categories: MOTUs with identity higher than 97% were determined at species level, MOTUs between 93 and 97% were assigned to genus, and MOTUs with identity below 93% were assigned to family.

Statistical Analysis

For each predator (*A. media* and *O. vulgaris*), we analyzed separately the MOTUs identified by different pairs of primers. Then, we calculated the proportion of reads for each MOTU in relation to the total number of reads (PR). We also calculated the frequency of occurrence for each MOTU (FM: percentage of number of samples tested positive for a given MOTU in relation to the total number of samples) and the frequency of the occurrence of each family (FF: percentage of number of samples tested positive for a given prey family in relation to the total number of samples).

Frequency of occurrence was calculated for higher taxonomic levels by combining information for all MOTUs falling within the relevant taxon (**Table 2**). Moreover, for those taxa that were detected with at least two pair of primers and to species level, we calculated the overall frequency of occurrence (percentage of samples which tested positive for a given taxon in relation to the total number of samples, **Table 2**).

Redundancy Analysis (RDA) was used to detect patterns in the diet of *O. vulgaris* and *A. media* and determine which explanatory variables influenced those patterns. We included the occurrences of each family (FF) detected in the analysis as response variables, considering the presence-absence of each family in each paralarva. All octopus paralarvae presented three suckers per arm and were therefore probably less than 10 days old (Villanueva, 1995; Garrido et al., 2016b). All squid paralarvae were less than 41 days old based on statolith ring measurements (Olmos-Pérez et al., unpublished data). However, since we did not have complete age data, size-at-age is very dependent on environmental (pre- and post-hatching) temperature, and food ingestion is likely more dependent on size that on age, we categorized DML into three different classes to facilitate detection of ontogenetic changes during paralarvae growing. Thus, *O. vulgaris* paralarvae were categorized as small (1.20–1.74 μm; $n = 21$), medium (1.75–1.98 μm; $n = 21$), or large (1.99–2.28 μm; $n = 22$), and *A. media* were categorized as small (1.42–1.99 μm, $n = 9$), medium (2.00–2.99 μm; $n = 15$), or large (3.00–6.02 μm; $n = 8$). Paralarval size class (i.e., small, medium, large), transects (i.e., transects T2, T3, T4, T5), seasons (i.e., summer, early autumn), and depth (z1, z2, z3, z4, and z5) were included as nominal explanatory variables. We used the correlation triplot ($\alpha = 0$, species conditional triplot), and the correlation matrix for the response variables. A significance test was applied with 4,999 permutations.

The effects of the season, transects, depth, and DML on dietary diversity and the presence of particular prey families in the diet were analyzed with generalized additive modeling (GAM) using a Poisson distribution for diversity and a binomial distribution for the other response variables and logit link function (link=logit), with season and transect as fixed factors and DML and depth effects fitted as smoothers (setting the bases dimension using $k = 4$ to avoid overfitting). Only the most frequently predated families (i.e., those detected in at least 10% of the predators, FF > 10) were used in this analysis. Models were fitted using backwards selection. The goodness-of-fit of the models was assessed with the Akaike Information Criterion (AIC). When the difference in AIC between two models (i.e., with and without one explanatory variable) was less than 2, an *F*-test was employed to select the best model (in case of a significant *F*-value the more complex model was preferred). All statistical analyses were performed with Brodgar 2.7.4. (Highland Statistics Ltd., UK).

Finally, "discovery curves" were plotted to determinate if the number of samples was sufficient to determine the importance of the most frequently detected prey species, for each combination of predator species (*O. vulgaris* and *A. media*), and primers (COI and 16sa). For each prey species, predator and primers, sets of 0, 1, 2... *n* samples were drawn at random from the available n samples and the proportional occurrence of the prey type was calculated for each sample size. Ten replicates were used to generate means and confidence limits, which were then plotted against sample size.

RESULTS

Bioinformatic Analysis

Of 8,274,658 raw reads, 5,734,163 were successfully demultiplexed and contained both read 1 and read 2. Then, 5,119,926 paired end reads were successfully merged, and 4,752,768 reads remained after quality filtering. A total of 4,124,464 reads was clustered into 1,155 MOTUs using a 97% threshold. Of the total reads, 3,189,247 corresponded to COI, 744,474 reads to the primers set 16Sa and 190,743 reads to the primer set 16Sb. Of these, 405 MOTUs (31,604 reads) did not match any GenBank sequence and 1,155 MOTUs, 750 (4,092,860 reads) had a match on GenBank database.

After meeting the thresholds of query coverage and identity, 465 MOTUs (131,843 sequences) were removed and 285 MOTUs (3,961,017 reads) were classified as: contamination (48 MOTUs, 41,422 reads), *O. vulgaris* (60 MOTUs, 2,150,967 reads), and *A. media* (55 MOTUs, 1,534,538 reads) and were removed prior to the analysis. In total, 122 MOTUs (234,090 reads) were then considered as potential prey.

Finally, 66 prey MOTUs (112,015 reads) were detected with the pair of primers COI, 53 prey MOTUs (122,029 reads) with pair of primers 16Sa and 3 prey MOTUs (46 reads) with pair of primers 16Sb (Supplementary Material 1). The total number of

TABLE 2 | Taxonomic groups identified with the different primers (COI, 16Sa or16Sb) in both predators.

Phyllum	Order	Family	Genus	Species	FT *O. vulgaris*				FT *A. media*			
					COI	16Sa	16Sb	O	COI	16Sa	16Sb	O
Arthropoda	Amphipoda	Hyperiidae			9.38							
	Calanoida	Calanidae	*Calanoides*	*C. carinatus*	1.56							
		Candaciidae	*Candacia*	*C. armata*						6.25		
		Clausocalanidae	*Clausocalanus*	*C. jobei*	4.69				6.25			
			Ctenocalanus	*C. vanus*					6.25			
			Pseudocalanus	*P. elongatus*					9.38			
									6.25			
		Euchaetidae	*Paraeuchaeta*	*P. hebes*					3.13			
		Paracalanidae	*Paracalanus*	*P. parvus*					3.13			
					39.63				31.25			
	Decapoda	Alpheidae				1.56						
		Carcinidae	*Carcinus*	*C. maenas*	4.69	32.81		32.81		9.38		
						4.69						
						7.81						
		Crangonidae	*Crangon*	*C. crangon*		3.13						
		Diogenidae	*Diogenes*	*D. pugilator*	4.69							
						4.69						
		Galatheidae	*Galathea*	*G. intermedia*	3.13							
				G. squamifera		1.56						
					3.13							
		Goneplacidae	*Goneplax*	*G. rhomboides*	23.44	12.50		26.56	9.38	6.25		12.50
					7.81							
					9.38							
		Inachidae	*Macropodia*	*M. parva*		4.69						
			Inachus	*I. dorsettensis*		3.13						
						7.81						
					1.56	1.56						
		Paguridae	*Anapagurus*	*A. hyndmanni*	3.13							
			Pagurus	*P. bernhardus*	7.81	6.25		10.93		3.13		
				P. prideaux	3.13	1.56		4.68	6.25			
					1.56	3.13						
		Pilumnidae	*Pilumnus*	*P. hirtellus*	46.88	67.19		76.56	6.25	18.75		25.00
						15.63						
					12.50	6.25						
						4.69						
		Pirimelidae	*Pirimela*	*P. denticulata*		7.81						
						3.13						
		Polybiidae	*Liocarcinus*	*L. navigator*	3.13	6.25		6.25		3.13		
					6.25	4.69						
			Necora	*N. puber*	1.56	10.94		10.94	3.13	3.13		6.25
						1.56						
						4.69						
		Porcellanidae	*Pisidia*	*P. longicornis*	6.25	9.38		10.93	6.25	21.88		25.00
		Portunidae			14.06				3.13			
		Processidae	*Processa*	*P. nouveli holthuisi*	1.56							
				P. edulis crassipes		3.13						
		Sesarmidae			1.56							
		Thiidae	*Thia*	*T. scutellata*		1.56						

(Continued)

TABLE 2 | Continued

Phyllum	Order	Family	Genus	Species	FT *O. vulgaris*				FT *A. media*			
					COI	16Sa	16Sb	O	COI	16Sa	16Sb	O
		Upogebiidae	*Upogebia*	*U. deltaura*	4.69							
						7.81				6.25		
		Varunidae				1.56				3.13		
		Xanthidae	*Xantho*	*X. pilipes*	1.56							
	Diplostraca	Podonidae	*Podon*	*P. intermedius*		9.38				6.25		
		Sididae	*Penilia*	*P. avirostris*	12.50							
						7.19						
	Euphausiacea	Euphausiidae	*Nyctiphanes*	*N. couchii*		4.69				18.75		
					1.56				12.50			
	Poecilostomatoida	Oncaeidae	*Oncaea*		4.69				3.13			
									6.25			
Chaetognatha	Phragmophora	Eukrohniidae			3.13				9.38			
Chordata	Perciformes	Gobiidae	*Crystallogobius*	*C. linearis*	1.56							
	Salpida	Salpidae	*Thalia*	*T. democratica*							3.13	
Echinodermata	Euryalida	Euryalidae			14.06				12.50			
	Ophiurida	Amphiuridae	*Amphiura*	*A. abyssorum*		7.81				3.13		
		Ophiactidae			4.69							
		Ophiuridae	*Ophiura*	*O. albida*						3.13		
					7.81				3.13			
									3.13			
Cnidaria	Leptothecata	Campanulariidae	*Obelia*	*O. geniculata*	1.56				46.88		12.50	
					1.56							
	Siphonophorae	Diphyidae	*Muggiaea*	*M. atlantica*	1.56				15.63			
			Muggiaea									
		Prayidae	*Rosacea*	*R. flaccida*			3.13				9.38	
Mollusca	Mytilida	Mytilidae	*Mytilus*	*M. galloprovincialis*		3.13						
	Architaenioglossa	Viviparidae			4.69							
	Veneroida	Lasaeidae			6.25							
		Montacutidae	*Tellimya*	*T. ferruginosa*	1.56							
Nermertina	Heteronemertea	Lineidae	*Cerebratulus*						3.13			

The frequency of occurrence of each Taxon (FT), detected by the primers individually (COI, 16Sa, 16Sb) and the combined information for taxa detected by at least two pair of primers (O, Overall frequency of occurrence).

sequences in each category detected in both predators by different pair of primers was, the average and the range are presented in Supplementary Material 2.

Prey Identification

Genetic analyses with primer COI revealed the presence of prey in the digestive glands of 56 *O. vulgaris* (56/64 = 87.5%) and 25 *A. media* (25/32 = 78.1%). Primers 16Sa revealed the presence of prey in the digestive tracts of 51 *O. vulgaris* (79.7%) and 10 *A. media* paralarvae (31.3%). Finally, 16Sb revealed the presence of prey in 2 *O. vulgaris* (3.12%) and 7 *A. media* paralarvae (21.87%). Primers COI amplified a wide spectrum of species detected in gut content of cephalopods paralarvae, belonging to a minimum of 7 phyla, 15 orders, and 32 families. Within these higher taxa, we were able to distinguish 28 genera and 27 species (**Table 2**). Primers 16Sa amplified mainly decapods, but also species belonging to other taxonomic groups. In total, they amplified taxa belonging to a minimum of 3 phyla, 6 orders, and 22 families. Within these, we were able to distinguish 21 genera and 24 species (**Table 2**). Primers 16Sb amplified in total 2 phyla, 3 orders, 3 families. Within these, we were able to distinguish 3 genera and 3 species (**Table 2**). In total, 21 families were exclusively identified by COI primers, 9 by 16Sa primers, and 2 by 16Sb. Thirteen families were detected with both primers COI and 16Sa (**Table 2**).

In total, considering all pair of primers together, prey were detected in 62 (96.9%) *O. vulgaris* and in 26 (81.3%) *A. media* paralarvae. The number of different prey taxa identified in individual *O. vulgaris* paralarvae range between 0 and 9 (mean ± standard error, 2.1 ± 0.267) with COI and between 0 and 7 (2.22 ± 0.232) with 16Sa. In individual *A. media* the number of prey taxa identified with COI primers were between 0 and 8 (2.09 ± 0.334) and between 0 and 11 (0.94 ± 0.378) with 16Sa.

O. vulgaris

The most abundant prey reads detected with primers COI (**Figure 2A**) matched with the crabs *Goneplax rhomboides* (order Decapoda), an unknown species of the family Portunidae (order Decapoda) and *Pilumnus hirtellus* (order Decapoda), as well as an unknown ophiuroid of the family Euryalidae (order Euryalida). With 16Sa primers (**Figure 2B**) the most abundant prey reads matched with the crabs *Carcinus maenas* (family Carcinidae) and *P. hirtellus* (family Pilumnidae) (Supplementary Material 1).

COI primers identified a total of 54 unique MOTUs belonging to 6 phyla, 14 orders, and 31 families. Within these, we were able to distinguish 28 genus and 20 species while 16Sa primers identified a total of 47 MOTUs belonging to 3 phyla, 5 orders, and 20 families. Within these, we were able to distinguish 18 genus and 19 species (**Table 2**).

Using COI primers, the most frequently detected MOTUs (FM) in *O. vulgaris* were the crab *P. hirtellus* (family Pilumnidae), the copepod *Paracalanus* sp. (family Paracalanidae) and the crab *G. rhomboides* (family Goneplacidae). With 16Sa, the most frequently detected MOTUs (FM) were the decapods *C. maenas* and *P. hirtellus*. The remaining MOTUs were detected in less than 10 octopus paralarvae (Supplementary Material 1).

Pilumnidae was the most frequently detected family in *O. vulgaris* with primers COI and 16Sa (FF =

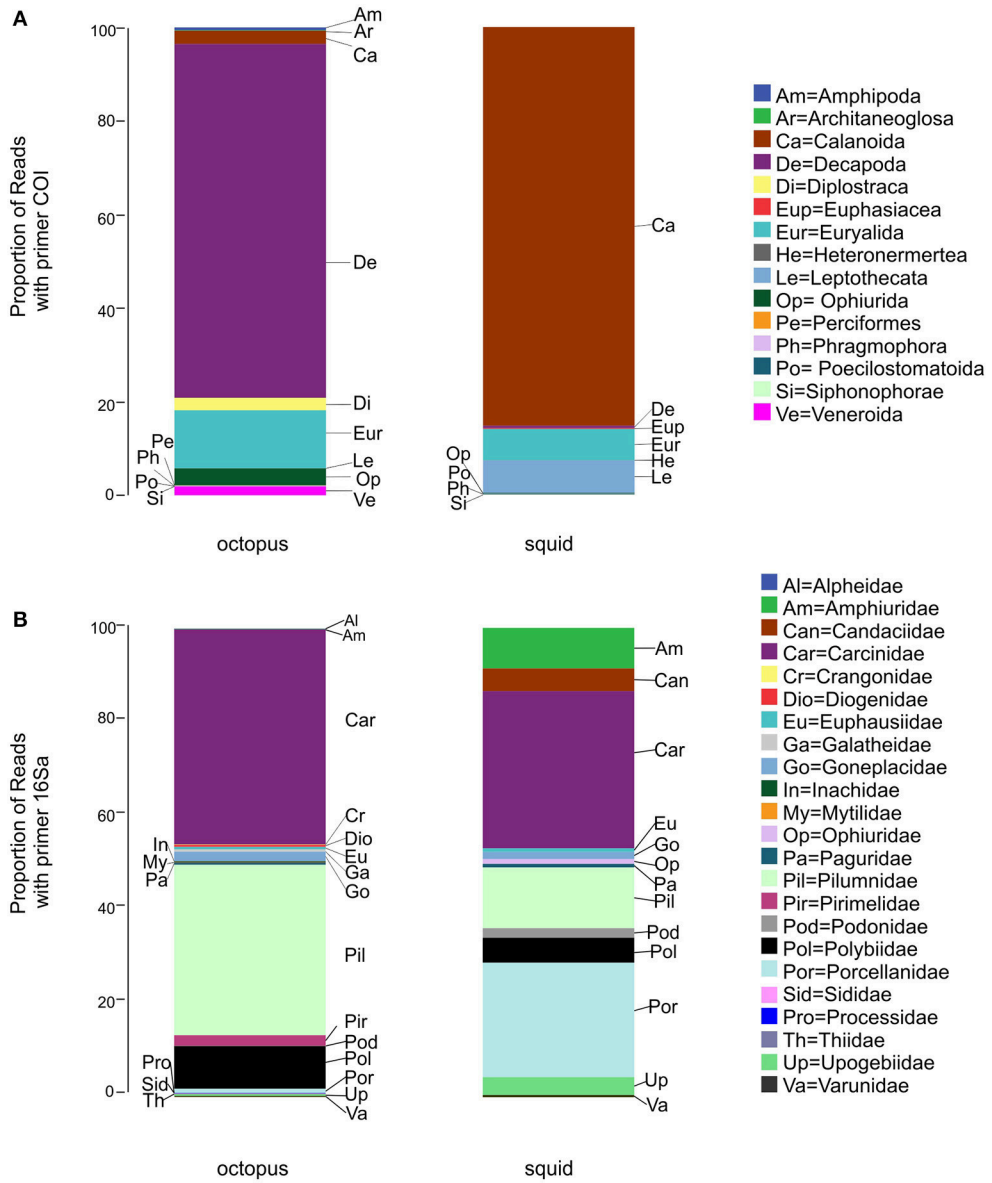

FIGURE 2 | Proportion of Molecular Operational Taxonomic Units (MOTU) prey reads (PR) detected with the primer COI (A) and the primer 16Sa **(B)** in *O. vulgaris* and *A. media*. MOTUs were clustered in orders **(A)** or families **(B)**.

47 and 67%, respectively) (**Figure 3**). With primers COI, other families detected in more than 10% of *O. vulgaris* paralarvae were Paracalanidae, Goneplacidae, Paguridae, Portunidae, Euryalidae, Sididae, and Polybiidae (**Figure 3A**). Primers 16Sa detected the families Carcinidae, Goneplacidae, Inachidae, Paguridae, and Polybiidae in more than the 10% of octopus paralarvae (**Figure 3B**).

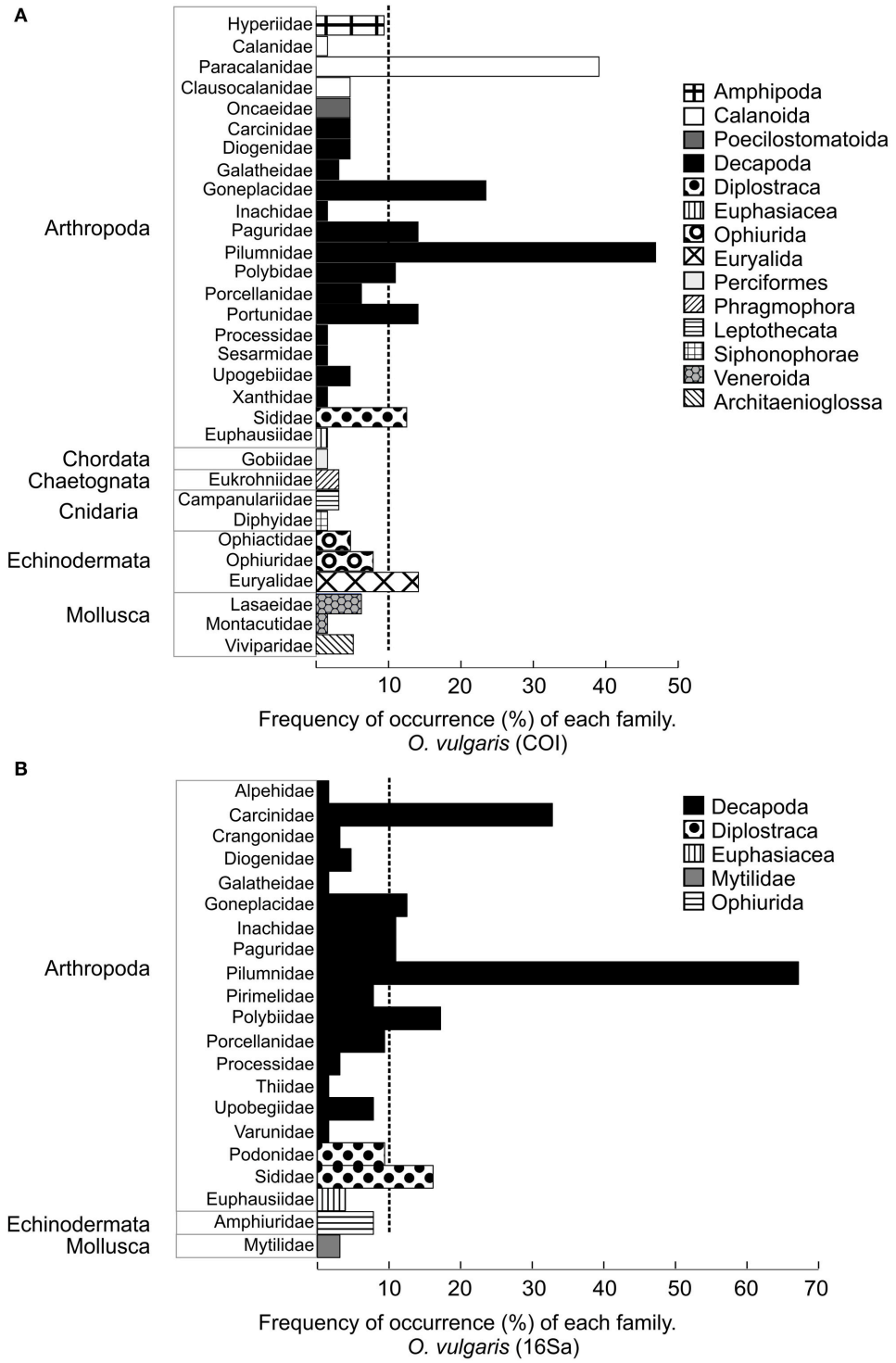

FIGURE 3 | Frequency of the occurrence of the families (FF) detected in *O. vulgaris* with primers COI (A) and primers 16Sa **(B)** with primer COI. Colors represent different orders. The corresponding phylum is indicated on the left. Vertical dashed line indicates the families detected in more than 10% of paralarvae.

Analysis with the primer 16Sb revealed that *Rosacea flaccida* (Order Siphonophorae) was present in two *O. vulgaris* paralarvae (four reads).

A. media

The most abundant prey reads with COI primers (**Figure 2A**) matched with the copepod *Paracalanus* sp. (order Calanoida), followed by the hydrozoan *Obelia geniculata* (order Leptothecata), and an unknown ophiuroid of the family Euryalidae (class Ophiuroidea). With 16Sa (**Figure 2B**), the most abundant prey reads matched with the crabs *C. maenas* (family Carcinidae), *Pisidia longicornis* (family Porcellanidae), and *P. hirtellus* (family Pilumnidae) (Supplementary Material 1).

In *A. media*, COI primers identified a total of 29 unique MOTUs, belonging to 5 phyla, 10 orders, and 17 families. Within these, we were able to distinguish 16 genera and 11 species, while 16Sa primers identified a total of 18 unique MOTUs, belonging to 2 phyla, 5 orders, and 13 families. Within these, we were able to distinguish 12 genera and 14 species (Supplementary Material 1).

The MOTUs most frequently detected (FM) in *A. media* were the hydroid *O. geniculata* (family Campanulariidae), the copepod *Paracalanus* sp. (family Paracalanidae), and the siphonophore *Muggiaea* sp. (family Diphyidae). The remaining 31 MOTUs were detected in less than 15% of squids (**Table 2**). 16Sa primers revealed that the most frequent detected MOTUs were *P. longicornis* and *P. hirtellus*. Seventeen MOTUs were detected in <10% of the squids (Supplementary Material 1).

The most frequently detected families detected with COI primers were: Campanulariidae (order Leptothecata),

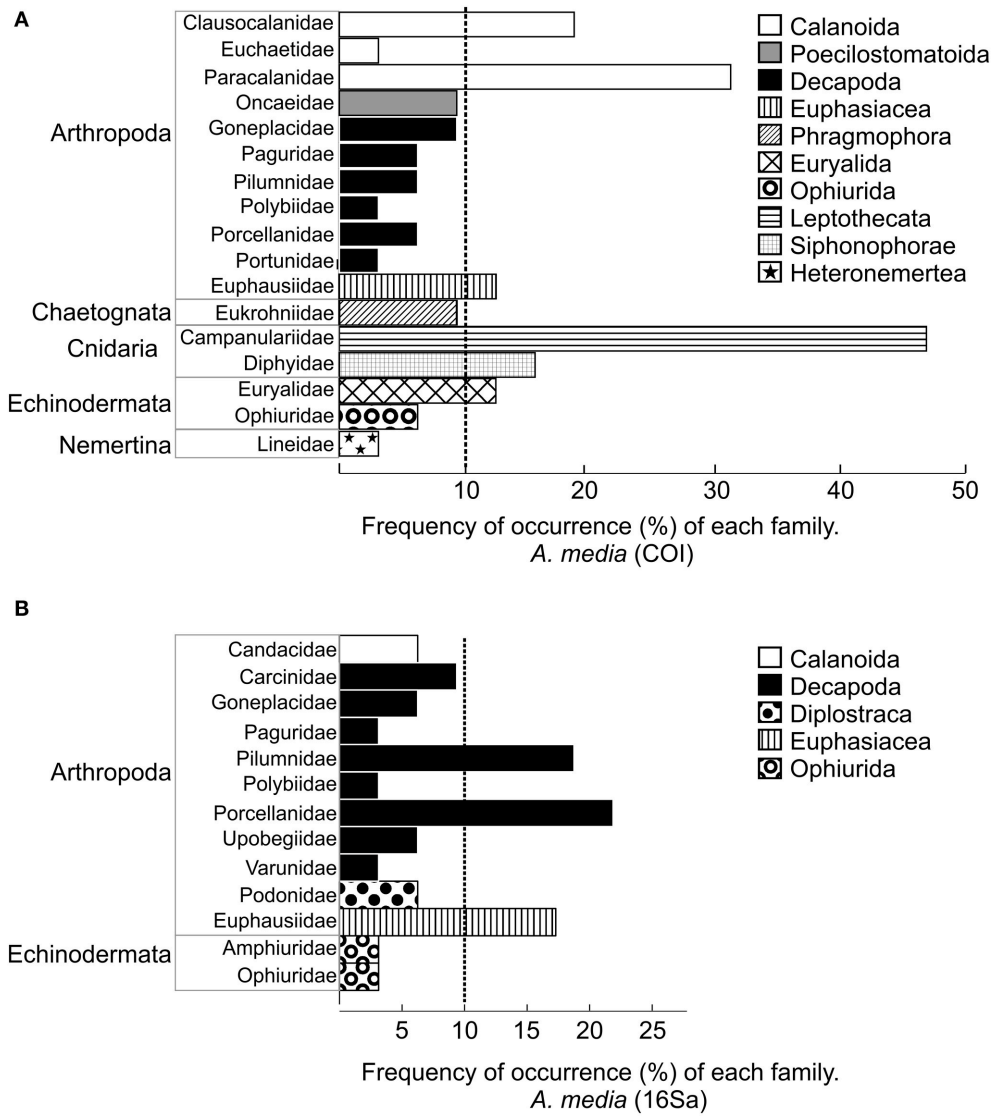

FIGURE 4 | Frequency of the occurrence of families (FF) detected in *A. media* with primers COI (A) and with primer 16Sa **(B)**. Colors represent different orders. The corresponding phylum is indicated on the left. Vertical dashed line indicates the families detected in more than 10% of paralarvae.

Paracalanidae, Clausocalanidae (order Calanoida), Diphyidae, Euphausiidae, and Euryalidae (**Figure 4A**). The most frequently detected families detected with 16Sa primers were Pilumnidae and Carcinidae (**Figure 4B**).

In *A. media*, 16Sb revealed the presence of the hydrozoan *O. geniculata* (FM = 12.5%, 32 reads), the siphonophore *R. flaccida* (FM = 9.3%, 7 reads), and the salp *Thalia democratica* (FM = 3%, three reads) (Supplementary Material 1).

Diet Selection

RDA analysis showed that season and transect, but not depth or individual size, significantly affected the prey families detected in the diet of *O. vulgaris* (**Table 3**). The sum of all canonical eigenvalues was 0.192 and the first two axes accounted for 51.53% of the fitted variation (i.e., the 9.91% of the total variation in the family data; **Figure 5**). In *A. media*, transect, but not season, size, or depth, significantly affected the families detected in their diet (**Table 3**). The sum of all canonical eigenvalues was 0.309, and the first two axes accounted for 48.73% of the fitted variation (i.e., the 15.05% of the variation in the family data; **Figure 5**).

The GAM analysis for the most frequently occurring families (detected in at least 10% of paralarvae) in *O. vulgaris* revealed that the copepod family Paracalanidae (FF = 39%) was more frequently predated in autumn than in summer ($p < 0.001$; **Table 4**). Predation on the crab family Pilumnidae (FF = 47%) differed across transects ($p = 0.028$; **Table 4**), being more frequent in T5 and T4 than the rest of transects (**Figure 6**). The ophiuroid family Euryalidae (FF = 14%) was more frequently predated in autumn than in summer ($p = 0.010$, **Table 4**) and was more frequent in smaller individuals ($p = 0.020$; **Figure 7**). The cladoceran family Sididae (FF = 13%) was more frequently found in small individuals ($p = 0.030$, **Figure 7**) and only detected in autumn (**Table 4**). The decapod families Goneplacidae (FF = 23%), Portunidae (FF = 14%), Paguridae (FF = 14%), and Polybiidae (FF = 11%) did not differ between seasons, among sizes or transects ($p > 0.05$ in all cases). The number of families in the diet of *O. vulgaris* differed between seasons ($p < 0.001$; **Table 3**) and among transects, ($p = 0.023$; **Table 3**). Thus, a wider range of families was predated in autumn than in summer, and in T3 than in T2 or T5 (**Table 3**). DML or depth did not affect the number of prey families detected in *O. vulgaris* ($p > 0.05$ in both cases).

The GAM analysis of the most frequent families (detected in at least 10% of paralarvae) in *A. media* revealed that the occurrences of the families Campanulariidae (FF = 47%), Paracalanidae (FF = 31%), Clausocalanidae (FF = 19%), Diphyidae (FF = 16%), and Euryalidae, (FF = 13%) in the diet were not affected by size, depth, transect, or season ($p > 0.005$ in all cases). However, family Euphausiidae (FF = 13%) was only detected in autumn. The number of families in the diet did not differ significantly with paralarval size, depth, transect, or season ($p > 0.05$ in all variables).

The discovery curves for *O. vulgaris* (**Figure 8**) showed stabilization of the proportional occurrence estimates, when at least 45 of 64 paralarvae were sampled. The discovery curves for *A. media* (**Figure 9**), did not show any stabilization for the whole number of samples analyzed ($n = 32$).

TABLE 3 | Summary of the RDA analysis.

Predator	Explanatory variable	Eigenvalue	*F*-statistic	*p*-value
O. vulgaris	Summer	21.17	2.632	**<0.001**
	T2	14.39	1.824	**0.002**
	T3	9.76	1.610	**0.032**
	T4	7.35	1.009	0.447
	Z1	10.64	1.020	0.428
	Z2	13.43	1.437	0.060
	Z3	8.87	0.744	0.617
	Small	9.69	1.101	0.323
	Medium	9.28	1.289	0.109
A. media	Summer	11.51	1.556	0.080
	T2	18.57	1.826	**0.017**
	T3	13.27	0.379	0.970
	T4	10.80	0.900	0.548
	Z1	1.78	0.569	0.724
	Z2	4.73	0.836	0.529
	Z3	8.55	0.396	0.977
	Z4	14.41	0.522	0.079
	Small	8.39	1.079	0.368
	Medium	8.39	0.893	0.593

Eigenvalues for single explanatory variables are expressed as a % of the sum of eigenvalues for the full set of explanatory variables (0.192 in O. vulgaris and 0.309 in A. media). All explanatory variables were included in the analysis: Season (Summer- Autumn); Transect (T2-T3-T4-T5); Depth (z1: 0-5 m; z2:5-10 m; z3: 10-20 m; z4: 20-35 m; z5:35-55 m); Size (small, medium, large). Significant values are in bold.

DISCUSSION

Overall, 107 MOTUs were successfully identified in *O. vulgaris*, which corresponded to 40 different families, 31 genera, and 32 species, while in *A. media*, 58 MOTUs were identified corresponding to 25 different families, 23 genera, and 21 species (Supplementary Material 1). The combination of the different primers targeting small DNA fragments, and comprehensive genetic databases, permitted us to identify up to 77 types of prey (**Table 2**). For the first time, a molecular approach was successfully applied to identify prey of wild *A. media* paralarvae, thereby increasing the range of known prey of wild *O. vulgaris* paralarvae during their first days of planktonic stage. Together, the results increased the knowledge of the prey predated by cephalopod paralarvae in their natural environment, suggesting more species to feed paralarvae in captivity conditions.

The amplification of the COI barcoding mitochondrial region with universal primers detected a broader taxonomic range of prey than the 16S primers (**Table 2**), and allowed the identification of 21 families that were not amplified with 16S primers. Additionally, 16Sa primers detected prey in digestive glands where no prey was detected with COI primers. 16Sa primers also amplified nine additional families not detected with COI primers. Of those, four families belonged to the class

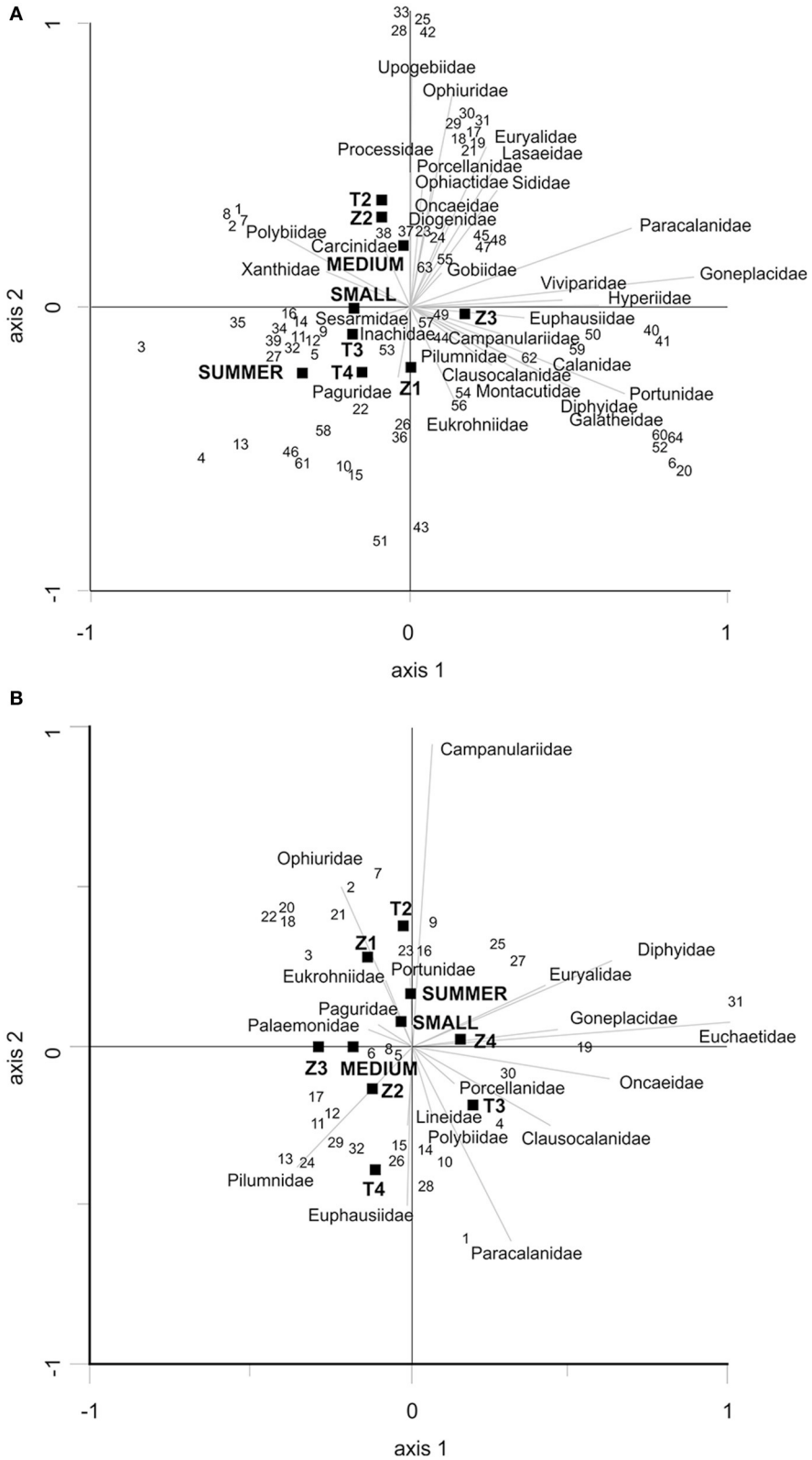

FIGURE 5 | (A) RDA triplot for the *O. vulgaris* families identified with COI gene. The correlation matrix was used. The first axis explains 29.57% and the second axis explains 21.96% of the total sum of all canonical eigenvalues (0.192). All the explanatory variables were used. **(B)** RDA triplot for the *A. media* families identified with COI gene. The correlation matrix was used. The first axis explains 27.26% and the second axis explains 21.47% of the total sum of all canonical eigenvalues (0.39). All the explanatory variables were used.

TABLE 4 | Summary of the best GAMs fitted to (A) dietary diversity (number of taxa detected) and (B) the occurrence of different families detected in *Octopus vulgaris*.

		Prey category	Model variables		Estimate	SE	Test				*p*-value	Dev.exp.	AIC
			Compared level	Reference level			z	χ^2	edf	df			
(A)													
		Number of families	Season					34.460		1	**<0.01**	46.500	227.510
			Summer	Autumn	1.207	0.206	5.870				**<0.01**		
			Transect					9.570		3	**0.023**		
			T3	T2	−0.753	0.264	2.857				**0.004**		
			T4	T2	−0.291	0.229	−1.270				0.204		
			T5	T2	−0.013	0.184	−0.073				0.942		
			T4	T3	0.462	0.308	1.500				0.134		
			T5	T3	0.740	0.275	2.689				**0.007**		
			T5	T4	0.243	0.243	1.142				0.253		
(B)													
Phylum Arthropoda	Order Calanoida	Paracalanidae	s(depth)					5.050	2.711		0.162	33.700	72.180
			Transect					5.985		3	0.112		
			Season					11.440		1	**0.001**		
			Summer	Autumn	2.350	0.696	3.383				**0.001**		
	Order Decapoda	Pilumnidae	s(depth)					7.287	2.641		0.059	24.900	79.730
			Transect					9.081		3	**0.028**		
			T3	T2	−1.276	1.195	−1.068				0.286		
			T4	T2	1.673	0.896	1.867				0.062		
			T5	T2	1.837	0.845	2.173				**0.030**		
			T4	T3	2.949	1.282	2.301				**0.021**		
			T5	T3	3.113	1.255	2.481				**0.013**		
			T5	T4	0.163	0.850	0.193				0.847		
	Order Diplostraca	Sididae	Season					0.000		1	1.000	59.400	34.880
			Transect					0.000		1	1.000		
			s(DML)					7.097	1.648		**0.030**		
			s(depth)					1.544	1.000		0.214		
Phylum Echinodermata	Order Euryalida	Euryalidae	Season					6.595		1	**0.010**	23.000	46.010
			Summer	Autumn	4.303	1.676	2.568				**0.010**		
			s(DML)					5.380	1.000		**0.020**		

Taxa listed are those for which the best GAM included a significant effect of at least one explanatory variable. Tests of significance are reported (χ^2 or z-values and associated probabilities) for all explanatory variables retained in the final models. Degrees of freedom (df) are presented for parametric terms (season and transect) and estimated degrees of freedom (edf) for smoothers (dorsal mantle length (DML) and depth). An edf of 1 indicates a straight line relationship. In addition, parameter estimates, ±standard error (SE), z-statistic and p-values are presented to allow the comparison between the different levels of the nominal explanatory variables (season and transects). Significant values are in bold. Percentage of deviance explained (Dev. exp.) by the model and AIC are also presented.

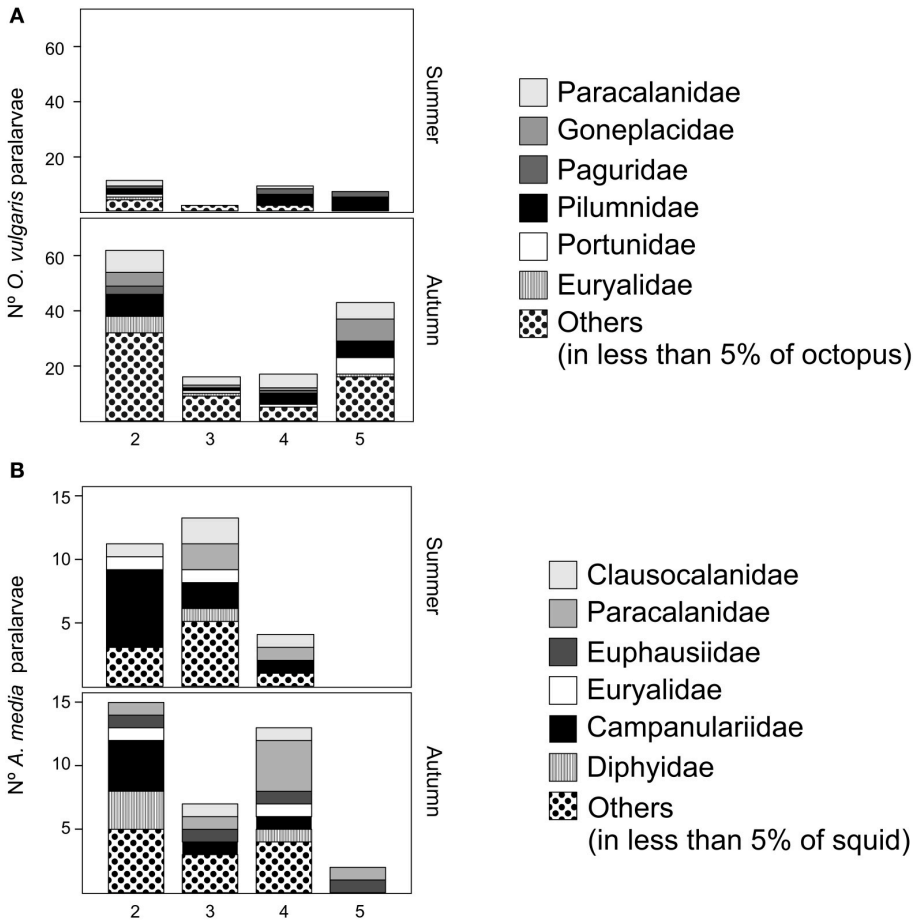

FIGURE 6 | Families detected in *O. vulgaris* paralarvae (A) and *A. media* paralarvae **(B)** in different transects (2, 3 4, 5) and different seasons (summer, autumn). The vertical axis represents the number of paralarvae that present a given family.

Malacostraca which was their target (Deagle et al., 2005), but 16Sa primers also amplified cephalopod DNA and five prey families belonging to ophiuroids, copepods, cladocerans, and mollusks (Amphiuridae, Ophiuridae, Candaciidae, Podonidae, and Mytilidae, respectively). Lastly, primers 16Sb were specifically designed to amplify teleost fishes (Deagle et al., 2009) but they amplified DNA from the predator species (i.e., *O. vulgaris* or *A. media*), two cnidarian species and urochordates (i.e., salps). When the same taxa were detected by two primer pairs, they were usually amplified unequally in the same predator (i.e., different occurrence for each prey in the same paralarvae and different number of reads). These results could be explained due to low prey DNA quantity and differential affinity of primers to prey DNA, supporting the usefulness of including more specific primers to increase taxonomic resolution of prey ingested (Blankenship and Yayanos, 2005; Deagle et al., 2009).

Previous studies (Piñol et al., 2014) showed that blocking primers are not essential in molecular dietary studies to detect small quantities of prey DNA. In our study, despite the large quantity of predator sequences (90% of sequences), the 7.5% of reads obtained from potential prey (Supplementary Material 2), provided prey information never uncovered by other methods employed to the date (i.e., visual, cloning, immunoassay) and highly increased our knowledge about the diet of paralarvae with many new prey taxa recorded. The addition of blocking primers, could have diminished predator sequences, increasing the number of prey reads (Vestheim and Jarman, 2008; Deagle et al., 2009; Leray et al., 2013a) and might have revealed additional prey species. However, additional studies comparing prey identification in diet analysis with both methodologies would be necessary to assess the utility of blocking primers to analyze the diet of cephalopod paralarvae. Owed to the high sensitivity of NGS methodologies, it is important to underline the possibility of detecting DNA of other organisms that were consumed by the prey ingested by the paralarvae, i.e., secondary predation (Sheppard et al., 2005). In addition, it may happen that some of the prey detected could be captured by the paralarvae inside of the net. If so, it should be expected to find prey remains in the proximal part of the digestive tract (esophagus, stomach, or crop). However, since only the digestive gland was dissected, we can assume that the prey detected in this study was ingested by the paralarvae before their capture.

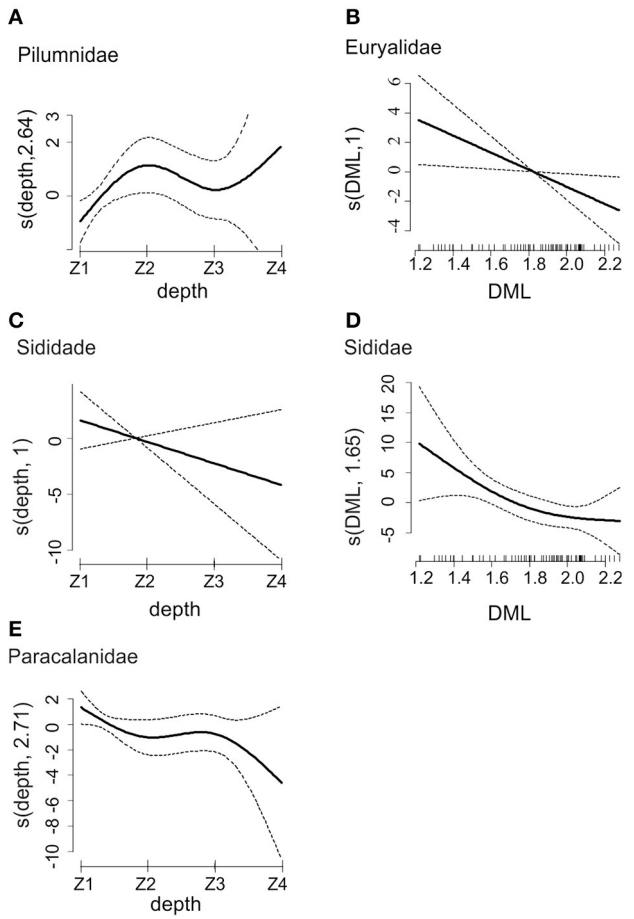

FIGURE 7 | Smooth curves for partial effects obtained by the Generalized Additive Modeling (GAM) of the occurrence of the families Pilumnidae (A), Euryalidae **(B)**, Sididae **(C,D)**, and Paracalanidae **(E)** as prey of *O. vulgaris* paralarvae. Explanatory variables used were dorsal mantle length (DML) and depth (Z1, Z2, Z3, Z4). Dotted lines are 95% confidence bands. The vertical axis represents the effect on the response variable.

Dietary Differences

Octopus vulgaris paralarvae mainly preyed on decapod species, that generally comprise <5% of the total zooplankton abundance in the Ría de Vigo (Roura et al., 2013; Buttay et al., 2015). Among decapods, the species most frequently detected in *O. vulgaris* were the crabs *C. maenas*, *P. hirtellus*, and *G. rhomboides* (families Carcinidae, Pilumnidae, and Goneplacidae, respectively), that are also the most abundant decapod species in the Iberian Peninsula coast (Paula, 1987; Fusté and Gili, 1991; Queiroga, 1996). Family Pilumnidae was less frequent in more oceanic transects (T5), probably because they migrate from estuarine zones to offshore waters during their larval development and there is higher concentration in more inshore waters. Moreover, species of this family were more frequently detected in paralarvae captured at depths between 5 and 10 m, probably because they migrate to the upper water layers at night (Dos Santos et al., 2008).

The second most frequently detected group in *O. vulgaris* gut contents were the Calanoid copepods, a group not detected in previous studies (Roura et al., 2012). In particular, *Paracalanus* sp. was the main copepod identified in *O. vulgaris* gut. In Galician zooplankton communities, Calanoid copepods in general represent more than 60% of total zooplankton abundance (Blanco-Bercial et al., 2006; Roura et al., 2013; Buttay et al., 2015). Zooplankton community studies in this area have also shown that high abundances of *Paracalanus* species are linked to low salinity values (Blanco-Bercial et al., 2006). In our study, this prey was more frequently detected in *O vulgaris* paralarvae captured in autumn. The upwelling conditions during this season (i.e., cold and low salinity waters), could have promoted high abundances of this species increasing their availability in the environment and thus facilitating the predation.

Brittle stars (family Euryalidae) and cladocerans (family Sididae) were both frequently detected in small *O. vulgaris* paralarvae, perhaps because they are an easier target than fast moving copepods and decapods. The cladoceran identified with COI primers was *Penilia avirostris*. This species has been highlighted as an indicator of warm waters, and high abundances have occasionally been described in the Ría de Vigo associated with an increase in water temperature (Figueiras et al., 2011). The sea surface warming trend observed in Galician coastal waters during recent years (Gómez-Gesteira et al., 2008) could be favoring the presence of this cladoceran species. Another cladoceran that is very abundant in the Ría de Vigo was detected by primers 16Sa, namely *Podon intermedius* (Roura et al., 2013; Buttay et al., 2015). It was identified also in small and medium individuals. The detection of abundant cladoceran species in octopus guts could suggest opportunistic predation on cladocerans, specifically by smaller paralarvae.

Only one fish species was identified with COI primers in a single *O. vulgaris* paralarvae, and no fish DNA was amplified with 16Sb primers that were specifically designed to amplify fish DNA (Deagle et al., 2009). This result suggests low predation on fish, perhaps because the high mobility of fish larvae makes it difficult for the paralarvae to capture them.

Regarding squids, in *A. media* different prey species and different frequencies of occurrence were detected compared to *O. vulgaris*: Cnidarians were detected in *A. media* paralarvae of all sizes. Cnidarians are not very abundant in zooplankton community in Galicia (Buttay et al., 2015). Thus, these results could suggest selective predation on cnidarians, as also observed in turtles and sunfish (Dodge et al., 2011; Sousa et al., 2016). In contrast, cnidarians were only detected in three *O. vulgaris.* Their rare presence might be explained as a secondary predation effect (Sheppard et al., 2005) because high resolution of NGS, can detect small DNA amount present in the digestive tract of a prey captured by the paralarvae. It is also possible that hydroids are predated by *O. vulgaris* because they are easy to capture for slow recently hatched paralarvae (<10 days old, Garrido et al., 2016b). Moreover, squid paralarvae ingested up to ten copepod species, while only four were detected in octopus. This difference between *A. media* and *O. vulgaris* might be related with their hunting skills, which are developed during initial life stages (Villanueva et al., 1997). *Alloteuthis media* also preyed on decapods, and species of this group were mainly detected with the primer pair 16Sa. Thus, this could imply that the amount of DNA present

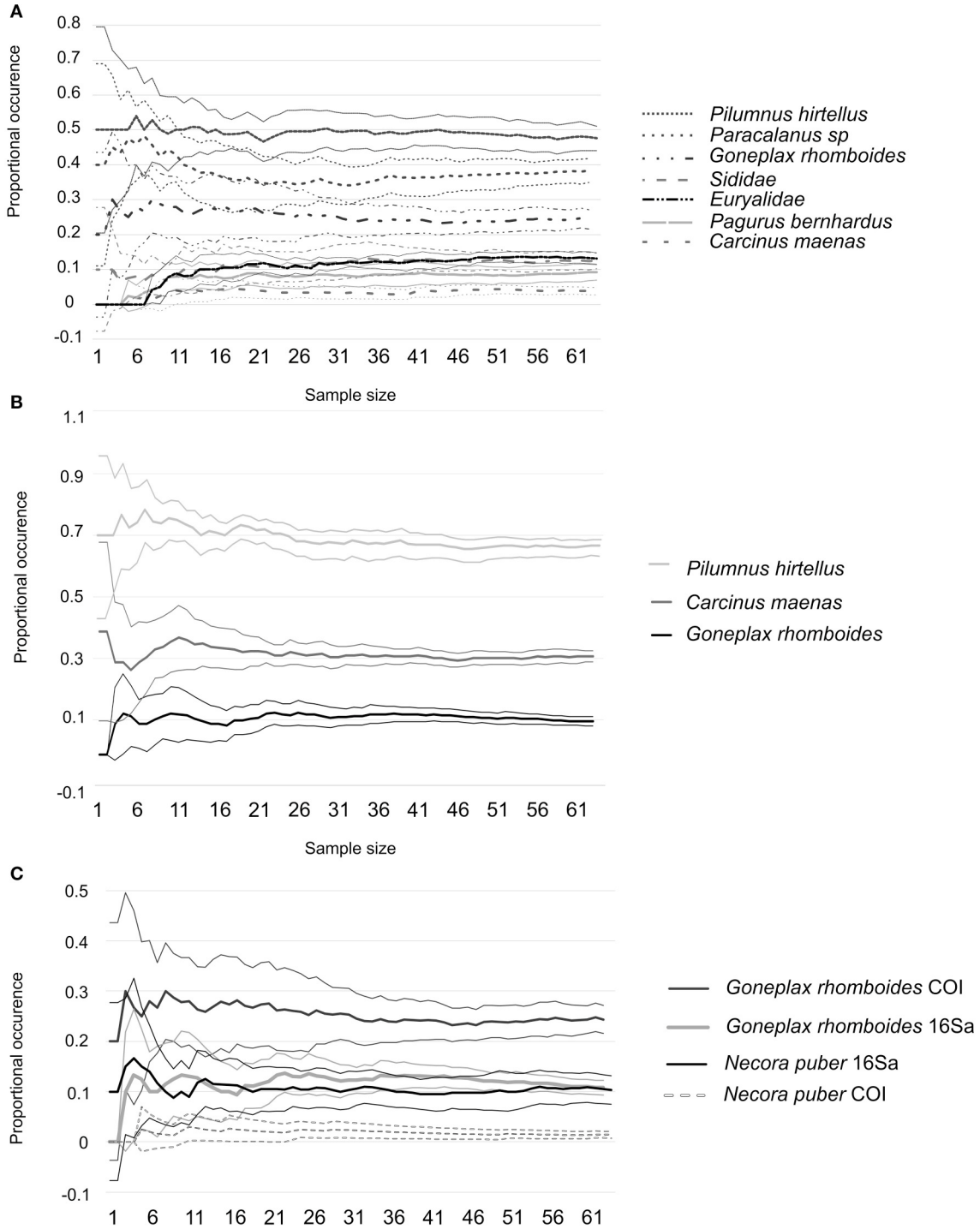

FIGURE 8 | Summary from 10 randomized sample sets of species identified in *O. vulgaris* with primers COI (A), primers 16Sa **(B)**, and both pair of primers **(C)**. Average values are presented as continues lines and Confidence Intervals (C.I.) as dashed lines.

was low and it was only possible to amplify decapod DNA with the specific pair of primers.

Other prey detected in both cephalopod species such as amphipods, cladocerans, euphausiids, and fishes, had been previously detected in the paralarval digestive system, but with a lower taxonomical resolution (Passarella and Hopkins, 1991; Vecchione, 1991; Venter et al., 1999; Vidal and Haimovici, 1999; Roura et al., 2012). Additionally, the gut contents of paralarvae

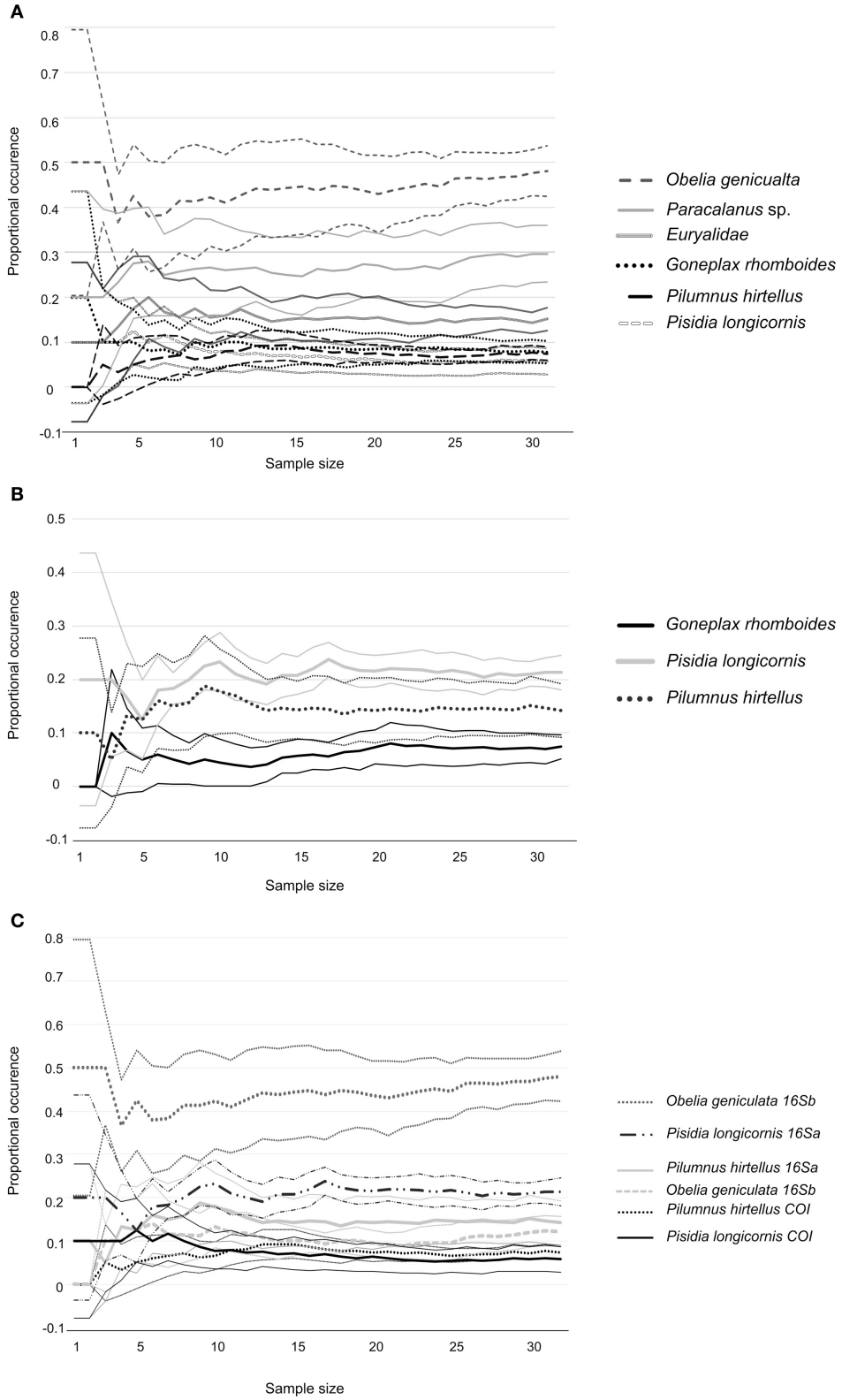

FIGURE 9 | Summary from 10 randomized sample sets of species identified in *A. media* with primers COI (A), primers 16Sa **(B)**, and both pair of primers **(C)**. Average values are presented as continues lines and Confidence Intervals (C.I.) as dashed lines.

of both species included molluscs, echinoderms, chaetognaths, and a nemertean that had never been previously identified in cephalopod paralarvae. Finally, DNA of chaetognaths and nemerteans was detected in a small number of paralarvae gut contents, and thus could reflect opportunist predation or alternatively, their DNA might be present in an organism ingested by the paralarvae, as an effect of secondary predation as explained above.

Diet diversity for *O. vulgaris* was influenced by the season and distance to shore. Numerous studies have shown that zooplankton communities in Galicia change according to oceanographic and meteorological conditions (Bode et al., 2009; Roura et al., 2013; Buttay et al., 2015). Thus, diet variability observed in *O. vulgaris* paralarvae might be related to zooplankton changes in prey availability in the zooplankton community. In contrast, no relationship could be established between the diet of *A. media* and the environmental explanatory variables or individual size. This may be related to small number of samples analyzed: discovery curves in *A. media*, showed very wide C.I. and no stabilization of the proportional occurrence estimates for the whole number of samples analyzed ($n = 32$). In contrast, *O. vulgaris* discovery curves, showed narrower C.I. and a stabilization of the proportional occurrence estimates, when at least 45 paralarvae are sampled. These results suggest that the number of *A. media* paralarvae analyzed was insufficient for a comprehensive dietary analysis of this species. In contrast, results suggest that the number of paralarvae of *O. vulgaris* analyzed in this study could be enough for this dietary analysis.

Our results showed that *O. vulgaris* prey on a wide variety of decapod species, but also frequently prey on other taxonomic groups, including mollusks, ophiuroids, amphipods, cladocerans, copepods, chaetognaths, or cnidarians. However, the low number of samples analyzed in previous research could have prevented the identification of rarely detected prey, that would likely only be identified when increasing the number of paralarvae analyzed. Moreover, the employment of several primers targeting different genes, could have favored the detection of additional species with broader taxonomic range that previous studies.

Overall, our results showed the usefulness of the NGS approach with several primers targeting different genes to dietary analysis of wild cephalopod paralarvae. Results have shown that they feed on a wide diversity of prey, mainly decapods, copepods, and cladocerans, but also other taxa that have not been previously identified in wild cephalopod paralarvae such as mollusks, echinoderms, chaetognaths, salps, cnidarians, and a nemertean. This study provides essential data to elaborate more suitable diets for captive cephalopod paralarvae, with the aim of increasing their survival for economically sustainable farming. Further studies are needed, including use of a wider variety of prey, mainly copepods from the genus *Paracalanus*, Cladocerans, and different decapod species, to test the effect on the digestive gland performance, growth and survival of recently hatched paralarvae.

AUTHOR CONTRIBUTIONS

ÁG: conceived the plankton sampling strategy and financially supported the project; ÁG, ÁR, and LO-P: undertook sampling surveys and contributed to the conception of the experiment; SB, GP, and LO-P: planned the experimental design; LO-P and SB: executed the laboratory work; SB: handled bioinformatic data analysis; GP and LO-P: did statistical analysis. All the authors have revised the manuscript critically for important intellectual content and have approval the final version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00321/full#supplementary-material

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You Are What You Eat: A Genomic Analysis of the Gut Microbiome of Captive and Wild *Octopus vulgaris* Paralarvae and Their Zooplankton Prey

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The common octopus (*Octopus vulgaris*) is an attractive species for aquaculture, however, several challenges inhibit sustainable commercial production. Little is known about the early paralarval stages in the wild, including diet and intestinal microbiota, which likely play a significant role in development and vitality of this important life stage. High throughput sequencing was used to characterize the gastrointestinal microbiome of wild *O. vulgaris* paralarvae collected from two different upwelling regions off the coast of North West Spain ($n = 41$) and Morocco ($n = 35$). These were compared to that of paralarvae reared with *Artemia* for up to 25 days in captivity ($n = 29$). In addition, the gastrointestinal microbiome of zooplankton prey (crabs, copepod and krill) was also analyzed to determine if the microbial communities present in wild paralarvae are derived from their diet. Paralarvae reared in captivity with *Artemia* showed a depletion of bacterial diversity, particularly after day 5, when almost half the bacterial species present on day 0 were lost and two bacterial families (Mycoplasmataceae and Vibrionaceae) dominated the microbial community. In contrast, bacterial diversity increased in wild paralarvae as they developed in the oceanic realm of both upwelling systems, likely due to the exposure of new bacterial communities via ingestion of a wide diversity of prey. Remarkably, the bacterial diversity of recently hatched paralarvae in captivity was similar to that of wild paralarvae and zooplankton, thus suggesting a marked effect of the diet in both the microbial community species diversity and evenness. This study provides a comprehensive overview of the bacterial communities inhabiting the gastrointestinal tract of *O. vulgaris* paralarvae, and reveals new research lines to challenge the current bottlenecks preventing sustainable octopus aquaculture.

Keywords: ***Octopus vulgaris*** **paralarvae, high throughput sequencing, gastrointestinal tract, microbial communities, core gut microflora, upwelling ecosystems, aquaculture microbiology**

INTRODUCTION

One of the most outstanding issues in microbial ecology of the gastrointestinal (GI) tract is understanding how biological and physical factors influence gut microbiota and their hosts (Sullam et al., 2012). The GI tract is occupied by a complex and dynamic ecosystem of organisms composed of an enormous variety of aerobic, facultative anaerobic and obligate anaerobic microbes that interact with the host and with each other (Nayak, 2010). The role of microbiota on host health is increasingly being recognized, for example, the GI microbiota of fish contributes to the development of its host through xenobiotic metabolism, microbially-mediated digestion of food, essential nutrient supply including vitamins, amino acids and fatty acids, immunity, and resistance toward intestinal pathogens (Kesarcodi-Watson et al., 2008; Ringø et al., 2016).

Until recently, most studies examining the microbiota associated with marine organisms have employed culture-dependent methods (Forney et al., 2004). This approach is somewhat limited, given that the vast majority of microorganisms present in a natural environment cannot be cultured *in vitro* (Fjellheim et al., 2007). For example, in marine biomes the percentage of unculturable organisms is estimated to be higher than 97% (Rappé and Giovannoni, 2003). Culture-independent methods, such as the detection and sequencing of the microbial-derived 16S small subunit ribosomal RNA (rRNA) gene, have been developed to overcome this limitation, and have been applied toward the study of hatchery-associated bacterial populations of Atlantic cod (*Gadus morhua*, Brunvold et al., 2007; Reid et al., 2009; Bakke et al., 2013), abalone (*Haliotis diversicolor,* Zhao et al., 2012), Atlantic halibut (*Hippoglossus hippoglossus* L., Verner-Jeffreys et al., 2003; Jensen et al., 2004) and great scallop (*Pecten maximus,* Sandaa et al., 2003). However, the focus on PCR amplification of the 16S rRNA gene alone may provide a biased estimate of species abundance, given that "universal primers" for 16S PCR are not necessarily universal, i.e., not all species can be detected due to unknown sequence variation, and that biases associated with primer mismatch, and preferential amplification of the most abundant groups have been described (reviewed in Forney et al., 2004).

High throughput sequencing (HTS) methods are increasingly being applied to the characterization of microbial communities, leading to a more comprehensive appreciation of extant biodiversity (Mock and Kirkham, 2012). Although, HTS approaches still commonly rely on the amplification of one or two hypervariable regions of the 9 hypervariable regions (V1–V9) present in the 16S rRNA gene, its significant advantage over conventional 16S rRNA sequencing is the dense sampling of a given community increasing the likelihood of capturing lowly abundant species (Mock and Kirkham, 2012). Moreover, the sample throughput of HTS is significantly higher than traditional approaches, mediated by sample barcoding and multiplexing (the number of samples limited largely by the number of unique barcodes available), enabling study designs thousands of times more robust than other PCR-based techniques (Zarkasi et al., 2014; Huang et al., 2016).

The efficiency and sustainability of any mariculture system will likely be significantly influenced by the microbial composition of the species in question in their natural environment. Characterization of species composition, relative quantities, and the potential sources of the core intestinal microbiota commonly associated with feed and larvae at different stages of development is essential for viability and vitality of the organisms (Ringø and Birkbeck, 1999; Olafsen, 2001), and aid in the identification of possible microbial pathogens affecting larval mortality (Star et al., 2013). In cephalopod mollusks (octopus, squids and cuttlefishes), only three studies have analyzed the microbial diversity of *Octopus* species (de la Cruz-Leyva et al., 2011; Iehata et al., 2015, 2016). These studies have used Denaturing Gradient Gel Electrophoresis (DGGE) techniques to analyse cultured bacterial diversity sampled from adults and eggs of the Chilean Gould octopus, *Octopus mimus* (Gould, 1852), revealing differences between males and females in the microbial families present (mostly Vibrionaceae and Streptococcaceae) and their nutritional enzymatic activities (Iehata et al., 2015). In addition, a relationship between egg-associated bacterial diversity and egg health condition (dominated by *Roseobacter*) was also detected (Iehata et al., 2016). Vibrionaceae were the main bacterial group, identified using RNA transcripts of the 16S rRNA gene, from metabolically active bacterial flora of adult octopuses collected in Mexico (de la Cruz-Leyva et al., 2011). Considering the low throughput approaches used in these studies, it is likely that they only account for a fraction of bacterial diversity present within octopods or cephalopods in general. The application of genomic methods will significantly enhance the characterization of the cephalopod paralarvae microbial communities, and may in turn provide useful insight toward improving the aquaculture conditions of commercially important species such as the common octopus, *Octopus vulgaris* Cuvier, 1797.

In spite of the plethora of experiments to solve it (reviewed in Vidal et al., 2014), rearing *O. vulgaris* paralarvae in captivity is difficult and remains a significant hurdle that prevents viable aquaculture. Little is known about the ecology of wild *O. vulgaris* paralarvae and their unusual planktonic strategy in the open ocean, largely due to difficulties in obtaining specimens (Roura, 2013). It has been recently suggested that *O. vulgaris* paralarvae undertake a unique planktonic strategy, compared with that of other coastal cephalopods with planktonic stages (Roura et al., 2016). They hatch close to the coast with only three suckers per arm, and after <10–15 days, they are transported away from the continental shelf by coastal upwelling filaments, finishing their development in the open ocean. Remarkably, 58 *O. vulgaris* paralarvae containing more than three suckers per arm were collected in zooplankton samples off the NW Iberian Peninsula (42 specimens) and Morocco (16 specimens), with bottom depths ranging between 787 and 3,110 m (Roura, 2013). These paralarvae are the only specimens larger than three suckers per arm ever collected in the Eastern Atlantic (Rocha et al., 1999; González et al., 2005; Moreno et al., 2009; Otero et al., 2009; Roura et al., 2016). These rare samples therefore provide a unique opportunity to study the ontogenic changes of their microbial biota from the coast to the ocean, and to compare the natural

microbiome against that found in aquaculture, with the aim to determine the importance of the GI microbiome on the health of captive paralarvae.

In this study, we have applied HTS to characterize the core gut microbiota of wild paralarvae collected in two different upwelling regions (NW Spain and W Morocco), and to identify the main microbial groups that differ between ecosystems. Furthermore, we have compared the GI microbiota of wild *Octopus* paralarvae against that of paralarvae reared with *Artemia* during 25 days in captivity. This enabled characterisation of the core gut microbiota of wild paralarvae and identification of bacterial groups that are not present in paralarvae reared in captivity, and to identify potential pathogens that may affect the health of paralarvae reared in captivity.

MATERIALS AND METHODS

Planktonic samples were collected during the multidisciplinary project "Canaries-Iberian Marine Ecosystem Exchanges (CAIBEX)" (**Figure 1**, red frames), off the coasts of North-Western Iberian Peninsula (CAIBEX-I: July 7 to 24, **Figure 1B**) and Morocco (CAIBEX-III: August 16 to September 5, **Figure 1C**) in 2009. Mesozooplankton samples were collected day and night with two 750 mm diameter bongo nets equipped with 375 μm mesh and a mechanical flow-meter. Three double-oblique towings were carried out (at a ship speed of 2.5 knots) per station over the continental slope (>200m depth): (i) at the deep scattering layer (DSL: 500 m), (ii) at 100 m, and (iii) at the surface (0–5 m). Over the continental shelf (<200 m) only two double oblique towings were collected at 100 m (when sea-bottom was <100 m, otherwise 10 m above it) and at the surface (0–5 m). The bongo net was first lowered to the desired depth, towed for 30 min and subsequently hauled at 0.5 m s–1. The net was recovered, cleaned on board and placed back into the sea for the next towing. Plankton samples were fixed with 96% ethanol and stored at −20°C to facilitate DNA preservation. All cephalopod paralarvae were sorted from the zooplankton samples and stored individually in 70% ethanol at −20°C. In total, 134 *O. vulgaris* paralarvae were collected during CAIBEX-I ($n = 99$ specimens) and CAIBEX-III ($n = 35$ specimens). Of these, 41 paralarvae were chosen from CAIBEX-I (ranging from 3 to 5 suckers per arm) and 35 from CAIBEX-III (ranging from 3 to 15 suckers per arm) to study the ontogenic changes of the microbiota in the wild.

The microbiota of paralarvae reared in captivity with *Artemia* in 2012 at the facilities of the Spanish Institute of Oceanography in Vigo (IEO-Vigo), was also analyzed using five replicates at ages 0, 5, 10, 15, 20, and 25 days post hatchling (ranging from 3 to 5 suckers per arm). Paralarvae were anesthetized at the end of the study by immersing them in a 1.5% $MgCl_2$dissolved in seawater at room temperature (18–21°C) for 10 min, after which the $MgCl_2$ concentration was increased to 3.5% for 30 min to kill them. The procedures applied herein comply with Directive 2010/63/EU, in terms of minimizing the number of animals used and animal sacrificing method employed (Fiorito et al., 2015). This study was performed in accordance with corresponding Spanish guidelines and regulations (Ley 32/2007, November 7th) and was exempt from an ethics review process.

The euphausiid *Nyctiphanes couchii*, the crabs *Pirimela denticulata* and *Pilumnus hirtellus,* and the copepod *Paraeuchaeta hebes,* were sorted from the zooplankton samples collected near the coast of NW Spain and the microbiome of their gastrointestinal tract was analyzed. Crabs and krill are known prey of wild *O. vulgaris* paralarvae (Roura et al., 2012), whereas *P. hebes* has not been described as part of the Octopus diet. However, this copepod is an important member of the coastal zooplankton (Roura et al., 2013) and has been recently identified in the digestive tract of *Alloteuthis media* paralarvae (Olmos-Pérez et al., 2017), and therefore, has been included as a potentially informative bioindicator of the environmental microbiota present.

Library Preparation and Sequencing

Genomic DNA was extracted from the dissected digestive tract of *O. vulgaris* paralarvae (including the esophagus, crop, stomach, caecum, digestive gland, and intestine) and zooplankton prey (including the internal contents of the cephalothorax after removing appendages and the carapace). DNA was extracted with QIAGEN DNeasy Blood and Tissue Kit according to manufacturer's instructions. A slight modification was made at the final elution stage; the elution was repeated twice using two 20 μL aliquots of 45°C ultrapure water, and stored as a combined 40 μL eluate prior to use.

A DNA fragment that spanned the V3 and V4 hypervariable regions of 16S rRNA (∼444 bp) was amplified with the primers S-D-Bact-0341-b-S-17 (341f)/S-D-Bact-0785-a-A-21(785r; Klindworth et al., 2013), since it is the optimal hypervariable regions to characterize bacterial communities (Mizrahi-Man et al., 2013; Sinclair et al., 2015). These primers included a modification to the 5′ end to include an Illumina-compatible adapter sequence to allow multiplexing (**Table 1** in bold). An evaluation of base-specific biases for the commonly used PCR primer sets used to amplify the 16S rRNA hypervariable regions compared with metagenomic data, revealed that <16% of 16S rRNA sequences are missed with the V3–V4 regions (Eloe-Fadrosh et al., 2016). They defined a subset of bases within the "universal" primers contributing to the percentage of metagenomic SSU rRNA gene sequences that would probably be missed in next generation PCR-based surveys. Accordingly, we modified one of these variable nucleotides by adding an inosine (I) to complement all four nucleotides (Geller et al., 2013) in the 3′ end of the universal primer 341f (**Table 1** in italics) to capture a greater fraction of the microbial diversity.

PCR reactions contained 0.35 μl of primer 341f and 0.2 μl of primer 785r (10 μM stock concentration), 6.25 μL REDTaq® ReadyMix (Sigma-Aldrich), 0.1 μL $MgCl_2$ and 1 μL of DNA (at a concentration of ∼20 ng) in a total reaction volume of 12.5 μL. Touchdown PCR cycle conditions included an initial denaturing step (95°C for 3 min), followed by 10 cycles at 95°C for 30 s, 58°C for 30 s (1° decrease per cycle) and 72°C for 30 s; followed by 15 cycles at 94 °C for 30 s, 48°C for 30 s, and 72°C for 30 s. Negative control reactions containing all components, but water instead of template, were performed alongside all PCR reactions to ensure that there was no contamination.

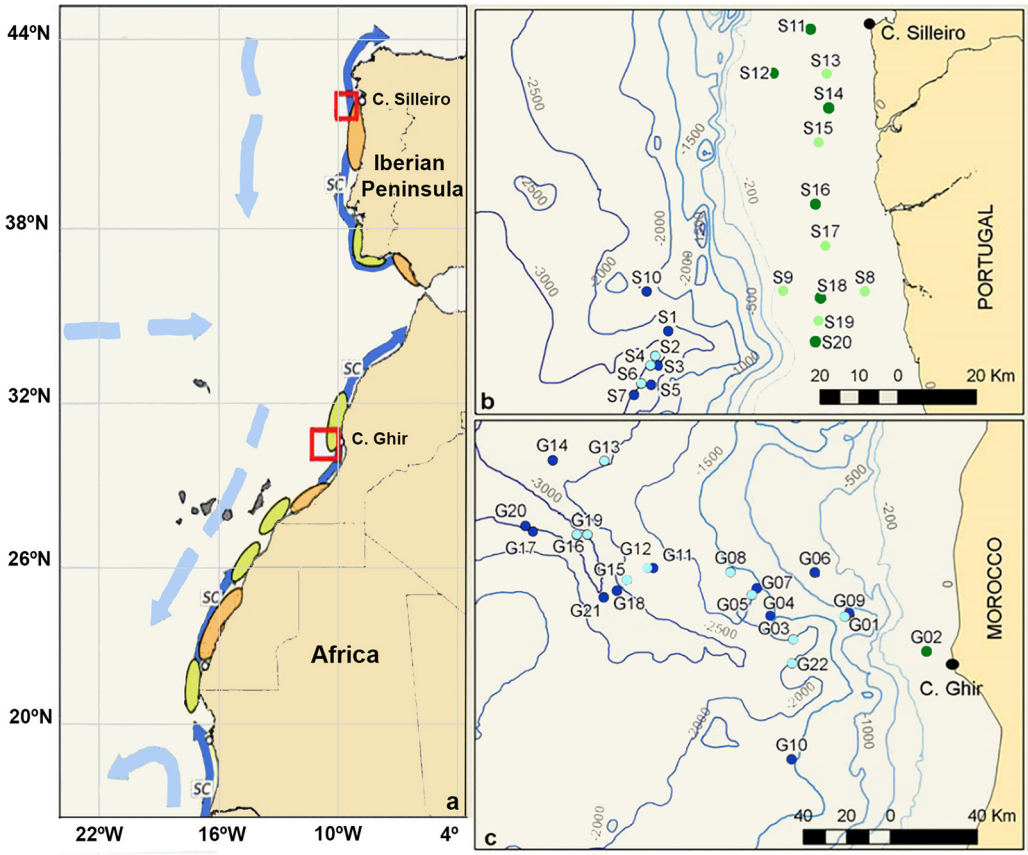

FIGURE 1 | (a) Schematic map of the Iberian Canary Current eastern boundary upwelling showing the areas sampled (red boxes) and the main currents (light blue: surface currents; dark blue: slope current = SC), retention (orange), and dispersion (green) zones on the shelf. **(b)** Zooplankton samples collected off the coast of the NW Iberian Peninsula. **(c)** Zooplankton samples collected off the Morocco coast. Samples collected over the continental shelf (green, <200 m depth) and in the open ocean (blue, >200 m depth), with light/dark colors representing day/night samplings.

TABLE 1 | Primers used in this study, modified from Klindworth et al. (2013) to include Illumina adapter overhang nucleotide sequences (in bold) and an inosine (I) in the 3′end of 341f primer instead of *N* (i.e., A or T or C or G).

Primer	Sequence 5′–3′
341fI	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG** CCTACGGG/GGCWGCAG
785r	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG** GACTACHVGGGTATCTAATCC

PCR products (2 μl) were visualized on a 1% (w/v) agarose gel. Five microliters of this PCR product was added to a second PCR reaction for 10 cycles (95°C for 10 s, 48°C for 15 s, and 72°C for 15 s), in order to incorporate Illumina dual index primers (4 μL of 1.25 μM) to the V3–V4 amplicon target by re-amplification. Amplified DNA solutions were purified using AMPure XP beads/PEG 6000 solution (1.1 × beads/DNA volume), quantified using a Qubit® 2.0 Fluorometer (Invitrogen) and pooled in equimolar concentrations (0.5 ng/μL). The library was diluted to 12.5 pM and sequencing was performed using a 600 cycle (paired-end) v3 MiSeq Reagent Kit on an Illumina MiSeq. PhiX sequencing library (Illumina) was spiked into the amplicon sequencing library (10%), to account for the limited sequence diversity among the 16S amplicons.

Quality Filtering and Bioinformatic Analysis

Quality filtering was carried out following recommendations for Illumina platforms (Bokulich et al., 2013). Reads that did not meet the following standards were removed: (i) Phred score below 30 (i.e., one error in 1,000 bases), (ii) less than 75% of target length, (iii) less than three consecutive low quality calls, and (iv) reads with ambiguous calls. The remaining paired-end reads were merged using PEAR v0.9.4 (Zhang et al., 2014). Merged reads were demultiplexed into individual sample read-sets based on their corresponding indexed adapter combination. Reads for which the indexes/primers did not match the expected sequences were discarded. The remaining reads were then filtered against a custom Kraken (v0.10.4) database to exclude archaeal and viral contamination (Wood and Salzberg, 2014). The UCHIME algorithm of USEARCH (v 6.0.307; Edgar et al., 2011) was used to check for chimeric sequences amongst the bacterial reads.

Bacterial reads were then classified using ClassifyReads, a high-performance naïve Bayesian classifier of the Ribosomal Database Project (RDP) described in Wang et al. (2007) available within the Illumina metagenomic analysis software 16S Metagenomics on BaseSpace (https://basespace.illumina.com). ClassifyReads uses a 32-character kmer word-matching strategy to determine the percentage of shared words between a query and the Greengenes taxonomy database (greengenes.secondgenome.com/downloads). This database is currently based on a *de novo* phylogenetic tree of 408,135 quality-filtered complete sequences calculated using FastTree (McDonald et al., 2012). Taxonomy was assigned to each read by accepting the Greengenes taxonomy string of the best matching Greengenes sequence (127,741 complete bacterial sequences; Werner et al., 2012). We selected this classification method due to favorable trade-offs among automation, speed, and taxonomic accuracy (Liu et al., 2008; Werner et al., 2012).

The RDP classifier uses a bootstrapping method of randomly subsampling the words in the sequence to determine the classification confidence (Wang et al., 2007). However, the error rate associated with a confidence threshold is dependent on several factors, including the taxonomic resolution of the prediction (kingdom vs. genus), the sequence length for classification, and the amplified region of the 16S rRNA gene. Consequently, the use of one overall "confidence" threshold for classification, for example 80% (Wang et al., 2007) or 50% (Claesson et al., 2009), often results in sub-optimal and unequal performance across regions and taxonomic ranks (Mizrahi-Man et al., 2013). In ClassifyReads, there is no bootstrapping procedure and confidence is statistically assigned based on the overall accuracy of the classification algorithm at different taxonomic levels (ranging from 100 to 98.24%, from kingdom to species). Reads that did not match a reference sequence were considered as unclassified and were included in the community analysis, since they represent an important source of bacteria particularly in anaerobic systems (Werner et al., 2012).

Multivariate Analysis of Microbial Communities

Relative abundances were calculated using the Greengenes classifications of the OTUs. Microbial community structure was examined with multivariate techniques using the software package PRIMER6 & PERMANOVA+ (Anderson et al., 2008). Genus relative abundances for all samples were log transformed (x + 1) to improve homogeneity of variance, and a Bray-Curtis similarity matrix was generated. A principal coordinate analysis (PCO) ordination was used to visualize the natural groupings of the samples using 2D and 3D plots. The natural groupings emerging from the PCO plot were further analyzed with PERMDISP, based on distances to centroids, to examine the dispersion among groups (Anderson, 2004). Subsequently, a non-parametric permutational ANOVA (PERMANOVA) analysis was used to test for statistical differences in the multidimensional space. PERMANOVAs were based on the Type III (partial) sum of squares and 999 permutations of residuals under a reduced model.

Relationships between the resemblance matrix of microbial families and explicative variables were explored with distance-based linear models (DistLM). We grouped the different variables in four sets: (i) *Run*: reads passing filter, reads classified, dilution/addition (2 categories); (ii) *Taxonomy*: bacterial, archaeal and viral reads, Shannon's species diversity index (H′), taxa identified (phyla, class, order, family, genus and species); (iii) *Experiment*: origin of samples (4 categories: Morocco, NW Spain, Aquaculture and zooplankton), day/night (categorical), strata (3 categories: 5, 100, and 500 m), coast/ocean (categorical); and (iv) *Octopus*: captive/wild (categorical), sucker number, dorsal mantle (DML), total length (TL), width, distance to coast, depth and age. Prior to modeling, all variables were tested for collinearity (Spearman correlation matrix) and those with determination coefficients (R^2) higher than 0.9 were omitted. The retained variables were then transformed to compensate for skewness when needed applying log (x + 1).

The contribution of these four sets of variables to the total variability found in the microbial resemblance matrix was determined using a step-wise selection procedure using the adjusted R^2 as selection criterion. All significant variables were introduced in the model with the "best" procedure of the DistLM model using the Bayesian information criterion (BIC), as it includes a more severe penalty for the inclusion of new predictor variables than Akaike's information criterion (AIC). Such a procedure permitted developing the simplest model to explain the microbial community structure. The output of the fitted model was visualized with distance-based redundancy analysis (dbRDA; Anderson et al., 2008).

The microbial families contributing most to similarities and dissimilarities among wild and captive paralarvae and the zooplankton were determined using the program SIMPER (Anderson et al., 2008). This analysis allowed recognizing the core gut microflora of wild and captive paralarvae, their contribution to the total community, and the discriminative power of the main families driving the differences between communities.

RESULTS

Octopus Samples

The 105 wild *Octopus* paralarvae analyzed in this study ranged from 1.30 to 5.01 mm in dorsal mantle length, contained 3–15 suckers per arm and were captured between 10 and 171 km off the coast (see more details on **Table 2**). The paralarvae found in the open ocean were thoroughly sampled in both upwelling systems, because they are essential to understand the ontogenic changes of the GI microbial communities during the transition from the coastal hatchling grounds ($n = 19$) to the oceanic realm ($n = 57$). The paralarvae grown in captivity showed high variability in size throughout their development, especially evident at days 15 and 25 (**Table 2**). One paralarva at day 20 was lost during the dissection and therefore, not included in the microbial analysis.

Sequence Analysis

A total of 13,688,392 HTS reads were generated from the amplicon sequencing and 10,260,748 were retained after quality

TABLE 2 | *Octopus vulgaris* paralarvae analyzed in this work from NW Iberian Peninsula (CAIBEX-I), Morocco (CAIBEX-III), and aquaculture, showing the averaged dorsal mantle length (DML), sucker number, depth sampled (wild paralarvae), and distance to coast.

Survey	Location	*n*	DML (mm)	Sucker *n*°	Depth (m)	Distance to coast (km)
NW Iberian Peninsula	Coast	10	1.97 ± 0.43	3	62–136	10–15
	Ocean	31	2.59 ± 0.81	3–5	1,940–3,105	62–75
Morocco	Coast	9	2.02 ± 0.26	3	88–90	19
	Ocean	26	2.78 ± 0.59	3–15	787–3,110	48–171
Aquaculture	Day 0	5	1.42 ± 0.08	3	–	–
	Day 5	5	1.86 ± 0.29	3	–	–
	Day 10	5	2.25 ± 0.24	3	–	–
	Day 15	5	2.50 ± 0.49	3–5	–	–
	Day 20	4	2.10 ± 0.16	3–5	–	–
	Day 25	5	2.67 ± 0.44	3–5	–	–

filtering. Kraken analysis revealed 0.023% and 0.007% of viral and archaeal sequences respectively, thus leaving 10,257,748 bacterial reads for further classification. The mean number of reads (± standard deviation) obtained per octopus sample was 96,406 ± 35,302 (range: 571–164,583) and 33,784 ± 18,495 in the zooplankton species (range: 9,840–50,974). Of these bacterial reads, 97.2% were successfully classified at phylum level (n = 28 phyla), 95.0% to class (n = 61), 93.4% to order (n = 123), 90.2% to family (n = 275), 83.7% to genus (n = 829), and 57.3% to species (n = 2,856) using the Greengenes taxonomy database. There was a consistent number of average reads, taxa identified and % of reads classified on the three types of octopus samples analyzed (aquaculture, NW Spain and Morocco) with no statistical differences among them (**Table 3**). However, the average number of reads and taxa identified were significantly lower in the zooplankton analyzed than in the octopus samples, but not the average % of reads classified (**Table 3**).

A statistical relationship between the number of reads and the concentration of PCR product (ng/μl) after the purification step was obtained (**Figure S1**), whereby samples with <0.75 ng/μl prior to pooling showed a direct relationship between the initial concentration and reads obtained ($R^2 = 0.78$). Interestingly, this relationship was not observed ($R^2 = 0.003$) for those samples with >0.75 ng/μl that were diluted before pooling the samples, suggesting that the most consistent results were obtained by starting with a higher DNA concentration and diluting it to a standard concentration prior to amplicon sample preparation for high throughput sequencing.

Microbial Community Structure and Ontogenic Changes

The microbial communities detected in *O. vulgaris* paralarvae collected in the wild were statistically different to microbiomes sampled from aquaculture paralarvae (PERMANOVA test, p = 0.001), with both communities pointing in opposite directions of the main axis of variation (**Figure 2A**). PCO1 accounted for 24.1% of the total variability detected in the resemblance matrix and was driven by the difference between captive (negative values) and wild paralarvae (positive values), and the DML of the paralarvae (thus showing ontogenic changes). PCO2 accounted for 17.3% of the total variation and was primarily driven by the number of species detected and number of reads, with positive/negative values indicating fewer/higher number of species and reads. PCO3 accounted for 8.5% of total variability and was driven by the two dominating bacterial families identified from the paralarvae in aquaculture, with positive values showing the samples dominated by Vibrionaceae and negative values Mycoplasmataceae (**Figure 2B**). In summary, wild paralarvae had on average more bacterial species and diversity than paralarvae reared in aquaculture and zooplankton (**Table 3**), while the percentage of reads identified was higher in captive paralarvae.

Analysis of bacterial families within each of the sample groups studied revealed qualitative differences between aquaculture and wild paralarvae/zooplankton groups (**Figure 3**). The bacterial families detected in the zooplankton differed to that of the paralarvae collected over the continental shelf of NW Spain, especially the families Corynebacteriaceae and Rivulariaceae, which were more abundant in the zooplankton. In addition, the microbial communities of wild paralarvae from both upwelling systems clearly differed depending on the location where the paralarvae were collected (shelf vs. ocean, **Figure 3**). These differences were consistent in both upwelling systems, NW Spain and Morocco (PERMANOVA test, $p = 0.011$), with families more evenly distributed on average in the ocean than the shelf regions.

The ontogenic changes in the microbial community were evident when the age of the paralarvae, measured as days in captivity and sucker number in the wild, was taken into account (**Figure 4**). Surprisingly, paralarvae hatched in captivity (day 0) had a diverse microbial flora (averaged number of species ± standard deviation, 636 ± 50), which was not significantly different (PERMANOVA test, p = 0.052) from that of recently hatched paralarvae in the wild (619 ± 189, marked with an asterisk in **Figure 4**). However, the microbial diversity recorded in aquaculture at day 0 was significantly higher (PERMANOVA test, p = 0.001) than the rest of the samples collected in aquaculture, with averages of 360 ± 149 (day 5), 321 ± 100 (day 10), 288 ± 62 (day 15), 367 ± 31 (day 20), and 385 ± 64 (day 25) bacterial species. The families Mycoplasmataceae and Vibrionaceae dominated the microbial communities of captive paralarvae from day 5 (68%) onwards, accounting for more than 82% of the total reads at day 25 (**Figure 4**).

The opposite trend was observed in the wild paralarvae, where the bacterial richness gradually increased to a maximum of 919 and 801 species in NW Spain and Morocco, respectively. Paralarvae caught close to the shore were found to have an even representation of bacterial families, similar to that of the zooplankton, whereas the GI of samples collected away from the shore (>4 suckers) were enriched with species of the family Comamonadaceae in both upwelling systems (**Figure 4**). The same interpretation can be drawn from the direction of the vectors DML and Comamonadaceae (**Figure 2**), pointing toward

TABLE 3 | Averaged number of reads (reads), taxa identified, and percentage of bacterial reads (%) classified to different taxonomic levels.

		Aquaculture ($n = 29$)	NW Spain ($n = 41$)	Morocco ($n = 35$)	Zooplankton ($n = 4$)
Phyla	Reads	81,955 ± 24,361	105,222 ± 34,435	89,882 ± 39,311	33,416 ± 18,229
	Taxa	19.59 ± 2.75	21.27 ± 2.55	20.80 ± 2.68	13.00 ± 1.63
	%	0.94 ± 0.05	0.98 ± 0.02	0.98 ± 0.01	0.99 ± 0.01
Class	Reads	79,347 ± 23,431	103,067 ± 33,631	88,147 ± 38,445	33,168 ± 18,095
	Taxa	33.79 ± 5.54	38.83 ± 5.24	38.26 ± 5.52	22.50 ± 2.08
	%	0.91 ± 0.05	0.96 ± 0.02	0.96 ± 0.02	0.98 ± 0.02
Order	Reads	78,192 ± 22,934	101,067 ± 32,824	86,875 ± 37,925	32,863 ± 17,802
	Taxa	72.17 ± 9.28	80.07 ± 10.29	79.20 ± 10.27	47.00 ± 4.90
	%	0.90 ± 0.05	0.94 ± 0.03	0.95 ± 0.03	0.98 ± 0.02
Family	Reads	76,090 ± 21,554	96,912 ± 31,615	84,180 ± 36,497	32,579 ± 17,776
	Taxa	154.86 ± 22.10	175.80 ± 27.26	174.46 ± 23.97	95.75 ± 14.17
	%	0.88 ± 0.05	0.91 ± 0.08	0.92 ± 0.04	0.97 ± 0.01
Genus	Reads	73,179 ± 19,904	89,748 ± 29,847	75,834 ± 33,424	31,912 ± 17,389
	Taxa	305.07 ± 72.02	390.61 ± 88.86	397.86 ± 72.89	162.75 ± 32.87
	%	0.85 ± 0.06	0.84 ± 0.08	0.82 ± 0.05	0.94 ± 0.01
Species	Reads	56,371 ± 16,415	58,778 ± 20,451	50,194 ± 21,556	20,296 ± 12,262
	Taxa	394.10 ± 141.04	583.80 ± 163.95	579.43 ± 151.08	267.25 ± 71.08
	%	0.66 ± 0.10	0.55 ± 0.07	0.55 ± 0.06	0.60 ± 0.12

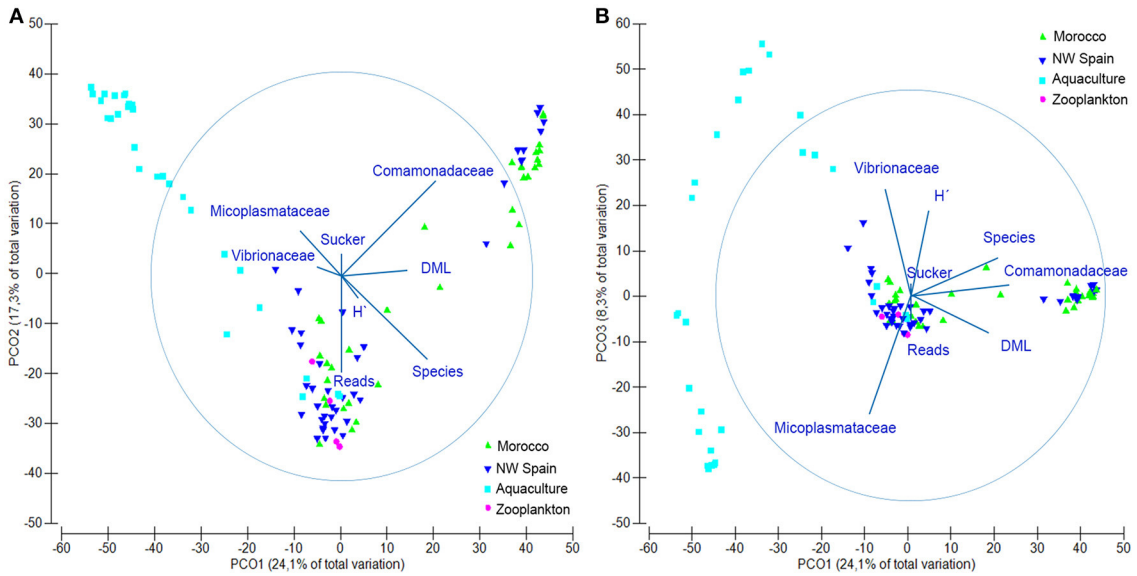

FIGURE 2 | Principal coordinate analysis (PCO) plot showing the microbial communities found in *Octopus vulgaris* paralarvae collected in the wild (green) and reared in captivity (dark blue), as well as their zooplankton prey (light blue). **(A)** Axes PCO1 vs. PCO2 showing the main drivers (vectors) of variation in the microbial communities. **(B)** Axes PCO1 vs. PCO3. Overlaid variable vectors represent the strength of the correlations with the different PCO axes obtained with the distance linear model, being the circle considered as the unity. DML, dorsal mantle length; H′, Shannon's diversity index; Reads, total reads passing filter; Species, number of bacterial species; Sucker, number of suckers.

the oldest paralarvae in the wild, thus showing that the main differences in the oceanic paralarvae were due to an increase in size (DML) and an incorporation of bacterial species of the family Comamonadaceae.

DistLM results showed that the examined variables accounted for 31.68% (*Octopus*), 28.12% (*Experiment*), 26.88% (*Taxonomy*), and 15.28% (*Run*) of the total variability found in the microbial communities. When considered altogether, they accounted for

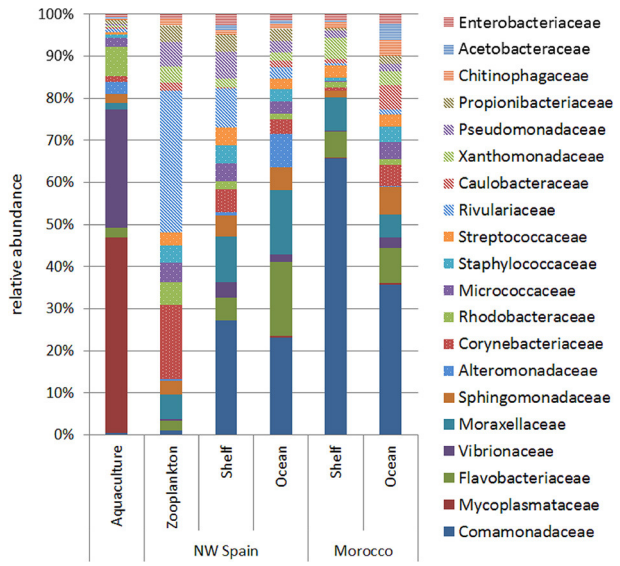

FIGURE 3 | Relative abundance of the main bacterial families (representing more than 1% of total reads) detected in the digestive tract of the zooplankton collected over the shelf (zooplankton) and in *Octopus vulgaris* paralarvae reared in captivity (aquaculture) and collected in the wild (NW Spain and Morocco). Wild paralarvae were classified according to their location, with samples collected over the continental shelf (<200 m) named as "shelf," and those collected over the continental slope (>200 m) named as "ocean."

up to 58.88% of the total microbial variability as follows: 31.68% (*Octopus*) + 15.76% (*Taxonomy*) + 6.85% (*Run*) + 4.59% (*Experiment*). The simplest model that accurately reproduces the microbial community structure obtained in this study (**Figure 5**), included five variables accounting for up to 50.4% of total variability: 21.42% (Comamonadaceae) + 15.75% (Mycoplasmataceae) + 9.28% (Vibrionaceae) + 2.32% (DML) + 1.62% (H′). This simple model reproduces both the variability found in the different samples analyzed as well as the ontogenic changes in bacterial communities. The contribution of the different variables to the different dbRDA axes showed that Comamonadaceae were characteristic of wild paralarvae, whereas Mycoplasmataceae and Vibrionaceae were largely found in captive paralarvae. This reduced model also highlighted the importance of *Octopus* DML and bacterial diversity (H′), since bacterial diversity was differentially correlated with size of the paralarvae between the wild and captive samples.

Core Gut Microflora

RELATE analyses revealed the main bacterial families driving both the similarities (**Table 4**) and the differences (**Table 5**) between the sample groups. Since paralarvae hatched in captivity (day 0) had a similar microbial community to wild paralarvae (**Figure 4**), we combined this group with the wild paralarvae to infer the "core" gut microbiota of healthy *Octopus* paralarvae (i.e., the common families to all paralarvae that declined in captivity). The importance of the families Flavobacteriaceae, Comamonadaceae, Moraxellaceae, and Sphingomonadaceae was evident in the wild paralarvae, with their contributions changing from one upwelling region to the other (**Table 4**). These differences are consistent with the statistical differences revealed by the PERMANOVA analysis among both upwelling systems, despite the main families being largely the same (**Figure 4**). In the zooplankton, the main bacterial family was Corynebacteriaceae which contributes up to 27.81% of the species present; in contrast, this family only represented between 2.89 and 5.16% of the bacteria found in the wild paralarvae collected in both upwelling systems and 0.85% in aquaculture (**Figure 4**).

Of the main families contributing to the dissimilarities between the groups analyzed (**Table 5**), the Family Comamonadaceae was determinant in the differentiation of all *Octopus* groups; this family was abundant in the paralarvae collected off Morocco but nearly absent in the paralarvae grown in captivity. These captive paralarvae were characterized by Mycoplasmataceae and Vibrionaceae families, while Rivulariaceae and Corynebacteriaceae were the main discriminant families of the zooplankton prey.

DISCUSSION

In this study, we present the first analysis of the GI microbiome of *O. vulgaris* paralarvae, characterizing both the complex microbial communities present in wild paralarvae and the ontogenic change in bacterial community composition based on diet and development in captivity. Paralarvae reared in captivity with *Artemia* showed a depletion of bacterial diversity, particularly after day 5 when almost half of the bacterial species present at day 0 were lost. In contrast, bacterial diversity increased in wild paralarvae as they developed in the ocean (**Figure 4**), likely due to the exposure of new bacterial communities via ingestion of a wide diversity of prey (Roura et al., 2012; Olmos-Pérez et al., 2017).

The number of bacterial sequences obtained per sample (average of 96,406 ± 35,302 *SD*) was almost 10 times the minimum sample depth needed to capture the structure of microbial communities (Caporaso et al., 2011). Only two samples had <10,000 sequences (833 and 8,574) and, despite their low depth, the main bacterial groups and their relative proportions were consistent with other samples. Despite using Greengenes, the most comprehensive microbial taxonomy database available (McDonald et al., 2012), we found a high percentage of unclassified sequences that may represent novel bacterial species present (between 20 and 60% per sample). High proportions of unclassified sequences have been described in other studies including mouse gut and anaerobic digester samples, where phylotypes unclassified at the genus level represented a greater proportion of the total community variation than classified OTUs, underscoring the need for greater diversity in existing reference databases (Werner et al., 2012). In our study, the percentage of unclassified reads explained up to 7.2% of the total variability found in the microbial communities. Interestingly, these unclassified OTUs were significantly more abundant in wild than captive paralarvae, indicating a high degree of novelty in the microbial species present in the digestive tract of wild paralarvae incorporated through the diet.

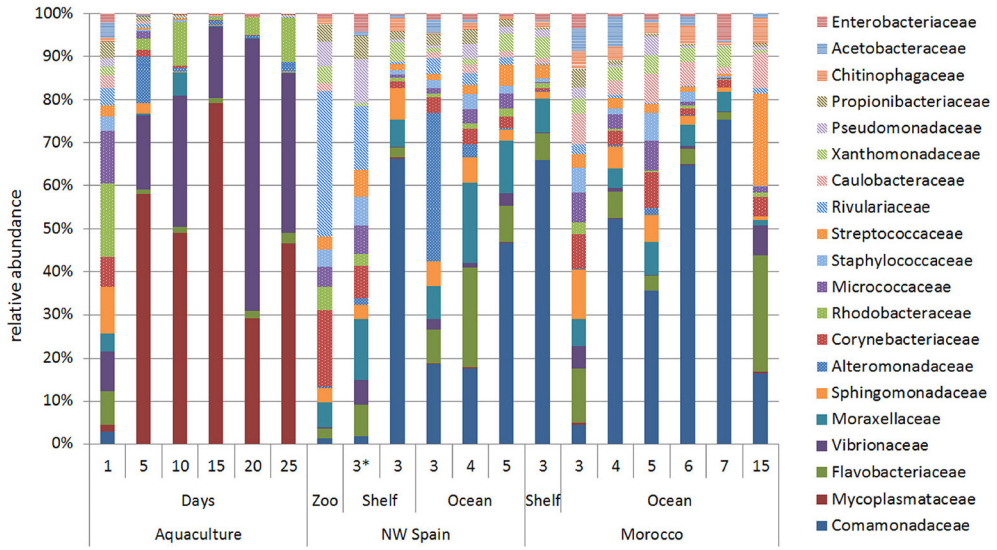

FIGURE 4 | Relative abundance of the main bacterial families showing the ontogenic changes in the microbial communities of *Octopus vulgaris* paralarvae reared in captivity for 25 days and in two upwelling regions of the North Eastern Atlantic: NW Spain and Morocco. The numbers present in the two upwelling regions represent the sucker number. The microbial community found in the zooplankton prey (Zoo) is represented together with the paralarvae collected in the same coastal sample (asterisk).

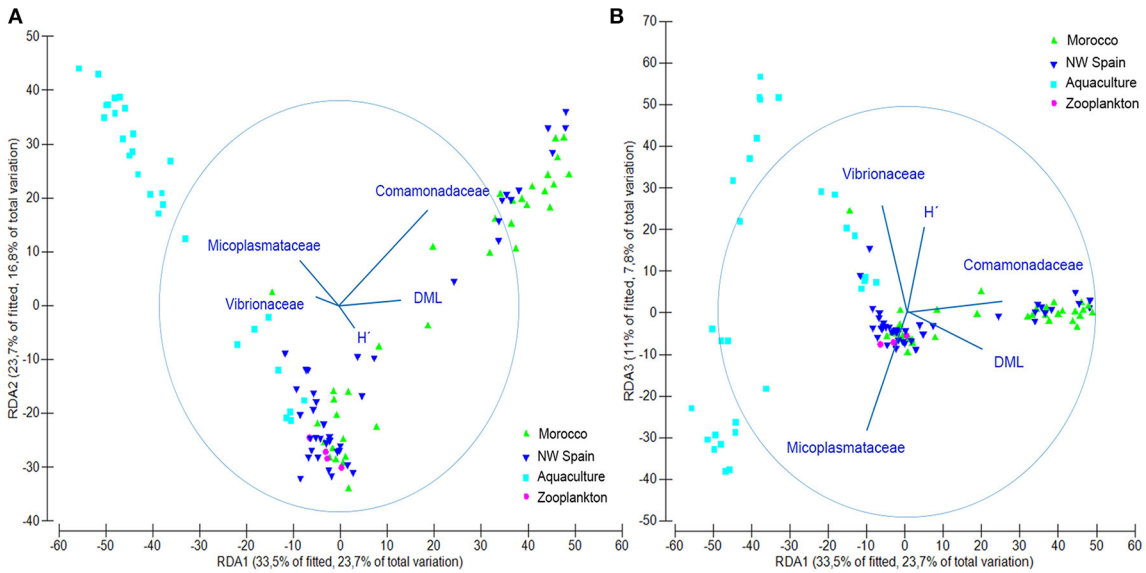

FIGURE 5 | Redundancy analysis (RDA) plot showing the output of the fitted distance linear model obtained with only 5 variables, accurately representing the microbial community structure found in this study. **(A)** RDA1 vs. RDA2 axes and **(B)** RDA1 vs. RDA3 axes. Abbreviations defined in **Figure 2**.

Consistent with this study, previous genomic studies have found that wild fish larvae have more diverse microflora than their captive relatives (e.g., Atlantic cod: Dhanasiri et al., 2011; olive flounder: Kim and Kim, 2013). This suggests that monospecific (*Artemia*) or even formulated diets are not as favorable as those diets encountered in nature, which seem to provide an important source of potentially beneficial microorganisms that might be exploited to supplement and diversify depleted microflora in captivity. The intestinal microbiota of a host can be classified as autochthonous (i.e., core bacteria in this study) or allochthonous bacteria (Ringø and Birkbeck, 1999). The autochthonous bacteria are those able to colonize the host's gut epithelial surface (microvilli), while the allochthonous bacteria are transient, associated with food or water, and cannot colonize except under abnormal conditions. Several studies have demonstrated

TABLE 4 | Top 10 most discriminant bacterial families of the different *Octopus vulgaris* paralarvae analyzed and their zooplankton prey.

Sample	Similarity	Families	Av. ab.	Con%	Cumulative
NW Spain	31.59	Flavobacteriaceae	0.10	16.99	16.99
		Comamonadaceae	0.13	15.49	32.49
		Moraxellaceae	0.08	13.55	46.04
		Sphingomonadaceae	0.03	6.11	52.15
		Corynebacteriaceae	0.03	5.16	57.31
		Staphylococcaceae	0.02	4.04	61.35
		Propionibacteriaceae	0.02	3.96	65.31
		Micrococcaceae	0.02	3.81	69.13
		Rivulariaceae	0.03	3.43	72.56
		Streptococcaceae	0.02	3.42	75.98
Morocco	42.49	Comamonadaceae	0.26	41.07	41.07
		Moraxellaceae	0.04	9.21	50.28
		Flavobacteriaceae	0.05	8.37	58.65
		Sphingomonadaceae	0.03	5.33	63.98
		Caulobacteraceae	0.03	3.91	67.90
		Xanthomonadaceae	0.03	3.85	71.75
		Chitiniphagaceae	0.02	2.90	74.65
		Corynebacteriaceae	0.02	2.89	77.54
		Bradyrhizobiaceae	0.02	2.12	79.65
		Streptococcaceae	0.02	2.08	81.73
Aquaculture	38.12	Mycoplasmataceae	0.31	52.56	52.64
		Vibrionaceae	0.20	29.60	82.15
		Rhodobacteraceae	0.05	5.67	87.83
		Flavobacteriaceae	0.02	2.70	90.53
		Alteromonadaceae	0.02	1.47	92.00
		Corynebacteriaceae	0.01	0.85	92.84
		Sphingomonadaceae	0.01	0.79	93.64
		Moraxellaceae	0.01	0.70	94.33
		Enterobacteriaceae	0.00	0.70	95.03
		Micrococcaceae	0.01	0.67	95.70
Zooplankton	37.72	Corynebacteriaceae	0.11	27.81	27.81
		Moraxellaceae	0.04	10.89	38.70
		Pseudomonadaceae	0.05	10.50	49.20
		Micrococcaceae	0.05	8.47	57.68
		Staphylococcaceae	0.03	7.05	64.73
		Propionibacteriaceae	0.03	6.44	71.17
		Sphingomonadaceae	0.03	6.34	77.51
		Streptococcaceae	0.04	3.89	81.40
		Rivulariaceae	0.14	3.62	85.02
		Microbacteriaceae	0.01	2.69	87.71

Averaged abundance (Av. ab.), contribution percentage to the total variability (Con%) and the cumulative variability explained by the families.

that the endogenous microbiota is an important component of the mucosal barrier, representing the first line of defense against pathogens (Gómez and Balcázar, 2008). The diverse core bacteria (autochthonous) detected in recently hatched *O. vulgaris* and wild paralarvae was rapidly modified and substituted by two opportunistic bacterial families, Vibrionaceae followed by Mycoplasmataceae (**Figure 4**). The same succession of opportunistic bacteria was also detected in cod larvae reared in captivity (McIntosh et al., 2008). Both families are known pathogens affecting many larviculture systems, with the family Vibrionaceae often found in parasitic or mutualistic associations with the gut of marine animals, where they provide diverse metabolic capabilities (Thompson et al., 2004; Sullam et al., 2012; Zhao et al., 2012).

Although certain *Vibrio* species are beneficial for the host (Austin et al., 2005; Fjellheim et al., 2007), this opportunistic group is responsible for high mortalities in larviculture (Brunvold et al., 2007; Zhao et al., 2012). Indeed, studies have shown that *Artemia* are important vectors of pathogens (mostly Vibrionaceae) that colonize fish (e.g., McIntosh et al., 2008; Reid et al., 2009) and abalone larvae after first feeding (Zhao et al., 2012). Interestingly, Vibrionaceae was identified using a culture-dependent method and 16S rDNA clone library in wild adult specimens of *O. mimus*, however most of the cloned sequences belonged to the family Mycoplasmataceae (Iehata et al., 2015). They suggested that *Mycoplasma* might be a autochthonous member of the octopus GI bacterial community with an unknown function, as is has also been found within the GI tract of wild specimens of Norway lobster (Meziti et al., 2010) and Atlantic salmon (Star et al., 2013). Our results indicated that this genus is present in both, wild and captive paralarvae, but their abundance is markedly different (**Table 5**). However, we suggest that the *Mycoplasma* species observed in captive *Octopus* paralarvae are opportunistic and, together with *Vibrio,* are candidate pathogens that may be responsible for the high mortalities observed in *Octopus* larviculture. *Mycoplasma* has also been detected in farmed salmon sporadically, but when present, it dominated the GI tract communities (Zarkasi et al., 2014). The sporadic nature of *Mycoplasma* suggests host factors at play that may influence GI tract community structure and contribute to dynamic changes. The saprophytic nature of *Mycoplasma*, with a fermentative metabolism, and its increasing abundance in captive octopus paralarvae may be related with the presence of dead paralarvae and *Artemia* at the bottom of the tank, which provide optimal conditions for this opportunistic genus. More research is needed to accurately identify the different *Mycoplasma* and *Vibrio* strains in order to test this hypothesis.

In our study, the diversity of the GI microbiota found in recently hatched paralarvae in captivity (day 0) was unexpectedly high (**Figure 4**). Olafsen (2001) suggested that a dense, diverse but non-pathogenic egg epiflora may be a barrier against colony formation by pathogens. One possible explanation is that the diverse microbiota in captive hatchlings of *Octopus* might be derived from bacteria attached to the egg capsule. This suggestion is supported by the observed biodiversity of culturable epiflora associated with healthy eggs of *O. mimus* (Iehata et al., 2016). Bacterial diversity of healthy eggs was higher than that of infected eggs (i.e., eggs from the same female that changed color from whitish to yellow-brownish indicative of infection), which were dominated by pathogenic genera like *Pseudoalteromonas*, *Vibrio,* and *Tenacibaculum*. In our study, the initial diversity rapidly decreased when *Octopus* paralarvae started exogenous feeding on *Artemia,* and opportunistic bacteria

TABLE 5 | Top 10 most discriminative bacterial families driving the differences between the groups studied, with the averaged abundances (Ab.) represented for each group and their contribution percentage to the total variability (Con%).

Contrast	Dissimilarity	Families	Ab. NW	Ab. Mo.	Con%	Cumulative
NW Spain vs. Morocco	66.63	Comamonadaceae	0.13	0.26	25.78	25.78
		Flavobacteriaceae	0.10	0.05	9.30	35.07
		Moraxellaceae	0.08	0.04	7.34	42.41
		Vibrionaceae	0.02	0.03	4.23	46.65
		Sphingomonadaceae	0.03	0.03	3.78	50.43
		Alteromonadaceae	0.03	0.00	3.39	53.81
		Corynebacteriaceae	0.03	0.02	2.96	56.77
		Caulobacteraceae	0.01	0.03	2.85	59.62
		Micrococcaceae	0.02	0.02	2.79	62.41
		Pseudomonadaceae	0.02	0.01	2.78	65.18
			Ab. Wild	**Ab. Aq.**		
Wild vs. Aquaculture	90.87	Mycoplasmataceae	0.26	0.37	28.11	28.11
		Vibrionaceae	0.04	0.24	17.43	45.54
		Comamonadaceae	0.05	0.00	12.99	58.53
		Flavobacteriaceae	0.03	0.01	4.97	63.51
		Moraxellaceae	0.03	0.01	4.76	68.27
		Rhodobacteraceae	0.03	0.04	3.56	71.83
		Alteromonadaceae	0.02	0.02	2.75	74.58
		Sphingomonadaceae	0.02	0.00	2.59	77.17
		Micrococcaceae	0.02	0.00	1.87	79.04
		Corynebacteriaceae	0.02	0.00	1.86	80.91
			Ab. Wild	**Ab. Zoo.**		
Wild vs. Zooplankton	72.78	Comamonadaceae	0.19	0.01	17.46	17.46
		Rivulariaceae	0.02	0.14	14.30	32.76
		Corynebacteriaceae	0.02	0.11	9.32	41.08
		Flavobacteriaceae	0.08	0.01	6.62	47.70
		Moraxellaceae	0.06	0.04	4.97	52.67
		Pseudomonadaceae	0.02	0.05	4.93	57.61
		Micrococcaceae	0.02	0.05	4.25	61.86
		Streptococcaceae	0.02	0.04	3.85	65.71
		Xanthomonadaceae	0.02	0.03	3.22	68.93
		Rhodobacteraceae	0.01	0.03	3.03	71.95
			Ab. Aq.	**Ab. Zoo.**		
Aquaculture vs. Zooplankton	88.23	Mycoplasmataceae	0.1	0.00	24.41	24.41
		Vibrionaceae	0.20	0.00	16.00	40.41
		Rivulariaceae	0.00	0.14	11.66	52.07
		Corynebacteriaceae	0.01	0.11	8.47	60.54
		Rhodobacteraceae	0.05	0.03	4.50	65.04
		Pseudomonadaceae	0.00	0.05	4.08	69.12
		Micrococcaceae	0.01	0.05	4.02	73.13
		Moraxellaceae	0.01	0.04	3.56	76.70
		Streptococcaceae	0.00	0.04	3.06	79.76
		Xanthomonadaceae	0.00	0.03	2.29	82.04

colonized the GI tract. In contrast, a gradual increase in species richness was observed among wild paralarvae as they migrated from their coastal hatchling grounds to the oceanic realm (**Figures 3**, **4**). This is the first time that this ontogenic change has been observed in *O. vulgaris* paralarvae and suggests a relationship between diversity of GI flora and paralarvae survival. This ontogenic change in the microbial community has also been observed in other marine organisms, including abalone

(Zhao et al., 2012), sponges (Cao et al., 2012), white shrimp (Huang et al., 2016) and fish aquaculture (reviewed in Ringø et al., 2016), and has been suggested to be a natural process that likely plays a role in the correct development of the host's immune system and GI tract, preventing pathogens from colonization.

Marine larvae are in constant interaction with bacteria during their first feeding (Olafsen, 2001), and compared to wild conditions, intensively cultured larvae experience stress due to inappropriate feeding (Iglesias et al., 2007) and higher larval densities than in their oceanic environment (Roura et al., 2016). Furthermore, the high organic load associated with rearing conditions may enhance the proliferation of opportunistic pathogenic bacteria (Lauzon et al., 2010), which can be detrimental to the paralarvae and is one potential cause for the highly unpredictable growth and reduced survival that limits *Octopus* aquaculture. Our results clearly demonstrate that the gut flora of captive paralarvae was distinctly different from the "healthy" gut flora community of wild paralarvae (**Figures 3**, **4**).

The bacterial families Comamonadaceae, Flavobacteriaceae, and Moraxellaceae were the most discriminating families enriched in the wild *Octopus* paralarvae core community, and could be a potential source of beneficial bacteria to test in captivity. This was the case of wild olive flounder (Kim and Kim, 2013), where wild fishes were an essential source of beneficial microbes that conferred resistance to pathogenic bacteria (Nayak, 2010). Bacterial composition in wild *O. vulgaris* (at the phylum level) was similar to carnivorous/herbivorous marine fishes (Sullam et al., 2012), with a composition hierarchy consisting of Proteobacteria>Actinobacteria>Bacteroidetes>Firmicutes. Interestingly, one of the core intestinal bacterial groups of wild *Octopus* paralarvae was the family Flavobacteriaceae (**Table 4**). Although this family was initially proposed to be exclusively found in herbivorous fishes (Sullam et al., 2012), this hypothesis was later rejected by a pyrosequencing study that found this group within the GI tract of wild Atlantic cod (Star et al., 2013).

Finally, it is remarkable the similarity of the microbial community found in wild zooplankton and that of the paralarvae growing near the coast (**Figure 4**). Although only four zooplankton species were analyzed in this study, the similarities observed support a close relationship between the microbial communities present in GI tract of the predator and that of its prey. Wild paralarvae continuously diversify their core gut microflora with a diverse diet (Roura et al., 2012; Olmos-Pérez et al., 2017), which provides a natural source of allochthonous bacteria. This diverse microbiota likely serve a variety of functions in the nutrition and health of the host by promoting nutrient supply, preventing the colonization of infectious agents, energy homeostasis and maintenance of normal mucosal immunity (Nayak, 2010). In summary, this study provides a comprehensive overview of the bacterial communities inhabiting the GI tract of *O. vulgaris* paralarvae, and reveals new research lines to challenge the current bottlenecks preventing sustainable octopus aquaculture.

AUTHOR CONTRIBUTIONS

AR, JS, and SD planned the work. AR captured the wild paralarvae and MN grew the paralarvae in captivity. AR, JS, and SD designed the genomic study. AR and SD performed the genomic analyses. AR, SD, JS, and MN wrote the manuscript. All authors have read and approved the content of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00362/full#supplementary-material

Figure S1 | Effect on the number of reads obtained per sample depending on the initial concentration of the PCR product after the cleaning step. PCR products with concentration below/above 0.5 ng/μL are shown in red/blue, respectively.

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Salivary Glands in Predatory Mollusks: Evolutionary Considerations

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Many marine mollusks attain or increase their predatory efficiency using complex chemical secretions, which are often produced and delivered through specialized anatomical structures of the foregut. The secretions produced in venom glands of *Conus* snails and allies have been extensively studied, revealing an amazing chemical diversity of small, highly constrained neuropeptides, whose characterization led to significant pharmacological developments. Conversely, salivary glands, the other main secretory structures of molluscan foregut, have been neglected despite their shared occurrence in the two lineages including predatory members: Gastropoda and Cephalopoda. Over the last few years, the interest for the chemistry of salivary mixtures increased based on their potential biomedical applications. Recent investigation with -omics technologies are complementing the classical biochemical descriptions, that date back to the 1950s, highlighting the high level of diversification of salivary secretions in predatory mollusks, and suggesting they can be regarded as a pharmaceutical cornucopia. As with other animal venoms, some of the salivary toxins are reported to target, for example, sodium and/or potassium ion channels or receptors and transporters for neurotransmitters such as, glutamate, serotonin, neurotensin, and noradrenaline, thus manipulating the neuromuscular system of the preys. Other bioactive components possess anticoagulant, anesthetic and hypotensive activities. Here, we overview available knowledge on the salivary glands of key predatory molluscan taxa, gastropods, and cephalopods, summarizing their anatomical, physiological and biochemical complexity in order to facilitate future comparative studies on main evolutionary trends and functional convergence in the acquisition of successful predatory strategies.

Keywords: molluscs, gastropods, cephalopods, predatory strategies, adaptations, evolution, salivary glands

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INTRODUCTION

Predation is a complex habit involving morphological, physiological, neural, and behavioral adaptations. Such lifestyle evolved multiple times in almost all molluscan classes, including the sessile Polyplacophora and Bivalvia. The veiled chiton *Placiphorella velata* uses its head flap and precephalic tentacles to capture small invertebrates (McLean, 1962), while in the bivalve order Anomalodesmata most of the species engulf small crustaceans with their eversible inhalant siphon (e.g., Morton, 1981, 1984). Apart from these remarkable cases it is undoubtable that some lineages

of Gastropoda and the whole class Cephalopoda fully exploited the opportunities offered by an active predatory lifestyle.

In Gastropods, and besides some "archaeogastropods," a predatory lifestyle evolved independently several times in a number of Caenogastropoda lineages, including (i) Neogastropoda (with about 40 families), (ii) Tonnoidea, (iii) Naticoidea, and (iv) Ficoidea. Fossil record suggests that predation evolved almost simultaneously in late Cretaceous, in the framework of the major reorganization of marine communities known as the Mesozoic marine revolution (Vermeij, 1977; Taylor et al., 1980; Tracey et al., 1993).

In contrast, Cephalopods are all carnivorous, coleoids being macrophagous predators, and *Nautilus* a scavenger. They emerged as predators since their major diversification event in middle-upper Paleozoic (Kröger et al., 2011) and evolved sophisticated techniques to search, capture, and kill their preys.

Both in Gastropods and in Cephalopods the physiology and sensory abilities allow the animals to seek diverse preys through a variety of feeding behaviors and predatory strategies (Hanlon and Messenger, 1996; Rodhouse and Nigmatullin, 1996; Modica and Holford, 2010). Their digestive system is arranged to form several compartments with morphological, structural, and functional specializations (Mangold and Bidder, 1989; Fretter and Graham, 1994). Among the anatomical and physiological adaptations of the digestive system enabling predation in these animals, a primary role can be attributed to the salivary glands. These discharge their secretion, via connecting ducts, into the buccal cavity, that—due to the morpho-functional characteristic of the molluscan *Bauplan*—corresponds to the immediate proximal space inside the mouth, allowing the closest proximity to the prey.

While earlier studies attributed only a lubricant role to salivary secretions, it is since the late nineteenth century that evidences begun to accumulate on the ability of salivary glands to produce bioactive substances. Modern approaches, including—omics technologies, have been confirming the high diversification of salivary secretions in predatory molluscs, identifying them as a very promising and still neglected taxon for the discovery and characterization of novel bioactive compounds.

Here, we summarize the available knowledge on salivary glands and their specialization in gastropods and cephalopods, in order to offer a framework to further detailed comparative studies aiming to elucidate how successful predatory strategies emerged in different molluscan lineages.

MORPHOLOGY AND ORGANIZATION OF THE SALIVARY GLANDS

Salivary glands are generally acinous in Caenogastropoda, but their anatomy and organization varies greatly, even in predatory taxa (Fretter and Graham, 1994). Evidences from ontogenetic comparative studies suggest that they may be considered homologous at least in "higher" Caenogastropods (Page, 2000).

Tonnoidea possess a single pair of large salivary glands differentiated in anterior and posterior lobes (see **Figure 1B**) that in the different families can be either morphologically separated or undivided, but with a proximal region resembling the anterior lobe. The anterior and posterior lobes discharge their secretion via a common duct (Barkalova et al., 2016), a characteristic that supports the interpretation of the two lobes as parts of a single gland. The anterior lobe is generally small and can be tubular or acinous, while the posterior lobe is more conspicuous, consists in morphologically uniform, radially arranged blind tubules and secretes sulfuric acid (Weber, 1927; Houbrick and Fretter, 1969; Fänge and Lidman, 1976; Hughes and Hughes, 1981; Andrews et al., 1999).

In Neogastropoda, which are almost all predators, both primary and accessory salivary glands are present. Primary salivary glands are acinous, generally paired and constituted of narrow branched ducts with a small lumen. They are joined in a single glandular mass in some species, but separate ducts are always maintained. The thin salivary ducts run along the esophagus until opening into the roof of the buccal cavity (**Figure 1A**). The secretory epithelium comprises two mixed cell types: superficial ciliated cells secreting mucus, and basal cells with apocrine secretion. The secretion is delivered through ciliary movement, as the outer muscular layer is poorly developed (Andrews, 1991). The accessory salivary glands are present in the basal family Cancellariidae, supporting the hypothesis that they are a synapomorphy of the Neogastropoda. Anyway, they are reduced to a single gland or absent in a number of families (Ponder, 1973; Andrews, 1991); even in families where they are generally well-developed (e.g., in Muricidae) cases of secondary loss are frequent. In most neogastropods accessory salivary glands are tubular and consist of a columnar secretory epithelium surrounded by a richly innervated sub-epithelial muscular coat. Gland cells, producing a peculiar granular secretion, lie outside the muscle layer and open via long necks in the central lumen of the gland, from which the secretion is delivered at the tip of the buccal cavity with non-ciliated ducts (Ponder, 1973; Andrews, 1991).

In cephalopods, three types of salivary glands are associated with the buccal mass: the submandibular gland, and the paired anterior and the posterior salivary glands (Mangold and Bidder, 1989; Budelmann et al., 1997).

The submandibular (or sublingual) gland, a non-paired organ lying below the salivary papilla and arranged into several lobes, is well-developed in octopods and *Vampyroteuthis* but reduced to small folds in *Nautilus*.

The paired anterior salivary glands are larger than the former, made by ramified tubules, and variable in different species. In *Nautilus* and cuttlefishes they are enclosed in the musculature (lateral lobes), while in octopuses they lay at both sides of the buccal mass (**Figures 1C,D**).

The posterior salivary gland lies behind the buccal mass. It is missing in nautiloids, paired in cuttlefishes and octopuses and unpaired in teuthoids and *Cirroteuthis*. It consists of numerous lobules made-up by tubules producing viscous secretions that are transported by muscular ducts to a common terminal canal opening into the anterior buccal cavity, nearby the apex of the salivary papilla (Mangold and Bidder, 1989).

In the posterior salivary gland two types of epithelia have been described. In type A, polarized columnar cells

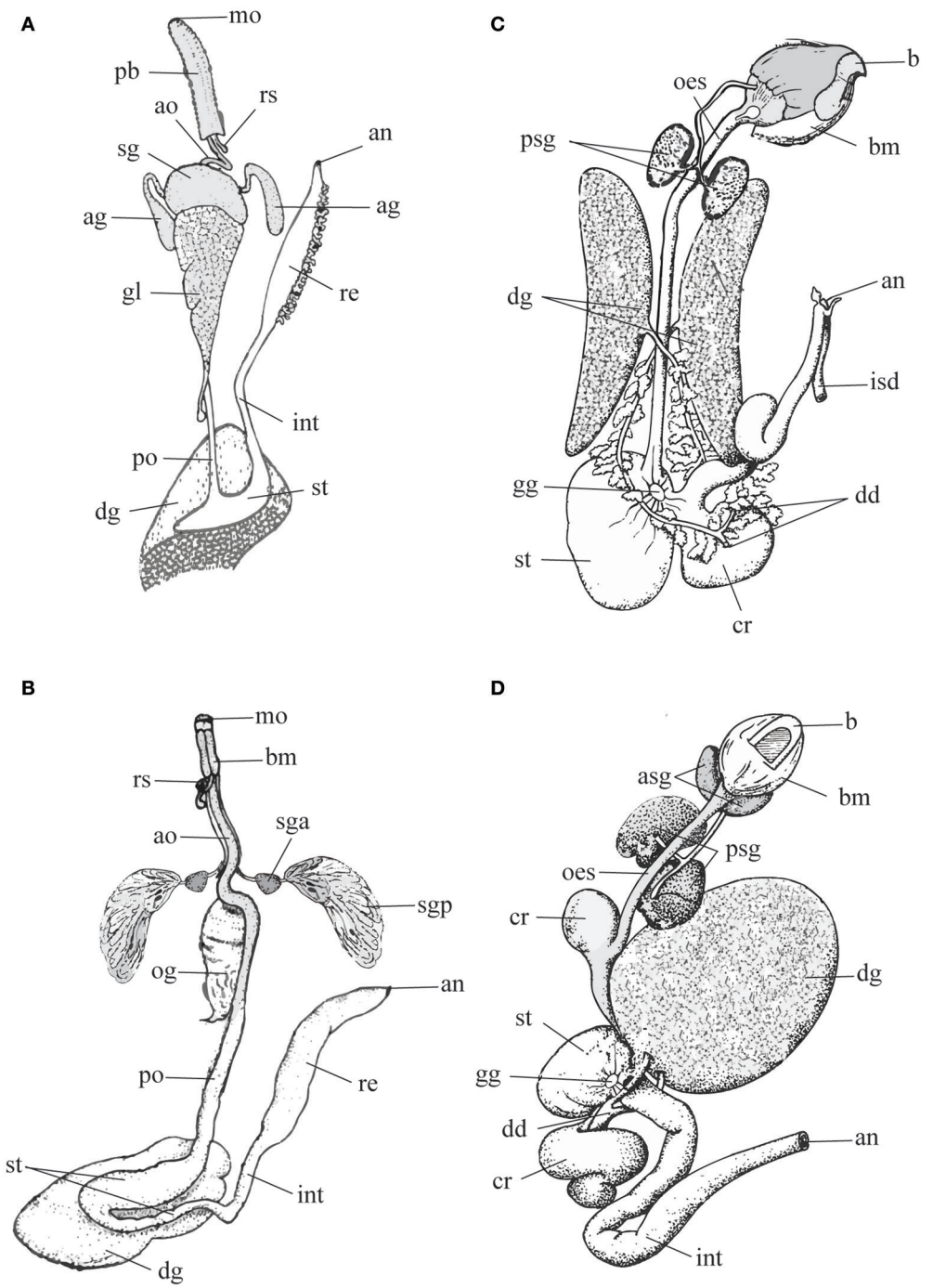

FIGURE 1 | Schematic representation of digestive tract in predatory gastropods **(A,B)** and cephalopods **(C,D)** to highlight differences in the morphology and arrangements of the salivary glands (gastropods: sga, sgp or sg; cephalopods: asg, psg). Left panel, Gastropods. **(A)** Neogastropoda Muricidae (modified after Wu, 1965); **(B)** Tonnoidea (modified after Barkalova et al., 2016). Right panel, Cephalopods. **(C)** *Sepia*, and **(D)** *Octopus* (modified after Budelmann et al., 1997). Terms and abbreviations follow original descriptions and despite analogies are not synonymized here. ag, accessory salivary gland; an, anus; ao, anterior esophagus; asg, anterior salivary gland; b, beak; bm, buccal mass; cr, crop; dd, digestive duct; dg, digestive gland; gg, gastric ganglion; gl, gland of Leiblein; int, intestine; isd, ink sac duct; mo, mouth; oes, esophagus; og, oesophageal gland; pb, proboscis; po, posterior esophagus; psg, posterior salivary gland; re, rectum; rs, radular sac; sg, salivary gland; sga, anterior lobe of the salivary gland; sgp, posterior lobe of the salivary gland; st, stomach.

containing few mitochondria are responsible of apocrine secretions. Type B, restricted to the duct area, is characterized by three types of cells, the most important being striated with abundant mitochondria and microvilli involved in active ion transport and excretion (Budelmann et al., 1997).

This assembly, typical of *Octopus* and *Eledone*, is simplified in *Sepia* where a single type of secretory cells, corresponding to *Octopus* type A is found.

The three salivary glands play different roles in feeding. The submandibular gland contributes to lubricating the passage of the food, the posterior salivary glands produce secretions used to paralyze the prey within a few seconds after capture (Ghiretti, 1959, 1960), while the secretion of the anterior salivary gland facilitates the action of the very viscous secretions of the posterior salivary glands.

PHYSIOLOGY OF THE SALIVARY GLANDS

Little is known about the nervous control of salivary secretion in molluscs: the most accurate review is provided by House (1980), and more recent updates are missing. Here, we summarize available knowledge on the topic to facilitate the understanding of its evolutionary relevance.

According to House (1980), the following sequence of events occurs in the salivary glands of several invertebrate taxa, not limited to molluscs: (i) neurohormone or transmitter release, (ii) receptor activation in gland cell, (iii) build-up of second messenger, (iv) electrical events (i.e., ion channels open, membrane potential change) activated by the receptor activation in the gland cell often synergistically to the build-up of second messenger; (v) secretory events (i.e., enzyme release, ion, and fluid secretion) initiated by the build-up of the second messenger. Evidence for a direct initiation of secretory events from the electrical ones appear possible, but research is missing (House, 1980; Barber, 1983). Besides the changes in membrane potential and conductance, neuro-modulators or neurotransmitters may provide uncoupling of neighboring gland cells, thus providing further regulation of the secretory event (House, 1980).

In gastropods, the neural control of salivary glands is quite simple (House, 1980). A resting potential is shown by secretory cells and not by muscle fibers in the gland. In fact, an electrogenic sodium pump distributes potassium ions, giving to the cell basal membrane physiological properties similar to those of certain muscle and nerve cells. High level of coupling is observed, and therefore synchronous, spontaneous action potentials are generated, resulting in an all-or-none action potential response. Studies on the ionic basis of the action potential indicate that the inward current is carried chiefly by calcium ions with a minor contribution due to sodium. Calcium entry triggers the exocytosis of granules from the cells. Because of coupling, the number of intervening cells alters the delay between the recorded action potentials, and spontaneous miniature depolarizations promote further massive release from the gland.

In contrast, the posterior salivary glands of cephalopods are known for their abundant innervation (at least 30,000 axons from the salivary nerves reach the glands, and about 10,000 axons in the salivary duct nerves control the muscular contraction of the duct; Young, 1965; review in House, 1980).

A dual innervation is reported for the posterior salivary gland (House, 1980). Larger axons originating from the subradular ganglia innervate circular smooth muscle cells surrounding the tubules. The neuromuscular junctions show membrane thickenings and at the nerve endings many small agranular vesicles and some large ones (predominantly cholinergic) are present. The muscle cells in the salivary duct receive innervation from presynaptic fibers that contain a heterogeneous population of vesicles (mostly monoamines).

The second innervating component consists of smaller axons derived from cell bodies in the superior buccal lobe (supra-oesophageal mass, part of the "brain"). These axons end close to the basal membranes of the tubular cells, with a synaptic cleft (20 nm) and apparently no synaptic membrane specializations. These nerve endings contain a mixed population of vesicles (i.e., small agranular vesicles, dense-cored, and granular vesicles), where catecholamines are found. Noradrenaline and 5-HT are considered to be transported along axons toward the glands from cell bodies in the superior buccal lobe. In analogy, and due to significant quantities of octopamine and tyramine found in the superior buccal lobe (Juorio and Molinoff, 1971; Juorio and Philips, 1975; Ponte and Fiorito, 2015), these amines appears to be transported to the glands where they are released on nerve stimulation.

BIOCHEMICAL COMPLEXITY OF SALIVARY SECRETIONS

Research on molluscan bioactive compounds have been mostly focused on cone snails, which are among the most studied and best understood of all venomous animals, and led to the pharmacological development of one commercially available drug (the ziconotide, a Ca^{2+} channel blocker) plus other compounds that are now in pre-clinical trials. Despite the discovery of alpha-conotoxins in the salivary secretion of *Conus pulicarius* (Biggs et al., 2008), in Conoidea toxin production is mostly due to venom gland, a synapomorphy of this superfamily evolved from the mid-esophageal gland of Leiblein (Ponder, 1973).

Studies on the biochemical properties of salivary secretion in other predatory molluscs are extremely reduced and mostly outdated (see **Table 1** for a summary).

In Gastropoda, a complex salivary secretion containing different toxins is reported for several Tonnoidea (Andrews et al., 1999; Barkalova et al., 2016), including neurotoxins and cytolytic-hemolytic echotoxins (Shiomi et al., 1994). Additionally, sulfuric acid bringing the saliva to a pH of 2 or less has been detected in nearly all tonnoideans (Barkalova et al., 2016).

In Neogastropoda a high quantity of tetramine, histamine, choline, and choline esters has been reported in whelks' salivary glands (Endean, 1972; Shiomi et al., 1994; Power et al., 2002). Tetramine blocks nicotinic acetylcholine receptors (Emmelin and Fänge, 1958) and has been responsible of a number of human intoxications (e.g., Fleming, 1971; Reid et al., 1988). In addition, salivary secretions of whelks include still unidentified inhibitors of neuronal Ca^{2+} channels (e.g., in *Neptunea antiqua,* Power et al., 2002). Cystein-rich glycoproteins were detected in some Nassariidae and Muricidae (Martoja, 1971; McGraw and Gunter, 1972; Minniti, 1986; Fretter and Graham, 1994). These may account for the observed effects of salivary secretion, including: (i) flaccid paralysis in *Mytilus edulis* and in barnacles (Huang and Mir, 1972; Carriker, 1981; Andrews, 1991; Andrews

TABLE 1 | A tabularized overview of cytolytic, hypotensive, neurotoxic and other enzymatic substances discovered as product of salivary glands of some gastropod and cephalopod species (not exhaustive list).

		Cytolitic		Hypotensive			Neurotoxins					Other enzymes						
		Echotoxins	n/a	Eleidosin	Oct-TK	Unknown	CAP	Cephalotoxin	Conotoxins	ShK toxins	Unknown	Carboxypeptidase	Chitinase	Hyaluronidase	Metalloprotease	Pacifastin	Phospholipase A2	S1 peptidase
Gastropoda	*Acanthinucella spirata*					✓					✓							
	Nucella lapillus					✓					✓							
	Stramonita haemastoma					✓					✓							
	Neptunea antiqua										✓							
	Cumia reticulata	✓					✓		✓	✓		✓			✓			✓
	Nassarius spp.										✓							
	Conus pulicarius								✓									
	Monoplex parthenopeum	✓																
Cephalopoda	*Sepia latimanus*		✓				✓				✓		✓				✓	✓
	Sepia pharaonis		✓				✓	✓					✓		✓	✓	✓	✓
	Sepia esculenta		✓					✓										
	Sepioteuthis australis		✓				✓	✓				✓	✓		✓	✓	✓	✓
	Uroteuthis noctiluca		✓				✓					✓	✓		✓	✓	✓	✓
	Octopus cyanea		✓				✓					✓	✓	✓		✓		✓
	Octopus vulgaris		✓		✓			✓										
	Octopus kaurna		✓		✓		✓				✓		✓			✓		✓
	Abdopus aculeatus		✓										✓		✓	✓		✓
	Hapalochlaena maculosa		✓								✓	✓	✓	✓		✓		✓
	Eledone moschata		✓	✓														
	Eledone cirrhosa		✓	✓														
	Pareledone turqueti		✓										✓					✓
	Adelieledone polymorpha		✓										✓					✓

Data from the following literature and references within: Biggs et al., 2008; Fry et al., 2009a,b; Modica and Holford, 2010; Ruder et al., 2013; Modica et al., 2015. See text for details.

et al., 1991; West et al., 1998); (ii) decrease of cardiac activity, vasodilatation, hypotension, and smooth muscle contraction in mammals (Huang and Mir, 1972; Hemingway, 1978) (iii) disruption of neuromuscular transmission in rat (West et al., 1998). In some Volutidae, the accessory salivary glands produce a narcotizing compound, with a very low pH, inducing muscular relaxation in the preys (Bigatti et al., 2009).

Besides these earlier studies, the only modern transcriptomic approach applied for the characterization of salivary secretion in a non-conoidean gastropod has been carried out on the hematophagous *Cumia (Colubraria) reticulata,* revealing a remarkable complexity of the salivary secretion. Neurotoxins, echotoxins, and several enzymes were detected, as well as putative inhibitors of hemostasis such as, TFPI-like protease inhibitors, the novel VWFA1 domain-containing proteins and ENPP-5 (Modica et al., 2015).

In Cephalopoda the posterior salivary gland is responsible for the production of a number of different biologically active substances, while the anterior salivary glands release large amount of mucus containing neutral glycoproteins (SH, S-S groups) and sialic acid, dipeptidase, and hyaluronidase, that probably facilitate the delivery of the viscous secretions of the posterior salivary gland and may be involved in external pre-digestion (e.g., Furia et al., 1975; Nixon, 1984; Hernández-García et al., 2000).

The toxic effects of posterior salivary glands secretion in Octopoda (including irreversible paralysis and death in crustaceans) were recognized in the late nineteenth century by Lo Bianco (1888). Toxicity was firstly attributed to the numerous biogenic amines produced by the posterior gland, including tyramine, histamine, acetylcholine, octopamine, and serotonin. Subsequently this was accounted to a protein component (Songdahl and Shapiro, 1974) named cephalotoxin (Ghiretti, 1959, 1960). In *Octopus vulgaris* two heavily glycosylated cephalotoxins, alpha and beta, have been characterized (Cariello and Zanetti, 1977), while a divergent SE-cephalotoxin was isolated from *Sepia esculenta* by Ueda et al. (2008). Reported effects of cephalotoxins include inhibition of respiration in crabs, inhibition of blood coagulation in both crabs and humans, and paralysis of crabs and cockroaches (Ghiretti, 1960).

Several hypotensive compounds have been also identified, including tachykinins such as, Eledoisin (Anastasi and Erspamer, 1962), originally isolated from *Eledone aldrovandi* and *Eledone moschata*, OctTK-1 and OctTK-2 from *O. vulgaris* (Kanda et al., 2003) and an OctTK-1 homolog from *Octopus kaurna* (Fry et al., 2009a).

CAP proteins have been detected in several cephalopod species, as well as novel putative toxins with no homology to any known peptide type (Fry et al., 2009a).

The active components of the posterior salivary gland secretion include also a range of enzymes identified in a number of cephalopods species, including S1 peptidase, hyaluronidase, carboxypeptidase, metalloprotease, phospholipase A2 (Romanini, 1952; Grisley and Boyle, 1990; Grisley, 1993; Fry et al., 2009a; Ruder et al., 2013).

Despite their potential, most of toxicological research in cephalopods has been focused on the TTX-like compounds produced by *Hapalochlaena*, which are responsible of human fatalities. *Hapalochlaena* TTX is not an endogenous salivary toxin, as it is produced by endosymbiotic bacteria in the salivary glands and in other parts of the body of the animal (Yotsu-Yamashita et al., 2007).

CONCLUDING REMARKS

Many competing hypothesis have been proposed for the phylogenetic relationships of the Mollusca, using morphological, molecular, and other characters (see Sigwart and Lindberg, 2015 for a critical review). According to the most recent reconstruction of evolutionary relationships of Molluscs (Smith et al., 2011), gastropods and cephalopods are paraphyletic, implying that a predatory lifestyle was independently acquired in these two well-diversified lineages.

Morphology of salivary glands displays different patterns in Gastropoda and in Cephalopoda. Cephalopods share a common arrangement, with a great uniformity in all the Coleoidea so far studied and minor variations (as expected) in *Nautilus,* congruently with the hypothesis that a carnivorous or predatory lifestyle is an ancestral characteristic of the group. Conversely, predatory gastropods developed a number of different morphological arrangements despite some shared characteristics, as expected in a group that evolved predation more recently multiple times in at least three main lineages, from an ancestral microphagous feeding ecology.

The remarkably higher complexity of the physiological regulation of salivary secretion in Cephalopoda compared to Gastropoda further confirms a major commitment toward predation since the early evolutionary history of the former group. In gastropods, the basic physiology of salivary secretion appears in agreement with a plesiomorphic condition of microphagous feeding.

If we consider the bioactive compounds secreted in the salivary glands of both groups, it should be noted that cephalopods evolved characteristic enzymes and neuropeptides belonging to families that are shared with many other venomous taxa, including snakes and spiders (Fry et al., 2009a,b; Casewell et al., 2013), while the reduced number of gastropods studied so far display a great inter-lineage variability and a reduced number of shared compounds with non-molluscan lineages. This condition, if confirmed by further studies on a broader range of taxa likely reflects independent evolution of predation in the different lineages of predatory Caenogastropods.

In summary, while in gastropods the onset of a predatory adaptation evolved recently (in the late Cretaceous) with respect to their evolutionary origin that dates back to the late Cambrian and had to cope with a *Bauplan* built for microphagy, cephalopods evolved predation from a scavenger ancestor at the time of their major diversification in middle-upper Paleozoic (Kröger et al., 2011).

In spite of the differences of salivary glands of gastropods and cephalopods we simplified in this review, a common feature emerged: the presence of multiple glands corresponding to an extremely rich chemical assemblage. This trait may have facilitated the specialization and differentiation of different cellular districts to achieve the final composition of the saliva. Several bioactive salivary components with cytolytic, hypotensive

and, above all, neuroactive activity are excellent candidates for biotechnological development, due to millions years of natural selection that have contributed to their specificity, a key factor in the evolutionary success of these predatory mollusks.

AUTHOR CONTRIBUTIONS

GP and MM developed the concept of the manuscript, searched the literature and wrote a draft. MM further developed the early draft. GP elaborated the figure.

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Digestive Physiology of *Octopus maya* and *O. mimus*: Temporality of Digestion and Assimilation Processes

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Digestive physiology is one of the bottlenecks of octopus aquaculture. Although, there are successful experimentally formulated feeds, knowledge of the digestive physiology of cephalopods is fragmented, and focused mainly on *Octopus vulgaris*. Considering that the digestive physiology could vary in tropical and sub-tropical species through temperature modulations of the digestive dynamics and nutritional requirements of different organisms, the present review was focused on the digestive physiology timing of *Octopus maya* and *Octopus mimus*, two promising aquaculture species living in tropical (22–30°C) and sub-tropical (15–24°C) ecosystems, respectively. We provide a detailed description of how soluble and complex nutrients are digested, absorbed, and assimilated in these species, describing the digestive process and providing insight into how the environment can modulate the digestion and final use of nutrients for these and presumably other octopus species. To date, research on these octopus species has demonstrated that soluble protein and other nutrients flow through the digestive tract to the digestive gland in a similar manner in both species. However, differences in the use of nutrients were noted: in *O. mimus*, lipids were mobilized faster than protein, while in *O. maya*, the inverse process was observed, suggesting that lipid mobilization in species that live in relatively colder environments occurs differently to those in tropical ecosystems. Those differences are related to the particular adaptations of animals to their habitat, and indicate that this knowledge is important when formulating feed for octopus species.

Keywords: ***Octopus maya, O. mimus*, digestive physiology, digestive gland, gastric juice, digestive enzymes, assimilation process**

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INTRODUCTION

For cephalopods, in particular for octopus, proteins are the main metabolic substrate characterized by a natural diet based mainly on crustaceans, molluscs, and fish (Alejo-Plata et al., 2009; Krstulovíc and Vrgoc, 2009; Estefanell et al., 2013). Previous studies have demonstrated the importance of crustaceans in octopus diets, showing that up to 19 crustaceans species can be found in the diet of

wild *Octopus vulgaris* paralarvae (Roura et al., 2012). In recent years and due to *Octopus maya* and *Octopus mimus* reaching high market value, these species were identified as two strong candidates for marine aquaculture as they adapt well to captivity and can eat freeze-dried diets, allowing for their cultivation in ponds and tanks.

Thanks to physiological digestion research, a semi-moist paste based on squid and crab meat was recently developed as a successful diet for *O. maya* juveniles and adults (Martínez et al., 2014; Tercero-Iglesias et al., 2015). With this diet, wild females were successfully acclimated, and pre adults were grown until spawning. Although, the diet was based on the digestive capacity of juvenile octopus, it was successfully used to cultivate hatchlings until they reached 250 g body weight, as requested by the gourmet market (Rosas et al., 2014). Research in nutrition is frequently dedicated to formulating diets for cephalopod species. For wild sub-adults of *O. vulgaris*, it was recently demonstrated that dehydration of raw materials at temperatures lower than 60°C induced similar growth rates when compared to freeze dried ingredients (Rodriguez-González et al., 2015), indicating that protein characteristics are correlated with the digestive capacity of the animals. Although, there are many papers related to important ingredients (amino acids, lipids) for *O. vulgaris* feed (Cerezo-Valverde et al., 2012a,b, 2013; Estefanell et al., 2013; Querol et al., 2013; Hamdan et al., 2014; Rodriguez-González et al., 2015) there is a general lack of knowledge of the physiological processes involved during nutrient digestion in octopus species. Considering that protein digestion is a key aspect of cephalopod nutrition, the study of the process by which proteins (and other nutrients) are digested and assimilated will determine diet design (Martínez et al., 2014).

According to Boucher-Rodoni et al. (1987), cephalopod digestion can be divided into two steps: extracellular and intracellular digestion. Extracellular digestion starts in the prey, where the chyme is formed after the action of salivary gland enzymes. After the chyme is ingested by the mouth, it flows to the anterior stomach (crop) and almost simultaneously to the posterior stomach, caecum and digestive gland (DG) where it is absorbed and intracellular digestion begins. The DG plays a key role in the digestive process, where chyme nutrients are hydrolysed and transformed into acyl-glycerides, amino acids (AA), or carbohydrates (Boucaud-Camou et al., 1976; Boucher-Rodoni et al., 1987; Budelmann et al., 1997). According to Linares et al. (2015) these processes are affected not only by the type of diet, but also by habitat temperature of each species.

Some years ago, it was proposed that *O. mimus* and *O. maya* were separated from the *O. vulgaris* co-family as a result of the separation of populations provoked by the emergence of Central America. The hypothesis suggests that this new geomorphology interrupted the genetic flow between Pacific and Atlantic populations, favoring their speciation (Perez-Losada et al., 2002; Porta, 2003). Recently the thermal tolerance of *O. mimus* embryos was established in the range of 14–21°C, explaining why this species is distributed from the tropical coastal zone of northern Peru (Tumbes) to sub-tropical zone of central Chile (Bahía de San Vicente) along a natural thermal range of 15–21°C (Uriarte et al., 2012). Conversely, *O. maya* inhabits a tropical ecosystem in the Yucatán Peninsula where the benthic thermal regime is in the range of 22–26°C (Noyola et al., 2013). So although both species probably have the same evolutionary origin, it is reasonable to suppose that environmental conditions in each habitat modulate their physiology, and in consequence the manner in which each species ingest, digest, and use the nutrients obtained from food.

This paper summarizes all the steps in the digestive physiology of *O. maya* and *O. mimus* particularly regarding the digestion timing of raw nutrients: absorption, transportation, storing and use of nutrients as a source of energy. Considering that crustaceans are the primary prey of both octopus species (Leite et al., 2009) and also the most complete food type (Rosas et al., 2013), this paper summarizes the studies performed on both octopus species fed with crab meat (Martínez et al., 2011, 2014; Linares et al., 2015). Using *O. maya* and *O. mimus* as models, the results obtained to date allow us to provide a general overview of (digestive physiology in these species, that we believe can be applied to other species living in the tropics (*O. maya*) and sub-tropics (*O. mimus*). In this context, we think that this summary is valuable for the development and management of a balanced feed that would allow maintenance of octopus species in captivity under the best nutritional conditions possible.

STARTING THE DIGESTION PROCESS

Protein digestion in octopuses starts in the prey when enzyme action, mainly chymotrypsin excreted by posterior salivary glands, initiates external digestion (Boucaud-Camou and Boucher-Rodoni, 1983). As in other octopus species, chymotrypsin activity in *O. maya* and *O. mimus* was detected as the principal enzyme of salivary glands involved in external digestion, where a pre-digestion of raw flesh produces soluble proteins that form the chyme when ingested (Aguila et al., 2007; Linares et al., 2015). Forty to eighty minutes after feeding, the first chyme rapidly fills the crop, stomach and octopus caecum. Besides the obvious role as the first nutritional molecules input, we hypothesize that this first chyme could activate zymogens (acidic and alkaline enzymes) located in the gastric juice (GJ) along the digestive tract and absorption sites in the DG where those enzymes are present. This hypothesis is based on the fact that the chyme composition, besides polypeptides produced by the pre-digestion in the prey, includes active enzymes from the prey that act on the zymogens (Boucaud-Camou and Boucher-Rodoni, 1983; Hedstrom, 2002). Results obtained in *Sepioteuthis lessoniana* demonstrated that, during digestion process, zymogens were newly released after the 1 h after feeding, increasing digestive efficiency in this species. From this perspective, Martínez et al. (2011) proposed that the first pulse of AA observed in the chyme could stimulate the brush border of the acinar cells in the octopus DG (Martínez et al., 2011) increasing, as in *S. lessoniana,* the digestive efficiency in octopus species. Although, it is unknown if AA content in the chyme can stimulate the digestive cells in the DG of octopus species, in chicken intestine some AA were observed to enhance the absorption of other AA, increasing the digestive efficiency of

proteins (Herzberg and Lerner, 1973). Therefore, we hypothesize that free AA in the first pulse of chyme registered in *O. maya* and *O. mimus* may facilitate nutrient absorption when more complex molecules are digested and absorbed in the second chyme pulse (Linares et al., 2015).

NUTRIENT PROCESS IN DIGESTIVE GLAND

Previous experiments with *O. maya* confirmed that glucose, synthesized via the gluconeogenic pathway, is the final energetic product of protein catabolism (Martínez et al., 2011; Rosas et al., 2011; Baeza-Rojano et al., 2013). Linares et al. (2015) showed that protein catabolism and glycogen synthesis in *O. maya* and *O. mimus* followed an inverse relationship throughout the digestive process. In that study, glycogen accumulation occurred at the end of the digestive process, when the AA and polypeptides were transformed into glycogen via the gluconeogenesis pathway. Linares et al. (2015) also identified lipids as a source of energy in *O. maya*, while glycogen was the first source of energy for *O. mimus*. Such differences could be related to the type of food that was used in each experiment; *O. maya* was fed crab of the genus *Callinectes* spp. because this is the favorite prey of this octopus species. Due to its role as a molting hormone precursor, cholesterol (Chol) is highly concentrated in the crustacean DG, (Teshima, 1997; Teshima et al., 1997; Pascual et al., 2003), and therefore is readily available to octopuses. It is possible that the two Chol peaks observed in the chyme of *O. maya* were obtained first from the haemolymph and later from that stored in the crab DG (Linares et al., 2015; **Figure 2**). Results obtained by Linares et al. (2015) also suggest that both Chol and AG were mainly used as a source of metabolic energy in the DG, because those authors did not detect changes in those nutrients in the haemolymph during their study. As a key digestive organ, the DG provides digestive enzymes and stores nutrients that are used as a metabolic energy source, with glucose (*O. mimus*) and lipids (*O. maya*) being the most important (Boucaud-Camou et al., 1976; Linares et al., 2015).

As was shown by Martínez et al. (2011), DG acini of *O. maya* are characterized by columnar cellular structures of a single cell type, with heterophagosomes, heterolysosomes, and residual bodies within the cells. Like *S. officinalis*, the DG cell kinetics in *O. maya* and *O. mimus* are related to the digestive process, in which cell metabolism and cell synthesis is directly associated with chyme pulses, which in turn are related to the ingestion of food (Boucaud-Camou et al., 1976; Boucher-Rodoni et al., 1987; Perrin et al., 2004). In *O. maya*, *O mimus* and other octopus species, asynchrony of the digestive cells depends on the digestive moment; the same digestive cells can be observed with different roles, either synthesizing enzymes or receiving the produced chyme for absorption and assimilation (Martínez et al., 2012; Linares et al., 2015). For that reason in the early stages of cephalopod research some researchers indicated that there were different types of cells in the DG (Budelmann et al., 1997).

As in other cephalopods species, the DG of *O. maya* and *O. mimus* perform intracellular digestion and release enzymes that will be used in the extracellular digestion (Semmens, 2002). During the digestive process the chyme provokes strong changes in the DG cells due to the biochemical reactions that occur in the heterolysosomes, where nutrients are digested by powerful acidic enzymes that cause cell wear. Under this dynamic, acinar cells will be replaced after each meal, requiring energy to complete this synthesis. Previous studies carried out in our laboratory demonstrated that the DG condition of *O. maya*, measured through its enzymatic activity depends on the type of diet. Aguila et al. (2007) fed octopus varied concentrations of fish meal and fish hydrolysed protein, and found that the enzymatic activity in the DG was higher in animals fed diets that provoked lower growth than obtained in animals fed crab, which produced the highest growth rate. That study demonstrated that animal diets made with fish meal and fish hydrolysed protein have low growth rates and lower DG energy content than octopuses fed crab, suggesting that the type of food determines not only the amount of energy directed to growth, but also the energy stored in the DG that will be used to process the next meal. Those results and others obtained from experiments performed with *O. maya* indicate that the type and level of protein are the principal source of metabolic energy in the muscle, while lipids are the principal source of energy for DG intracellular metabolism (Martínez et al., 2011; Rosas et al., 2011; Baeza-Rojano et al., 2013). Similar results were observed in *O. mimus*, indicating that the biochemical pathways observed in *O. maya* could be generalized to other octopus species (Linares et al., 2015).

DIGESTIVE ENZYMES

Acidic enzymes in the crop, stomach and DG were first observed in *O. vulgaris* (Morishita, 1974). Studies by Martínez et al. (2011) and Linares et al. (2015) indicate that acidic enzymes are not only present in *O. vulgaris*, but also in *O. maya* and *O. mimus*. Those enzymes were also observed in other cephalopod species such as squid and cuttlefish (Perrin et al., 2004; Cardenas-Lopez and Haard, 2005, 2009), suggesting that this type of enzyme has a key role in the digestive capacity of cephalopods. A partial characterization of the digestive enzymes in the GJ and DG of *O. maya* (Martínez et al., 2011) found that cathepsin D, which requires an acidic environment to develop maximum activity, is 18 and 72% inhibited in the GJ and DG, respectively; this indicates that as shown by Morishita (1974), acidic enzymes have an important role in the digestive process of this octopus species. However, that family of enzymes (cathepsin and pepsin) has been demonstrated to be quite sensitive to the biochemical structure of the ingested protein. In a study of myofibrillar protein susceptibility to proteases (pepsin) when meat is exposed to heating, the cooking process was observed to affect protein digestibility via a reduction of attack enzyme sites in the denatured protein (Santé-Lhoutellier et al., 2008). To test if ingredients cooked at a high temperature also affect their digestibility for octopus (via the reduction of cathepsin attack sites in cooked protein), seven experiments carried out to study the effects of several industrial cooked fish, clam and squid meal, and laboratory cooked crab meat on growth and survival of *O.*

maya juveniles (Rosas et al., 2013). Results of that study showed that diets based on fresh crab paste, lyophilized crab, and squid promoted better growth rates than those observed in animals fed diets made with cooked meal. Also, the *in vitro* enzyme activity was higher in the DG of animals fed cooked ingredients than in the DG of animals fed fresh pastes, indicating that a secretagogue effect was induced in those animals as a consequence of reduced diet digestibility. Therefore, lyophilisation was considered the method that maintained native protein in octopus diets, through facilitation of cathepsin enzyme activity, and in consequence better diet digestibility (Martínez et al., 2014; Tercero-Iglesias et al., 2015). Although, the effect of the pH on the GJ and DG enzymes was only established in *O. maya* (Martínez et al., 2011), Linares et al. (2015) observed that high enzymatic activity can be obtained when the gastric juice of *O. mimus* is assayed in relatively low pH (5.5). Considering that only an 18% enzyme inhibition was observed when pepstatin A was used in *O. maya* GJ, it is possible to hypothesize that there are other cathepsins working in the acidic environments of the GJ. For example, cathepsin L activity was demonstrated in the giant squid *Dosidicus gigas* (Cardenas-Lopez and Haard, 2009), suggesting that if the DG intracellular pH is acidic in other cephalopods, then other cathepsins in addition to cathepsin D (Martínez et al., 2011) may also be present in the GJ.

Results obtained until now indicate that there is synchronization between DG enzymes pulses and the GJ enzyme activity. In *O. maya* two pulses were observed (20–80 and 80–180 min), while only one pulse was noted (80–180 min) in the enzyme activity of *O. mimus,* suggesting strong differences in digestive dynamics between species (Linares et al., 2015). These differences could be due to the different environmental temperature in the habitat of each species, with more frequent enzyme release in tropical species (e.g., *O. maya*) than in subtropical or temperate species (*O. mimus*). Therefore, temperature could be regulating all the digestive activity including ingestion rate, chyme formation, intracellular digestion, and enzyme production. Digestive physiology of *O. maya* and *O. mimus* are similar in many aspects to the process described by Boucaud-Camou et al. (1976) for *O. vulgaris*. Considering the available information, a conceptual model showing the most important aspects of the *O. maya* and *O. mimus* digestive physiology was developed, which we think can be applied as a general model to other octopus species (**Figure 1**).

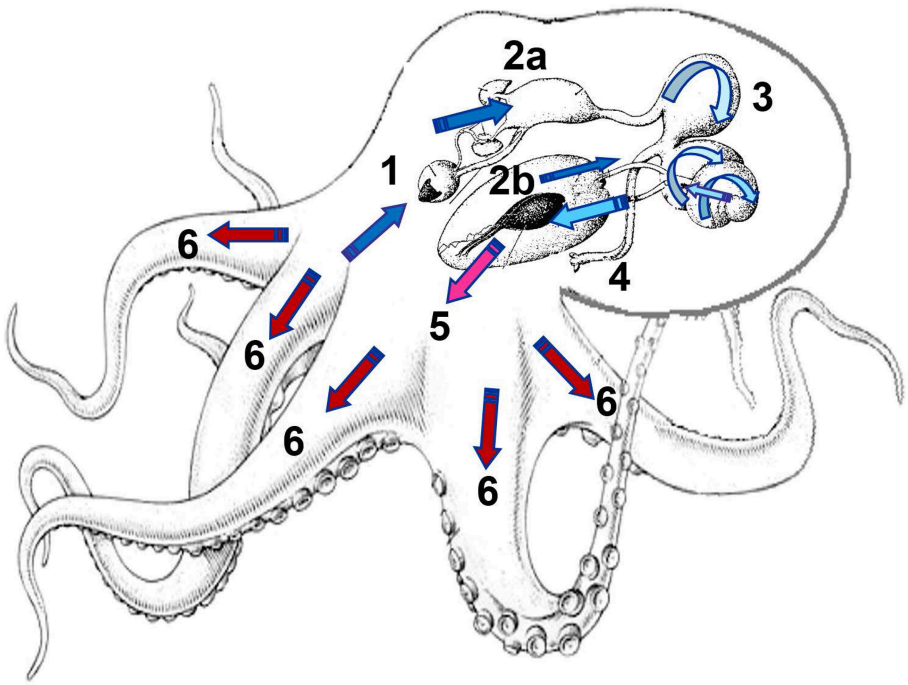

FIGURE 1 | Digestive physiology of *Octopus maya* and *O. mimus*, showing the food route and the time required by the chyme to reach each part of digestive tract. 1: Mix of enzymes and soluble protein enter into the crop after the external digestion of the prey, activating zymogens in gastric juice (GJ) located along the digestive tract. 2a: Almost at the same time, soluble proteins are rapidly launched into the digestive gland (DG) activating the absorption sites, while the DG enhances its activity, absorbing nutrients and releasing enzymes in pulses (2b). Depending on the timing, alkaline and acidic enzymes act in the stomach and caecum (3). The change of color of the arrow (dark blue to clear blue) indicates changes in digestive environment, modulated by the type of enzymes involved along the digestive process changing from raw ingested food (dark blue) to assimilated and stored nutrients (red). 4: Cell debris and undigested material forming feces are sent to the anus. 5: As a result of internal digestion in the DG, amino acids (AA) and other nutrients are transported by the hemolymph to tissue to be used as a source of energy and molecules to restore tissues. In *O. maya* two peaks of AA were recorded at 20 min and 180 min after feeding (Linares et al., 2015). 6: Finally, 400–480 min after feeding nutrients are stored in tissues as muscle. Illustration: M. Linares and C. Rosas.

OCTOPUS MAYA AND *O. MIMUS* DIGESTIVE TIMING

The digestive physiology timing in *O. maya* and *O. mimus* is different and is probably associated with differences in habitat. *O. maya* is found in habitats where temperature fluctuates between 22 and 30°C, while *O. mimus* lives in thermal regimes that are between 14 and 22°C. Considering that type of diet and the living weight can modify the digestive timing, Linares et al. (2015) carried out their study using as a food two similar crustacean species (the blue crab *Callinectes sapidus* and *Cancer setosus*) that inhabit the adult zones of both octopus species (810 ± 116 g for *O. maya*; 1048 ± 180 g for *O. mimus*).

In that study, two digestive step processes were observed in *O. maya*: the first one was characterized by production of soluble nutrients in the prey that were rapidly ingested and absorbed, filling the digestive tract and used for muscle protein synthesis (**Figure 1**). After, a second slower process was identified, where more complex nutrients were obtained from muscle flesh of the prey, transformed into soluble nutrients, then transported to the DG to be catabolized and placed into muscle or stored temporarily to be used as a source of energy for the next meal (**Figure 1**). The digestive process of *O. mimus* was slower than in *O. maya*, showing a peak of muscle glycogen accumulation at the end of the digestive process (400 min after feeding), indicating that each species has its own timing and physiological process, related to the thermal regime in which species has evolved (Linares et al., 2015).

Figure 2 summarizes the digestive process occurring in each section of the digestive tract of *O. maya* (**Figure 2A**) and *O.*

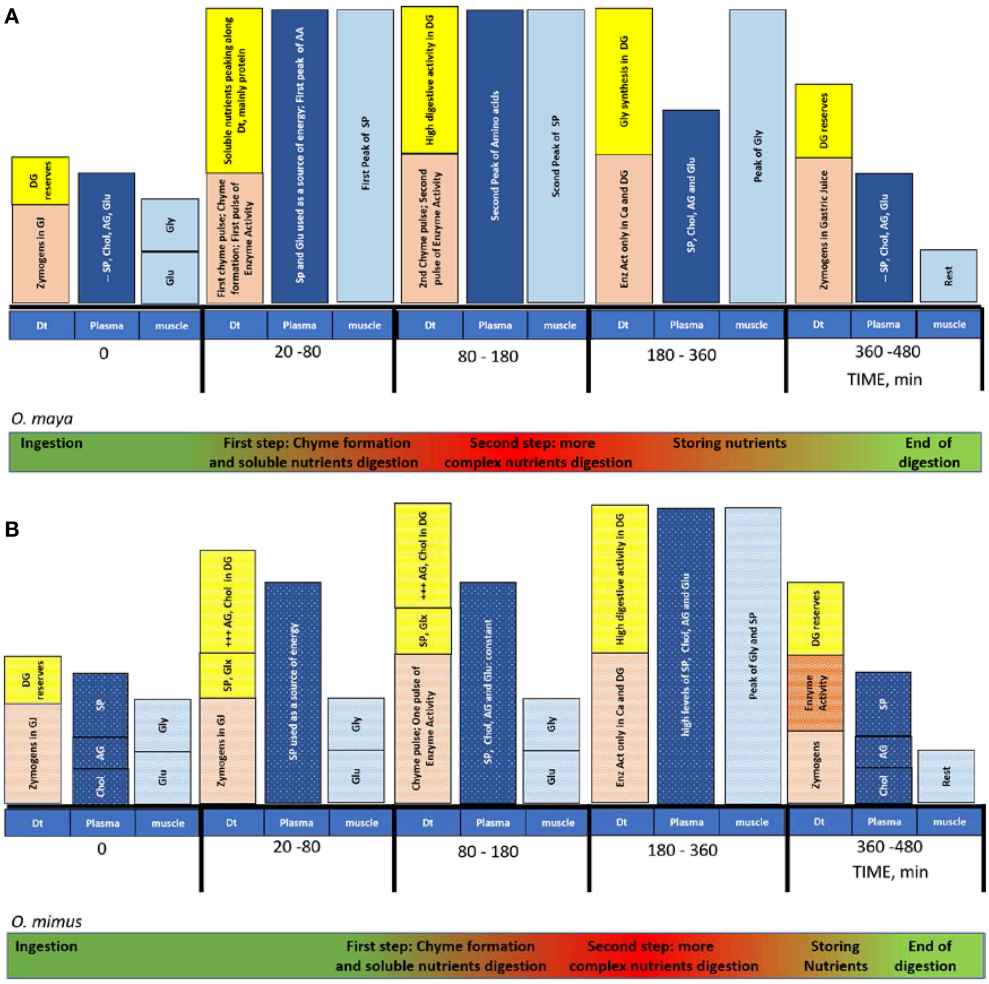

FIGURE 2 | Timing of the digestive process, absorption and assimilation in adults of *O. maya* **(A)** and *O. mimus* **(B)** of the food. Before the ingestion, gastric juice (GJ) is located along the digestive tract: crop, stomach and caecum. Reserves in the DG are constant. Once the prey was offered *O. maya* took 20 min to ingest food while *O. mimus* took 140 min. While *O. maya* stored protein, *O. mimus* stored AG and Chol. The peak of the digestive process was recorded around 180 min after feeding in *O. maya* and 360 min after feeding in *O. mimus*. The end of the process was registered between 360 to 480 min in both species. Dt, digestive tract; DG, digestive gland; Ca, caecum; SP, soluble protein; Chol, cholesterol; AG, acyl glycerides; Glu, glucose; Gly, glycogen; GLx, glucose and glycogen mix; Enz, digestive enzymes; AA, amino acids. Symbol + indicates the magnitude of metabolites accumulated in DG.

mimus (**Figure 2B**). In that figure, all the digestive sequences that occur at the same time and along the digestive tract during digestion of each species are encapsulated. From this figure it is evident that although the general process is similar between species, there are differences in the timing of the process and the form in which DG reserves are used. In *O. mimus*, lipids were mobilized faster than proteins while in *O. maya* an inverse relationship between proteins and lipids was observed, suggesting that mobilization of lipids could be a priority in temperate octopus species (Mukhin et al., 2007). Also, it was observed that in *O. mimus* the acidic enzyme activity in the digestive tract was greater than in *O. maya,* suggesting that through modulation by low temperatures *O. mimus* could require more enzymes to digest the meal. Consequently, we think that those differences should be considered by the nutritionists who design dry foods for these octopus species. This review shows that although different octopus species have digestive systems with similar functions, each species has a unique way of digesting the food it consumes. This reflects the thousands of years of evolution that each one has experienced in different habitats, and these differences should be considered for the maintenance of healthy organisms in captive conditions.

Both *O. maya* and *O. mimus* prepare their digestive tracts for digestion. Martínez et al. (2011) and Linares et al. (2015) found digestive enzymes and zymogens in GJ along the digestive tract before meal ingestion, indicating that these GJ were secreted by the DG in preparation for the next meal. Linares et al. (2015) showed that protein, fatty acids and cholesterol in the haemolymph were the nutrients required to maintain the fasting octopus period (**Figure 2A**), indicating that to be prepared, octopuses not only secrete zymogens, but maintain energetic substrates in the haemolymph to be used as a source of energy between meals. This is because octopuses depend directly on the food they consume to obtain energy, as they do not have many tissue reserves to mobilize (Rosa et al., 2005), as was also shown in *O. vulgaris* (García-Garrido et al., 2011). In *O. vulgaris* 3 d of fasting was enough to induce mobilization of mantle lipids, cholesterol (Chol), acyl glycerides (AG), soluble proteins (SP), and amino acids (AA), which were used as a source of metabolic energy (García-Garrido et al., 2011). Results obtained in *O. maya* showed that this species is adapted to tolerate only short fasting periods; after 2 d of fasting a strong AA mobilization was observed (George-Zamora et al., 2011). Although, there is no information on histological changes in *O. mimus* during digestion or the use of reserves, we suspect that AA mobilization dynamics similar to those observed in *O. maya* could be present.

Both octopus species reacted immediately to offered meals, although there were differences between them. In *O. maya* adults, 20 min is enough to ingest a crab of around 100 g at 26°C (Martínez et al., 2011; **Figure 2A**). During this time, *O. maya* injects saliva to the prey which contains chymotrypsin and a neurotoxic fraction that causes paralysis and postural changes in the crab (Pech-Puch et al., 2016). After, the first pulse of chyme containing soluble nutrients is absorbed, initiating the digestive process. In this first chyme, high levels of soluble protein were recorded in both species along the digestive tract including the DG, where intracellular digestion begins (**Figure 2A**). This first pulse of chyme is the result of the chymotrypsin digestion in the prey, and is a mixture of soluble nutrients, octopus chymotrypsin, and many types of activated and inactivated prey enzymes (**Figure 1**; Martínez et al., 2012). Once this first pulse of chyme is ingested, it is mixed with the acidic gastric juice previously stored in the crop, stomach, and caecum resulting in the start of the acid digestion in the digestive tract. Although, a similar process was identified in *O. mimus* (**Figure 2B**), Linares et al. (2015) showed that adults of this species maintained at 14°C needed 80 min to ingest the food, demonstrating that temperature make more slow the digestive dynamics in these species.

As was mentioned earlier, the role of the salivary glands at the beginning of digestion process is important (Boucaud-Camou and Boucher-Rodoni, 1983; Pech-Puch et al., 2016). Enzymes from salivary glands in *O. maya* participating in the first chyme pulses are found 20 min after the meal (Pech-Puch et al., 2016). In *O. mimus* this process occurs 140 min after the meal (Linares et al., 2015), indicating again that habitat temperature of each species modulates this process (**Figure 2B**). Taking into consideration that the first chyme pulse could be acting as a zymogens activator (Martínez et al., 2011), timing differences in the start of digestion between species (**Figure 2**) could be indicating that the activation of zymogens and the timing of absorption in *O. mimus* also occurs later than in *O. maya* (Linares et al., 2015; **Figure 2**).

Once the digestive system in the DG is activated, heterophagosomes in the acinar cells transport energetic molecules to the haemolymph, where they are transported to muscle and other tissues (Linares et al., 2015; **Figures 2A,B**). Results obtained in *O. maya* and *O. mimus* provided evidence that during DG cells activation, nutrients previously stored in the DG were presumably directed to fuel the intracellular digestion (**Figure 2**). Reductions in DG glycogen and increments of soluble glucose in *O. maya* and *O. mimus* (Linares et al., 2015) support that idea. It is interesting to note that while soluble AG and Chol were also used as a source of energy in *O. maya* (**Figure 2A**), in *O. mimus* those nutrients were accumulated, indicating the importance of lipid metabolism in temperate species (**Figure 2B**), where lipids have a key role in maintenance of membrane fluidity in addition to their energetic role (Estefanell et al., 2013).

Differences between species can be also observed in relation to the use and destination of nutrients in the DG and other tissues (**Figure 2**). Haemolymph glucose levels changed significantly during the digestive process in *O. maya*, indicating that this nutrient is mobilized to support the energetic demands in different tissues of the animal (**Figure 2A**). The higher mobility could be necessary to satisfy the muscle energy demands in their tropical environment (Noyola et al., 2013). In contrast, glycogen was stored in *O. mimus* (Linares et al., 2015), indicating that glycogen reserves could be critical in temperate environments to maintain the DG, instead of the muscle activity observed in

tropical species (**Figure 2B**). As in other marine invertebrates, whether they are tropical or temperate species, glycogen is an energetic product of the intermediate metabolism of non-essential AA, which is part of the muscle energy pathways (Loret, 1993) and for that reason is a key reserve molecule for the digestive process in cephalopods (Rosa et al., 2005). The role of AA in octopus energetics was demonstrated when two peaks of essential and non-essential AA were observed in *O. maya* haemolymph (Linares et al., 2015; **Figure 2A**). The first one was noted in conjunction with the first chyme pulse (40 min after meal) and the second one during the peak of digestive enzyme activity 140–180 min after the meal (Linares et al., 2015). This fast mobilization of AA indicates that those molecules were coupled with the digestive process, and presumably directed to tissues to be used for protein synthesis (growth) and/or glycogen synthesis (energy) (**Figure 2A**). In *O. maya* it was demonstrated that phenylalanine, isoleucine, alanine, glutamine, and serine are used as metabolic fuel, while histidine, arginine, and lysine are accumulated as reserves in muscle during starvation (George-Zamora et al., 2011). It is interesting to note that the observed peaks of AA in haemolymph occurred at the same time that chyme soluble protein peaked; effectively suggesting a strong mobilization of AA was the result of soluble protein catabolism (Linares et al., 2015). The role of AA as a source of energy in cephalopods was also observed in 14 other species of nektobenthic, benthic, and benthopelagic cephalopods (Rosa et al., 2005). In their study, Rosa et al. (2005), showed that proline and arginine were used as a source of energy, supporting our idea that AA are the principal energetic substrates in cephalopods via amino acid catabolism and glycogen synthesis (Rosas et al., 2002; Miliou et al., 2005). Results obtained by Linares et al. (2015) showed that glycogen peaks follow a peak of soluble protein, presumably following the digestive process:

$$\begin{aligned}\text{Proteins in prey} &> \frac{\text{Soluble proteins in the chyme}}{\text{Digestion in crop, stomach and DG}}\\ &> \frac{\text{AA in haemolymph transported to muscle}}{\text{Intracellular enzymes in DG}}\\ &> \frac{\text{Glucose}}{\text{Synthesis in DG and/or muscle}}\\ &> \frac{\text{Glycogen}}{\text{Synthesis in DG and/or muscle}}\end{aligned}$$

Although, Linares et al. (2015) did not evaluate the haemolymph AA of *O. mimus,* considering that glycogen was also accumulated in its muscle after feeding, we conjecture that as observed in *O. maya*, the muscle glycogen in *O. mimus* was synthesized from AA, reaching its maximum value 400 min after feeding (**Figure 2B**).

At the end of the digestive process (480 min after feeding), a DG pH reduction was reported in *O. maya* and *O. mimus* (Martínez et al., 2011), suggesting a release of digestive enzymes in preparation for a new digestive cycle. Those enzymes could be sent to the digestive tract, where a reduction of pH was also registered (Linares et al., 2015). Reduction of enzymatic activity could also indicate that enzymes in the new gastric juice were zymogens that require the chyme to be totally activated.

Following the histological dynamics of DG in *O. maya*, Martínez et al. (2011) also observed an increment of residual body density 360 min after feeding, indicating that the feces and cellular debris removal process reached its maximum level at that time. Posteriorly, all the activity in the digestive system was reduced, with low production of residual bodies in the DG cells indicating that digestive cycle had ended (**Figure 2A**). At that time, nutrient reserves were accumulated in wait for the next meal (Martínez et al., 2011; **Figure 2B**).

As was previously stated for *O. vulgaris* by Boucaud-Camou and Boucher-Rodoni (1983), is evident the digestive physiology of *O. maya* and *O. mimus* is a fast and strongly dynamic process. In adults, this process takes around 480 min to be completed, indicating that this type of animal should be fed at least every 8 h to maintain its health in captivity (Linares et al., 2015). At a semi-pilot scale, this feed protocol has been followed for more than 5 years (Rosas et al., 2014); adults of *O. maya* were fed every 8 h using fresh scraps of marine fish or fresh crab (Caamal-Monsreal et al., 2015) or a diet formulated to stimulate spawning in laboratory conditions (Tercero-Iglesias et al., 2015). Under these conditions the number of eggs spawned was quite similar to those observed in wild spawns (Vidal et al., 2014), indicating that laboratory animals fed every 8 h reach a similar healthy condition to those on the continental shelf of the Yucatán Peninsula, where this species lives (Avila-Poveda et al., 2016; Angeles-Gonzalez et al., 2017). *O. maya* and *O. mimus* are well adapted, as are the majority of cephalopod species, to digest a high-quality animal protein diet using a mix of acidic and alkaline enzymes. This allows them to efficiently obtain the energy and molecules necessary to maintain their physiological functions according to the environment where they live, as shown for the tropical (22–30°C; *O. maya*) and temperate (14–22°C; *O. mimus*) species.

AUTHOR CONTRIBUTIONS

CR, PG, AO, RM, and CC designed and ran the experiments in Mexico and Chile. CR, PG, PD, MM, CP, and AS, performed the laboratory analysis and processed the data. All authors contributed to write the paper.

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MINI REVIEW
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The Role of the Cephalopod Digestive Gland in the Storage and Detoxification of Marine Pollutants

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The relevance of cephalopods for fisheries and even aquaculture, is raising concerns on the relationship between these molluscs and environmental stressors, from climate change to pollution. However, how these organisms cope with environmental toxicants is far less understood than for other molluscs, especially bivalves, which are frontline models in aquatic toxicology. Although, sharing the same basic body plan, cephalopods hold distinct adaptations, often unique, as they are active predators with high growth and metabolic rates. Most studies on the digestive gland, the analog to the vertebrate liver, focused on metal bioaccumulation and its relation to environmental concentrations, with indication for the involvement of special cellular structures (like *spherulae*) and proteins. Although the functioning of phase I and II enzymes of detoxification in molluscs is controversial, there is evidence for CYP-mediated bioactivation, albeit with lower activity than vertebrates, but this issue needs yet much research. Through novel molecular tools, toxicology-relevant genes and proteins are being unraveled, from metallothioneins to heat-shock proteins and phase II conjugation enzymes, which highlights the importance of increasing genomic annotation as paramount to understand toxicant-specific pathways. However, little is known on how organic toxicants are stored, metabolized and eliminated, albeit some evidence from biomarker approaches, particularly those related to oxidative stress, suggesting that these molluscs' digestive gland is indeed responsive to chemical aggression. Additionally, cause-effect relationships between pollutants and toxicopathic effects are little understood, thus compromising, if not the deployment of these organisms for biomonitoring, at least understanding how they are affected by anthropogenically-induced global change.

Keywords: aquatic toxicology, mollusca, Cephalopoda, biomarkers, toxicological pathways, bioaccumulation

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INTRODUCTION

Cephalopods are a particular group of invertebrates that share many important features with high-order animals as a result of convergent evolution, with emphasis on nervous system function. These features are, nonetheless, analogs to those of chordates, as cephalopods hold the basic molluscan body plan. Molluscs, however, form a cunningly diverse group of animals, ranging from sedentary filter feeders like bivalves to the giant predator squid *Architeuthis*. Among predators, cephalopods are of special interest in terms of anthropogenic impacts onto food webs, as they feed on a wide range of live prey and have high growth and metabolic rates (see Mangold, 1983), which poses important questions regarding bioaccumulation and tolerance to chemical

stressors. However, the physiological and molecular mechanisms underlying toxicopathological effects and detoxification processes in cephalopods are not well understood, albeit the importance of other molluscs, especially bivalves, in biomonitoring and substance testing. Overall, most studies concerning exposure of cephalopods to marine contaminants relate to the accumulation of trace elements, in most cases being limited to commercial species (see Penicaud et al., 2017).

It is suggested that storing metals in various tissues can be an important strategy to cope with metal toxicity (see Miramand and Bentley, 1992; Bustamante et al., 2000; Raimundo et al., 2005 plus the recent review by Penicaud et al., 2017). This efficient strategy, which likely minimizes energetic costs, is seemingly common among Nautiloid and Coleoid cephalopods (Bustamante et al., 2000). However, the mechanisms involved even in these basic processes are not fully resolved, particularly in the case of organic contaminants. This gap noticeable for invertebrates in general, bivalves included, albeit the attention the digestive gland has been receiving as target for bioaccumulation and biomarker analysis for being the analog the vertebrate liver. While there is indication that detoxification and excretion processes may indeed occur with the assistance of specialized cells (Costa et al., 2014), this issue is not entirely consensual as the digestive gland is able to provide long-term storage of both toxic and essential metals, such as Cd and Zn (Bustamante et al., 2002b) The growing level of genomic annotation for bivalves and a few cephalopods also indicates that CYP-like enzymes and respective organic xenobiotic pathways are active in molluscs (Cheah et al., 1995).

As such, the present review aims at summarizing the state-of-the-art on the role of digestive gland in the detoxification of organic and inorganic contaminants in cephalopods, emphasizing the comparative microanatomy, physiology, and molecular processes among various groups of molluscs that, however, indicate that toxicological pathways in cephalopods may be more diverse and complex than anticipated.

FORM AND FUNCTION OF THE CEPHALOPOD DIGESTIVE GLAND

The molluscan digestive gland is a multi-task annex to the digestive tract, involved in secretion of digestive enzymes, extra— and intracellular digestion, substance storage and excretion (Bidder, 1966). As in many invertebrates, organs are called to perform multiple functions due to reduced differentiation comparatively to vertebrates. The basic structure of the digestive gland is well-conserved among molluscs, being formed by blind-end indigitations called "tubules" or more accurately, *diverticula*, being connected to the gut (specifically to the caecum, in cephalopods) by ducts (Budelmann et al., 1997). Refer to **Figure 1** for a comparative overview of the molluscan digestive gland. In cephalopods, albeit the lack of an absolute consensus, three distinct cells types have been identified. Digestive cells are the most abundant, followed by basal (replacement) and the more elusive excretory cells, characterized by a single, large hydropic vacuole that may bear mineral precipitates. Note that basal cells are commonly termed crypt and basophilic cells in gastropods and bivalves, respectively. Although demonstrated in cephalopods (e.g., Boucaud-Camou, 1968; Costa et al., 2014), the existence of specialized excretory cells is not consensual in other molluscs and their specific function in cephalopods is not well understood. There are, nonetheless, reports on changes in size and number of hydropic vacuoles of digestive gland cells of gastropods and bivalves as a result of exposure to mixed metallic and organic toxicants (Zaldidar et al., 2007; Lobo et al., 2010).

Cephalopod digestive gland epithelia are more complex than other molluscs' with respect to specialized endosomes. In fact, structures such as "boules" (vacuoles involved in digestion and enzyme secretion) and "brown bodies" (excretion of crystalline salts and amorphous materials) are seemingly exclusive. The lack of detailed studies integrating digestive gland histology and cytology with molecular pathways, as well as the lack of comparative studies between molluscan taxa hinders understanding how molluscs evolved to handle hazardous substances.

Accumulation of toxicants in the digestive gland depends on their mechanisms of apical entry. Bustamante et al. (2002b) revealed that Cd and Zn enter the cephalopods' digestive gland directly *via* food and indirectly *via* blood, in the latter case if uptake occurs from seawater. Nonetheless, the same authors disclosed that the elimination of these metals is faster if uptaken through water. It must be noted that the existence of a closed circulatory system in these molluscs, likely render the organ particularly efficient for nutrient absorption and as a filtering system for peripheral fluids. Indeed, unlike bivalves for instance, the cephalopod digestive gland possesses an intricate network of arteriole-like blood vessels (e.g., Swift et al., 2005; Costa et al., 2014). To these features is added the ability to form (and eventually release) mineral corpuscles called *spherulae* (spherocrystals) in the basal cells, first noticed by Martoja and Marcaillou (1993) and more recently described by Costa et al. (2014) which may thus have an important role in metal homeostasis, similarly to what has been suggested for some gastropods (Volland et al., 2012).

The first descriptions of the microstructure of the cephalopod digestive gland are almost as old as histology itself (refer to the pioneer works by Frenzel, 1886 and Cuénot, 1907). These were complemented by important histochemical descriptions being made from the 1960s onward that favored structural and digestion-related aspects (e.g., Boucaud-Camou, 1968; Semmens, 2002; Martinez et al., 2011). However, even for bivalves, which are the most investigated invertebrates by toxicologists, there are many gaps about the relation between form and function of the digestive gland and toxicant metabolism.

THE DIGESTIVE GLAND IN BIOACCUMULATION AND DETOXIFICATION OF METALS

The vast majority of literature focusing on toxicants in cephalopods relates to metals, as cephalopods are known to bioaccumulate impressive amounts of hazardous elements, like Cd, albeit others, such as Hg, appearing to be less significant,

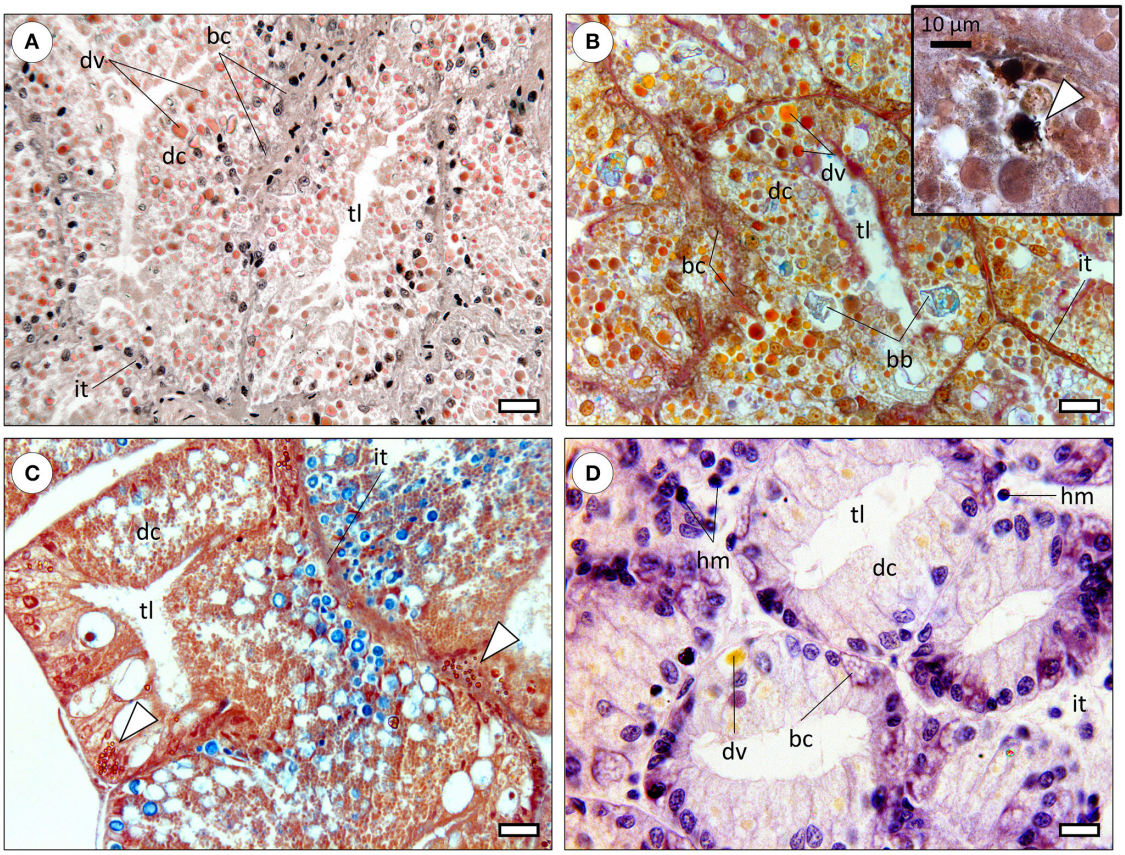

FIGURE 1 | Comparative histology of the molluscan digestive gland (paraffin sections). bc, basal cells (also called replacement; crypt, basophilic or pyramid cells); dc, digestive cells; dv, digestive vacuoles; hm, haemocytes; it, intertubular tissue; tl, tubule lumen. **(A)** Digestive gland of the common octopus (*Octopus vulgaris*) evidencing large digestive tubules (*diverticula*) formed mostly by digestive cells. The distinctive digestive vacuoles of cephalopods are naturally pigmented and traditionally referred by the French term "boules." Haematoxylin & Eosin. Scale bar: 25 µm. **(B)** Micrograph of the digestive gland of a cuttlefish (*Sepia officinalis*), showing a similar structure to that of *Octopus*. Brown bodies (bb) are distinctive of sepioids, being comprised of amorphous, undigested, materials. Tetrachrome stain. Scale bar: 25 µm. *Inset*: Basal cells were observed to hold calcic *spherulae* that include other metals as well, embedded in a proteinaceous matrix but the issue needs further research. The presence of calcium in *spherulae* in basal cells is here determined histochemically (stained black) through the von Kossa reaction, counterstained with Nuclear Fast Red (arrowhead). **(C)** Section through the digestive gland of the marine gastropod *Onchidella celtica* (Pulmonata), evidencing a similar structure and to that of cephalopods, albeit differences in the histochemical signal of digestive vacuoles, here predominantly blueish (from sugars), likely due to the herbivore feeding regime. The staining is similar to that of the preceding panel. The specimen was fixated in Zenker's solution, which contains (potassium) bichromate that reacts with metallic compounds originating yellow-orange deposits (arrowheads), once again visible in basal cells. Scale bar: 25 µm. **(D)** Section across the digestive gland of a bivalve (*Ruditapes decussata*), stained with Haematoxylin and Eosin. The tubules are smaller than previous examples and digestive cells less intricate with respective to variety, quantity and natural coloration of digestive vacuoles, regardless of digestive phase (which is similar among all panels). Basal cells are again evident and bear vesicular-like structures, potentially *spherulae* or similar. Note the wider and sparser intertubular tissue within which haemocytes can be found, as bivalves have an open circulatory system. Scale bar: 12 µm.

which indicates metal- and organ-specific pathways (Penicaud et al., 2017). It has been shown that the digestive gland holds the highest concentrations of essential (like Cu and Zn) and non-essential (such as Ag, Cd and Pb) metals in several cephalopods, with emphasis on *Sepia* and *Octopus*, comparatively to other organs, mantle and arms included, the latter of which raise particular concerns regarding human consumption (e.g., Raimundo et al., 2004, 2005; Seixas et al., 2005; Bustamante et al., 2006; Pereira et al., 2009). However, the digestive gland has been identified as the main metal accumulation organ even in the Nautiloidea (Pernice et al., 2009). Available data suggests that the cephalopod digestive gland is particularly efficient in the retention of these elements, likely as a function of chelating agents, especially proteins. In fact, there have been a few works that related concentrations of metals in this organ with different types of largely undisclosed proteins distributed through several subcellular partitions. For instance, Raimundo et al. (2010a) noticed that, in the common octopus, Pb was associated to unknown high molecular weight proteins while Zn, Cu and Cd showed high affinity to both high and low molecular weight proteins. Somewhat similar associations were recorded in the digestive glands of red arrow squid (*Nototodarus gouldi*) by Finger and Smith (1987) and in the common cuttlefish by Bustamante et al. (2006). These authors suggested that

these associations may relate to different metal detoxification mechanisms, in many cases likely modulated by metal burden *per se*, through the induction of chelating proteins. Interestingly, Costa et al. (2014), demonstrated histochemically that the metal-containing *spherulae* in cuttlefish digestive gland basal cells are formed by a matrix of proteinaceous materials, being released into the lumen of tubules as cells differentiate. However, there seems to be some selectivity in the accumulation of metals in *spherulae*. Unlike Cu, which appears to be accumulated mostly in these structures, Fe accumulates essentially in the cytosol of digestive cells, which has been confirmed histochemically in the independent works by Martoja and Marcaillou (1993) and Costa et al. (2014) with *Sepia officinalis*. Altogether, basal cell *spherulae* may be common in molluscan digestive glands, even though their role and formation is not well understood. Volland et al. (2012), for instance, demonstrated the existence of these structures in basal cells in the marine gastropod *Strombus*, albeit being seemingly not involved in Cd bioaccumulation upon induced exposure. In agreement, Bustamante et al. (2002a) reported that, in several different cephalopods, Cd is mostly cytosolic.

It has also been suggested that metallothioneins (MTs), well described in bivalves, play an important role in the formation of *spherulae* (Martoja and Marcaillou, 1993). However, in cephalopods, MT induction in the digestive gland may not be entirely consistent with exposure to metals, even in the case of exposure to strong inducers such as Cd (Bustamante et al., 2002a; Raimundo et al., 2010b; Rodrigo et al., 2013). It is possible that elements such as Cd, are not involved in detoxification via *spherulae* (thus remaining in the cytoplasmic fraction), which in its turn, relies on MT and, potentially, unknown high molecular weight proteins (see Penicaud et al., 2017, for a summary). This interesting perspective, indicates that there can be novel mechanisms of toxicity and detoxification of non-essential metals like Cd in cephalopods that need to be unraveled. As such, the known mechanisms for MT expression, described essentially for vertebrates, namely those relying on metallothionein transcription factor (MTF) mediation may not apply entirely or at all. In **Table 1** are summarized the most relevant among the very few publications on biomarker responses in cephalopod digestive gland in an ecotoxicological context, which includes also the MT response as potential indicator of exposure to metals.

EVIDENCE FOR THE METABOLISM OF ORGANIC TOXICANTS

Although far less common than for metals, some studies addressed the issue of bioaccumulation of various organic hazardous substances in the cephalopod digestive gland, from polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) to amnesic shellfish toxin (Costa et al., 2005, 2009; Danis et al., 2005; Storelli et al., 2006; Semedo et al., 2014). Nevertheless, the pathways of detoxification and elimination of organic substances, pollutants and toxins (endogenous or exogenous) that are called bioactive compounds are strikingly more complicated than metal chelation and expression of chelators. These mechanisms have been described mostly for mammals and are not consensual for invertebrates, once again indicating important knowledge gaps, in spite of the relevance of molluscs for ecotoxicologists.

The pathways for drug metabolism, meaning phases I (biotransformation) and II (conjugation), to which is now added a phase III (elimination) have been described in the vertebrate liver (see Ferreira et al., 2014, and references therein). Their functioning in invertebrates is not entirely consensual, in part due to the differential response of common biomarkers between vertebrates and invertebrates. For instance, one of the most important systems involved in phase I, the cytochrome P450 (CYP) monooxygenase complex (involved in the detoxification of many bioactive xenobiotics), has long been shown to hold similarities between invertebrates and vertebrates using cDNA probes and Western blotting techniques (Livingstone, 1994). This includes evidence that at least some octopodid cephalopods express CYP isoenzymes, albeit at reduced levels comparatively to vertebrates (Cheah et al., 1995). The CYP systems are localized mainly in the microsomes of the digestive gland cells of molluscs, although it was also found in other tissues like gills and even in haemocytes (Oehlmann and Schulte-Oehlmann, 2003). Still, the relevance of CYP1A in molluscs (involved in the metabolism of important PAHs, polychlorinated biphenyls and dioxins, as examples) has been disputed through works with bivalves, in favor of other forms, such as CYP4 (Chaty et al., 2004). In contradiction, there is some evidence for CYP1A activity and induction in terrestrial molluscs exposed to toxicants (Snyder, 2000, for a review). It is also important to mention that the pathways leading to increased CYP expression, usually involving xenobiotic-activated nuclear receptors (XANRs), are not well understood in invertebrates (see Richter and Fidler, 2014). In fact, the cephalopod equivalent for the aryl hydrocarbon receptor (Ahr) pathway, which is responsible for increased expression of CYP1A by PAHs and similar in vertebrates, remains a mystery. Still, there are promising findings with bivalves regarding its expression in gills and digestive glands exposed to Ahr agonists like benzo[a]pyrene (e.g., Châtel et al., 2012).

Schlenk and Buhler (1988) suggested that CYPs are more important in the metabolism of endogenous substrates rather than xenobiotic metabolism in Polyplacophora digestive glands, whilst other works evidenced some induction in bivalves (concerning enzyme content and activity) by agents ranging from pharmaceuticals to acrylamide, although unable to metabolize compounds that can be biotransformed in vertebrates or induced in a reduced extent (see for instance Galli et al., 1988; Larguinho et al., 2014). Also, Cheah et al. (1995) disclosed modest induction of CYPs in *Octupus pallidus* digestive gland after exposure to known inducers like β-naphtoflavone and Aroclor, with increased activity of ethoxycoumarin-*O*-deethylase (ECOD) but not for ethoxyresorufin *O*-deethylase (EROD), which is one of the best accepted biomarkers of exposure to bioactive pollutants in vertebrates. This finding is in agreement with the work by Schlenk and Buhler (1988) with chitons, even though Semedo et al. (2014) found negligible activities of both enzymes in wild *Octopus vulgaris*.

TABLE 1 | A compilation of different biomarkers and other indicators of exposure to environmental toxicants measured in cephalopods.

Species	Approach	Target	Biomarker responses	Substances of interest	Geographical area	Main outcomes	Reference
Sepia officinalis	*In vitro* bioassay	Digestive gland cells	Enzyme activity (trypsin, collagenase, hyaluronidase and pronase) plus cell viability	Cu, Zn, Ag	U.K.	Ag can be highly toxic as it inhibited digestive enzymes, whereas Zn only caused adverse effects at higher concentrations. Cu had no significant effects.	Le Bihan et al., 2004
Sepia officinalis	Field sampling	Adults (digestive gland and gills)	LPO, GST, GSHt, GSH/GSSG, MT	Mixed environmental toxicants (metallic and organic)	Portugal	Lipid peroxidation was one of the most significant biomarkers in the digestive gland, together with GST. Gills probably more sensitive.	Rodrigo et al., 2013
Sepia officinalis	Bioassay	Juvenile (whole-organism sampling)	LPO, GST, SOD, Catalase, MT	Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, V, Zn and Zn alone	France	Maternal transfer of metals in the cuttlefish. Evidence for deficient Zn regulation.	Le Pabic et al., 2015
Sepia elegans *Sepia officinalis* *Sepia orbignyana* *Loligo vulgaris* *Illex coindetii* *Todarodes sagittatus* *Eledone cirrhosa*	Field sampling	Adults (digestive gland)	MT	Cd	France (Bay of Biscay) and Faroe Islands.	No differences between size and gender were noted. Cadmium was mainly located in the soluble fraction of digestive gland cells, probably bound to cytosolic proteins. Limited participation of these proteins in the detoxification of Cd.	Bustamante et al., 2002a
Octopus pallidus	Bioassay	Adults (digestive gland)	CYP, EROD, ECOD, NADPH cytochrome c oxidase	β-naphthoflavone, Aroclor 1254	Australia	Cytochrome P450 appears to be inducible by Aroclor 1254, as measured by ECOD activity and CYP quantitation.	Cheah et al., 1995
Octopus vulgaris	Field sampling	Adults (various tissues, including the digestive gland)	MT	Mixed environmental metals	Portugal	Apparent MT-Cd and MT-Cr associations in digestive gland. No such association was obtained between Co, Ni and As in analyzed tissues.	Raimundo et al., 2010b
Octopus vulgaris	Field sampling	Adults (multiple organs, including the digestive gland)	DNA-SB (comet assay)	Zn, Cu, Cd and Pb	Portugal	No significant differences effect of animal size. DNA damage could be associated to Cd but was too high in digestive gland for clear associations.	Raimundo et al., 2010a
Octopus vulgaris	Field sampling	Adults (arms and digestive gland)	CAT, SOD, GST, LPO, PCO	Cu, Zn, Pb, Cd, As	Portugal	Effective anti-oxidant pathways in the digestive gland, including the activity of GST as a scavenger for lipid hydroperoxide radicals.	Semedo et al., 2012

(Continued)

TABLE 1 | Continued

Species	Approach	Target	Biomarker responses	Substances of interest	Geographical area	Main outcomes	Reference
Octopus vulgaris	Field sampling	Adults (arms and digestive gland)	EROD, ECOD	PAHs	Portugal	Low levels of PAHs in the digestive gland and low levels of EROD and ECOD activities.	Semedo et al., 2014
Octopus vulgaris	Bioassay	Paralarvae (whole organism)	CAT, GST, HSP70 and SOD gene expression (by qRT-PCR)	Mn, Cd	Spain	Gene expression suggests that Cd has an oxidative stress potential greater than Mg with hazardous effects and mortality at relatively low concentrations, triggering stress-related responses as defense.	Nicosia et al., 2015

CAT, catalase activity; CYP, cytochrome P450; DNA-SB, DNA strand breakage; ECOD, ethoxycoumarin O-deethylase; EROD, ethoxyresorufin O-deethylase; GSH, glutathione (reduced); GSSG, glutathione (oxidized); GST, glutathione S-transferase activity; HSP70, heat-shock protein 70 KDa; LPO, lipid peroxidation; MT, metallothionein induction; NADPH, nicotinamide dinucleotide (reduced); PAH, polycyclic aromatic hydrocarbon; PCO, protein carbonyl content; SOD, superoxide dismutase activity; qRT-PCR, quantitative real-time polymerase chain reaction.

The activity of phase II enzymes, such as glutathione *S-transferase* or UDP-glucosyltransferase have been found in cephalopod digestive gland, with evidence that they can be induced by environmental toxicants but their specificity toward a specific class of substances has yet to be demonstrated (Schlenk and Buhler, 1988; Rodrigo et al., 2013; Le Pabic et al., 2015), since phase II enzymes can be induced by many factors, oxidative stress in particular. Inclusively, they were found to be promising targets in *Octopus* paralarvae exposed to metals (Nicosia et al., 2015). Altogether, less specific biomarkers, therefore responsive unspecific physiological challenge, yield more promising outcomes in the cephalopod digestive gland, among which are included superoxide dismutase (SOD) and catalase activities or glutathione induction (Semedo et al., 2012; Rodrigo et al., 2013; Nicosia et al., 2015). Note, though, that there are indications that the activity of oxidative stress enzymes can be significantly modulated by age in cephalopods (Zielinski and Pörtner, 2000). Interestingly, Oellermann et al. (2012) found that cuttlefish may adjust cardiac mitochondrial metabolism *per se* to adjust to thermal challenge, which indicates the ability to fine tune mitochondria-mediated oxidative functions under stress. This may explain the relevance of oxidative stress biomarkers in these animals, despite the missing link between mitochondrial function and toxicant-induced oxidative stress.

Another interesting target could be heat-shock proteins (HSPs) and similar chaperones that interfere with gene expression pathways. They have been described at the transcriptome level in several molluscs, including a few cephalopods (reviewed by Wang et al., 2013), and can be pointed as promising biomarkers (refer also to Oehlmann and Schulte-Oehlmann, 2003). In spite of the absence of similar work on cephalopods, with the exception of the work by Nicosia et al. (2015) with *Octopus* paralarvae (whose transcript levels increased with Cd exposure concentration), HSP70 induction has provided interesting results in other molluscs (bivalves in particular) subjected to several environmental stressors, from bacterial infection to metals, PAHs, pharmaceuticals, and biotoxins (Köhler et al., 1996; Boutet et al., 2003; Cellura et al., 2006; Song et al., 2006; Mello et al., 2012; Gust et al., 2013).

Altogether, detoxification of organic toxicants in the cephalopod digestive gland does occur, albeit potentially better adapted to dispose of toxins and unwanted by-products from feed and basal metabolism. It must also be considered that endpoints that are common biomarkers in vertebrates may not be the most adequate in molluscs, with particular respect to phase I. At the present, responses related to phase II and oxidative stress appear to be more viable biomarkers of exposure to bioactive organic toxicants, together with ECOD activity (refer to **Table 1**). Phase III of detoxification is still a subject of debate, being essentially reported for vertebrates, fish included. The action of specific transporters, like ATP-binding cassette (ABC) transporters, located in digestive tract cells, is described for the elimination of xenobiotic metabolites (reviewed by Ferreira et al., 2014). Even though not yet scrutinized in cephalopods, ABC transporters have been investigated in other molluscs and their action in gills during metal-induced challenge has already been reported (e.g., Della Torre et al., 2015). It would be of relevance

to attempt to relate form and function of excretory vacuoles in digestive gland cells with phase III activity. Additionally, while the lack of genomic annotation is a clear drawback to unravel potentially novel mechanisms of detoxification through phases I to III in cephalopods, with the advent of state-of-the-art proteomics, metabolomics, (epi)genomics and transcriptomics tools (next-generation sequencing of genomic DNAs and RNAs), plus bioinformatics, more mechanistic information could be retrieved regarding detoxification pathways in cephalopods and other molluscs while assisting biomarker discovery.

CONCLUDING REMARKS

Even though it remains to be seen whether cephalopods can have such a significant role in biomonitoring as bivalves, the importance of these animals in ecosystem functioning, fisheries and more recently aquaculture dictates the relevance of investigating how these animals cope with anthropogenic pressures than endanger their habitats. The still scarce literature on physiological and molecular pathways related to detoxification of noxious substances suggests, however, that the digestive gland plays a major role, much in similarity with the vertebrate liver. As genomic annotation for these animals is slowly coupled with traditional biomarkers approaches that until recently were considered more or less exclusive to vertebrates, it may now be inferred that the toxicological pathways in cephalopods are more complex than expected for both metallic and organic xenobiotics, steering toward a new direction to understand the adaptative mechanisms of cephalopods to impacted marine ecosystems.

AUTHOR CONTRIBUTIONS

AR and PC are responsible for the conceptual design and writing of the manuscript. PC supervised the work.

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Hypoxic Induced Decrease in Oxygen Consumption in Cuttlefish (*Sepia officinalis*) Is Associated with Minor Increases in Mantle Octopine but No Changes in Markers of Protein Turnover

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The common cuttlefish (*Sepia officinalis*), a dominant species in the north-east Atlantic ocean and Mediterranean Sea, is potentially subject to hypoxic conditions due to eutrophication of coastal waters and intensive aquaculture. Here we initiate studies on the biochemical response to an anticipated level of hypoxia. Cuttlefish challenged for 1 h at an oxygen level of 50% dissolved oxygen saturation showed a decrease in oxygen consumption of 37% associated with an 85% increase in ventilation rate. Octopine levels were increased to a small but significant level in mantle, whereas there was no change in gill or heart. There were no changes in mantle free glucose or glycogen levels. Similarly, the hypoxic period did not result in changes in HSP70 or polyubiquinated protein levels in mantle, gill, or heart. As such, it appears that although there was a decrease in metabolic rate there was only a minor increase in anaerobic metabolism as evidenced by octopine accumulation and no biochemical changes that are hallmarks of alterations in protein trafficking. Experiments with isolated preparations of mantle, gill, and heart revealed that pharmacological inhibition of protein synthesis could decrease oxygen consumption by 32 to 42% or Na^+/K^+ ATPase activity by 24 to 54% dependent upon tissue type. We propose that the decrease in whole animal oxygen consumption was potentially the result of controlled decreases in the energy demanding processes of both protein synthesis and Na+/K+ ATPase activity.

Keywords: European cuttlefish, *Sepia officinalis*, HSP70, octopine, polyubiquitinated protein, ventilation frequency

INTRODUCTION

The European cuttlefish (*Sepia officinalis*) is a dominant species in the north-east Atlantic Ocean and Mediterranean Sea. It is a benthic species that occurs to depths of 200 m. Sexually mature and juvenile individuals migrate to coastal grounds in the spring; the latter in pursuit of abundant food sources (Pierce et al., 2008; Bloor et al., 2013). These geographic areas are at risk of becoming

hypoxic because of the increase of nutrients and organic matter into the coastal waters (Van Den Thillart et al., 1994; Grantham et al., 2004; Chan et al., 2008). Hypoxic conditions occur naturally in nature (Ekau et al., 2010); however, there are reports of a global oceanic oxygen decline (Schmidtko et al., 2017), which is becoming a problem for marine biodiversity (Vaquer-Sunyer and Duarte, 2008) and an important environment stressor to marine species (Cosme and Hauschild, 2016; Townhill et al., 2017). Hypoxia is also a potential challenge under intensive aquaculture conditions (Brett, 1979; Delaney and Klesius, 2004; Burt et al., 2013) due to the high density of animals and because of the costs associated with keeping oxygen saturation at normal levels. Routine oxygen consumptions of sepioids are within the same range of those of octopus species (Boyle, 1991) and optimal oxygen saturations for *Octopus vulgaris* are reported to be in between 100 and 65% dissolved oxygen saturation (DO_2) for proper food intake and growth, while suboptimal (modest hypoxia) values range from 65 to 35% DO_2 (Cerezo Valverde and García García, 2005). These suboptimal values may be reached with a combination of high densities and temperatures in Mediterranean countries, where cuttlefish culture will take place. This is relevant because oxygen availability limits thermal tolerance in *S. officinalis* (Melzner et al., 2006), O_2 consumption rates rise with increasing temperature up to a metabolic threshold (Melzner et al., 2007) related to the oxygen-binding properties of haemocyanin, which lowers with increasing temperature (Brix et al., 1994). As such, it is important to have an understanding of the response of cuttlefish to challenges of a suboptimal oxygen environment that is considered to be either ecologically and/or practically relevant.

S. officinalis, as with other cephalopods, exhibit a decrease in oxygen consumption below a critical environmental oxygen level (Houlihan et al., 1982; Johansen et al., 1982; De Wachter et al., 1988; Cerezo Valverde and García García, 2005; Seibel et al., 2014). Severe hypoxia induces a mobilization of mantle glycogen pools in this species with concomitant accumulation of the anaerobic end product octopine (Storey and Storey, 1979; Storey et al., 1979). The production of octopine is catalyzed by the enzyme octopine dehydrogenase (ODH) that occurs in particularly high levels in mantle, ventricle, and tentacle relative to other tissues (Storey, 1977). Despite this existing information, the biochemical responses of this species, at the tissue level, to environmentally relevant levels of hypoxia have not been fully characterized, so the importance of anaerobic octopine production under these conditions is unclear. In addition to octopine accumulation, more recent studies with the exceptionally hypoxia tolerant Humboldt (jumbo) squid, *Dosidicus gigas*, reveal other aspects of the response to low oxygen levels that include a decrease in mantle contraction frequency (i.e., gill ventilation) and increases in mantle heat shock protein 70 (HSP70) and ubiquinated proteins (Trübenbach et al., 2013a,b, 2014). Here we question if these responses occur in *S. officinalis* as well, as it is considered a hypoxia-sensitive species (Storey et al., 1979).

Decreases in oxygen consumption that are not due to reduced spontaneous swimming activity must be related to decreases in other components of energy demand. Aside from muscle contraction, the two most energy demanding processes in cells are the maintenance of ionic gradients via Na^+/K^+ ATPase and protein turnover (synthesis and degradation) (Wieser and Krumschnabel, 2001). The rapid growth rates of cephalopods have been suggested to occur due to high rates of protein synthesis and high efficiency of protein retention, which implies low protein degradation (Houlihan et al., 1990; Carter et al., 2009; Moltschaniwskyj and Carter, 2010). It is possible to measure the relationship between these processes and oxygen consumption by pharmacological inhibition of Na^+/K^+ ATPase with ouabain and protein synthesis with cycloheximide (Wieser and Krumschnabel, 2001; Agin et al., 2003). Here we use these pharmacological tools on isolated preparations to assess the potential tissue specific contribution of Na^+/K^+ ATPase activity and protein turnover to the whole animal decrease in oxygen consumption.

Mantle is the largest tissue of *S. officinalis*, corresponding to approximately 30–40% of body mass (Castro et al., 1992; Speers-Roesch et al., 2016). We focused our attention on this tissue for assessment of biochemical changes and to determine its potential contribution to alterations in metabolic demand under hypoxia. Although gill and heart represent only about 2 and 0.1% of body mass, respectively (Speers-Roesch et al., 2016), these tissues were also included in our study as they are essential organs to supply oxygen to the rest of the animal, which must maintain function under hypoxia, and have been previously connected to high fractional rates of protein synthesis (Houlihan et al., 1990).

MATERIALS AND METHODS

Ethical Statement

All the procedures were approved by CCMAR Animal Welfare Committee (ORBEA CCMAR-CBMR) and Direcção-Geral de Alimentação e Veterinária (DGAV) of the Portuguese Government, according to National (Decreto-Lei 113/2013) and EU legislation (Directive 2010/63/EU) on the protection of animals used for scientific purposes. In addition, protocols were approved by institutional Animal Care Committees at each of the Canadian Universities where authors of this paper are based. Procedures were only applied to live animals by authorized users.

Animals

Cuttlefish (*S. officinalis*) were reared according to the latest culture technology described in Sykes et al. (2014). All experiments used juveniles produced from eggs laid by a F6 captive stock. The mass of group 1 was 59.3 ± 5.4 g (N = 18) and of group 2, used only in mantle oxygen consumption experiments to avoid limiting oxygen diffusion across thick mantle tissue, was 1.90 ± 0.14 g (N = 7). Experiments were done during May 2016, at CCMAR's Ramalhete Aquaculture Station (Ria Formosa, Portugal—37°00′22.39″N; 7°58′02.69″W). Temperature, salinity, and DO_2 were measured daily, at 9 h 30, in the stock tank. Both temperature and DO_2 were measured with a VWR DO220 probe, while salinity was measured with a VWR EC300 salinity meter. Water temperature was 20.5 ± 1.27 (S.D.)°C, salinity was 35.9 ± 0.9 (S.D.) g L^{-1} and DO_2 level

was 101.1 ± 1.2 (S.D.)%. Cuttlefish were fed frozen grass shrimp (*Palaemonetes varians*) *ad libitum* on a daily basis.

Oxygen Uptake and Ventilation Frequency in Whole Animals

Two closed respirometry (Lefevre et al., 2016) acrylic chambers (48.5 × 28.5 × 29 cm), each with an external recirculating pump (Maxi-Jet PH MP400) and a PASCO PasPort PPS-2169 multiparameter sensor were used. The recirculating system had a total volume of 41 L. Seawater entered and exited on opposite sides of the chamber at a flow of 200 L h^{-1}. Dissolved oxygen saturation data were collected in the upper right section of the chamber at 1 Hz in the PASCO Capstone software and at a temperature of 19.9 ± 1.36 (S.D.) °C. The chamber was completely isolated from any visual stimuli (both researchers and light) by the application of matte black stickers on the outer walls, except from above and one of the 48.5 cm sides. This side had a rectangular LED stripe (50 × 30 × 50 × 30 cm) attached that emitted red light (wavelength peak of 630 nm) until the chamber was completely filled with water and, thereafter, white light (wavelength peaks at 460, 520 and 630 nm) with an intensity of 80 lux. These conditions allowed for the recording of movement (from above) with a Logitech C615 HD camera, and of behavior and ventilation rates (from the side) with a GoPro Hero 3^+ Black Edition. Video recordings were made for the duration of each experiment at 1,920 × 1,080 pixel resolution and 24 frames per s (fps), from above, and 1,920 × 720 pixel and 50 fps, from the side.

Initially, 6 animals were individually exposed to a DO_2 of 100% and 6 other animals to a DO_2 of 50% oxygen saturation for 60 min. Each condition was tested pairwise, 1 cuttlefish at 100% DO_2 vs. another at 50% DO_2. At the end of this procedure all the animals were euthanized. Samples of mantle, gill, and heart were rapidly removed and frozen in liquid nitrogen for biochemical analysis. Rates of oxygen consumption $\dot{M}O_2$ were determined for animals exposed to 50% DO_2 during 60 min. In addition, 6 further control animals (DO_2 of 100%) were used to collect $\dot{M}O_2$ during 90 min, to increase the number of available data points for regression fitting. Background $\dot{M}O_2$ (resulting from consumption of bacteria and electrode) was also assessed with no animal ($N = 3$) in the chamber for 90 min and was found to be negligible.

Hypoxia at 50% DO_2 was achieved by gassing seawater with pure N_2 using a ceramic stone in a closed 90 L reservoir. This water was then injected into the chamber, until the 41 L respirometry recirculating system was filled, and where the animal was already resting. Animals were removed from the stock tank with a black net and transported in a 10 L black bucket filled with seawater to the weighing station. They were individually weighed in a black plastic tray and quickly transported to the chamber area of the experimental room which was illuminated with 1 lux red light conditions (Philips TL-D Colored 18W Red 1SL/25). An outer room containing the data collection stations was isolated from the holding chambers. To avoid inking in the experimental chambers, animals were left resting in the bucket for 5 min before being lightly sedated (mild anesthesia) by adding ethanol to a concentration of 2.5% (Fiorito et al., 2015). After sedation (≈2 min), animals were transferred to the chamber, which had 2 cm in height of seawater and thereafter filled with seawater from the 90 L reservoir (either 100 or 50% DO_2). As soon as the chamber was filled (≈2 min) and air purged, the system was put in recirculating mode, light switched to white and data acquisition was started on all the probes. This filling time was enough for recovery from sedation at this concentration (Gonçalves et al., 2012).

Ventilation rates were determined from video recordings, according to the following sampling scheme for each animal: (a) first and last 10 min—10 samples of 1 min each; b) from 10 to 50 min in hypoxia and normoxia (control)—1 sample every 10 min. After the procedure, the chamber was emptied, the animal removed, and euthanized with 10% ethanol in seawater (Sykes et al., 2012), the brain was then bisected downwards and forwards, followed by two lateral cuts to sever the brain from the optical lobes (Lewbart and Mosley, 2012). No relevant movements in the chamber nor extreme behavior reactions (Gonçalves et al., 2012) were recorded in any video sampling.

Biochemical Assays

Octopine, Glucose, and Glycogen

Tissues were homogenized and then sonicated (QSonica q55) in 6% $HClO_4$ (mantle 1 g tissue: 9 mL $HClO_4$; gill 1 g tissue: 4 mL $HClO_4$), and subsequently centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant was neutralized with 2 M $KHCO_3$. Octopine was assayed in buffer containing 100 mM TRIS, 8 mM NAD^+, and excess octopine dehydrogenase (ODH) at pH 9.3. The increase in absorbance at 340 nm, following addition of enzyme, was monitored in a microplate reader. Octopine was calculated based on the NADH extinction coefficient of 6.22 and the pathlength of the plate reader assay was experimentally determined in near IR using the absorbance of water at 975 nm according to the following equation (Lampinen et al., 2012).

$$\text{pathlength} = \frac{OD975_{assay\ buffer} - OD900_{assay\ buffer}}{0.173} \times 10\ mm$$

ODH was isolated from frozen scallop, *Placopecten magellanicus*, adductor muscle by a modification of the method of Gäde (1985). Tissue was homogenized (25% w/v) in 100 mM TRIS (pH 7.5, 0.1 mM EDTA, 0.1 mM DTT) and centrifuged at 17,000 × *g* for 10 min at 4°C. Ammonium sulfate was then added to the supernatant up to 65% saturation and stored at 4°C for 7 days to precipitate ODH. The sample was centrifuged, the pellet was re-suspended in 100 mM TRIS (pH 7.5, 0.1 mM EDTA, 0.1 mM DTT), the sample was desalted on a Sephadex G-50 column, and fractions containing ODH activity were retained. Contaminating dehydrogenases were removed by passing the sample through a Cibachrome Blue F3GA agarose column (Affi-Gel Blue, Bio-Rad Laboratories, Hercules, California, USA). Fractions containing ODH activity were combined and subsequently desalted on a Sephadex G-25 column to ensure removal of all residual metabolites. The resulting sample yielded 2 bands on an SDS-PAGE gel, one at ∼43 kDa matching the molecular weight of ODH, and a second band of unknown identity at approximately

90 kDa. Lactate dehydrogenase activity was undetectable in the purified sample.

Glucose was analyzed in buffer containing 250 mM imidazole, 5 mM $MgSO_4$, 10 mM ATP, 0.8 mM NADP+, excess glucose 6-phosphate dehydrogenase and thereafter treated with excess hexokinase. A standard curve was created for glucose analysis. Assays were conducted in a microplate reader at a wavelength of 340 nm. Glycogen was assessed as glucosyl units following amyloglucosidae treatment of the neutralized supernatant (Keppler and Decker, 1974). Free glucose was subtracted from the total glucosyl equivalents to yield glycogen levels. All organic reagents were purchased from Sigma-Aldrich.

Polyubiquitin and HSP70

Polyubiquitinated proteins and HSP70 were determined by western blots. Frozen tissues were sonicated in 9 vol of 50 mM TRIS buffer (pH 7.4) and then centrifuged for 5 min at 14,000 × *g* at 4°C. The protein concentration in the supernatant was determined using the Bradford assay (Bio-Rad) and then adjusted to 3 μg/μL with the homogenization buffer. Tissue extracts were added to Laemmli sample buffer (1:1), boiled for 5 min and 4.5 μg of proteins were resolved by SDS-PAGE (TGX Stain-Free FastCast 10%, Bio-Rad). Resolved proteins were subsequently transferred to PVDF membranes, the membranes were blocked using 5% BSA before being incubated with the primary antibody. To analyze the levels of polyubquitinated proteins, we used an antibody that only recognized proteins containing K48-linked polyubiquitin chains (Abcam ab190061) but had no cross-reactivity to monoubiquitin or polyubiquitin of other linkages. Levels of HSP70 were quantified by loading known quantities of a recombinant HSP70 (Enzo Life Sciences, #ADI-SPP-758, standard levels of 15, 30, and 60 ng per lane) along with the samples and using an HSP70 antibody (#AS05 083A, Agrisera). In all cases, secondary detection was done using an anti-rabbit HRP-linked antibody (for polyubiquitin: #7074, Cell Signaling Technology; for HSP70: SAB-300, Enzo Life Sciences). The chemiluminescence signal was detected using a CCD camera system (ImageQuant LAS 500, GE Life Sciences). Band intensity was quantified using ImageJ (imagej.nih.gov).

Oxygen Uptake by Isolated Preparations

Tissue $\dot{M}O_2$ was measured in 0.20 μm filtered seawater containing an additional 10 mM KCl, 200 mM taurine, and 1 mM glucose. The concentration of taurine used here is representative of measured tissue taurine levels in this species (Maccormack et al., 2016). Cuttlefish were euthanized as described above and 5–10 mg samples of gill, systemic heart, or mantle muscle were collected, weighed, and cut into 2–3 mg pieces using a razor blade. Tissue preparations from the same animal were then transferred to paired respirometry chambers (OX1LP, Qubit Systems Inc., Kingston, ON, Canada) containing 750 μL incubation medium maintained at 20 ± 0.1°C with a recirculating refrigerated water bath. Chambers were calibrated daily and background O_2 consumption rates were negligible without tissue. Baseline $\dot{M}O_2$ was recorded for 300 s before addition of pharmacological agents and recordings were continued for an additional 300 s before experiments were terminated. Treatments consisted of 5 mM ouabain (e.g., Postel et al. (2000)) or 25 μM cycloheximide, a known antibiotic inhibitor of protein synthesis (Giuditta et al., 1968; Prozzo and Giuditta, 1973; Wieser and Krumschnabel, 2001) that has previously been applied in studies associated with learning in the species by blocking protein synthesis (Agin et al., 2003). Stock solutions were prepared in DMSO and delivered to the chambers in 10 μL. A tissue preparation from the same animal was run simultaneously with each drug treatment and exposed to 10 μL DMSO as a vehicle control. Chambers were thoroughly rinsed with 95% ethanol, ddH_2O, and incubation medium between runs and treatments were alternated between respirometry chambers to control for potential chamber effects. Respirometry chambers were interfaced to a LabQuest data acquisition system and DO_2 readings were collected over 10 min using LoggerPro V3.8 software (Vernier Software and Technology, Beaverton, OR, USA). Initial tissue $\dot{M}O_2$ prior to treatment was determined from the linear decrease in DO_2 between 120 and 300 s and post-treatment $\dot{M}O_2$ was determined between 400 and 600 s. $\dot{M}O_2$ was linear with tissue mass (data not shown) and consistent with previous experiments (Maccormack et al., 2016).

Data Analysis and Statistics

All values are expressed as mean ± s.e.m with the exception of water temperature, salinity, and DO_2 that are expressed as mean ± standard deviation. Statistical tests applied are stated in either the Results section or the legends to the figures. All data were tested for normal distribution with the Shapiro-Wilk test as well as for homogeneity of the variances with the Levene's test (Zar, 1999). Statistical difference was considered for $P < 0.05$.

RESULTS

Whole Animal Experiment

Figures 1A,B show examples of dissolved oxygen variation over the sampling period for individual cuttlefish either exposed to 100 or 50% DO_2 over 90 or 60 min, respectively. Oxygen consumption by hypoxic animals was 37% lower than animals held at 100% air saturation (189 ± 23 vs. 119 ± 6 nmol/g min) (**Figure 1C**). Cuttlefish maintained under control normoxic conditions exhibited a decrease in ventilation rate over the 60 min holding period ($y = -0.52x + 88$) (**Figure 1D**). Animals held under hypoxia also displayed a decrease in ventilation rate but to a lesser extent ($y = -0.1x + 102$). After 60 min ventilation rate was 85% higher in hypoxic than normoxic individuals.

Octopine was significantly higher in mantle than in gill or heart regardless of oxygenation condition. Octopine levels were significantly higher in mantle of hypoxic than normoxic animals but there was no significant difference between levels in normoxic or hypoxic animals in either gill or heart. (**Figure 2A**). There was no significant difference in either free glucose or glycogen levels in mantle between normoxic or hypoxic held animals (**Figure 2B**). HSP 70 was significantly higher in gill than in mantle or heart regardless of oxygenation condition (**Figure 2C**). There was no significant difference between levels in normoxic or hypoxic animals in any of the three tissues. Similarly, there

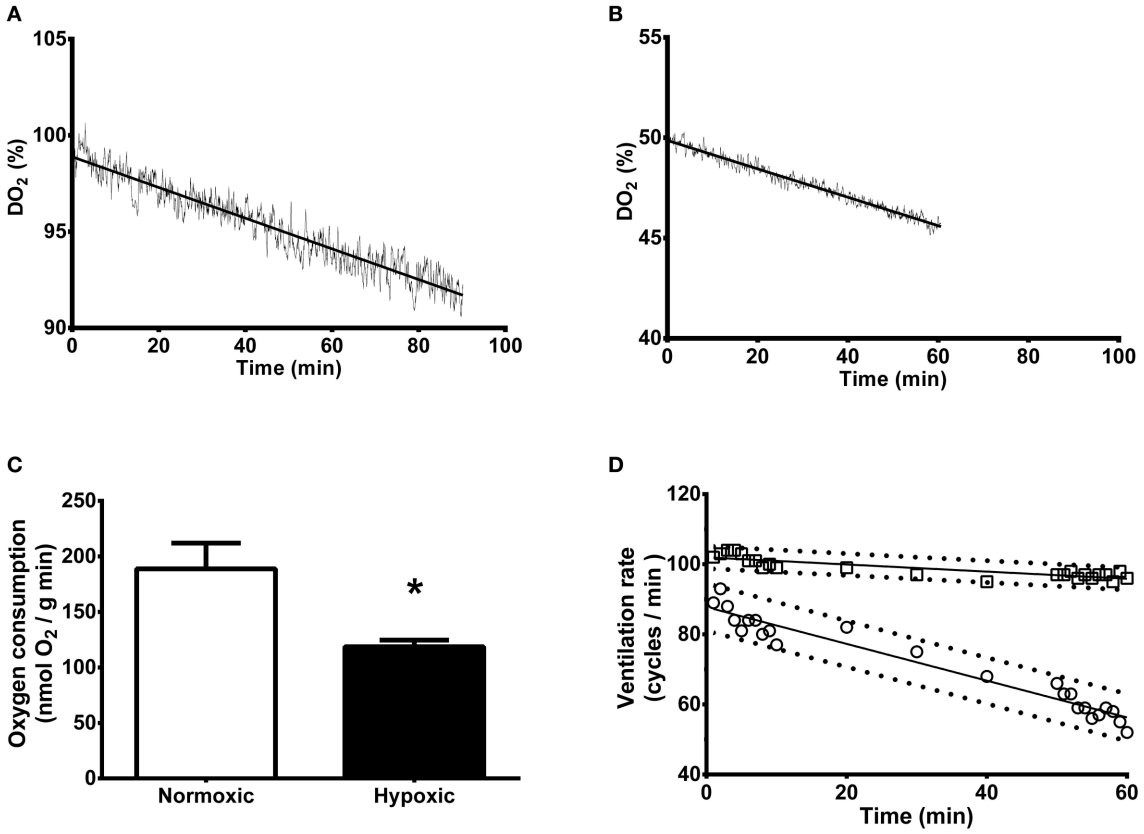

FIGURE 1 | Whole animal rate of oxygen consumption and ventilation of *Sepia officinalis* under either normoxic or hypoxic (50% dissolved oxygen saturation, 1 h) conditions. (A) Dissolved oxygen saturation concentration (DO_2) variation expressed as percentage over a 90 min sampling time in cuttlefish exposed to 100% DO_2 (control). **(B)** Dissolved oxygen saturation concentration (DO_2) variation expressed as percentage over a 60 min sampling time in cuttlefish exposed to 50% DO_2 (mild hypoxia). **(C)** Oxygen consumption expressed as nmol O_2/(g wet weight animal) min. *Indicates a statistically significant difference between normoxic and hypoxic conditions (2 tailed *t*-test; $P = 0.025$). $N = 6$ for normoxic and $N = 5$ for hypoxic conditions. **(D)** Ventilation rate expressed as cycles/min. Control, circles; Hypoxic, squares. $N = 4$ for all time points. The slopes are significantly different ($P < 0.001$).

was no significant difference in levels of polyubiquinated protein in mantle, gill or heart between treatment groups (**Figure 2D**).

Isolated Tissue Experiment

Pre-treatment oxygen consumption rates for gill, heart, and mantle are presented in **Figure 3A**. There was no significant difference in rates amongst the three tissues with values for all being approximately 500 nmol O_2/gm wet weight. min. Rates for gill and heart are from animals weighing approximately 59 g; while those for mantle are from smaller/younger animals with a mass of approximately 2 g. Preparations were treated with cycloheximide and ouabain to assess how much of the decrease in oxygen consumption noted in the whole animal study could potentially be attributed to protein synthesis and Na^+/K^+ ATPase, respectively. **Figure 3B** shows the percentage decrease in oxygen consumption for each of the tissues following correction for any DMSO effect. Cycloheximide had a substantial effect upon oxygen consumption with average decreases ranging from 32% for gill to 42% for mantle. Similarly, ouabain treatment resulted in average decreases in oxygen consumption from 24% for mantle to 54% for heart.

DISCUSSION

Changes in $\dot{M}O_2$ and Ventilation Frequency under Hypoxia

Cuttlefish remained sedentary in the respirometry chambers under both normoxic and hypoxic conditions. As such, any difference in oxygen consumption between groups cannot be attributed to spontaneous swimming activity. When corrected for differences in temperature and body mass, the $\dot{M}O_2$ values reported here under control conditions are in the same range as rates previously noted (Johansen et al., 1982; Melzner et al., 2006, 2007; Grigoriou and Richardson, 2009; Lamarre et al., 2016). Here, exposure to 50% DO_2 for 60 min resulted in a 37% decrease in oxygen consumption. Although limited to 2 individuals of *S. officinalis* with a mean mass of 153 g, a decrease in $\dot{M}O_2$ of about 29% at a similar level of hypoxia was previously observed by De Wachter et al. (1988). Under comparable conditions, *S. officinalis* of body mass between 100 and 1,500 g showed a decrease in oxygen consumption of approximately 50% (Johansen et al., 1982). It is clear that a substantial reduction in $\dot{M}O_2$ is a common response to modest hypoxia

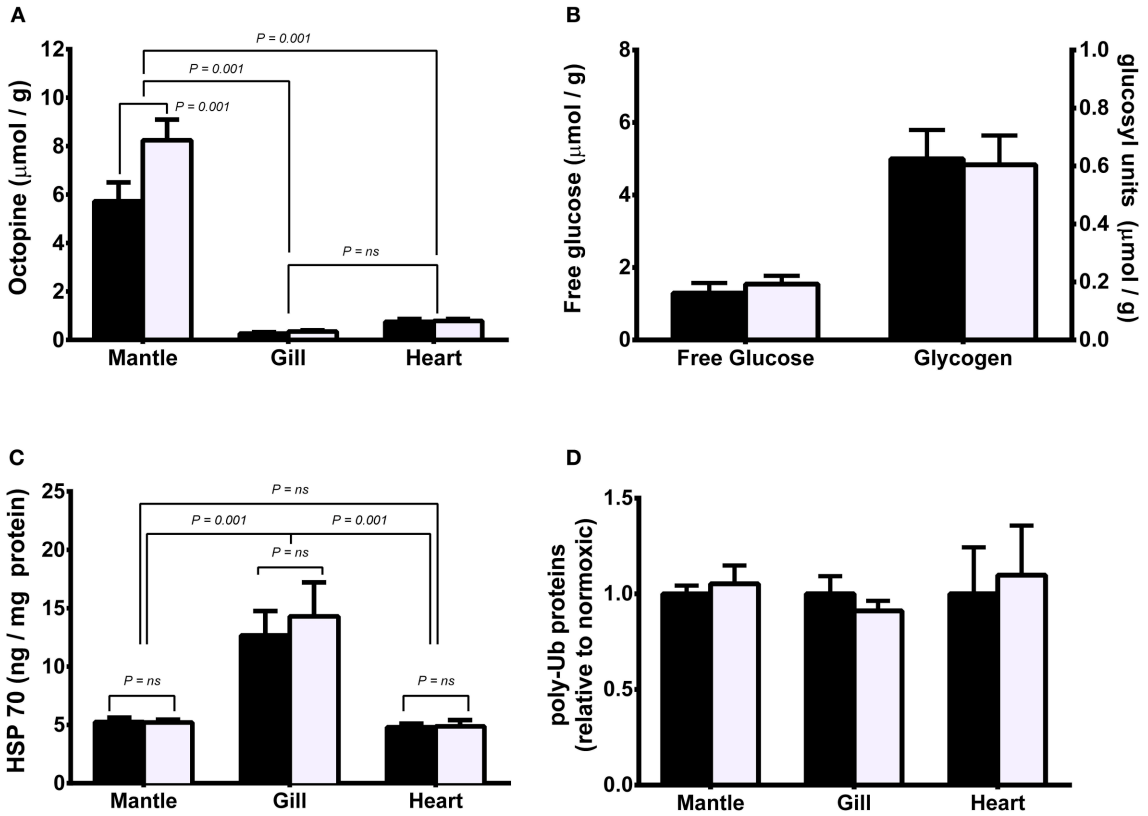

FIGURE 2 | Metabolite and protein levels in mantle, gill, and heart of *Sepia officinalis* under either normoxic (open bars) or hypoxic (closed bars) (50% dissolved oxygen saturation, 1 h) conditions. (A) octopine; **(B)** mantle free glucose and glycogen; **(C)** HSP70; **(D)** polyubiquitinated proteins. Statistical significance for octopine, HSP70, and polyubiquitinated proteins, was assessed with a 1-way ANOVA and for differences between glucose or glycogen levels with a *t*-test. $N = 6$ for all conditions except for free glucose in hypoxic mantle where $N = 4$. Differences between means or grouped means represent statistical difference (Tukey's multiple comparison test; $P < 0.001$). No differences were found in mantle free glucose and glycogen nor polyubiquitinated proteins ($P > 0.05$).

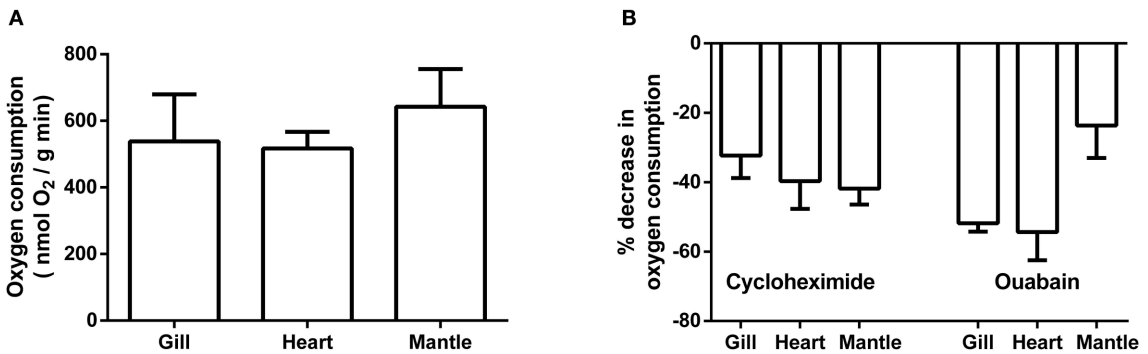

FIGURE 3 | Basal rate of oxygen consumption and impact of cycloheximide and ouabain on isolated preparations of gill, heart, and mantle from *Sepia officinalis.* Gill and heart values are from animals of approximately 59 g; while mantle is from animals of ~2 g. **(A)** Rates of oxygen consumption. Statistical significance was assessed with a 1-way ANOVA and no differences were found. $N = 7$ for gill and $N = 6$ for heart and mantle. **(B)** % decrease in oxygen consumption following treatment with cyclohexamide and ouabain $N = 6$ for all experiments. All values were calculated following correction for any impact of DMSO.

in *S. officinalis*; whether life could be sustained indefinitely at this level of hypoxia and if this will impact on the welfare (pain, suffering, distress, and lasting harm) of the animal remains unknown.

Ventilation rate under normoxic conditions decreased over the 60 min holding period in the same fashion as previously shown (Boal and Ni, 1996) and is presumably related to the stress of transferring the animals to the experimental chamber.

After 60 min the ventilation rate recorded here was in the same range as previously noted in other studies (Bone et al., 1994; Boal and Ni, 1996; Boal and Golden, 1999; Melzner et al., 2006). Ventilation rate in hypoxic animals remained relatively stable with time and was 85% higher in hypoxic than normoxic cuttlefish after 60 min. To our knowledge this is the first report of rate of ventilation in hypoxic *S. officinalis* or any other cuttlefish species. The response differs from inactive, juvenile Humbolt squid where deep hypoxia resulted in a decrease in ventilation frequency, as well as contraction strength and ventilation volume per min (Trübenbach et al., 2013a). The mantle muscle of squid and cuttlefish consists of a main mass of circular fibers that are mitochondria-poor and primarily anaerobic, as well as inner and outer layers of mitochondria-rich aerobic fibers (Bone et al., 1981; Mommsen et al., 1981). The central fibers are used in escape jetting contractions while the fibers of the inner and outer layers are responsible for rhythmical respiratory ventilation (Bone et al., 1981). It is probable that the increase in ventilation frequency observed here places additional demand to supply ATP to the contractile mechanisms under hypoxia even in the face of reduced oxygen consumption.

Anaerobic Metabolism is Minimal

Octopine dehydrogenase is the dominant opine dehydrogenase in *S. officinalis*. We were unable to detect activity of alanopine, tauropine, or strombine dehydrogenase in this species' tissues (unpublished data). The level of ODH in mantle, heart and gill was 35, 20, and 2 μmol/min g wet weight, respectively (Speers-Roesch et al., 2016). The level of octopine in mantle was substantially higher than in heart or gill, consistent with the high activity of ODH in mantle and lower activity in the other two tissues.

Sixty min of 50% hypoxia was associated with an increase in mantle octopine of 2.5 μmol/g. There was no change in octopine level in either gill or heart following the hypoxic period. The observed increase in octopine in mantle was low relative to the capacity to produce this metabolite. For instance, the increase in mantle octopine in *S. officinalis* forced to exercise and then subjected to hypoxia was 13 μmol/g (Storey and Storey, 1979). Another study found a drastic elevation of octopine in the haemolymph of *S. officinalis* exposed to <30% O_2 hypoxia for about 60 min (Storey et al., 1979). A similar increase in mantle octopine occurred in hypoxic Humbolt squid (Seibel et al., 2014) and squid (*Lolliguncula brevis*) forced to swim to exhaustion (Finke et al., 1996). The modest increase in octopine observed in this study is in line with the lack of change in glycogen levels during the hypoxic challenge. Based on ATP equivalents that may be generated by aerobic and anaerobic metabolism, the contribution of octopine production to overall energy production is minimal.

The relative quantity of polyubiqutinated protein was similar in mantle, gill, and heart under control conditions. There was no impact of the hypoxic challenge in any of the tissues tested. This finding is in contrast to that for juvenile Humbolt squid where severe hypoxia resulted in a three-fold increase in ubiquitinated proteins with a size of approximately 100 kDa (Trübenbach et al., 2014). These authors proposed that the increase in polyubiquintinated protein in association with a decrease in levels of at least two abundant proteins in the same size range (HSP90 and alpha-actinin) provides protection under hypoxia via a number of mechanisms, including the provision of certain amino acids for anaerobic energy production (Trübenbach et al., 2014). In *S. officinalis* food deprivation results in almost total depletion of digestive gland triglyceride, $\dot{M}O_2$ decreases by approximately 30%, and mantle polyubiquitin mRNA and polyubiquitinated proteins increase, suggesting an increase in protein degradation via the ubiquitin-proteosome pathway to provide amino acids for energetic purposes (Lamarre et al., 2012, 2016). Although in this species there appear to be mechanisms to increase protein breakdown in association with decreased $\dot{M}O_2$, we found no evidence that this occurs under modest hypoxia, at least based on levels of polyubiquinated protein.

HSP70 is a well-recognized stress protein that plays a role in maintaining protein integrity via chaperoning newly synthesized and unfolding proteins (Balchin et al., 2016). HSP70 levels were significantly higher in gill than in mantle or heart and did not change in any tissue with exposure to hypoxia. Relatively high HSP70 levels in *S. officinalis* gill may be related to the high rate of protein synthesis in gill relative to mantle based on direct measurements (Lamarre et al., 2016), and relative to heart and mantle based on total RNA levels (Lamarre et al., 2012). Furthermore, since gill is at the interface with the aquatic environment it may experience more perturbations than other tissues and so has higher constitutive levels of HSPs as protection. HSP70 levels increased in mantle of hypoxic juvenile Humbolt squid (Trübenbach et al., 2013b) but not in adult animals (Seibel et al., 2014). Thus, the generality of a HSP70 response to hypoxia in cephalopods is unresolved but it is clear that under the conditions of the current study there was no change in HSP70 level.

The minimal increase in octopine content, along with stability in glycogen pools, polyubiquitinated protein level, and HSP70 levels in cuttlefish subject to 50% hypoxia for 1 h suggests that anaerobic metabolism is activated to only a limited extent and that the challenge is not particularly stressful to the animal. The 37% decrease in $\dot{M}O_2$ must be associated with an equivalent decrease in energy demand. Given that the animals are quiescent under both control and hypoxic conditions, the question becomes as to what metabolic processes are decreased to maintain energy balance.

Potential Sites of Energy Reduction—Insights from Isolated Tissue Experiments

There was no difference in the rate of oxygen consumption between tissue slices of gill, heart, and mantle. The *in vitro* activity of citrate synthase, a qualitative indicator of aerobic metabolism, was approximately 20-fold higher in *S. officinalis* heart than in mantle or gill which were similar to one another (Speers-Roesch et al., 2016). The relatively low rate of oxygen consumption in heart noted here is likely due to the non-contractile nature of

the preparation and reflects the basal metabolism of quiescent cells. The heart and gill samples were from juvenile animals and the mantle from hatchlings. Smaller animals have higher mass specific rates of oxygen consumption than larger animals (Johansen et al., 1982; Grigoriou and Richardson, 2009). It is likely that if oxygen consumption of mantle had been conducted on tissue removed from animals the same size as that used for gill and heart that the rate for mantle would have been relatively lower.

One of the major energy demanding processes in non-contracting cells is protein synthesis. In food deprived cuttlefish, both $\dot{M}O_2$ and protein synthesis are decreased in concert. A 36% decrease in whole animal $\dot{M}O_2$ was associated with a 63% decrease in fractional rate of protein synthesis in both mantle and gill (Lamarre et al., 2016). The importance of these findings in the context of the current study is that it demonstrates that protein synthesis is a regulatable element of the energy demand machinery in *S. officinalis*. Here we show that the inclusion of cycloheximide decreases $\dot{M}O_2$ in isolated gill, heart, and mantle preparations. Based on studies with goldfish and trout it is likely that the level of cycloheximide utilized here resulted in a total shut down of protein synthesis (Wieser and Krumschnabel, 2001), as previously suggest by the Agin et al. (2003) results on the species and because the amount used here was more than 3 times higher than the dosages used in *Octopus vulgaris* (Prozzo and Giuditta, 1973) and *Loligo pealii* (Giuditta et al., 1968), which resulted in 80% protein synthesis inhibition in the optic globe with 100 μg/mL and 95–96% inhibition in squid axons with 200 μg/mL, respectively. If it is assumed that all tissues in the quiescent animals are consuming oxygen at similar rates, a total inhibition of all protein synthesis would be required to meet the whole animal decrease in $\dot{M}O_2$ of 37%. This is highly unlikely given that even in an anoxic resistant fish, oscar (*Astronotus crassipinnis*), protein synthesis is decreased by only 55% and 60–85% in muscle and liver, respectively, at a DO_2 of 10% (Lewis et al., 2007). Therefore, although a decrease in protein synthesis could potentially contribute to the decrease in whole animal $\dot{M}O_2$, there must be additional mechanisms to reduce energy expenditure.

Na^+/K^+ ATPase is a major driving force for many energy dependent ion transport processes and is considered one of the major cellular sites for ATP utilization.

Na^+ /K^+ ATPase has been identified in numerous tissues of *S. officinalis* (Donaubauer, 1981) and is presumably ubiquitous. A ouabain sensitive Na^+/K^+ ATPase has been identified in gill of squid (*Doritheutis plei*) (Proverbio et al., 1988) and more recently, Na^+/K^+ ATPase in *Sepia* gill was shown to increase in response to increases in water CO_2 level (Hu et al., 2011). In the hypoxia tolerant intertidal clam, *Mercenaria mercenaria*, a severe hypoxic exposure resulted in a decrease in the maximal *in vitro* enzyme activity of Na^+/K^+ ATPase (Ivanina et al., 2016). As with protein synthesis, these studies illustrate that Na^+/K^+ ATPase is a site that can be controlled to alter energy demand. Here it is shown with isolated tissues that inhibition of Na^+/K^+ ATPase with ouabain decreased $\dot{M}O_2$ by ∼20% in mantle and 50–60% in gill and heart. Given that the Na^+/K^+ ATPase is one of the largest consumers of ATP in cells, it is likely that a curtailment of this process occurs in *S. officinalis* under hypoxia.

CONCLUSIONS

A hypoxic challenge to *S. officinalis* of 50% DO_2 results in only a minor activation of anaerobic metabolism as assessed by octopine accumulation. There were no changes in levels of HSP70 and polyubiquitinated proteins suggesting no alteration in rates of protein breakdown. The animal responds with an increase in ventilation and an overall decrease in metabolic rate, potentially due to decreases in both rates of protein synthesis and Na^+/K^+ ATPase activity. The relatively high level of HSP70 in gill compared to mantle and heart is a novel finding and may be related to the high rates of protein synthesis in this tissue.

AUTHOR CONTRIBUTIONS

SL, TM, AS, and WD participated in the conception and design of research; JC and AS performed the animal husbandry; all authors performed the experiments, acquired and analysed data of work; SL, TM, AS, and WD performed interpretation of data; all authors drafted, edited and approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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CORRECTION
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Corrigendum: Hypoxic Induced Decrease in Oxygen Consumption in Cuttlefish (*Sepia officinalis*) Is Associated with Minor Increases in Mantle Octopine but No Changes in Markers of Protein Turnover

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Keywords: European cuttlefish, *Sepia officinalis*, HSP70, octopine, polyubiquitinated protein, ventilation frequency

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A Corrigendum on

Hypoxic Induced Decrease in Oxygen Consumption in Cuttlefish (*Sepia officinalis*) Is Associated with Minor Increases in Mantle Octopine but No Changes in Markers of Protein Turnover

by Capaz, J. C., Tunnah, L., MacCormack, T. J., Lamarre, S. G., Sykes, A. V., and Driedzic, W. R. (2017). Front. Physiol. 8:344. doi: 10.3389/fphys.2017.00344

In the original article, there was a mistake in **Figure 2B** as published. The right Y-axis of **Figure 2B**, concerning the amount of glycogen, was incorrectly printed resulting in glycogen levels 10-fold too low. The axis should read "0–8" and not "0–1." The corrected **Figure 2** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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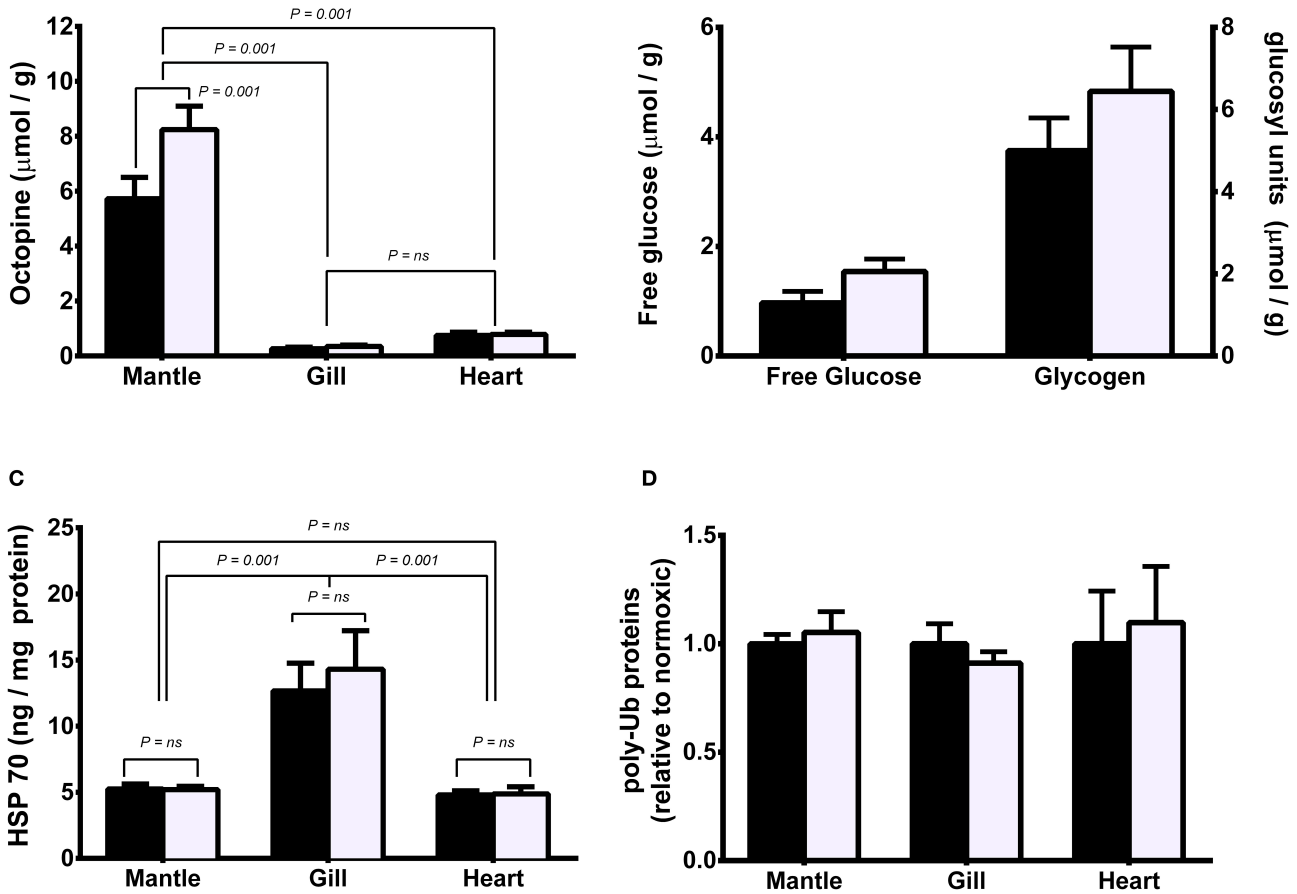

FIGURE 2 | Metabolite and protein levels in mantle, gill, and heart of *Sepia officinalis* under either normoxic (open bars) or hypoxic (closed bars) (50% dissolved oxygen saturation, 1 h) conditions. **(A)** octopine; **(B)** mantle free glucose and glycogen; **(C)** HSP70; **(D)** polyubiquitinated proteins. Statistical significance for octopine, HSP70, and polyubiquitinated proteins, was assessed with a 1-way ANOVA and for differences between glucose or glycogen levels with a *t*-test. $N = 6$ for all conditions except for free glucose in hypoxic mantle where $N = 4$. Differences between means or grouped means represent statistical difference (Tukey's multiple comparison test; $P < 0.001$). No differences were found in mantle free glucose and glycogen nor polyubiquitinated proteins ($P > 0.05$).

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ORIGINAL RESEARCH
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The Gastric Ganglion of *Octopus vulgaris*: Preliminary Characterization of Gene- and Putative Neurochemical-Complexity, and the Effect of *Aggregata octopiana* Digestive Tract Infection on Gene Expression

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The gastric ganglion is the largest visceral ganglion in cephalopods. It is connected to the brain and is implicated in regulation of digestive tract functions. Here we have investigated the neurochemical complexity (through *in silico* gene expression analysis and immunohistochemistry) of the gastric ganglion in *Octopus vulgaris* and tested whether the expression of a selected number of genes was influenced by the magnitude of digestive tract parasitic infection by *Aggregata octopiana*. Novel evidence was obtained for putative peptide and non-peptide neurotransmitters in the gastric ganglion: cephalotocin, corticotrophin releasing factor, FMRFamide, gamma amino butyric acid, 5-hydroxytryptamine, molluscan insulin-related peptide 3, peptide PRQFV-amide, and tachykinin–related peptide. Receptors for cholecystokinin$_A$ and cholecystokinin$_B$, and orexin$_2$ were also identified in this context for the first time. We report evidence for acetylcholine, dopamine, noradrenaline, octopamine, small cardioactive peptide related peptide, and receptors for cephalotocin and octopressin, confirming previous publications. The effects of *Aggregata* observed here extend those previously described by showing effects on the gastric ganglion; in animals with a higher level of infection, genes implicated in inflammation (NFκB, fascin, serpinB10 and the toll-like 3 receptor) increased their relative expression, but TNF-α gene expression was lower as was expression of other genes implicated in oxidative stress (i.e., superoxide dismutase, peroxiredoxin 6, and glutathione peroxidase). Elevated *Aggregata* levels in the octopuses corresponded to an increase in the expression of the cholecystokinin$_A$ receptor and the small cardioactive peptide-related peptide. In contrast, we observed decreased relative expression of cephalotocin, dopamine β-hydroxylase, peptide PRQFV-amide, and tachykinin-related peptide genes. A discussion is provided on (i) potential roles of the various molecules in food intake regulation and digestive tract motility control and

(ii) the difference in relative gene expression in the gastric ganglion in octopus with relatively high and low parasitic loads and the similarities to changes in the enteric innervation of mammals with digestive tract parasites. Our results provide additional data to the described neurochemical complexity of *O. vulgaris* gastric ganglion.

Keywords: digestive system, *Octopus vulgaris*, gastric ganglion, parasite, *Aggregata octopiana,* gene expression

INTRODUCTION

The gastric ganglion is a prominent feature of the peripheral nervous system in coleoid cephalopods. Gross morphological descriptions of the ganglion are available for *Sepia officinalis* (Alexandrowicz, 1928), *Idiosepius paradoxus* (Shigeno and Yamamoto, 2002), *Octopus vulgaris* (Young, 1967, 1971), and *Eledone cirrhosa* (Isgrove, 1909); illustration and a brief description during development is available for *Sepioteuthis sepioidea* (Shigeno et al., 2001) and *Euprymna scolopes* (Kerbl et al., 2013). In contrast to the single gastric ganglion in coleoid cephalopods, in *Nautilus* a pair of small ganglia distributing nerves to the viscera emerge from the visceral nerves (Owen, 1832).

The gastric ganglion (see original description for *O. vulgaris* in: Chéron, 1866; Bogoraze and Cazal, 1946) innervates most of the digestive tract, i.e., the crop, stomach, intestine, and caecum. It also connects with the central nervous system via the sympathetic nerves, the visceral nerves through rectal and intestinal nerves and through the abdominal nerves (Young, 1967). The complex structure of the gastric ganglion and its relationships support the view that it functions both independently and integrating information originating from, for example, the crop and intestine (Young, 1967), thus appearing to act not only as a simple relay but also as an integrative center (Andrews and Tansey, 1983). The intricate connectivity and complexity of the ganglion is further revealed by intense tubulinergic immunoreactivity of the neuropil (e.g., Shigeno and Yamamoto, 2002).

The well-defined innervation of the cephalopod digestive tract and the fact that it often hosts parasites (review in: Hochberg, 1983; Castellanos-Martínez and Gestal, 2013) raise the possibility that the presence of parasites may induce physiological responses (e.g., Gestal et al., 2002b) in the innervation, as occurs in mammals (see below).

In mammals, digestive tract pathogens (i.e., bacteria, viruses or parasites) can induce a range of responses including local inflammation, sensitization of visceral afferent nerves (peripheral terminal, cell body and central nervous system levels) and modulation of enteric nervous system (ENS) functionality (for review see: Halliez and Buret, 2015; Guarino et al., 2016; Obata and Pachnis, 2016). Examples are provided by the bacteria *Campylobacter jejuni* (Goehler et al., 2005), *Clostridium difficile* (Wadhwa et al., 2016) and *Salmonella typhimurium* (Gabanyi et al., 2016), rotavirus (Lundgren et al., 2000; Istrate et al., 2014) and the parasites *Giardia duodenalis*, *Nippostrongylus brasiliensis*, *Trypanosoma cruzi* and *Trichinella spiralis* (for review see Halliez and Buret, 2015). Mucosal damage of the digestive tract, such as occurs with an ulcer, can also produce sensory neuron sensitization (Bielefeldt et al., 2002), as can intestinal inflammation (Stewart et al., 2003).

With parasitic infections, changes observed in the gut innervation in rodents (mice or rats) include increased levels of the tachykinin substance P (e.g., *Trichinella spiralis*, Swain et al., 1992; *Nippostrongylus brasiliensis*, Masson et al., 1996), reduced acetylcholine release (e.g., *Trichinella spiralis*, Collins et al., 1989), and acute and chronic selective damage to the ENS (*Trypanosoma cruzi*, Campos et al., 2016). The sensitization of visceral afferent neurons and damage to the enteric innervation produced by pathogens contribute to post-infectious syndromes in humans (e.g., post-infectious gastroparesis in children, Naftali et al., 2007; post-infectious irritable bowel syndrome in adults, Schwille-Kiuntke et al., 2015; Wadhwa et al., 2016). Thus, the innervation of the mammalian digestive tract is affected both acutely and chronically by a range of pathogens including parasites.

In *O. vulgaris* the presence of parasites (review in: Hochberg, 1983; Castellanos-Martínez and Gestal, 2013) may induce responses either locally or systemically. The parasite most frequently found in octopus digestive tract is *Aggregata octopiana* (Estévez et al., 1996; Castellanos-Martínez and Gestal, 2013), a microscopic Coccidian, spore-forming, single-celled obligate intracellular parasite. It is one of the various species of *Aggregata* belonging to apicomplexan Protozoa (Apicomplexa: Aggregatidae). *A. octopiana* may reach incidences higher than 90% in some populations of *O. vulgaris* (e.g., West Mediterranean, Mayo-Hernández et al., 2013). In the digestive tract of octopus, *A. octopiana* is found in both non-cuticularized (caecum and intestine), and cuticularized (esophagus and crop) structures, in the digestive gland and other nearby organs (Gestal et al., 2002a,b). In infected animals, cysts are visible with the naked eye as small white patches embedded in the muscular wall of the digestive tract (Mayo-Hernández et al., 2013). Therefore, they are in close proximity to the enteric neurons located in the serosa and between the circular and longitudinal muscle layers of the digestive tract in cephalopods (Alexandrowicz, 1928; Graziadei, 1960).

Histological and ultrastructural lesions associated with *A. octopiana* infection include hypertrophy with nuclear displacement of the host cell, inflammation, phagocytosis, ulceration and final damage of the organ architecture (Gestal et al., 2002a). *Aggregata* appears to be particularly abundant in the caecum and intestine in the octopus, and has been proposed to be responsible for a "malabsorption syndrome" (Gestal et al., 2002b). *A. octopiana* has also been suggested to induce a systemic immune response (Castellanos-Martínez et al., 2014a; Gestal and Castellanos-Martínez, 2015).

AIM OF THIS STUDY

The effects of the lesions associated with *A. octopiana* infection in *O. vulgaris* have been explored at different levels, but the impact on neural structures has never been investigated. Here we ask whether *Aggegata* affects one component of the digestive tract innervation, namely the gastric ganglion. Our hypothesis is based on the assumption that the gastric ganglion is connected, through both sensory and motor neurons, to all regions of the digestive tract, which can be affected by *Aggregata,* and therefore the damage and local inflammation induced by the parasite will cause functional modification reflected in gene expression. The neurons of the gastric ganglion could also be affected by substances (e.g., TNFα) released as components of the systemic immune response to *Aggregata* (Castellanos-Martínez et al., 2014a,b).

To explore our working hypothesis and to expand current knowledge of the neural complexity of this ganglion, we: (i) surveyed the neurochemical diversity of the gastric ganglion in *O. vulgaris* by a combination of approaches including *in silico* molecular characterization and immunohistochemistry with a particular focus on ligands and receptors likely to be involved in neurotransmission; (ii) used real time RT-qPCR to compare the expression of selected target genes in the gastric ganglion from octopuses with relatively "high" and "low" *Aggregata* parasite loads, to provide insights into the potential impact of infection on the control of the digestive tract.

MATERIALS AND METHODS

Animals and Tissue Sampling

Octopus vulgaris Cuvier, 1797 (Mollusca, Cephalopoda) of both sexes (males, $N = 10$ − body weight: mean ± SEM = 185 ± 22 g; females, $N = 12$ − body weight: mean ± SEM = 272 ± 19 g) were obtained from local fishermen (Bay of Naples, Italy). Twelve octopuses were used for immunohistochemistry, and an additional 10 utilized for analysis of the potential effects of *Aggregata* infection on the gastric ganglion (see below). All animals originated from a larger sampling study measuring the incidence of octopus' parasite load (*A. octopiana*) in the Mediterranean.

Killing animals solely for tissue removal does not require authorization from the National Competent Authority under Directive 2010/63/EU and its transposition into national legislation. Samples were taken from local fishermen, by applying humane killing following principles detailed in Annex IV of Directive 2010/63/EU as described in Fiorito et al. (2015). In brief, octopuses were immersed in freshly made 3.5% magnesium chloride hexahydrate (Sigma Aldrich, CAS Number: 7791-18-6) dissolved in sea water**.** After 30 min immersion, the animals were unresponsive to handling and a noxious mechanical stimulus, and ventilation completely stopped; killing was completed by destruction of the brain.

Tissue Removal

All dissections were carried out on an ice bed. The digestive tract was approached via incision of the dorsal mantle and of the capsule covering the digestive gland. The gastric ganglion was identified on the right-hand side at the junction of the crop, stomach, caecum and intestine (**Figure 1**). The ganglion was removed by transection of nerve trunks projecting to adjacent structures and cutting the connective tissue capsule adherent to the serosa of the digestive tract. The ganglion was placed initially in either fixative (for immunohistochemistry) or RNA Later Stabilization Solution (RNALater, Thermo Fisher Scientific; for gene expression studies) as indicated below.

In addition, the entire digestive tract was removed by transection of the esophagus as it exited the brain and the rectum at the level of the anal sphincter. Blunt dissection freed the gastric ganglion from adherent structures.

For all animals, the digestive tract was inspected for the presence of white cysts indicative of *A. octopiana* infection and rapidly frozen on dry ice and then stored at −20°C for subsequent quantification of the magnitude of *A. octopiana* infection (see below).

Immunohistochemistry and Staining

Excised gastric ganglia from animals with no visible *Aggregata* infection were immersed in fixative appropriate for each of the employed antisera and/or treated for the specific purpose of the required staining (**Table 1**). In most cases for paraffin and cryostat sections ganglia were placed in 4% paraformaldehyde (PFA) in seawater (4°C for 2 h). After fixation, the tissue was treated as briefly summarized below.

(a) Transferred to 70% ethanol (in distilled H_2O) until processing and paraffin-embedding (∼2 weeks) according to standard protocols (i.e., slow dehydration in increasing concentrations of ethanol, clearing in xylene overnight, three changes through liquid paraffin, and embedding). The embedded ganglia were then sectioned at 5–7 μm and dried overnight at 60°C.

(b) For cryosectioning after several washes in PBS, ganglia were cryoprotected in sucrose 30% and embedded in OCT compound (Sakura Tissue-Tek© OCT Compound; Gentaur, San Jose, CA). The embedded ganglia were then sectioned at 20 μm.

(c) To allow detection of noradrenaline and octopamine, we followed the protocol developed by Ponte and Fiorito (2015) for octopus. In brief, the tissue was fixed (6.25% glutaraldehyde, 75% picric acid, 5% glacial acetic acid, and 1% sodium metabisulfite in distilled water; 3 h), washed in sodium metabisulfite solution (SBM) and dehydrated in an ascending ethanol series until permeabilization (mixture of ethanol/methyl salicylate, 5 min) and then rehydrated in a descending ethanol series (in Tris-HCl-SMB) and embedded in agarose. The embedded ganglia were then sectioned at 50 μm (Vibratome VT1200S, Leica Microsystems, Germany) and collected as free-floating sections. No antigen retrieval was required (for details see Ponte and Fiorito, 2015).

For fluorescence immunohistochemistry (IHC) on paraffin tissue, sections were deparaffinized, rehydrated and subjected to heat-mediated antigen retrieval in Sodium Citrate buffer (pH

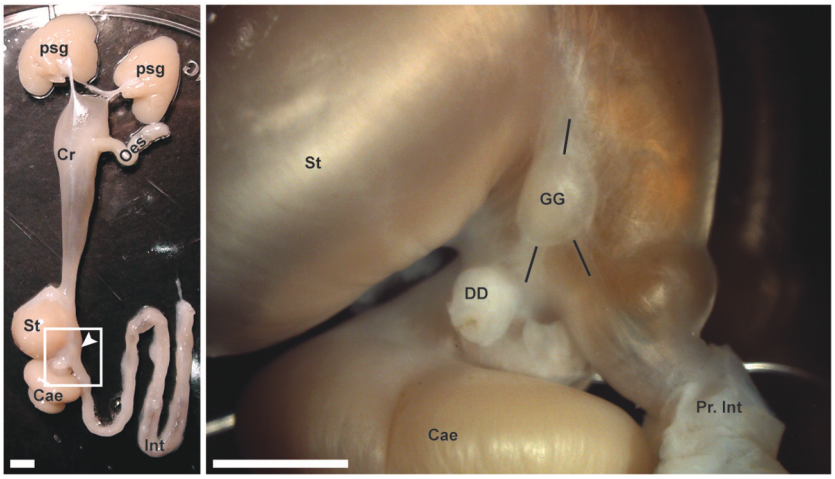

FIGURE 1 | The gastric ganglion of *Octopus vulgaris*. **(Left panel)** A dissected digestive tract from an octopus. From top (anterior) to bottom (posterior): Posterior salivary glands (Psg), crop (Cr), esophagus (Oes), stomach (St), caecum (Cae), and intestine (Int). Within the square (see magnification in the **Right panel**), the arrow points to the gastric ganglion. **(Right panel)** In higher magnification, the same structures are identified together with the gastric ganglion (GG) and surrounding nerve bundles (see black lines to highlight their relative positions) running toward the stomach and crop (up), and toward the caecum and digestive duct (bottom left) or toward the intestine (bottom right). The proximal part of the intestine (Pr. Int) and the cut end of the digestive duct (DD) are also evident. Scale bars: 500 μm.

TABLE 1 | List of antibodies utilized in this study and their source.

Antigen			Methods	Supplier	Catalog number	References
Full name	**Abbreviation**	**Ab type**				
Acetylated alpha-tubulin	Ac-Tub	M	PFA – Paraf	Sigma	T 6793	Shigeno and Yamamoto, 2002; Wollesen et al., 2009
Neurofilament 200	NF	M	PFA – Paraf	Sigma	N 5389	Imperadore et al., 2017
Neuronal nuclear antigen (RNA binding protein fox-1 homolog 1)	NeuN	P-Cy3 Conjugate	PFA – Paraf[a]	Millipore	ABN78C3	Present study
Corticotropin Releasing Factor	CRF	P	PFA – Cryo	Peninsula Labs	T-4037	Suzuki et al., 2003
FMRFamide (FMRF-BTg)	FMRFamide	P	PFA – Cryo	Immunostar	20091	Di Cristo et al., 2003; Wollesen et al., 2012
Tyrosine Hydroxylase	TH	P	PFA – Cryo	Millipore	AB152	Baratte and Bonnaud, 2009; Ponte, 2012
Noradrenaline (NA-G-BSA)	NA	P	(Ponte and Fiorito, 2015; see also Methods section)	GemacBio	AP006	Ponte, 2012; Ponte and Fiorito, 2015
Octopamine (OA-G-BSA)	OA	P	(Ponte and Fiorito, 2015; see also Methods section)	GemacBio	AP007	Ponte, 2012; Ponte and Fiorito, 2015
γ-Aminobutyric acid (GABA-BSA)	GABA	M	PFA – Paraf	Sigma	A0310	Cornwell et al., 1993; Ponte et al., 2010
5-Hydroxytryptamine (5-HT-BSA)	5-HT	P	PFA – Paraf	Sigma	S5545	Ponte et al., 2010; Wollesen et al., 2010, 2012
Substance P (TAC1-BSA)	TAC1	M	PFA – Cryo	Novusbio	NB100-65219	Springer et al., 2004
Common type of Choline Acetyltransferase	cChAT	P	(Casini et al., 2012)[b]	Dr JP Bellier (gift)		Casini et al., 2012

Name and abbreviation and type of antibody (monoclonal: M, polyclonal: P) utilized are included together with supplier and catalog number. Cited references refer to previous studies where the same antibody (or with identical epitope) has been utilized for nervous tissue in cephalopods.

[a]*Antibody applied after antigen retrieval as described by Herculano-Houzel and Lent (2005): 1 h (70°C) in 0.2 M boric acid solution (pH 9). The sections were then washed in PBS and incubated with a primary antibody against NeuN (rabbit polyclonal; Cy3 conjugate).*

[b]*To detect cChAT-ir we followed recommendations provided by Casini et al. (2012).*

6.0). Sections were blocked in 10% goat serum (2h at RT) and then incubated at 4°C with primary antibody overnight. After washing (PBS, 3 × 5 min) tissues were incubated with AlexaFluor-conjugated secondary antibodies as appropriate for 2 h at 37°C in the dark. For the final 15 min, DAPI was added for detection of DNA/nuclei. Tissue was again washed in

PBS (3 × 5 min), and then mounted with fluorescent mounting medium (Fluoromount™ Sigma Aldrich).

For NeuN detection we applied antigen retrieval to paraffin sections as described by Herculano-Houzel and Lent (2005), i.e., boric acid solution (see **Table 1** for details) and then incubated with a primary antibody against NeuN (rabbit polyclonal; Cy3 conjugate).

To detect cChAT immunoreactivity we followed the standard protocol utilized for octopus (Casini et al., 2012).

The omission of each primary antibody was used as a negative control for each immunostaining procedure.

Classic histological staining was utilized to examine the general organization of the octopus' gastric ganglion. We applied Hematoxylin and Eosin (H&E) on deparaffinized/rehydrated tissues incubated in Mayer's hematoxylin (Sigma) for 5 min, then washed in running tap water (5 min), followed by incubation with Eosin Y (Sigma) solution (1 min), and then rapidly dehydrated, cleared in xylene, and mounted (Mayer, 1893; e.g., Shigeno et al., 2001). Serial sections (50 μm) were also collected on chrome-alum-gelatin-coated slides, and stained using with Picro-Ponceau with hematoxylin following Kier (1992).

Classical histological sections were examined using bright-field microscopy (Zeiss Axiocam 2, with Zen-Blue software) and photographed with a Zeiss105 color camera. For IHC we utilized a Leica DMI6000 B inverted microscope and a Leica TCS SP8 X confocal microscope (Leica Microsystems, Germany). Tile Z-stacks were performed using a 0.2 μm step size. Images were processed using LAS X software (Leica Microsystems, Germany). IHC figures have been assembled following guidelines for color blindness provided by Wong (2011).

Assessment of *Aggregata octopiana* Infection

The entire digestive tract (esophagus to rectum) of *O. vulgaris* ($N = 10$) was removed immediately *post mortem*, weighed and after vigorous washing in homogenization buffer processed according to Gestal and co-workers (Gestal et al., 1999; see also Gestal et al., 2007; Tedesco et al., 2017). In brief, after homogenization, sporocysts of *A. octopiana* were counted by a Neubauer chamber and their number expressed as sporocysts per gram of tissue. Samples considered in this study were taken from a larger sampling study aimed to describe possible differential parasite loads in different octopuses. We selected samples to assure a significant difference in parasite load between animals (see section Results).

Transcriptome Analysis and *in Silico* Characterization of the Gastric Ganglion

A preliminary characterization of the complexity of the gastric ganglion of *O. vulgaris* was conducted utilizing recent transcriptome data derived from Drs. R. Sanges' and G. Fiorito's Research Groups at the Stazione Zoologica Anton Dohrn (Napoli, Italy). We utilized a dataset based on two separate RNA-seq studies (Petrosino, 2015; Zarrella et al., unpublished data) carried out on *O. vulgaris* central nervous system (i.e., optic lobes, supra-esophageal and sub-esophageal masses), distal extremities of arm (including muscular and nervous tissues), stellate and gastric ganglia (for technical details see: Musacchia et al., 2015; Petrosino, 2015). The resulting transcriptome (Drs. R. Sanges and G. Fiorito Labs: Stazione Zoologica Anton Dohrn; see also Petrosino, 2015) identified more than a hundred thousand transcripts from different neural structures, significantly extending previously available transcriptome data for this species (Zhang et al., 2012).

We applied a biased strategy, based on Gene Ontology (http://www.geneontology.org/), to these datasets and mined for gene-transcripts potentially involved and/or implicated in the response to infection, inflammation, immune/stress responses.

Analysis of Gene Expression

RNA Extraction and cDNA Synthesis

Gastric ganglia were dissected from animals and stored in RNA Later Stabilization solution for 24 h (+4°C) and transferred to −80°C until further processing. Total RNA was extracted using SV Total RNA Isolation System (Promega, Z3100) according to manufacturer instructions. Quality and quantity of extracted RNA was assessed through UV absorption measurements (Nanodrop ND-1000 UV-Vis spectrophotometer, Nanodrop Technologies). Absence of DNA contamination was verified through PCR (ubiquitin primers) followed by gel electrophoresis. For cDNA synthesis, 500 ng of total RNA from each sample were processed with iScript-cDNA Synthesis Kit (Bio-Rad, 1708891).

Primer Design: Efficiency and Specificity

Primers were designed by Primer3Plus software (www.bioinformatics.nl/primer3plus) using target sequences deduced from *O. vulgaris* transcriptome (Petrosino, 2015). Primer parameters were set to 20 nucleotides in length, product size 100–200 base pairs and melting point 58–60°C. Primers were also analyzed with the Multiple Primer Analyzer[1] to estimate presence of, and possibly avoid, primer-dimers.

The efficiency of each pair of primers (**Table 2**) was calculated according to standard methods curves, and following Sirakov et al. (2009). Briefly, five serial dilutions (1:5, 1:10, 1:20, 1:40, 1:80) of a standard sample were made to determine the efficiency of reactions conducted with each pair of primers. Standard curves were generated for each sample/gene combination using the *Ct* value vs. the logarithm of each dilution factor (Pfaffl et al., 2002; Radonić et al., 2004).

Each amplification reaction was conducted in a volume of 25 μl containing: 2 μl of diluted cDNA template, 2.5 μl of 10× PCR reaction buffer (Roche), 2.5 μl of dNTP mix (0.2 mM), 1 μl of each primer (25 ρmol/μl), 0.25 μl of Taq DNA polymerase (5U/μl), and sterile H_2O. The amplification cycles were conducted by Peltier Thermal Cycler PTC-200 (MJ Research). After denaturation at 95°C (2 min) 34 amplification cycles were carried out as follows: denaturation (94°C, 15 s), annealing (60°C, 30 s), extension (72°C, 1 min). Finally, an extension cycle was carried out at 72°C for 7 min to complete

[1] https://www.thermofisher.com/it/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html

TABLE 2 | *O. vulgaris* genes ($n = 33$) identified and utilized in this study.

Protein name	Gene name	AN	Primer sequence 5′-3′		AS	E	GO annotation		
							Process	Function	Component
Glutathione synthetase	Gss	P48637	F2	CAGGATTGGGAAACTCGGAAAA	218	2.00	0006520	0000166	0005829
		(MF467271)	R2	ACAGCTTCCTCTCCTTCAGG					
Nuclear factor NF-kappa-B p100 subunit	Nfkb2	Q9WTK5	F	ACTGCAGCAAGGGTATTTGC	96	1.86	0000122	0000977	0005634
		(MF467265)	R	TACGCTTTGTCACGTGCATC					
Adenosine receptor A2a	ADORA2A	P29274					0001963	0001609	0005882
		(MF467261)							
Tool like receptor 3	Tlr3	Q0PV50	F	CGCTCTTTGGATCTTTCTGG	134	2.00	0001819	0003723	0005768
		(MF467268)	R	AATGTGTTGCTGCTGACACG					
Toll-like receptor 13	Tlr13	Q6R5N8					0002376	0003723	0005737
		(MF467260)							
Allograft inflammatory factor 1-like	Aif1l	Q9EQX4	F	TGTTGTTGTGATCCCCCTCT	139	1.74	0008150	0003779	0005737
		(MF414196)	R	TTGCCTGCAAGTGTTACTCG					
Protein singed	Sn	Q24524	F	AGGGCAAATATGCACTGGTC	117	1.95	0007015	0003779	0005737
		(MF467264)	R	AGATTTGACCCGACTTGAGC					
Neurabin-1	PPP1R9A	Q9ULJ8					0007015	0003779	0005737
		(MF467258)							
Dopamine beta-hydroxylase	Dbh	Q64237	F	ATCCGGAAGGAGTGAATGTG	185	2	0001816	0003824	0005615
		(MF414198)	R	GAAGCGCTGGAAGTTTTGTC					
Peroxiredoxin 6	Prdx6	Q5ZJF4	F	CCTTCTGTGCGTGAAGATGA	104	2	0006629	0003824	0005615
		(MF467270)	R	TTTGGACAAGCTGTCATTCG					
Endothelin-converting enzyme 1	ECE1	P97739					0006508	0004222	0005886
Glutathione S-transferase omega-2	Gsto2	Q6AXV9	F2	CCGCATGAGATAGTGAACATCA	173	2	0006749	0004364	0005737
		(MF467266)	R2	TCAGTGGGAGTTAATGGTGGA					
Glutathione peroxidase	GPX	U5XKR1	F	ACGTGCAGATGAACAAGCTG	198	2	0001659	0004601	0005737
		(KF233998)	R	GAGCGTTTTCTCCGTTGACT					
Superoxide dismutase Cu-Zn	Sod1	P80566	F	CCACAGGCAAGTCTTTTCCC	192	2	0006801	0004784	0005623
		(MF467269)	R	CGACCTTGGCAATGTTGAGT					
Serpin B10	Serpinb10	P48595	F2	GAGGTCAATGAAGAAGGTACGG	91	1.93	0010466	0004867	0005615
		(MF467263)	R2	ATTTCAGTGGTGGCATCCTC					
Gastrin/cholecystokinin type B receptor	Cckbr	P46627	F	ATCGAGTGGCACGTTTATCC	101	1.96	0007165	0004871	0005886
			R	GAAGTAACACCGCGAAGCTC					
Cholecystokinin receptor type A	Cckar	Q63931 (KY994648)	F	CTAGACCCATGGCCGTTCTA	141	1.88	0007165	0004871	0005886
			R	CTGCTGGCCTACACCTCTTC					
Octopressin receptor	OPR	Q5W9T5	F	CGCCTTGGATCGTTACATCT	124	1.74	0007165	0004871	0005886
		(AB116233)	R	CCAGTTGTGGCAATGAACAC					
Orexin receptor type 2	Hcrtr2	O43614	F	CAAGCAAGGACGAAGGTAGG	105	1.96	0007165	0004871	0005886
		(MF467262)	R	GTCATCAAAAACGGCCAGAT					
Corticotropin-releasing factor receptor 2	Crhr2	Q60748					0007165	0004871	0005783
		(MF467256)							
Cephalotocin receptor 1	CTR1	Q7YW31					0007165	0004871	0005886
		(AB158493)							
Lipopolysaccharide-induced TNF-alpha factor	Litaf	Q8QGW7	F	CTCGTTGCCCCAAATGTAAC	105	2	0001816	0005125	0005737
		(MF467267)	R	AACTGAGGAGGAGGCAAATG					
Molluscan insulin-related peptide 3	Mirp	P80090	F	TGAGAGGGGATGAGGAGATG	190	2	0008284*	0005158*	0005576*
			R	AACTGGAGTGGGATCGTTTG					

(Continued)

TABLE 2 | Continued

Protein name	Gene name	AN	Primer sequence 5′-3′		AS	E	GO annotation		
							Process	Function	Component
Cephalotocin	CT	Q76DN0	F	ACCCCTTTCACAGAACATGC	142	1.92	0001975*	0005179*	0005576*
		(AB162925)	R	TGTATGTCTTGCGGACCAAA					
Fatty acid-binding protein homolog 9	lbp-9	Q965W1	F	AACGACCATGTCCCTAAACG	122	1.92	0006810	0005215	
		(MF414199)	R	CGGGCTGCTCTATACCACTC					
High-affinity choline transporter 1	CG7708	Q9VE46 (MF467259)					0003333	0005215	0005887
Anoctamin-1	Ano1	Q8BHY3	F	GAACTCATTCGCCAAGAAGC	171	2	0006810	0005227	0005737
		(MF414197)	R	AGGCAGTTTCGTCTGCAGTT					
Sodium- and chloride-dependent glycine transporter 2	SLC6A5	Q9Y345					0006810	0005328	0005826
		(MF414200)							
Rab effector Noc2	Rph3al	Q58D79	F	CCATGAAGGTGGCAAGTTTT	133	1.94	0006886	0005509	
		(MF467257)	R	AACCTTCCTTGTTGCCATTG					
Small cardioactive peptide-related peptide	SCPRP	Q2V2G5	F	AACCCGTATTTGGCGTAGTG	173	2	0007218		0005576
		(AB198190)	R	GTTGCCCTGGTCTGAAAAAG					
Protein PRQFV-amide	PRQFV	Q86MA7	F	CAGTGTTGCGGAGAGATGAA	159	1.89	0007218		0005576
			R	CTGCAGCATGGTCTGACTGT					
Tachykinin-related peptide	Octtkrpre	Q6F6I8	F1	CGTTTAGTTGGGGCTTTTCA	118	1.92	0007218*		0005576*
		(AB037112)	R1	GGGTTCCCGAGGTAAAAGAG					
Hypothetical protein OCBIM_22024610mg	OCBIM_22024610mg	A0A0L8H1B4					0009607		0016020

*Transcripts are given together with valid protein and gene names, and Uniprot accession number (AN); GeneBank accession numbers for O. vulgaris orthologs of these genes are indicated in parenthesis. Whenever available and for nucleotidic sequences that have been validated we provide GenBank accession number (AN, in parenthesis). The table also include sequences of primers (n = 24, Forward and Reverse) used for RT-qPCR experiments, amplicon size (AS) and efficiency (E). Gene ontology (GO) annotation for biological processes, functions and components are included. GO identifiers marked with *refer to cases where GO were attributed following the identification of similar proteins known in vertebrates or invertebrates to have analogous functions and when GO does not provide any result based on the valid Protein Name.*

all the strands. PCR products were run on 2% agarose gel in TBE buffer 0.5× (45 mM Tris-borate, 1mM EDTA) and detected expected bands were isolated. DNA was extracted using GenElute Gel Extraction Kit (Sigma-Aldrich, NA1111) and analyzed using an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems).

Real-Time qPCR

In order to analyze expression levels of specific genes of interest, a panel of putative reference genes was first screened to find the most stable genes for these experimental conditions (see the approach utilized for octopus in Sirakov et al., 2009). In our experiments, we utilized: eukaryotic translation initiation factor 4 (*EIF4G1*), LIM and SH3 domain protein (*F42H10.3*), lamin-B1 (*Lmnb1*), cytoplasmic FMR1 (*Sra-1*), ubiquitin-40S ribosomal protein S27a (*RPS27A*), elongation factor 1-alpha (*eef1a*), 40S ribosomal protein S18 (*RPS18*). The gene expression stability of the candidate reference genes for our samples was evaluated with BestKeeper (Pfaffl et al., 2002) and NormFinder (Andersen et al., 2004), following Sirakov et al. (2009). We identified the three most stable reference genes as *Lmnb1*, Sra-1 and *RPS27A*.

For gene expression experiments, samples from 10 octopuses were processed in triplicate. Polymerase chain reactions were carried out in an optical 384-wells plate with Applied Biosystems ViiA7 (Life Technologies) using Fast SYBR Green Master mix (ThermoFisher Scientific) to monitor dsDNA synthesis. Reactions (total volume: 10 μl) contained: 1 μl cDNA, 5 μl SYBR Green Master mix reagent, 4 μl of forward and reverse primers mix (0.7 pmol/μl each). The following thermal profile was used: 95°C for 10 min; 95°C for 15 s, 60°C for 1 min, 40 cycles for amplification; 72°C for 5 min; one cycle for melting curve analysis, from 60° to 95°C to verify the presence of a single product. Specificity of PCR products was checked by melting curve analysis followed by gel electrophoresis and DNA sequencing. PCR data were analyzed using the ViiA™ 7 Software (Life Technologies) to determine cycle threshold (*Ct*) values. Each assay included a no-template control for every primer pair. All sequences have been deposited in GenBank after validation (**Table 2**).

Data Analysis

Relative expression of the genes of interest, identified by *in silico* analysis of the octopus transcriptome, in the gastric ganglion and other tissues was analyzed through hierarchical clustering and principal component analysis (PCA) using Multi-experiment Viewer (MeV) software (Saeed et al., 2003). Quantitative

real-time PCR experiments, carried out to evaluate the response of the octopus gastric ganglion to different levels of *A. octopiana* infection ("high" vs. "low" parasite load), were analyzed through Multivariate Analysis of Variance (MANOVA) following Tsai and Chen (2009). Gene expression changes are expressed as Log_2 fold-changes following common practice (Friedman et al., 2006; Fundel et al., 2008). For all statistical analyses we used SPSS (rel. 18.0, SPSS Inc. - Chicago, 2009), with the exceptions mentioned above, and following Zar (1999). All tests were two-tailed and the alpha was set at 0.05.

RESULTS

In Silico Comparison of Gastric Ganglion Transcripts with Other Tissues

Figure 2 illustrates the transcriptional profiles of 33 genes expressed in *O. vulgaris* gastric ganglion (**Table 2**), selected through Gene Ontology (GO) Biological Functions search of the available transcriptome data. In brief, we counted over 65,000 nucleotide transcripts selectively expressed in the octopus' gastric ganglion. Our biased GO searching strategy probed for transcripts considered of particular relevance to the response to infection (e.g., neurotransmitter-synthesizing enzymes, receptors for transmitters and hormones), or implicated in inflammatory and/or immune/stress responses (e.g., superoxide dismutase, toll-like receptors, fatty acid-binding homolog 9); this provided a reduced number of potential candidate genes.

A more comprehensive analysis of the transcriptome fingerprints of *O. vulgaris* gastric ganglion is beyond the scope of this study, and this list should be considered preliminary.

We found that *in silico* relative abundance (counts) of the 33 transcripts in the octopus gastric ganglion varied. These ranged from <1 CPM (endothelin converting enzyme 1) to >1,000 CPM for fatty acid-binding homolog 9 (Lbp-9), the highest count for any transcript considered in any tissue. We

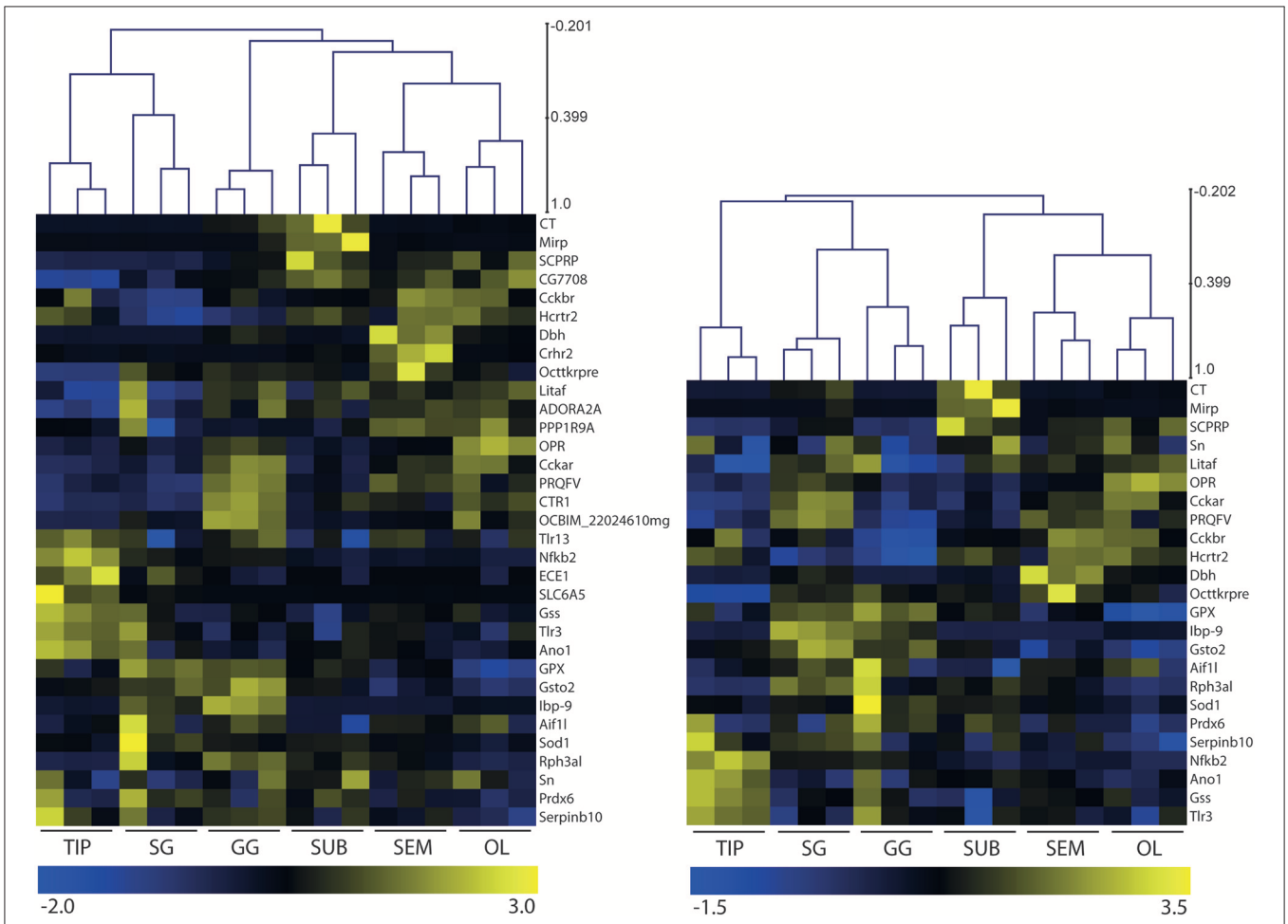

FIGURE 2 | Heatmaps and hierarchical clustering of gene expression levels in octopus brain (OL, SEM, SUB), stellate (SG) and gastric (GG) ganglia, and arm tip (TIP). Thirty-three **(Left panel)** and 24 transcripts **(Right panel)** mined from *Octopus vulgaris* RNAseq data were selected with a focus on neurotransmitters, signaling machinery and inflammation. Data were normalized per gene and subsequently analyzed by hierarchical clustering of the samples (tissues) and transcripts. Differences in the relative expression and clustering revealed a differential pattern between the gastric ganglion and other tissues considered independently from the number of transcripts included in this preliminary *in silico* characterization of octopus gastric ganglion. See **Table 2** and text for details.

also found 16 gene transcripts with counts <10 CPM, and nine in the range 10–100 CPM (allograft inflammatory factor, SCPRPamide, Protein PRQFV-amide, glutathione S-transferase, glutathione peroxidase, superoxide dismutase, peroxiredoxin 6 protein, glutathione synthase-like isoformX3; hypothetical protein OCBIM). Finally, 6 of the 33 genes considered had transcript counts in the range 100–1,000 CPM (dopamine beta-hydroxylase, tachykinin related peptide, Rab effector Noc2, cephalotocin, molluscan insulin-related peptide 3, protein singed). *In silico* relative expression of these 33 transcripts are overviewed in **Figure 2**.

A subset of 24 of the 33 genes (**Table 2**) was validated through real time RT-qPCR (see also **Figure 6**). In addition, and to confirm homogeneity between the two sub-sets of *O. vulgaris* gastric ganglion transcripts, we produced heatmaps and performed hierarchical clustering analysis (**Figure 2**). Differences were identified in the transcriptional fingerprint of *O. vulgaris* gastric ganglion when compared with other tissues, namely the stellate ganglion, arm tip and brain regions (**Figure 2**). The differences emerged independently from the number of transcripts considered (i.e., either 33 or 24 genes; **Figure 2**).

Principal Component Analysis (PCA) confirmed that the gastric ganglion profile differs from the other tissues (**Figure 3**). The two first principal components accounted for less than 50% of the total variance, and the cut-off eigenvalue (set to 1) was achieved only with principal component 5 (for PC analysis of either 33 or 24 transcripts, data not shown). Nevertheless, the gastric ganglion segregated into a different quadrant from the stellate ganglion. Furthermore, the analysis showed that the arm tip and the regions belonging to octopus' central nervous system also segregated in different quadrants (**Figure 3**), thus confirming that the genes considered here have a specific expression profile, and revealing that the three different brain regions (optic lobes and supra- and sub-esophageal masses) cluster together, but distant from the peripheral tissues considered.

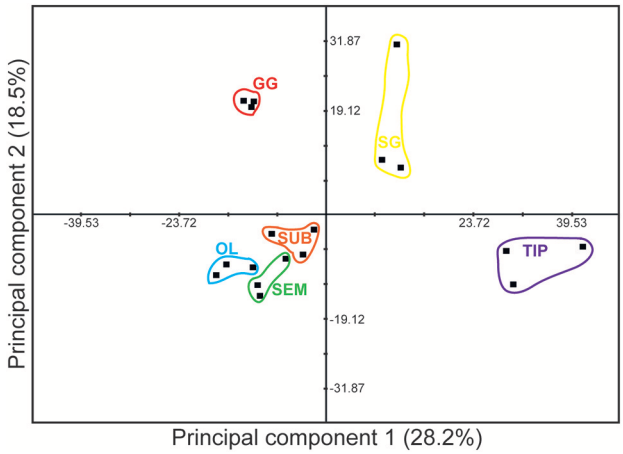

FIGURE 3 | Principal Component Analysis of gene expression data based on transcript levels (*in silico*, see **Figure 2**) of 33 genes selected for this study (see **Table 2** for full list). Data are plotted for tissue taken from three ($N = 3$) different animals. Dissimilarity between peripheral and central nervous system tissues emerged from the analysis with a clear distinction of the octopus gastric ganglion signature from other peripheral structures (i.e., stellate ganglion and arm). See text for details.

Extending Current Knowledge of *O. vulgaris* Gastric Ganglion Structural Complexity

O. vulgaris gastric ganglion is an ovoid structure prominently located at the junction of the crop, stomach, caecum and proximal intestine at the point where they share a common lumen (**Figure 1**). The well-circumscribed nature of the ganglion was particularly evident following histological examination, which confirmed previous data reporting that it is almost entirely encapsulated in connective tissue (Bogoraze and Cazal, 1946; **Figures 4A–C**). The ganglion itself consists of very densely packed nerve cell bodies distributed in a cortical layer of the oblate spheroid structure, with axons coursing centrally, bundling, and allowing the ganglion to be connected with other structures including the subjacent digestive tract (**Figures 4A–C**). Following Bogoraze and Cazal (1946), the peripheral layer of cells appears relatively larger than those that are more closely distributed toward the internal neuropil (see also Young, 1971).

Numerous cells also appear in the neuropil. A group of them are clustered together, toward the center of the ganglion, creating a wall apparently separating it into two sections (**Figure 4J**, arrowheads; see description in Young, 1971). In addition, some cells are dispersed in the neuropil and have been described by Bogoraze and Cazal (1946) as forming a glio-vascular network (**Figures 4E,I**). In our samples, we found cells with somata diameters ranging from 7 to 30 μm.

In the following section, a further description of the cellular components and fibers constituting the octopus gastric ganglion, together with information derived from attempts to localize modulators and other characteristic markers, is provided through immunohistochemistry (IHC). The results should be considered preliminary. This because the currently available commercial antibodies we utilized are not designed for octopus or cephalopods. However, as shown in **Table 1** (consider also exceptions therein) these have been already applied to cephalopod tissues in a series of studies, despite that in some cases standardized ways to biologically validate them have not being applied. Therefore, observations provided below should be considered as an indication of "-like immunoreactivity" (referred hereunder as like-IR or IR). These limitations are considered further in the Discussion.

We utilized NeuN for the first time in octopus. It positively identified the great majority of cells in the gastric ganglion (**Figures 4J–M**) confirming its general architecture (see above). In addition, there were a few cells negative for the NeuN-antibody (**Figure 4M**, arrowheads), suggesting also the existence of supporting cells (e.g., glia-like cells) in the gastric ganglion. A complex organization of fibers was revealed through neurofilament-IR; these appeared mostly ordered and compacted into bundles in the part proximal to the sympathetic (**Figure 4D**, *sn*) nerve (i.e., superior and dorsal bundles, *sensu* Bogoraze and Cazal, 1946). On the other hand, fibers appeared greatly intertwined toward the posterior area of the neuropil

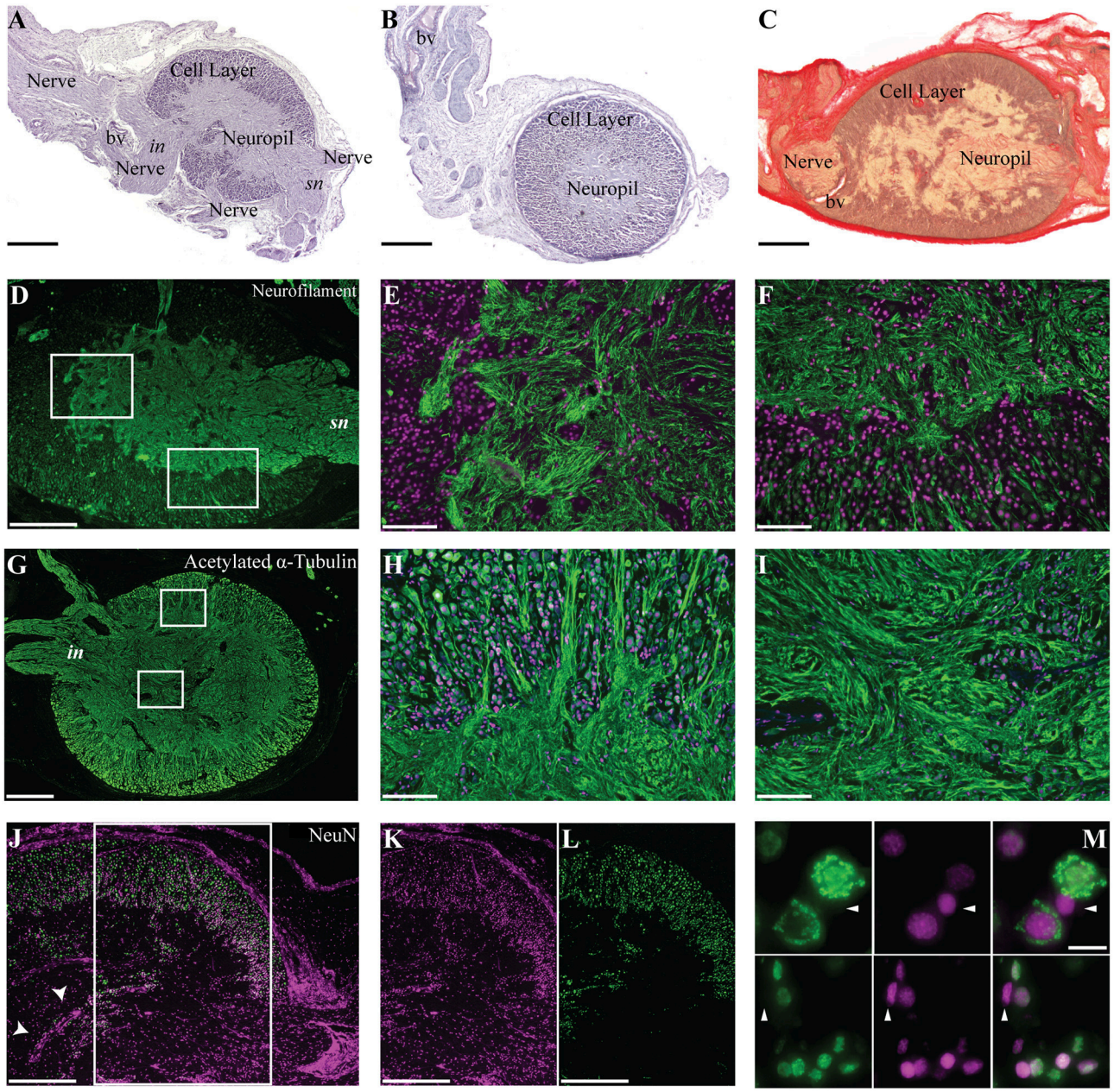

FIGURE 4 | Morphological description of the octopus gastric ganglion. Hematoxylin and eosin **(A,B)** and Picro-Ponceau with hematoxylin **(C)** served to characterize the ganglion, with its cortical layer of neural cells (Cell layer) and internal neuropil. Nerve bundles are highlighted; in **(A)** intestinal (in) and sympathetic (sn) nerves are indicated; staining also revealed blood vessels (bv). Connective tissue and the cell layer surrounding and enveloping the ganglion is clearly identified after Picro-Ponceau with hematoxylin staining **(C)**. **(D–M)** Fluorescent immunohistochemistry for (in green) neurofilament **(D–F)**, acetylated alpha-Tubulin **(G–I)**, and NeuN **(J–M)**, DAPI is utilized as nuclear stain (magenta). Neurofilament marks the inner neuropil **(D)** and the intricate organization of fibers (enlargement in **E**), with some neural processes entering the cellular layer (enlargement in **F**). Acetylated alpha-tubulin **(G–I)** shows the external group (external layer) of cells larger than those distributed internally (internal layer) **(H)** with several processes progressing toward the internal neuropil **(H,I)**. NeuN and nuclear stain (DAPI) merged image **(J)** are provided as separate channels in **(K,L)**, respectively for areas corresponding to the white rectangle. A cell wall dividing the neuropil of the ganglion is clearly visible (**J**, arrowheads). Higher magnification to show the neurons positive for NeuN (green), DAPI (magenta), and merged channels in **(M)**. The cells not positive for NeuN are indicated with arrowheads. Scale bars: 500 μm **(D,G,J,K,L)**; 200 μm **(A–C)**; 100 μm **(E,F,H,I)**; 10 μm **(M)**.

(**Figure 4D**, left rectangle in; enlargement in **Figure 4E**), before the emergence of the ventral nerve bundles (see also intestinal nerves in Young, 1967). It is in this area that neurofilament-IR revealed fibers forming polygonal, round or ovoid networks resulting from the convergence of numerous fiber bundles (**Figures 4D–F**).

Using acetylated alpha-tubulin antibody we found positive cells and fibers (**Figures 4G–I**). The fibers appeared intertwined and arranged in different directions and orientations. Most of them appeared to emerge from larger cells forming networks where cells appeared dispersed into bundles of different size and complexity (**Figures 4F,H**).

To gain a broader understanding of the molecular complexity and signaling potential of the gastric ganglion, the expression of a range of putative neurotransmitters was also analyzed by IHC.

We observed numerous nerve fibers positive for tyrosine hydroxylase antibody (**Figures 5A,B**). These are identified as ordered bundles in the neuropil of the ganglion and toward the anterior pole in areas belonging to the sympathetic nerve bundles. This pattern contrasts with the diffuse and apparently disorganized arrangement observed toward the posterior end, corresponding to the area where neurofilament-like IR revealed intricate round (or ovoid) network of fibers.

A diffuse, but evident and widely distributed IR-signal occurred in sections using octopamine antibody. Octopamine immunoreactivity revealed small "button-like"-vesicles (**Figures 5C,D**) widely distributed in the cytoplasm and neuronal processes, again suggesting the existence of an intricate network.

5-hydroxytryptamine-like positive nerve fibers (**Figures 5E,F**) were clearly visible in the neuropil, in some cases forming a clustered network (**Figure 5F**, arrowheads). Furthermore, we identified several processes originating from larger neurons and progressing toward the neuropil, in some cases surrounding cells belonging to the internal layer of the ganglion (**Figure 5F**, arrows).

Common type of choline acetyltransferase (cChAT) antibody identified a few sparse, but clustered positive cells (**Figures 5G,H**), and several positive fibers dispersed in the neuropil.

GABA-like IR revealed an intricate widely distributed reticulum surrounding the great majority (almost all) of cells; this appeared particularly evident in the cells belonging to the more external layers of the cortical zone (**Figure 5I**). It overlapped with the noradrenaline-like IR we observed (**Figure 5J**), although appearing less intricate when compared with GABA-like IR. Noradrenaline-IR is also found in fibers of the nerve bundles (**Figure 5K**).

Corticotrophin releasing factor (CRF-like IR) was identified in a subset of the cells within the cortical layer of the gastric ganglion (**Figure 5L**) and also in a few CRF-like IR positive nerve fibers.

FMRFamide-like immunoreactivity was widely distributed in the ganglion with numerous positive fibers identified in various parts of the neuropil and in several cells distributed in the surrounding cortical layer (**Figures 5M,N**). In several areas, strong FMRFamide-like IR was seen in the neuropil in a cluster of fibers forming a beaded appearance (**Figure 5M**). Furthermore, positive FMRFamide-like fibers have been identified in the nerves that connect the gastric ganglion with other structures.

Various cells of the internal zone of the cortical layer of the gastric ganglion were positive for tachykinin–like immunoreactivity (**Figure 5O**). We also observed numerous tachykinin-like positive fibers both in the neuropil and nerves (**Figure 5P**).

Gene Expression in the Gastric Ganglion as a Result of *Aggregata octopiana* Infection

We found diversified expression of the selected 24 genes in the gastric ganglion after RT-qPCR experiments linked to parasite load in the digestive tract samples (**Figure 6**; see also **Table 3**). We compared gene expression in gastric ganglia of octopuses with an elevated number of *A. octopiana* sporocysts ["high," sporocysts/tissue(g): median $= 1.37*10^6$, 95% CI $\pm$ 1.80; $N = 5$] in their digestive tract, with those from *O. vulgaris* with a relatively low parasite load ["low," sporocysts/tissue(g): median $= 0.26*10^6$, 95% CI $\pm$ 0.21; $N = 5$; parasite incidence, "high" vs. "low": $Z = 2.6$, N1, N2 $= 5$, $p = 0.009$ after Mann-Whitney U test]. In the latter group, we counted fewer than 6,000 sporocysts per gram of tissue; in "high" group we found the highest value of *Aggregata* sporocysts [sporocysts/tissue(g) $= 5.8*10^6$]. In three out of ten animals we found a very few (<3/octopus) larval forms of other parasites, i.e., cestodes and nematodes. There is no evidence for effects of these parasites on the functioning of the cephalopod digestive system, to the best of our knowledge (Hochberg, 1990).

We observed a significant change in gene expression in the gastric ganglia in response to infection [high vs. low-−24 genes, MANOVA: $F_{(4,7)} = 202.59$, $p < 0.001$]. **Figure 6** plots gene expression as fold changes observed in octopus gastric ganglia from "high" relative to those sampled from "low" parasite load animals. Seventeen out 24 genes had significant pairwise changes (after Bonferroni *post-hoc* comparison; **Figure 6**; **Table 3**). Under these computational circumstances, six genes increased their relative expression (high vs. low: *Sn*, *Nfκb2*, *Cckar*, *SCPRP*, *Serpinb10*, *Tlr3*) while decreased gene expression was observed in the others ($n = 11$; high vs. low: *Litaf*, *Dbh*, *OPR*, *CT*, *Mirp*, *PRQFV*, Tk, *Sod1*, *Prdx6*, Gpx1, *Rph3al*). Furthermore, genes for Ov-Nuclear factor NF-κB p100 subunit and Ov-Toll like receptor 3 were found with at about one-fold increase (**Figure 6**). At least a one-fold decrease was observed for Ov-Lipopolysaccharide-induced tumor necrosis factor-alpha factor, Ov-Molluscan insulin-related peptide 3, Ov-Superoxide dismutase [Cu-Zn] (Ov-*Sod1)*, and Ov-Rab effector Noc2 (**Figure 6**).

DISCUSSION

This study provides an update of the knowledge on the complexity of the gastric ganglion of the cephalopod mollusc *O. vulgaris* using a combination of immunohistochemical and gene expression analyses. Recent transcriptome analyses of octopus and other cephalopods (e.g., Zhang et al., 2012; Albertin et al., 2015; Petrosino, 2015; Salazar et al., 2015; Liu et al., 2016; Liscovitch-Brauer et al., 2017; Tian et al., 2017) have greatly facilitated this study. The data we accessed (R. Sanges and G. Fiorito Laboratories *O. vulgaris* transcriptome; see Materials and Methods) enabled characterization of the molecular fingerprint of octopus' gastric ganglion. Despite limitations imposed by the set of transcripts considered (33 out over 60,000 in total),

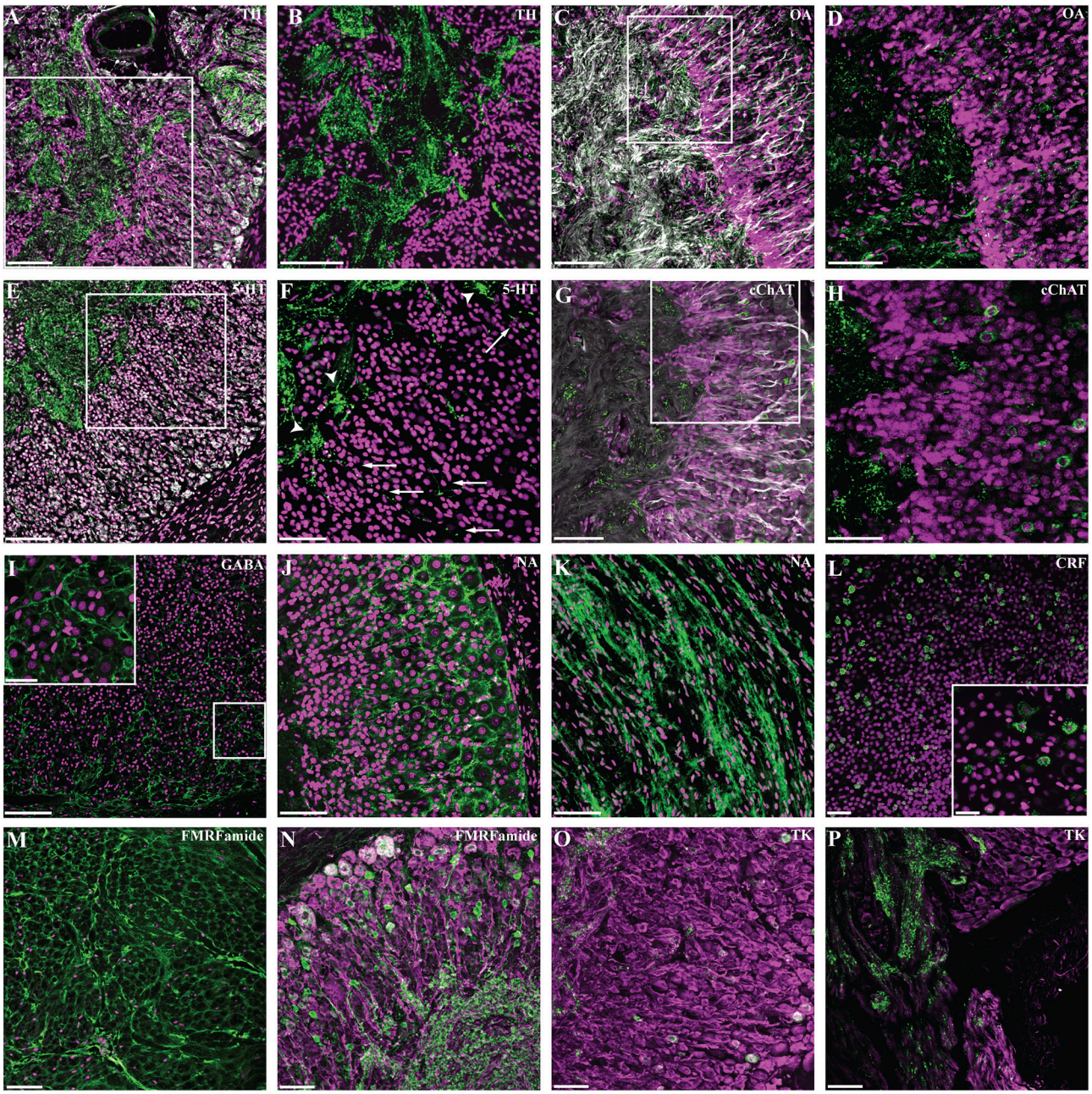

FIGURE 5 | Neurochemical complexity of *Octopus vulgaris* gastric ganglion. Fluorescent immunoreactivity (in green) for: Tyrosine Hydroxylase (TH, **A,B**), Octopamine (OA, **C,D**), 5-hydroxytryptamine (5-HT, **E,F**), common Choline Acetyltransferase (cChAT, **G, H**), GABA **(I)**, Noradrenaline (NA, **J, K**), Corticotrophin releasing factor (CRF, **L**), FMRF-amide **(M,N)**, and Tachykinin (TK, **O**,**P**); DAPI is utilized as nuclear stain (magenta, except in **N–P**). Co-localization with acetylated alpha-tubulin (AcTub) is shown in **(A,E,N,O,P)** with neurofilament (NF) in **(C,G)**. TH-IR fibers as they appear in the internal neuropil of the gastric ganglion **(A)** co-localized with AcTub (gray). The white square area in **(A)** is enlarged in **(B)** to show the strong TH immunoreactivity at the level of neural processes. **(C)** The intricate network of octopamine positive fibers and button-like positivity as seen in the neuropil and cellular layer (colocalized with NF, gray). The high magnification in **(D)** shows details of octopamine-IR fibers inside the cellular layer and their prolongation within the internal neuropil. **(E)** 5-HT-IR and AcTub (gray) revealed fibers in the internal neuropil with several processes progressing toward the cellular layer. The high magnification detail in **(F)** shows some of these processes in the cellular layer (arrows), and some clustered network fibers (arrowheads). **(G)** The common Choline Acetyltransferase immunoreactivity seen in cells and fibers, co-localized with NF (gray). The magnification in **(H)** to highlight cChAT-IR in cells. GABA-IR **(I)** identified an intricate network surrounding the cells of the cortical layer; a magnification is provided in the enlargement. **(J)** Noradrenaline immunoreactivity revealing an intricate reticulum surrounding cells of cellular layer, and at the level of the sympathetic nerve **(K)**. CRF-IR positive cells **(L)** and detail in the higher magnification (square) of the cellular layer. FMRFamide-IR fibers are observed in the intestinal nerve (transverse section, **M**). FMRFamide-IR fibers in the neuropil of the gastric ganglion **(N)** with processes and cells in the cellular layer (co-localized with AcTub, magenta). Tachykinin-like IR in cells and fibers in the neuropil **(O,P)**; co-localization with AcTub (magenta). See text for details. Scale bars: 100 μm (**A,B,D**,**E**,**H**,**I,J**,**K**,**L**); 50 μm (**C**,**G**,**F,M,N,O,P**); 25 μm (higher magnification in **L**,**I**).

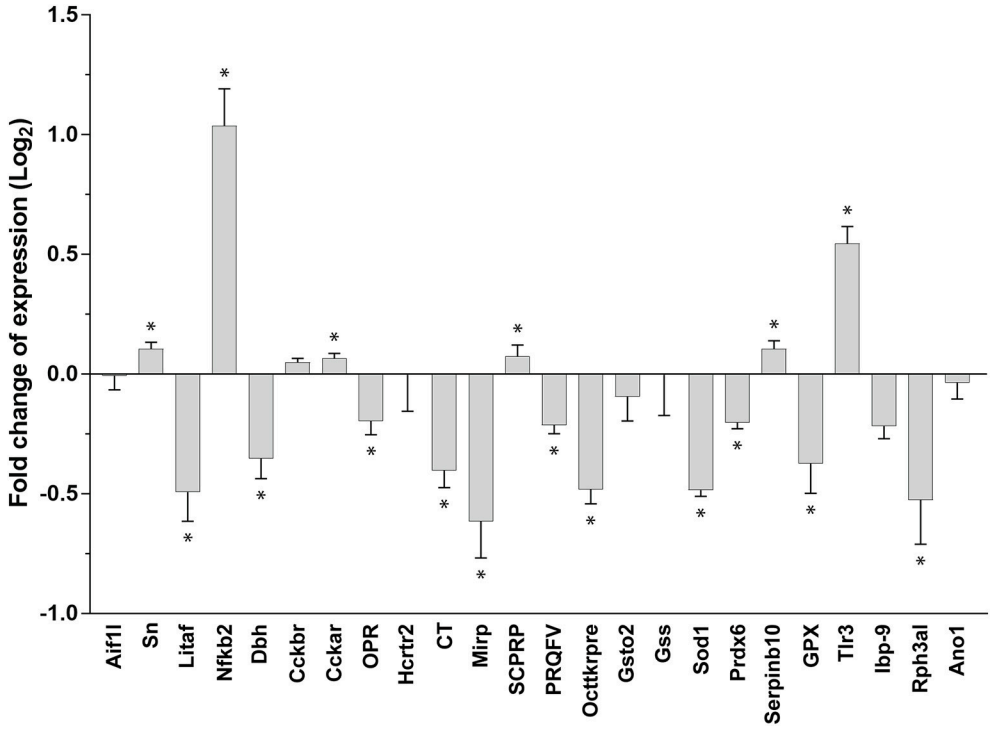

FIGURE 6 | Relative gene expression using real-time RT-qPCR. Fold changes of expression (log_2, error bar: 95% confidence interval). A differential pattern of up- and downregulation of 24 genes in the octopus gastric ganglion of animals infected by *Aggregata octopiana* ("high" level) emerged when compared ("high"/"low") with their expression in samples taken from octopus where the parasite load from *Aggregata* was defined as "low." Asterisks mark significant changes; see text for details and **Table 3**.

in silico gene expression analysis revealed that the ganglion is clearly distinguishable from other central and peripheral nervous system tissues, when the same set of genes is considered. This is confirmed by PCA (**Figure 3**), with the gastric ganglion segregated into a different quadrant in respect to the stellate ganglion, and the arm tip and the octopus' brain structures clustered in other quadrants (**Figure 3**).

Below we will first summarize our data and the literature to depict the neurochemical complexity of *O. vulgaris* gastric ganglion, and then discuss the effects of *Aggregata* infection on gene expression in the ganglion.

O. vulgaris Gastric Ganglion: A Contribution to the Understanding of Its Neural Complexity

In *O. vulgaris* the gastric ganglion appears as a white, oval-shaped, encapsulated structure (see **Figure 1**) about 3 mm long (in a 500 g body weight animal Andrews and Tansey, 1983). It is located on the serosa of the digestive tract at the junction of the crop, stomach, caecum and proximal intestine (Bogoraze and Cazal, 1946; Young, 1967). The cephalopod gastric ganglion is characterized by a dense neuropil surrounded by cell bodies, encapsulated by connective tissue. This structure was apparent from the H&E staining (**Figure 4C**), which also showed axon bundles exiting the ganglion for adjacent regions of the digestive tract, and confirmed previous descriptions (see Bogoraze and Cazal, 1946; Young, 1967, 1971). The dense layer of nerve cell bodies surrounds a central neuropil, with axons forming the nerve bundles linking, through an intricate network, to the adjacent structures: crop, stomach, caecum, hepatic ducts and intestine—(Bogoraze and Cazal, 1946; Young, 1967). The major nerve bundles have been identified as: superior bundle (3 nerves: anterior- and posterior-gastric, and gastro-esophageal nerves), dorsal bundle (spreading in a fan innervating the caecum), and ventral bundle (3 nerves) emerging from the posterior end (**Figure 4G**: *in*) of the gastric ganglion and projecting toward the intestine (see descriptions in Bogoraze and Cazal, 1946; Young, 1967).

We observed nerve cell bodies larger (external layer) than those closer (internal layer) to the neuropil (**Figures 4H**, **5N**), in analogy to other ganglia in octopus as described by Bogoraze and Cazal (1944; 1946; see also Young, 1971). However, very small cells (less than 5 μm diameter) were not confirmed by our observations.

In the neuropil a dividing partition made of cell bodies (**Figure 4J**, arrowheads) was also noted, as reported by Young (1967, 1971). In *Nautilus* there are paired gastric ganglia (Owen, 1832) and we hypothesize that the partition observed in octopus' gastric ganglion is a remnant of the fusion of paired ganglia in an ancestral cephalopod to form a single ganglion in modern cephalopods.

TABLE 3 | Pairwise comparison of gene expression in the gastric ganglion between "high" vs. "low" *Aggregata octopiana* infected octopuses.

Protein	Gene	*P* (after Bonferroni)
Allograft inflammatory factor 1-like	Aif1l	0.729
Protein singed	Sn	**<0.001**
Lipopolysaccharide-induced TNF-alpha factor	*Litaf*	**<0.001**
Nuclear factor NF-kappa-B p100 subunit	*Nfκb2*	**<0.001**
Dopamine beta-hydroxylase	*Dbh*	**0.001**
Gastrin/cholecystokinin type B receptor	*Cckbr*	0.051
Cholecystokinin receptor type A	*Cckar*	**0.01**
Octopressin receptor	*OPR*	**<0.001**
Orexin receptor type 2	*Hcrtr2*	0.318
Cephalotocin	*CT*	**<0.001**
Molluscan insulin-related peptide 3	*Mirp*	**<0.001**
Small cardioactive peptide-related peptide	*SCPRP*	**0.002**
Protein PRQFV-amide	*PRQFV*	**0.009**
Tachykinin-related peptide	*Octtkrpre*	**<0.001**
Glutathione S-transferase omega-2	*Gsto2*	0.341
Glutathione synthetase	*Gss*	0.821
Superoxide dismutase Cu-Zn	*Sod1*	**<0.001**
Peroxiredoxin 6	*Prdx6*	**0.01**
Serpin B10	*Serpinb10*	**0.001**
Glutathione peroxidase	*GPX*	**0.002**
Toll like receptor 3	*Tlr3*	**<0.001**
Fatty acid-binding protein homolog 9	Ibp-9	0.06
Rab effector Noc2	*Rph3al*	**<0.001**
Anoctamin-1	*Ano1*	0.449

*Results after MANOVA are computed after Bonferroni correction. See **Figure 6** for direction of changes.*
Bolded p values show statistically significant differences.

The characterization of *O. vulgaris* gastric ganglion was further extended by identifying neuronal markers that can be utilized for studying the morphology of the cephalopod ganglia moving forward.

We identified cell bodies using NeuN antibody, a neuronal nuclear marker known to recognize neurons in the both the central and peripheral parts of the vertebrate nervous system including the autonomic innervation of the digestive tract (Mullen et al., 1992). The epitope of this antibody (see **Table 1**) matched at least seven nucleotide sequences (average length >1,000 bp) belonging to the *O. vulgaris* transcriptome available to us. In particular, we identified it by BLAST as the RNA binding protein fox-1 homolog 3 (RBFOX3). To the best of our knowledge, RBFOX3 gene encodes a member of the RNA-binding FOX protein family, which is involved in the regulation of alternative splicing of pre-mRNA. It has an RNA recognition motif domain, and is known to produce the neuronal nuclei (i.e., NeuN) antigen that has been widely used as a marker for post-mitotic neurons (see https://www.ncbi.nlm.nih.gov/gene/146713; see also Nikolić et al., 2013 where is reported to identify neural cells in *Helix*).

In our experiments, NeuN antibody identified a population of cells in the octopus gastric ganglion. According to the original description of Bogoraze and Cazal (1946), the nerve cells forming the cortical layers (4 to 5, *sensu* Bogoraze and Cazal, 1946) are all of "ganglionic type (plasmo- or somatochromes), large in size, abundant in cytoplasm, rich in Nissl bodies, with vesicular and nucleolus nuclei" (Bogoraze and Cazal, 1946, p. 123; **Figure 4M**, upper row). Following the authors' description, there are no karyo-chrome cells expected in the octopus gastric ganglion (Bogoraze and Cazal, 1946). In addition, neural cells of the cortical layers are described as placed in a neuronal "lodge" formed by fibers and agglutinated neuroglia proposed to be part of the intricate glio-vascular network characterizing typical ganglia in octopus (see Bogoraze and Cazal, 1944, 1946, p. 123). Based on the NeuN, noradrenaline and GABA immunoreactivities we observed, it is suggested that our data match with the description provided by Bogoraze and Cazal. Interestingly, the intricate network seen for noradrenaline and GABA (**Figures 5I,J**) resembles the original drawings of the gastric ganglion histology (see Figure XIII in Bogoraze and Cazal, 1944).

It was not possible to investigate the glia-like cells in the gastric ganglion of octopus, as specific markers are not available for cephalopods. Imperadore et al. (2017) were unsuccessful using antibodies against vimentin and glial fibrillary acidic protein in octopus neural tissues; this contrasts with previous results (Cardone and Roots, 1990). Furthermore, transcripts for glial fibrillary acidic protein in *O. bimaculoides* genome or in *O. vulgaris* transcriptome do not seem to occur (Imperadore et al., 2017).

We were unable to use synaptic markers (e.g., synapsin and synaptogamin) due to the lack of specificity of the commercial antibodies available. However, previous investigation of the *O. vulgaris* transcriptome (Zhang et al., 2012) identified the presence of synaptophysin and synaptotagmin-7 in central nervous system of *O. vulgaris,* and the genome of *O. bimaculoides* includes 13 synaptotagmin genes (Albertin et al., 2015).

O. vulgaris gastric ganglion cells were also positive for the vertebrate neuronal marker acetylated α-tubulin, consistent with the finding of Shigeno and Yamamoto (2002) of intense tubulinergic immunoreactivity in the gastric ganglion of the pygmy cuttlefish (*Idiosepius paradoxus*). Acetylated α-tubulin staining revealed a very intricate net of fibers that appear to contribute to the connectivity within and outside the gastric ganglion.

In *O. vulgaris* the gastric ganglion is connected to the brain by a pair of sympathetic nerves (*sensu* Young, 1967). These arise from the inferior buccal ganglion (in the supra-esophageal mass) and run to the gastric ganglion embedded in the esophagus and crop muscle, giving off branches and forming a plexus in their wall *en passant* (Young, 1967, 1971). Additionally, connection to the central nervous system is via the abdominal and intestinal nerves, which in turn connect with the visceral nerve (*sensu*, Young, 1967) originating in the palliovisceral lobe (in the posterior sub-esophageal mass). A study reconstructing the intricate intra-ganglionic network will provide a simple proof-of-concept for future studies aimed at constructing a "connectome" of the octopus brain (see, Choe et al., 2010; Marini et al., 2017).

A number of putative neurotransmitters have previously been identified in the gastric ganglion of *O. vulgaris*: dopamine

(Juorio, 1971; Andrews and Tansey, 1983), octopamine (Juorio and Molinoff, 1974), noradrenaline (Andrews and Tansey, 1983), acetylcholine (Andrews and Tansey, 1983), small cardioactive peptide-related peptide (Kanda and Minakata, 2006), and octopressin (Kanda et al., 2003c; Takuwa-Kuroda et al., 2003). Receptors for cephalotocin (Kanda et al., 2003b), octopressin (Kanda et al., 2005) and gonadotrophin releasing hormone (i.e., Oct-GnRH, Kanda et al., 2006) have also been reported. To the best of our knowledge, endocrine cells have not been identified in the gastric ganglion of any cephalopod, so here we assume that the substances identified originate from neurons (or possibly supporting cells) and are neurotransmitters rather than hormones. On the other hand, Bogoraze and Cazal (1946) described a "Juxta-ganglionnaire tissue" in the gastric ganglion of *O. vulgaris* (p. 121 and following pages, Bogoraze and Cazal, 1946), and this may suggest the existence of "neurosecretory tissue" in the ganglion as shown in other neural structures of cephalopods (e.g., Barber, 1967; Bellows, 1968; Martin, 1968; Froesch, 1974).

We have extended knowledge of the diversity of signaling molecules and receptors in the gastric ganglion of *O. vulgaris*. By using *in silico* gene expression analysis, real-time polymerase chain reaction experiments and immunohistochemistry, we provide for the first-time evidence for the presence in the octopus gastric ganglion of: Cephalotocin, Corticotrophin releasing factor, FMRF-amide, Gamma amino butyric acid, Molluscan insulin-related peptide 3, Protein PRQFV-amide, Small cardiac peptide-related peptide, and Tachykinin-related peptide-like IR.

As mentioned above, the currently available commercial antibodies have been in most of the cases already used on cephalopod tissues, despite not being designed for octopus or cephalopods, and they have not been validated according to current criteria (**Table 1**, but see exceptions therein). The results of this study should therefore be considered as preliminary and future detailed immunohistological studies using these antibodies will require more sophisticated approaches.

We comment below briefly on each of the substances.

Cephalotocin (CT; Figures 2, 6). The expression of the gene for the nonapeptide cephalotocin was demonstrated, complementing previous studies showing cephalotocin receptors (CTR_1 and CTR_2) in the gastric ganglion (Kanda et al., 2003b, 2005). Expression of Ov-CT was down regulated (>8-fold) in octopuses with "low" *Aggregata* parasite load (compared with those where the number of sporocysts was very limited/negligible; data not shown). Interestingly, octopus with elevated parasite load show only a limited down-regulation of CT gene expression (**Figure 6**), suggesting that it is further modulated in highly infected octopuses.

Cephalotocin is a member of the oxytocin/vasopressin superfamily of peptides which is widely distributed in vertebrates and invertebrates (Hoyle, 2011; Gruber, 2014), with the other member present in octopus being octopressin (Takuwa-Kuroda et al., 2003). Our findings contrast with a previous study of the gastric ganglion in *O. vulgaris* (Takuwa-Kuroda et al., 2003), which failed to show the presence of cephalotocin mRNA in the ganglion, although octopressin mRNA was clearly present. Takuwa-Kuroda et al. (2003) provided evidence for cephalotocin in the *O. vulgaris* sub-esophageal mass, which includes the lobes from which most of the extrinsic nerves supplying the digestive tract originate (Young, 1971). In cuttlefish pro-sepiatocin and sepiatocin are both found in the sub-esophageal lobes (Henry et al., 2013).

We are unable to reconcile the difference in the evidence for the presence of cephalotocin between the above octopus studies, but both provide evidence for a member of the oxytocin/vasopressin superfamily in the gastric ganglion, and the presence of the cephalotocin receptor (Kanda et al., 2003b) supports a role for a member of this family as a neurotransmitter in the ganglion. However, in *O. vulgaris*, Takuwa-Kuroda et al. (2003) found no effect of cephalotocin on rectal contractions. Cephalotocin is considered to play a role in the neurosecretory system of the vena cava (Takuwa-Kuroda et al., 2003) so it is possible that it acts as a hormone (i.e., transported via the vasculature) on the receptors identified in the gastric ganglion.

Corticotrophin Releasing Factor (CRF; Figures 2A, 5). CRF-like IR has been reported in *O. vulgaris* brain tissue and co-localized with neuropeptide Y-like substance (Suzuki et al., 2003). CRF receptor has been included in the list of Class B (secretin-type) G-protein-coupled receptors in the genome of *O. bimaculoides* (see Supplementary Note 8.5 in Albertin et al., 2015). We identified a subset of neural cells belonging to the cortical layers in the gastric ganglion that were CRF-like positive, along with some positive fibers.

FMRFamide (Figures 5M,N). We identified several cells and numerous fibers positive for the FMRFamide antibody utilized. There is considerable evidence for the presence of FMRFamide and/or FMRFamide-related peptides in the central (Di Cosmo and Di Cristo, 1998; Suzuki et al., 2002; Di Cristo et al., 2003; Zatylny-Gaudin et al., 2010; Cao et al., 2016) and peripheral divisions (e.g., chromatophore motorneurones, Loi et al., 1996; stellate ganglion, Burbach et al., 2014) of the cephalopod nervous system. The demonstration of pronounced FMRFamide-like IR in octopus gastric ganglion nerve fibers is consistent with other findings reporting the presence of FMRF-amide like peptides in the palliovisceral lobe (*Sepiella japonica*, Cao et al., 2016), the site of origin of the visceral nerve that connects the brain and gastric ganglion (Young, 1967, 1971), and in rectal nerve endings (*Sepia officinalis*, Zatylny-Gaudin et al., 2010) that may originate from the gastric ganglion (Young, 1967). The FMRFamide and RFamide-like peptides are widely distributed amongst the Mollusca including cephalopods (Walker et al., 2009; Zatylny-Gaudin and Favrel, 2014).

RFamides (including FMRFamide) have been implicated in inhibition of feeding in gastropods (Bechtold and Luckman, 2007) and a similar role has been proposed for cephalopods (Zatylny-Gaudin et al., 2010; Zhang and Tublitz, 2013; Cao et al., 2016), but no direct evidence has been provided. Peptide GNLRFamide increased tone, contraction frequency and

amplitude in the rectum from *S. officinalis,* but was without effect on either the esophagus or contractile regions of the male or female reproductive tracts (Zatylny-Gaudin et al., 2010).

Molluscan Insulin-Related Peptide 3 (Mirp, Figures 2A,B, 6). Peptides with insulin-like structures have been identified in molluscs including *Aplysia* (Floyd et al., 1999) and *S. officinalis* (Zatylny-Gaudin et al., 2016), but as far as we are aware this is the first time that a member of this family has been identified in a cephalopod ganglion. The occurrence in the gastric ganglion is consistent with a neurotransmitter, rather than the more conventional endocrine role for insulin. However, the latter has not been demonstrated in cephalopods (see Goddard, 1968).

In the mollusc *Aplysia*, food deprivation decreases insulin mRNA expression in the cerebral ganglia, and injections of insulin lowers hemolymph glucose levels (Floyd et al., 1999). However, further studies in *Aplysia* using human insulin showed that it was able to hyperpolarize neurons most likely via ion channels (Shapiro et al., 1991). Thus, a potential neuromodulatory role for molluscan insulin-related peptide 3 in the gastric ganglion should not be excluded**.**

It is noteworthy to report that in *Aplysia,* the same transcript has been identified as a precursor of opioid-like peptides known to be specific modulators in molluscan neurons (i.e., putative enkephalin, Moroz et al., 2006). *in silico* analysis of the *O. vulgaris* and *Aplysia californica* (Moroz et al., 2006) transcriptomes revealed that the two orthologs are very similar each other. Our gene expression experiments show that Ov-*Mirp* was downregulated in *Aggregata* infected animals (high vs. low, **Figure 6**). However, when gene expression data are compared against samples where the number of sporocysts was negligible Ov-*Mirp* appeared at least 8-fold upregulated in gastric ganglia belonging to octopus with "low" parasite loads, and about 3-fold upregulated in the "high" load condition (data not shown).

Peptide PRQFVamide (PRQFV, Figures 2A,B, 6). This pentapeptide was first identified in *Aplysia* where immunostaining was demonstrated in axons in the wall of the digestive tract and the stomatogastric ring (Furukawa et al., 2003). In *Aplysia*, PRQFV-amide inhibits contractions of the digestive tract and modulates neurons in the buccal feeding system. The expression of Ov-PRQFV appeared significantly decreased ("high" vs. "low," **Figure 6**). Octopuses with "low" *Aggregata* parasite load show an upregulation (>7-fold) of this gene when compared to animals with a limited/negligible load (data not shown). Higher levels of parasite infection induced a significant depression of gene expression (data not shown).

Based upon its location and reported functions in *Aplysia*, its presence in the gastric ganglion of *O. vulgaris* is perhaps not surprising. Incomplete PRQFV-amide prohormones have been identified in the neuropeptidome of *S. officinalis* and related mature peptides (e.g., PMEFL amide) are present in the hemolymph (Zatylny-Gaudin et al., 2016). We are not aware of functional data which would provide insights into the function of PRQFV-amide in the cephalopod gastric ganglion.

Small Cardioactive Peptide-Related Peptide (SCPRP, Figures 2A,B, 6). Kanda and Minakata (2006) reported the presence of oct-SCPRP (a decapeptide) in the gastric ganglion using Southern blot, and our results provide further biological validation. A low parasite load in the digestive tract induced an upregulation of Ov-SCPRP expression, but it was reduced when the levels of *Aggregata* were elevated (comparing "high" or "low" with samples where the number of sporocysts was limited/negligible; data not shown; refer also to **Figure 6**).

A small cardioactive decapeptide peptide has been identified in the *S. officinalis* neuropeptidome (gastric ganglion not analyzed), but interestingly was not detected in the central nervous system (Zatylny-Gaudin et al., 2016). Contraction of the radula protractor muscle in response to SCPRP has been reported in *O. vulgaris* (Kanda and Minakata, 2006) and is consistent with a possible role for this peptide in control of digestive tract motility.

Tachykinin-related Peptide (OcttKrpre, Figures 2A,B, 6). We identified various cells positive to the antibody utilized for this study (TAC1 in **Table 1**) distributed in the internal (smaller diameter) cortical layer of neural cells in the gastric ganglion (**Figure 5O**). In addition, fibers in the nerve bundles and in the neuropil, were also positive (**Figure 5P**). Ov-OcttKrpre appeared downregulated ("high" vs. "low," **Figure 6**). However, gene expression data showed that in octopus with "low" *A. octopiana* parasite load in the digestive tract it was upregulated (when compared with samples with limited/negligible load, data not shown), confirming the view that modulation of gene expression is linked to differing parasitic loads.

Tachykinin related peptides are one of the two families of tachykinin-type peptides occurring in protostomes. Seven members of the tachykinin-related peptide family have been characterized from *O. vulgaris* (Kanda et al., 2003a, 2007) and molecular studies of the brain in both *S. officinalis* and *O. vulgaris* have identified a single precursor molecule that encodes nine peptides amidated at the C-terminal (Zatylny-Gaudin et al., 2016). The molecular data in this study extends the distribution of TKRPs to the visceral innervation in octopus. Further support for TKRPs as putative transmitters in the gastric ganglion comes from the presence of the oct-TKRP receptor (oct-TKRPR) in the ganglion (Kanda et al., 2007). The properties of the cloned oct-TKRPR have been investigated in *Xenopus* oocytes and this revealed that the receptors were most sensitive to oct-TKRP II and III. Interestingly they were insensitive to substance P (Kanda et al., 2007). Further support for TKRPs in the regulation of the digestive tract comes from stimulation of contractions in the esophagus, stomach and crop of octopus by oct-TKRP II and III (H. Minakata et al. unpublished results, cited in Kanda et al., 2007). Our results further extend IHC studies in *Nautilus* (in the heart, Springer et al., 2004) and *Sepia* (referred to as squid, Osborne et al., 1986) where Substance P-like immunoreactivity is reported for the optic lobes (processes and cells).

All the evidence for acetylcholine as a neurotransmitter in the gastric ganglion of *O. vulgaris* is indirect. It is

based upon the presence of the common type of choline acetyltransferase (present study, see **Figures 5G,H**) and acetylcholinesterase (Andrews and Tansey, 1983). We identified a few dispersed clusters of cells in the cortical layers and several fibers in the neuropil where cChAT-IR has been identified. There is extensive evidence for acetylcholine as a neurotransmitter in other parts of both the central and peripheral components of the nervous system in cephalopods (for review see Messenger, 1996; but see also Bellanger et al., 1997, 2005; Kimura et al., 2007; D'Este et al., 2008; Casini et al., 2012; Sakaue et al., 2014) and its role as a neurotransmitter is further supported by genomic evidence for acetylcholine receptor subunits in *O. bimaculoides* (Albertin et al., 2015).

The previous studies using neurochemistry (Juorio, 1971) and histochemistry (Andrews and Tansey, 1983) to demonstrate the presence of adrenergic and dopaminergic neurons in the gastric ganglion of octopus are supported by our gene expression experiments for dopamine β hydroxylase (Dbh in **Figure 6**), catalyzing the reaction that produces noradrenaline from dopamine, and by the tyrosine hydroxylase IR (**Figures 5A,B**). In addition, Class A, rhodopsin has been reported in *O. bimaculoides* genome (Albertin et al., 2015). Acetylcholine has a stimulatory effect on the cephalopod esophagus, but an inhibitory effect (most likely via nicotinic receptors) on the crop and stomach (Wood, 1969; Andrews and Tansey, 1983) Both 5-HT and adrenaline enhance contractile activity in the crop, stomach and intestine (Wood, 1969; Andrews and Tansey, 1983).

Whilst octopamine has been demonstrated in the gastric ganglion by histochemistry (Juorio and Molinoff, 1974), it has not previously been shown using immunohistochemistry. Our study confirms its presence and distribution for the first time in the ganglion (but see for the octopus brain: Ponte, 2012; Ponte and Fiorito, 2015). Octopamine-IR is observed in bouton-like structures suggesting the existence of an intricate octopaminergic network in *O. vulgaris* gastric ganglion. This resembles findings in other invertebrates where the octopaminergic distribution in nervous structures has been described in detail (e.g., Kononenko et al., 2009). In the octopus' central nervous system octopamine-positive neurons are prominent in some lobes (i.e., basal and peduncle lobes; see Ponte, 2012; Ponte and Fiorito, 2015).

Tyrosine Hydroxylase (TH) positive neurons have been reported to be localized in discrete areas of the cephalopod nervous system including cerebral and gastric ganglia of the developing cuttlefish (Baratte and Bonnaud, 2009), and mostly in the posterior buccal lobe of the adult octopus brain (Ponte, 2012; see also Ov-Dopamine transporter in Zarrella et al., 2015), suggesting the existence of a dopaminergic modulatory system in cephalopods. In the gastric ganglion, TH-IR appears clearly in fibers and we cannot exclude the possibility that this contributes to local synthesis of final products (i.e., dopamine, noradrenaline or octopamine) as recently demonstrated in other organisms (e.g., Gervasi et al., 2016; Aschrafi et al., 2017).

GABA has not previously been reported to occur in the octopus gastric ganglion, but is widely distributed in the central nervous system (Cornwell et al., 1993; Ponte et al., 2010; Kobayashi et al., 2013). We found a distributed GABA-IR positivity revealing an intricate network appearing to surround the great majority of neural cells belonging to the external cortical layer of the ganglion. This resembled the description provided by Bogoraze and Cazal (1946), who observed "the cortical ganglion cells placed in a neuronal box formed by 'agglutinated neuroglia' in clear dependence with a glio-vascular network" [Our translation from French]. Noradrenaline-IR seemed to overlap partially with this GABA-IR network surrounding large neural cells. We can only speculate that this resembles the network described by Bogoraze and Cazal (1944) and that this network may represent the "juxta-ganglionic" tissue that seems to be a major component of octopus ganglia. Future studies are required to further support this hypothesis.

Recent findings extend further the role of GABA, providing evidence for functions supplementary to its classic one as a major inhibitory neurotransmitter (i.e., excitatory and inhibitory, Swensen et al., 2000; activation of glial cells, Serrano et al., 2006; "gliotransmitter," Yoon and Lee, 2014). A close dialogue between neuro-glia modulatory systems has been also reported for norepinephrine (e.g., Gordon et al., 2005).

Functionally speaking however, neither GABA nor octopamine had an effect on the esophagus, crop or stomach in *O. vulgaris* (Andrews and Tansey, 1983).

Apart from the octopressin (OPR; **Figures 2A,B**, **6**) and cephalotocin receptors (CTR1, **Figure 2A**; see also above), we identified, for the first time, cholecystokinin$_A$ and cholecystokinin$_B$ receptors and the orexin$_2$ receptor (Hcrtr2; **Figures 2A,B**, **6**) in the octopus gastric ganglion. CCK-like peptides and receptors are present in invertebrates (e.g., sulfakinin [SK] family, Yu and Smagghe, 2014). *In silico* studies have identified members of the CCK/SK family in molluscs (Zatylny-Gaudin and Favrel, 2014). In addition, a member of the CCK family has been identified in the brain and hemolymph of *S. officinalis* (Zatylny-Gaudin et al., 2016). The latter observation raises the possibility that the CCK receptors identified in the gastric ganglion in the present study may be responsive to CCK acting as a hormone.

The putative orexin$_2$ receptor we identified (Hcrtr2, **Figures 2A,B**, **6**) is a GPCR, 7TM domain, rhodopsin-like receptor. This finding is potentially problematic as the ligand orexin, implicated in vertebrates in food intake regulation and digestive tract motility (e.g., Kirchgessner, 2002; Volkoff, 2016), is reported to not be present in invertebrates (Scammell and Winrow, 2011). However, the orexin and allatotropin receptors are proposed to be related to each other, and allatotropins are present in protostomes (Mirabeau and Joly, 2013) including cuttlefish (Zatylny-Gaudin et al., 2016). The allatotropin receptor is not annotated in the *O. vulgaris* transcriptome as such (Baldascino and Fiorito, unpublished), but the sequence of orexin we found has a relative similarity with *Sepia*-allatotropin (55.8%; data not shown). Functional studies of the allatotropin and orexin family of peptides in cephalopods

TABLE 4 | Summary of evidence from the literature and the present study for putative neurotransmitters, neurotransmitter/hormone receptors and neurotransmitter synthesis/destruction enzymes in the gastric ganglion of *O. vulgaris*.

Ligand*	References	Techniques	Receptor	Transmitter synthesis/enzyme release/uptake/destruction	Effect of ligand on *O. vulgaris* digestive tract tissue *in vitro*
NON-PEPTIDES					
Acetylcholine	Andrews and Tansey, 1983	HC, Phy		Acetylcholinesterase	Increased tone: oesophagus
	Present study	IHC		Common type choline acetyl transferase	Decreased tone/inhibition of contractions: Crop, stomach
Dopamine	Juorio, 1971	NC			
	Andrews and Tansey, 1983	HC, Phy			Tone increase: crop
	Present study	IHC		Tyrosine hydroxylase	
Gamma amino butyric acid	Andrews and Tansey, 1983	Phy			No effect on esophagus, stomach or rectum
	Present study	IHC			
5-hydroxytryptamine	Andrews and Tansey, 1983	Phy			Decreased tone and contractions: esophagus
	Present study	IHC			Increased tone contraction amplitude: intestine
Noradrenaline	Juorio, 1971	NC			
	Andrews and Tansey, 1983	HC, Phy			Contraction stimulation: oesophagus, crop, stomach, intestine, rectum
	Present study	RT-qPCR		Dopamine β-hydroxylase	
Octopamine	Juorio and Molinoff, 1974	NC			
	Andrews and Tansey, 1983	Phy			No effect on any region
	Present study	IHC			
PEPTIDES					
Cephalotocin	Kanda et al., 2003b	Cloning	Cephalotocin		
	Takuwa-Kuroda et al., 2003	Phy			No effect on rectum
	Present study	RT-qPCR			
Cholecystokinin/gastrin	Andrews and Tansey, 1983	Phy			No effect (pentagastrin) on any region
	Present study	RT-qPCR	$Cholecystokinin_A$		
Cholecystokinin	Andrews and Tansey, 1983				No effect (pentagastrin) on any region
	Present study	RT-qPCR	$Cholecystokinin_B$		
Corticotrophin releasing factor	Present study	IHC			
FMRF-amide	Present study	IHC			See discussion
Gonadotrophin releasing hormone (GnRH)	Kanda et al., 2006	Cloning, Phy	GnRHR		Contraction of radula retractor muscle
Molluscan insulin-related peptide 3	Present study	RT-qPCR			
Octopressin (OP)	Takuwa-Kuroda et al., 2003	Cloning, Phy			Increased tone and contraction amplitude: rectum
	Kanda et al., 2005	Cloning	OPR		
	Present study	RT-qPCR	OPR		
Orexin	Present study	RT-qPCR	Orexin receptor type 2		
PRQFV-amide	Present study	RT-qPCR			
Small cardioactive peptide-related peptide	Kanda and Minakata, 2006	Cloning			Contraction of radula protractor muscle
	Present study	RT-qPCR			
Tachykinin related peptide (TKRP)	Kanda et al., 2003a	Cloning			
	Kanda et al., 2007	Cloning	TKRPR		

(Continued)

TABLE 4 | Continued

Ligand*	References	Techniques	Receptor	Transmitter synthesis/enzyme release/uptake/destruction	Effect of ligand on *O. vulgaris* digestive tract tissue *in vitro*
	Minakata et al., unpublished (cited in Kanda et al., 2007)	Phy			TKRPII/III stimulation of contractions in esophagus, crop and stomach
	Present study	IHC, RT-qPCR			

Non-peptides are shown in the upper part of the table and peptides in the lower half. In the absence of functional studies on the gastric ganglion data is included showing the effect of the ligand (or a closely related molecule) on tissue from the digestive tract in O. vulgaris and studied in vitro. Note that functional studies are very limited and all results, particularly negative findings, should be treated with caution until confirmed. HC, histochemistry; IHC, immunohistochemistry; NC, neurochemistry; Phy, physiological experiments; RT-qPCR, quantitative polymerase chain reaction. See section Discussion for more detailed analysis of ligands identified in O. vulgaris gastric ganglion.
**Italics in the ligand column indicates that evidence for the presence of the ligand is indirect based on the presence of the presumed receptor or synthetic/destructive enzymes.*

are required to characterize the putative octopus orexin$_2$ receptor.

Octopus Gastric Ganglion Responses to *Aggregata octopiana* Infection

We found changes in the relative expression of 24 genes present in the transcriptome of *O. vulgaris* when we analyzed the mRNAs of animals with relatively "low" vs. "high" *A. octopiana* loads (**Figure 6**). Differences were observed for genes responsible for the synthesis and release of molecules implicated in neurotransmission and in those with a potential role in inflammation and oxidative stress.

In octopus with higher levels of *Aggregata* infection, a relative increase of gene expression was found (**Figure 6**) for: the CCK$_A$ receptor and the small cardioactive peptide-related peptide (*SCPRP*), *Nfkb2* Ov-*Tlr3*. *Nfkb2*is known to be the endpoint of a series of signal transduction events initiated by biological processes including inflammation, immunity, cell differentiation and growth, tumorigenesis and apoptosis. A member of the toll-like receptor family (Ov-*Tlr3*) plays a fundamental role in pathogen recognition and activation of innate immunity.

In contrast, we observed a reduced gene expression for dopamine β-hydroxylase, cephalotocin, tachykinin-related peptide, PRQFV-amide and the orexin$_2$ receptor (**Figure 6**). This was also the case for *Litaf,* considered to play a role in endosomal protein trafficking and in targeting proteins for lysosomal degradation, thus contributing to downregulation of downstream signaling cascades.

The above functions are deduced from Universal Protein Resource (UniProt).

The paucity of functional studies in cephalopods makes it difficult to predict the functional consequences; the following discussion is speculative.

The higher expression of CCK$_A$ receptors and a similar directional change in CCK$_B$ receptors combined with lower levels of the orexin$_2$ receptor gene is interesting since in vertebrates activation of the orexin receptor stimulates food intake (e.g., for mammals, Wong et al., 2011; for fish, Volkoff, 2016) while CCK is inhibitory (e.g., for mammals, Dockray, 2014; for fish, Volkoff, 2016). The other potential effect of the differences in gene expression would be on the movements of the digestive tract by altering gastric ganglion outputs. The reduced expression of the genes for dopamine β-hydroxylase, tachykinin-peptide related peptide and orexin$_2$ receptor would be predicted to reduce the overall contractile activity of the digestive tract, which may be advantageous for the parasite to reduce expulsion. However, the relatively lower expression of PRQFV-amide is not consistent with this overall effect of *Aggregata,* assuming that PRQFV-amide is inhibitory in octopus digestive tract as is the case in *Aplysia* (Furukawa et al., 2003). Finally, SCPRP stimulates contractions of the radula in *O. vulgaris*. Our findings of increased expression levels in octopuses with high levels of infection appear to be inconsistent with inhibition of digestive tract motility.

The products of the Rab effector Noc2 gene are implicated in exocytosis and the lower levels in the gastric ganglion with increased *Aggregata* infection would be anticipated to negatively impact release of signaling molecules.

The molluscan insulin-related peptide 3 gene showed the largest difference between the two groups with relatively reduced gene expression in the highly infected group. Nothing is known of the function of this peptide in cephalopods, but a role either in regulation of food intake or regulation of metabolism appears likely.

Functional studies are required to resolve the above speculations, but the cluster of gene changes observed should focus attention on control of food intake and digestive tract motility. Whilst reduction of growth in *Aggregata*-infected *O. vulgaris* has been attributed to "malabsorption syndrome" induced by the pathological and physiological effects on the digestive tract (Gestal et al., 2002a,b), this may be further exacerbated by impaired motility and suppression of food intake resulting from effects on the innervation of the digestive tract.

Previous studies of the molecular responses to *Aggregata* infection in *O. vulgaris* have focused on the changes in the hemolymph (Castellanos-Martínez et al., 2014a,b), gills and caecum (Castellanos-Martínez et al., 2014a), but not neural tissue, although the caecum is likely to have contained some enteric neurons. In the gastric ganglion, we demonstrated a relative increase in the expression of several genes with products related to tissue inflammatory responses including *NFκB2*, *Tlr3*, *Sn*, and *Serpin b10*. Toll-like receptors and NFκB pathways have previously been identified in *O. vulgaris* and the Tlr-2 appeared upregulated in hemocytes, gills and the caecum of *Aggregata* infected octopus (Castellanos-Martínez et al., 2014a).

These findings are consistent with the present study and indicate a systemic inflammatory response.

Amongst the potential inflammatory mediators, the NFκB gene showed the largest magnitude relative difference (high infection > low infection) of all the genes studied with Tlr-3 receptor next. The demonstration of the expression of the Tlr-3 gene in the gastric ganglion of *O. vulgaris* is of particular relevance as the same receptor has been identified in the ENS and dorsal root ganglia of mice (Barajon et al., 2009). The relatively higher levels of Serpin B10 would be expected to reduce peptidase activity contributing to the prevention of inflammatory damage. In the hemolymph of healthy octopus, fascin mRNA (i.e., protein singed; **Table 2**) was expressed at higher levels than in animals with a high level of *Aggregata* (Castellanos-Martínez et al., 2014a) which is not consistent with the present study, but may indicate tissue-specific responses. It is interesting that the proteomic part of the same study showed a significant increase in fascin in the animals with a high level of *Aggregata* (Castellanos-Martínez et al., 2014b).

Among the pro-inflammatory genes, only lipopolysaccharide-induced TNFα factor was at a relatively lower level in the highly infected group. This gene has been implicated in the cephalopod immune response (Gestal and Castellanos-Martínez, 2015).

Three genes which have been implicated in oxidative stress (superoxide dismutase, peroxiredoxin 6 and glutathione peroxidase) were expressed at relatively lower levels in the gastric ganglion of animals with the higher level of *Aggregata* infection. These findings are consistent with the previous hemolymph proteomic study which showed a downregulation of peroxiredoxin in octopuses with a higher level of *Aggregata* infection (Castellanos-Martínez et al., 2014b). As reactive oxygen species are one of the host defense mechanisms, relatively lower levels in more highly infected animals may be due to the actions of the parasite to enhance its survival. *Aggregata* is an Apicomplexan parasite and this group appears to be particularly sensitive to oxidative stress (Bosch et al., 2015).

Although the difference between the groups ("high," "low") is clear, we do not know how long the animals in the present study had *Aggregata*, whether the changes we observed are acute or chronic, if neural tissue other than the gastric ganglion (e.g., brain) is affected by the systemic immune changes or if the changes are reversible if the infection is cleared.

CONCLUSIONS

This combined *in silico*, molecular and immunohistochemistry study, although preliminary, has provided additional evidence for a complex neurochemical fingerprint of the octopus gastric ganglion including the identification of a number of peptide ligands/receptors for the first time in a cephalopod. By providing for the first-time evidence that the parasitic load of the digestive tract in octopus results in differences in the molecular profile in neural tissue regulating digestive tract function (i.e., the gastric ganglion), our data suggest that the possible pathophysiological effects of *Aggregata* extend beyond epithelial damage and systemic immune responses (cf. digestive parasites in mammals) to include the peripheral nervous system.

We summarized knowledge from the literature and results from the present study to illustrate the diversity of ligands and receptors in the *O. vulgaris* gastric ganglion (see **Table 4**) and functional effects on the motility of the digestive tract.

The structural and neurochemical complexity of the gastric ganglion together with its relatively large size suggest that it is not a simple relay between the brain and the digestive tract and it is likely to have an integrative role analogous to the ganglia in the stomatogastric systems of crustaceans (e.g., Swensen et al., 2000; Hedrich et al., 2011; Dickinson et al., 2015; Daur et al., 2016, and molluscs, Jing et al., 2007). Coordination of the passage of digestive tract contents from one region to another, as digestion proceeds, would be one such integrative function to explore.

AUTHOR CONTRIBUTIONS

PA and GP conceived and designed the experiments; EB, GD, TS, and GP performed experiments; PT and CH carried out some of the experiments; GD provided *in silico* analysis of the transcriptome; EB, GD, GP collected and analyzed the data. All authors discussed the results, contributed to writing and commented on the manuscript at all stages. All authors read and approved the submitted manuscript.

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The Digestive Tract of Cephalopods: Toward Non-invasive *In vivo* Monitoring of Its Physiology

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Ensuring the health and welfare of animals in research is paramount, and the normal functioning of the digestive tract is essential for both. Here we critically assess non- or minimally-invasive techniques which may be used to assess a cephalopod's digestive tract functionality to inform health monitoring. We focus on: (i) predatory response as an indication of appetitive drive; (ii) body weight assessment and interpretation of deviations (e.g., digestive gland weight loss is disproportionate to body weight loss in starvation); (iii) oro-anal transit time requiring novel, standardized techniques to facilitate comparative studies of species and diets; (iv) defecation frequency and analysis of fecal color (diet dependent) and composition (parasites, biomarkers, and cytology); (v) digestive tract endoscopy, but passage of the esophagus through the brain is a technical challenge; (vi) high resolution ultrasound that offers the possibility of imaging the morphology of the digestive tract (e.g., food distribution, indigestible residues, obstruction) and recording contractile activity; (vii) needle biopsy (with ultrasound guidance) as a technique for investigating digestive gland biochemistry and pathology without the death of the animal. These techniques will inform the development of physiologically based assessments of health and the impact of experimental procedures. Although intended for use in the laboratory they are equally applicable to cephalopods in public display and aquaculture.

Keywords: cephalopods, digestive tract, Directive 2010/63/EU, feces, food intake, nutrition, ultrasound, welfare assessment

INTRODUCTION

Interest in cephalopods and their welfare is stimulated by significant policy changes initiated at European Union (EU) level, that are recognized to have an impact beyond Europe (Di Cristina et al., 2015). Such European Policies and Directives have implications for cephalopods in scientific research, aquaculture or public display, and relevance for fisheries and ecology, i.e., Directive 2010/63/EU, EU Common Fisheries Policy, Marine Strategy Framework Directive, the revision of IUCN Red List (review in: ICES, 2014; Di Cristina et al., 2015; Xavier et al., 2015).

Cephalopods are a special case in a "regulation" context since: (i) it is the first time that research on an entire class of invertebrate has been regulated in the European Union (Smith et al., 2013), and (ii) the species are very diverse in terms of ecological, biological, physiological, and behavioral adaptations (e.g., Packard, 1972; Borrelli and Fiorito, 2008; Grasso and Basil, 2009; Kröger et al., 2011; Albertin et al., 2015).

Daily assessment of animal well-being is required under Directive 2010/63/EU (Fiorito et al., 2015), but few indicators of digestive system physiology are available. Fiorito and co-workers list five (out of 27) signs potentially linked to the digestive system that may indicate an alteration in normal behavior or physiology (see Table 5 in Fiorito et al., 2015).

How can the functionality of the digestive tract be assessed routinely at the "tank–side"?

Here we review and suggest a series of non-invasive and/or minimally invasive approaches (**Table 1**).

PREDATORY RESPONSE AND FOOD INTAKE

The vast majority of cephalopod species are active predators, and prey attack is considered to be a good indicator of overall health (Fiorito et al., 2014). Latency to attack either a live- or an artificial crab is used as an indication of health in *Octopus vulgaris* (Amodio et al., 2014), and a prompt feeding response to a live fish is proposed for cuttlefish and squid as an indication of full recovery after transport (Oestmann et al., 1997). Latency to attack may vary between individuals (Lee et al., 1991) which probably denotes different temperaments (e.g., Sinn et al., 2001; Sinn and Moltschaniwskyj, 2005; Borrelli, 2007; Carere et al., 2015), and these differences should be taken into account (see discussion in Borrelli and Fiorito, 2008). In addition, it may be influenced by housing, as shown by laboratory reared juvenile cuttlefish that have a shorter attack latency (three times faster) if housed together (Warnke, 1994), and if there is more available space (Boal et al., 1999).

Ingestion of a normal amount of food (assuming "normal" can be defined for species, age, sex, and temperature) at a regular frequency is probably the most useful indicator of health. However, monitoring food intake requires knowledge of the amount of food provided and of possible remains. Therefore, tanks should be inspected for empty carapaces, shells, and other residues as well as uneaten food. For prepared diets whenever accepted, allowance needs to be made for portions of the food pellet lost, due to leaching and disaggregation, and not ingested.

If animals are fed live prey, uneaten specimens must be removed to avoid possible welfare issues; the prey may become the "predator," particularly if the size difference between the two individuals is not large (e.g., a small cuttlefish and a shrimp). In addition, leaving uneaten prey may lead to inaccurate estimation of intake over a particular time period.

An additional issue is to ensure that every animal has sufficient food. This can be challenging for species which may be housed in groups such as *S. officinalis*, *Loligo vulgaris* (or other squid) or *O. vulgaris* that are kept under culture conditions. This may be assisted by observation, but feeding hierarchies should be taken into consideration when animals (e.g., cultured cuttlefish) are housed together (see discussion in Warnke, 1994). To circumvent these issues, and again in an aquaculture context animals are fed *ad libitum*, but accurate monitoring of food remains is required.

Food intake may decrease with increasing size in cephalopods, and depends on food availability, its quality and size, the duration of digestion, maturation, and temperature (Mangold, 1983).

Feeding behavior and food intake may be affected by experimental procedures; resumption of the normal status should be assessed at individual animal level. A few examples from the literature are provided below.

Octopus tetricus attacked and ate crabs within 15 min after brief anesthesia used to facilitate handling for weighing (Joll, 1977). Similarly, *S. officinalis* resumed feeding on grass shrimp 7 min after recovery from anesthesia used for handling (Gonçalves et al., 2012). Cuttlefish resumed feeding 48 h post-surgery after kainic acid lesion of the vertical lobe (Graindorge et al., 2008). *O. vulgaris* performed a normal predatory response 1 h after anesthesia and arm surgery (Shaw et al., 2016). However, recovery of the attack response following cold water "anaesthesia" in the same species is prolonged with respect to circumstances when magnesium chloride is used as agent (Agnisola et al., 1996).

Cephalopod paralarvae or other early stages provide further challenges. Ingestion of food and estimation of food intake may be assessed by direct observation or through the use of microfluorospheres (around 10 μm diameter) included in the food (see Villanueva and Norman, 2008, and below), since at early stages most species are transparent.

BODY WEIGHT AND DEVIATION FROM "NORMALITY"

Loss of body weight or growth below particular pre-set limits are frequently used as humane end points (see discussion in: Smith et al., 2013; Fiorito et al., 2015) in regulated procedures. However, setting limits requires knowledge of the normal variations in body weight for the species studied and the housing conditions (particularly water temperature). Total body length is not considered a valid index of growth in cephalopods because of variables including the elasticity of tentacles and arms, and differences in relative growth linked to sex and season (e.g., Bello, 1991; Cortez et al., 1999; Pierce et al., 1999; Sivashanthini et al., 2009).

Gross growth efficiency is dependent on food quality, its ingestion and water temperature (Mangold, 1983). Growth curves for several cephalopod species in captivity show a reduction in growth rate over Winter, due to a temperature decrease in open aquaria (e.g., Nixon, 1966; Joll, 1977; Boyle and Knobloch, 1982; Domingues et al., 2002). However, in constant temperature aquaria, a reduction in growth rate is most likely due to reduced food intake.

Additionally, the available bottom area of the tank and the stocking density may also influence growth rates (see for example: Forsythe et al., 2002; Correia et al., 2005; Delgado et al., 2010; Domingues and Márquez, 2010; Domingues et al., 2010).

TABLE 1 | Summary of parameters that could be used to monitor cephalopod digestive tract functioning by non-invasive or minimally invasive techniques to provide either a direct or indirect insight into the physiology of the digestive tract. Examples are taken mainly from studies on S. officinalis or Nautilus pompilius and/or octopus (mostly O. vulgaris), but all techniques appear equally applicable to squid.

Parameter monitored	Technique	Hypothesized potential welfare significance in relation to digestive tract function	References
Body weight	Regular weighing (preferably in water)	Indicates normal food intake, digestive tract functionality, and metabolism	Nixon, 1966; Joll, 1977; Boyle and Knobloch, 1982; Domingues et al., 2002; Semmens et al., 2004; García-Garrido et al., 2010; Sykes et al., 2014
Predatory behavior and interest in food	Behavioral observations Food seeking behaviors, time to capture live or alternative prey	Normal appetitive drive, major digestive tract pathology, unlikely	Oestmann et al., 1997; Borrelli and Fiorito, 2008; Amodio et al., 2014
Food intake (amount, normal time course of ingestion, and feeding frequency)	Behavioral observations (including video recording) Inspection of tank for uneaten food and residues	Ingestion mechanisms functional, major digestive tract pathology, unlikely Visceral pain (if present in cephalopods), unlikely	Guerra and Nixon, 1987; Grisley et al., 1999; Semmens et al., 2004; García-Garrido et al., 2010; Lamarre et al., 2012
Oro-anal transit time	Dye, indigestible, or radio-opaque markers in the food	Normal digestive tract functionality	Bidder, 1957; Westermann et al., 2002
Presence of food in the digestive tract, normally distributed and contractile activity present	Ultrasound, X-ray (±contrast medium) Direct observation in transparent paralarvae	Indicates ingestion of food and, if normally distributed, shows lack of structural defects and indirectly normal motility (visible with ultrasound, see **Figure 1**)	Westermann et al., 2002; **Figure 1** (ultrasound), *this paper*
Appearance of digestive tract mucosa	Endoscopy (digestive tract)	Identification of mucosa defects and presence of parasites in the lumen	No publications in cephalopods (but see Sykes et al., 2017) Utilized in finfish Moccia et al., 1984, crabs Heinzel et al., 1993, and veterinary medicine Sladakovic et al., 2017
External appearance of rectum	Endoscopy (mantle)	Cysts of some parasites (e.g., *Aggregata* ssp) may be visible	No publications in cephalopods For *Aggregata* see Mayo-Hernández et al., 2013
Digestive gland size (% body weight) and density	High resolution ultrasound	Global indicator of metabolic status and functionality of digestive tract Increased water content indicates catabolism and should be detectable during sonographic examination	García-Garrido et al., 2010; Speers-Roesch et al., 2016 Supplementary Figure 1, *this paper*
Digestive gland biochemistry	Needle biopsy	Indicator of overall metabolic status and normal functioning of the digestive tract[a]	No publications on needle biopsy in cephalopods, but well established in humans (e.g., Kim and Shin, 2017) For biochemistry approaches see: Lopes et al., 2014; Lamarre et al., 2016; Speers-Roesch et al., 2016; Penicaud et al., 2017
Mantle muscle thickness and gill morphology (remodeling)	Ultrasound	May indicate severe food deprivation or chronic failure of digestive tract function	No formal studies in cephalopods, but for mantle/gill morphology during food deprivation see Lamarre et al., 2012 See **Figure 1**, *this paper*
Regurgitation of food	Behavioral observation (including video recording)	May indicate toxic food, digestive tract obstruction, or disordered neural control	Unpublished observations cited in Andrews et al. (2013) A. Sykes and E. Almansa, unpublished observations

(Continued)

TABLE 1 | Continued

Parameter monitored	Technique	Hypothesized potential welfare significance in relation to digestive tract function	References
Defecation frequency change	Behavioral observation (including video recording)	Indirect measure of digestive tract functionality, but only reflects handling of previous meal May indicate stress, digestive tract infection, or alteration in secretion, absorption of motility, defective control	No formal studies in cephalopods
Fecal form, color, composition change	Behavioral observation (including video recording) Fecal collection (molecular and/or cytological analysis)	Form may reflect motility of the lower digestive tract; color most likely related to diet; composition reflects epithelial water transport, but parasites/bacteria may be present and cytology may eventually reveal disease	No formal studies (see main text for incidental observations in the literature)
Urine composition: Tank water [ammonia]	Chemical or electrochemical detection (potentially real time, continuous monitoring)	Indication of nitrogenous metabolism reflecting protein intake and overall metabolic status	Boucher-Rodoni and Mangold, 1985; Segawa and Hanlon, 1988; Katsanevakis et al., 2005; García et al., 2011
Oxygen consumption	Metabolic chamber	Assessment of normal metabolism and indication of catabolic state of O_2:N_2 ratio	Boucher-Rodoni and Mangold, 1988; Valverde and García, 2005; Capaz et al., 2017
Circulating nutrients, metabolites	Hemolymph sampling	Indicates normal functioning of the digestive tract and particularly the digestive gland	Aguila et al., 2007; Linares et al., 2015
Circulating hormones, including those regulating the digestive tract	Hemolymph sampling	Insights into the control of the digestive tract, but also potential larkers of immune response (e.g., TNFα) or stress	No formal studies of digestive tract hormones in cephalopods, but see Zatylny-Gaudin et al., 2016 for peptidome study including haemolymph for candidates
Circulating inflammatory markers	Hemolymph sampling	May indicate the presence of a pathogen in the digestive tract	e.g., Castellanos-Martínez et al., 2014a,b; Gestal and Castellanos-Martínez, 2015

Obstruction of the hepatopancreatic duct would prevent both water and nutrient absorption by the digestive gland (Wells and Wells, 1989).

The contribution of total body water (intra- and extracellular) to body weight should not be overlooked. Cephalopods ingest and absorb water from the sea and from the diet via the digestive tract, and prevention of fluid absorption from the digestive tract leads to ∼10% loss in body weight in 24 h in *O. vulgaris* (Wells and Wells, 1989, 1993).

Body weight loss should always be investigated, and if a reduction in food intake is identified as the primary cause then this also needs investigation.

Body weight and food intake decrease in several species with maturation (e.g., Mangold and Boletzky, 1973; Lee, 1995) and around the time of egg laying, and since this may affect females, their reproductive status should be checked in cases of evident reduction of body weight/food intake. In females of many, but not all species, reproductive status is accompanied by loss of body weight due to reduced or absent food intake (e.g., Wodinsky, 1977; but see Sykes et al., 2013b), and reduction of food intake may also occur in senescent mature males (Anderson et al., 2002).

Individual tissues may be affected to a greater degree than is apparent from body weight. For example, the proportionate weight loss of the digestive gland in food deprived *O. vulgaris* is greater than might be suspected from the change in body weight (see Supplementary Figure 1).

If an animal is losing weight, but food intake is within normal limits then the cause is likely to be the functioning of the digestive tract. However, as the pathophysiology of the cephalopod digestive tract has not been studied in any detail, we can only hypothesize about the causes by analogy with vertebrates (see **Table 1**).

Body weight is probably the best overall indicator of adequate nutrition, but it may not be the most practical parameter to use for routine health assessment. Repeated measures of body weight show variability (Nixon, 1971) and may induce effects linked to handling (e.g., Locatello et al., 2013). Indeed, the effects of repeated measures of body weight on animal welfare have not been properly investigated, to the best of our knowledge. Repeated handling of the animal may be stressful (for example see: Malham et al., 1998; Grimaldi et al., 2013). According to Nixon (1966) frequent handling of *O. vulgaris* may reduce growth rate, but this does not appear to be the case in cuttlefish (Sykes et al., 2003, 2013a,b).

Weighing usually requires removal of the animal from its tank for measurement in air, although animals can be weighed in water (Aronson, 1982). Body weight may not be a sufficiently sensitive welfare indicator for daily assessment, although it may be useful over longer intervals. However, for species housed in groups reliable identification of individuals is required, and several methods are described for cephalopods (e.g., Huffard et al., 2008; Ikeda et al., 2009; Zeeh and Wood, 2009; Byrne et al., 2010; Barry et al., 2011; Estefanell et al., 2011; Sykes et al., 2017).

ORO-ANAL TRANSIT TIME

This is the time from food ingestion until exit as feces, and provides an overall measure of the key digestive tract functions of motility, secretion, and absorption. The most common method is to mark the food with a chemical marker (e.g., carmine in Bidder, 1957) or to incorporate an indigestible marker into the food (e.g., glass beads encapsulated in *Artemia* nauplii, Villanueva and Norman, 2008). However, if particular dietary constituents are associated with a fecal color change then by diet switching it would be possible to estimate transit time for that food.

A study in *Nautilus pompilius* applied X-ray imaging of food labeled with contrast medium (barium sulfate) to monitor the time course of digestion (about 12 h at 18–19°C Westermann et al., 2002). This method has been also explored in *S. officinalis* (**Figure 1H**). However, this requires brief but repeated anesthesia which, together with restraint and handling, is likely to affect the oro-anal transit time of the animal.

The relatively few studies undertaken measured oro-anal transit times (see Supplementary Table 1) ranging from 2–10 h in squid (temperatures 16–22°C) to 8–24 h *in S. officinalis* (14–23°C), and 8–30 h in octopus (10–30°C). Overall, transit is faster in animals living at higher water temperatures; the slowest time we were able to find was for *Benthoctopus levis* (>30 h at 6°C: Mangold and Lu, unpublished, cited in Mangold and Bidder, 1989).

High resolution ultrasound may provide a method for monitoring movement of digestive tract contents (see below), provided that the animal could be adapted to remain relatively quiescent during repeated imaging sessions lasting a few minutes. This approach is tractable in cuttlefish and octopus, but may be problematic with squid because of their preference to moving in the water column rather than remaining quiet on the bottom of a tank. It is important that measurements are taken in non-sedated and unrestrained animals as this will modify the results.

FECAL OUTPUT

Fecal analysis provides a non-invasive method for monitoring digestive tract functionality. Defecation and fecal composition are considered separately.

Defecation

Based upon transit times (see above) and tank inspection it is usually assumed that defaecation takes place at least once daily in *S. officinalis* and *O. vulgaris,* but this has yet to be confirmed by a direct study.

Defecation is highly likely to be under central nervous system control as "reflex" expulsion of feces, in the presence of a predator when an animal is attempting to hide, would be disadvantageous. Central control of defecation is likely to be via the visceral nerve originating in the palliovisceral lobe (part of the posterior sub-oesophageal mass) which in *O. vulgaris* is described as supplying specific branches with relatively large axons (∼5 μm) to the terminal rectum and anal flaps (Young, 1967). Injection of 5-hydroxytryptamine (5-HT) into the brain blood supply (via an implanted cannula in the dorsal aorta) evoked defecation in *O. vulgaris.* This was not induced by nicotine, gamma amino butyric acid, and L-glutamate injections (Andrews et al., 1983). Defecation was not evoked by 5-HT following removal of the supra-oesophageal lobes, suggesting that it may be under "higher" motor control.

Descriptions of defecation in cephalopods are rare; in both cuttlefish and *O. vulgaris* feces are expelled from the anus in a "rope" and are ejected from the mantle cavity via the siphon by mantle contraction referred, for octopus, as a "cough" (Wells, 1978).

Studies of defecatory behavior are needed to establish normal patterns for each laboratory housed species in relation to the diet. In this way criteria can be set for when a change (increase or decrease) requires investigation or intervention.

Fecal Appearance and Composition

Descriptions of the appearance of cephalopod feces are scant. In *N. pompilius* fed on shrimp feces are described as "red-brown threads, 2 cm in length and with no solid components" (Westermann et al., 2002, p. 1620). These appear "long filiform, but quite variable in size and in color" in *O. vulgaris* (Taki, 1941), and grayish brown in color when the animals are fed bivalves. An orange brown color is more characteristic of the feces in *O. vulgaris* fed on crabs, and an orange/red color is characteristic of feces of *S. officinalis* fed on grass shrimp or crabs most likely due to carotenes. Feces after feeding animals with fish or prepared diets not rich in crustaceans will lack any obvious pigmentation and may be white. The "fecal ropes" have an obvious mucus coating and presumably contain excretory products of metabolism from the digestive gland and any undigested or unabsorbed food. Feces may contain "chips of cuticle" and fish scales (Wells, 1978) and dead cells (a possible source of cells for genotyping) sloughed from the digestive tract

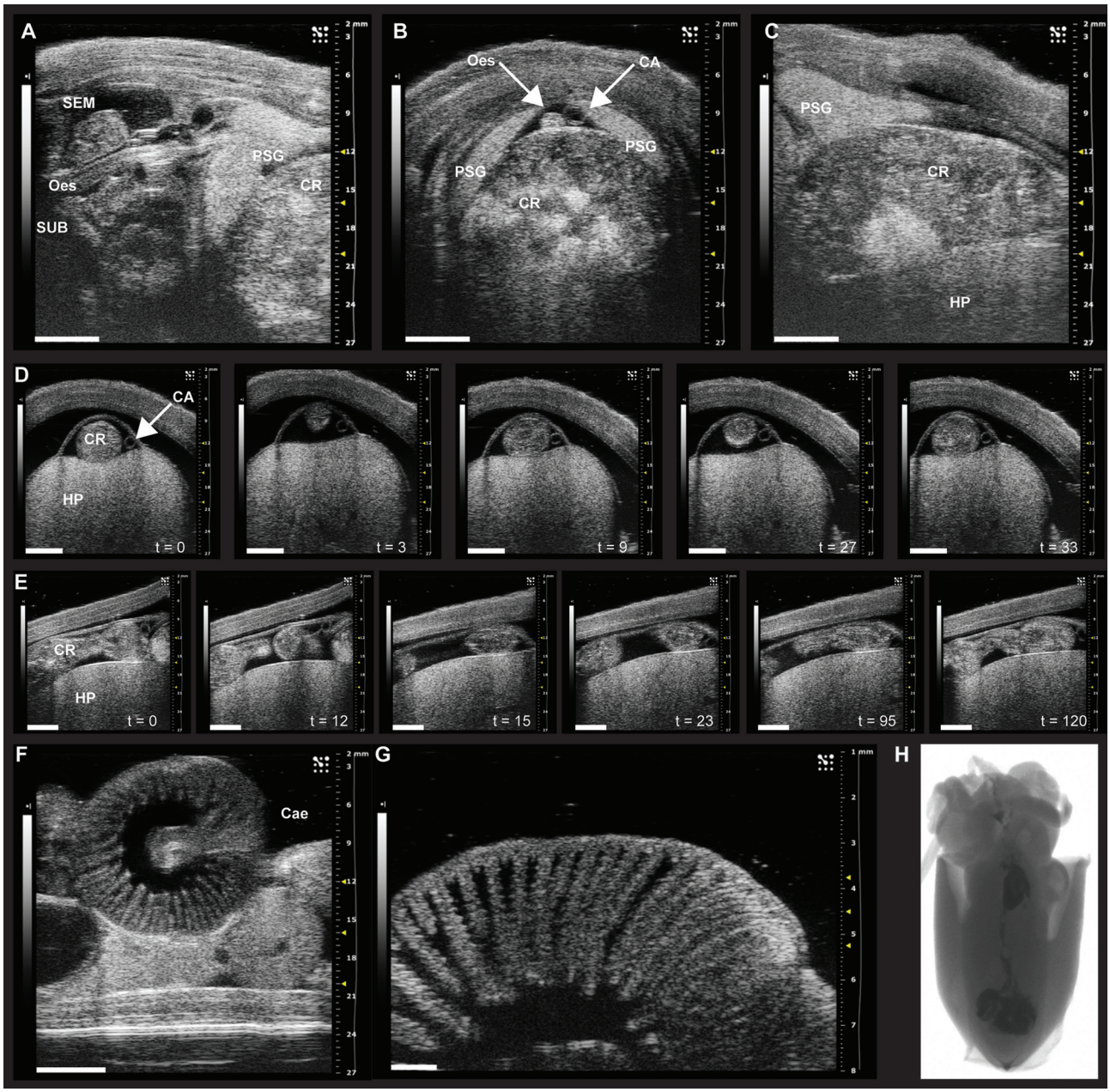

FIGURE 1 | Sonographic scanning of the digestive tract of *Octopus vulgaris* **(A–G)** and X-ray imaging in juvenile *S. officinalis* **(H)**. **(A–G)** The digestive tract of *O. vulgaris* as it appears during ultrasound examination (VEVO 2100, VisualSonics). **(A–C)** The anterior part of the digestive system (note the crop full of food) and its relationship to other parts within the mantle. **(A)** Ultrasound examination in the longitudinal plane with supra- (SEM) and sub-oesophageal masses (SUB, sagittal view) and the esophagus (Oes) and the crop (CR, on the right). The posterior salivary glands (PSG) are also clearly identifiable. **(B)** Sonographic scanning using a transverse plane reveals a distended crop (CR) full of food, the esophagus (Oes), and the cephalic aorta (CA) lying on its dorsal surface between the posterior salivary glands (PSG). **(C)** Sonographic examination (longitudinal plane) showing one posterior salivary gland (PSG) with its typical leaf-shaped appearance, the distended crop (CR), and the hepatopancreas (HP), ventrally. **(D)** A sequence of frames from the sonographic examination (transversal plane) of the octopus digestive tract octopus reveals the crop of an animal fed 6 h before the ultrasound scan (about 30 s); the peristaltic motility of the crop is evident through the sequence of snapshots (from t = 0 to 33 s) with contractions and relaxations moving the crop contents. **(E)** The sequence of frames taken from the same animal during ultrasound examination in the longitudinal plane identifies contraction and relaxation of the crop dividing the bolus. **(F)** The caecum with its characteristic spiral organization as it appears during sonographic scanning. **(G)** High resolution (48 MHz) ultrasound scanning of the caecum showing the "villi-like" structures. **(H)** X-ray imaging of food labeled with contrast medium (barium sulfate) to monitor the course of digestion in juvenile *S. officinalis*. Scanning performed with a Kodak DXS-4000 Pro system on anesthetized individual. CA, cephalic aorta; Cae, caecum; CR, crop; Oes, esophagus; HP, hepatopancreas; PSG, posterior salivary gland; SEM, supra-oesophageal mass; SUB, sub-oesophageal mass. Images provided here resulted from examinations carried out in compliance with local regulations, and for veterinary purposes. Scale bar, **A–F**: 5 mm; **G**: 1 mm.

epithelium or the digestive gland, digestive tract flora, and shed parasites and cysts.

Steroid hormones have been detected in the feces of *Enteroctopus dofleini* (Larson and Anderson, 2010), further illustrating the potential of feces as source of biomarkers thus serving as indicators of animals' health and welfare. Overall, the feces are an overlooked potential source of information about the physiology of the digestive tract and their utility as a non-invasive monitor of animal health should be investigated.

ENDOSCOPY AND ULTRASOUND

Endoscopy is a technique widely used for human and veterinary clinical investigation of the digestive tract (for example see Fritscher-Ravens et al., 2014; Sladakovic et al., 2017) to examine the mucosa for abnormalities (e.g., polyps, parasites) or to perform a biopsy for subsequent analysis and for some surgical procedures. The technique has also been used to investigate finfish (Moccia et al., 1984) and crab digestive tract (Heinzel et al., 1993). Endoscopy requires sedation or general anesthesia so the potential stress of this must also be taken into account when considering welfare implications.

The size of endoscopes is a limiting factor in the application to investigate the cephalopod digestive tract with the restriction placed on the esophagus by the supra- and sub-oesophageal lobes and their connecting circum-oesophageal structures being a particular issue. The lower digestive tract is, in theory, accessible to endoscopic inspection via the anal sphincter, but the size of the endoscope will again be a limiting factor. In addition, as far as can be ascertained in all cephalopods, the intestine exits the gastro-caecal junction running caudally and dorsal, but during its course turns rostrally and ventral to exit the mantle near the siphon. Therefore, inspection of the proximal intestine would require a very flexible endoscope.

Ultrasound is utilized for non-invasive imaging of the mantle, vasculature, brain and arms of cephalopods. Ultrasonographic examination can be undertaken without sedation or anesthesia as carried out in *S. officinalis* (King et al., 2005; King and Adamo, 2006) or in *O. vulgaris* (Grimaldi et al., 2007). However, in other circumstances light anesthesia is required to ensure stable images for quantitative analysis of arm or brain morphology (Grimaldi et al., 2007; Margheri et al., 2011).

If an animal stops eating for no apparent reason, ultrasound may help to investigate the digestive tract and search for an obstruction; it should also be possible to view contractile activity of the crop, stomach, caecum or intestine as illustrated in **Figure 1**. Since the digestive gland decreases in weight, but has an increased % water with increasing duration of food deprivation (see Supplementary Figure 1) it may be possible to use ultrasound measurements of size and density as an index of the metabolic status of the animal, contributing to overall welfare assessment.

As cephalopods experiencing severe food deprivation mobilize lipids from the digestive gland and proteins from muscle and the gills (Lamarre et al., 2012, 2016; Speers-Roesch et al., 2016), ultrasound examination of these structures may provide insights into the overall health status of the animal, particularly if the same structures were imaged on arrival in the laboratory.

CLOSING REMARKS

The assessment of cephalopod digestive tract function through non-invasive methods needs to be developed further but the above overview highlights key areas (**Table 1**). For example, a specific analysis of the oro-anal transit times will facilitate species comparisons, investigation of the effects of environmental change, assessment of the impact of pathogens, investigation of neural and hormonal control, and provide standardized methods for comparison of experimental diets for use in aquaculture. Deviation of body weight from normality is considered a key welfare indicator, and the impact of prolonged food deprivation on welfare should be taken into account. Analysis of fecal composition will also give insights into absorption and secretion in the digestive tract especially if combined with measurements of metabolites in the haemolymph and/or digestive gland using minimally invasive techniques (Lamarre et al., 2012, 2016; Speers-Roesch et al., 2016). Simple methods for assessment of digestive tract function will also facilitate comparative studies of a wider range of species including those found more frequently in public aquaria.

Although we focused our attention on a series of possible markers of digestive tract function to be monitored through routine assessment at the "tank-side," daily assessment of health and welfare largely relies on observation of the animal. Understanding the external manifestations, including behavioral changes, of underlying digestive tract pathophysiology will be essential to improve welfare assessment tools for cephalopods.

AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript and agreed on the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00403/full#supplementary-material

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The Digestive Tract of Cephalopods: a Neglected Topic of Relevance to Animal Welfare in the Laboratory and Aquaculture

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Maintenance of health and welfare of a cephalopod is essential whether it is in a research, aquaculture or public display. The inclusion of cephalopods in the European Union legislation (Directive 2010/63/EU) regulating the use of animals for scientific purposes has prompted detailed consideration and review of all aspects of the care and welfare of cephalopods in the laboratory but the information generated will be of utility in other settings. We overview a wide range of topics of relevance to cephalopod digestive tract physiology and their relationship to the health and welfare of these animals. Major topics reviewed include: (i) Feeding cephalopods in captivity which deals with live food and prepared diets, feeding frequency (*ad libitum* vs. intermittent) and the amount of food provided; (ii) The particular challenges in feeding hatchlings and paralarvae, as feeding and survival of paralarvae remain major bottlenecks for aquaculture e.g., *Octopus vulgaris*; (iii) Digestive tract parasites and ingested toxins are discussed not only from the perspective of the impact on digestive function and welfare but also as potential confounding factors in research studies; (iv) Food deprivation is sometimes necessary (e.g., prior to anesthesia and surgery, to investigate metabolic control) but what is the impact on a cephalopod, how can it be assessed and how does the duration relate to regulatory threshold and severity assessment? Reduced food intake is also reviewed in the context of setting humane end-points in experimental procedures; (v) A range of experimental procedures are reviewed for their potential impact on digestive tract function and welfare including anesthesia and surgery, pain and stress, drug administration and induced developmental abnormalities. The review concludes by making some specific recommendations regarding reporting of feeding data and identifies a number of areas for further investigation. The answer to many of the questions raised here will rely on studies of the physiology of the digestive tract.

Keywords: cephalopods, digestive tract, Directive 2010/63/EU, *Octopus vulgaris*, *Sepia officinalis*, welfare

INTRODUCTION

Normal development, growth and the maintenance of health and well-being are only possible if all the digestive tract functions (e.g., motility, digestion, and absorption) operate normally and in concert. Understanding the physiological processes and the impact of external factors (e.g., handling, temperature, diet quality including exposure to food toxins, exposure to viral/bacterial infections and parasites) is important for normal laboratory maintenance of the animal in a research setting, as well as for optimizing conditions for aquaculture at each life stage.

The study of the physiology of the cephalopod digestive apparatus has mainly focused on *Sepia officinalis* (Bidder, 1966; Boucaud-Camou and Boucher-Rodoni, 1983; Mangold and Bidder, 1989; Quintela and Andrade, 2002a,b; Sykes et al., 2013; Costa et al., 2014), *Octopus vulgaris* (Boucher-Rodoni and Mangold, 1977; Boucaud-Camou and Boucher-Rodoni, 1983; Andrews and Tansey, 1983b; Mangold and Bidder, 1989), *Octopus maya* (Martínez et al., 2011a,b, 2012; Rosas et al., 2013; Linares et al., 2015; Pech-Puch et al., 2016). Few studies have been carried out in *Loligo vulgaris* and other squid (Bidder, 1950; Mangold and Bidder, 1989). Furthermore, the morphology, motility and absorptive functions of the digestive tract of *Nautilus pompilius* have been the subject of limited investigation (Westermann and Schipp, 1998a,b, 1999; Ruth et al., 1999; Westermann et al., 2000, 2002).

The inclusion of all "live cephalopods," taken to mean all living species (about 700), at all life stages after hatching, in Directive 2010/63/EU (European Parliament and Council of the European Union, 2010) covering the use of animals in scientific research and education poses a number of challenges for research (Smith et al., 2013; Fiorito et al., 2015) including that aimed at optimizing practices in aquaculture (Sykes et al., 2012; Smith et al., 2013; Fiorito et al., 2015). Whilst the Directive regulates studies in the Member States of the European Union, the principles it enshrines and the approaches to care and welfare required for compliance are likely to impact on cephalopod research outside the European Union (see Fiorito et al., 2014, for discussion of wider implications). In comparison to the commonly studied vertebrate laboratory species and commercially exploited vertebrates such as salmon and trout, chickens, cows, and pigs (Stevens, 1988; Grosell et al., 2010; Rønnestad et al., 2013), knowledge of the physiology of the cephalopod digestive tract at all life stages is limited.

Cephalopods are also kept for education and display purposes and, as in the laboratory and aquaculture, the normal functioning of the digestive tract is essential for good health and wellbeing (Fiorito et al., 2015). In this review, we will highlight a number of specific aspects of the relationship between feeding behavior and the physiology of the cephalopod digestive tract where increased understanding is required to ensure animal welfare. We will also discuss areas where further study is required.

FEEDING CEPHALOPODS IN CAPTIVITY

The vast majority of known cephalopods are carnivorous, obtaining food either by scavenging (e.g., *N. pompilius*) or predation of live food (e.g., *S. officinalis*, *L. vulgaris, O. vulgaris*), with a few exceptions such as the vampire squid (*Vampyroteuthis infernalis*) that is a detritivore of "marine snow," possibly requiring different digestive processes to obligate cephalopod carnivores (Hoving and Robison, 2012).

Information on diets in the wild is limited, but insights can be obtained through analysis of isotopes or stomach contents among other techniques (e.g., Hobson and Cherel, 2006; Villegas et al., 2014; Scheel et al., 2016; Regueira et al., 2017). In captivity, individual animals may show a preference for one type of prey over another even when both are available (e.g. crustaceans vs. fish for *S. officinalis*, crustaceans vs. mussels for *O. vulgaris*). Captive conditions, especially during key developmental periods may influence food preference across the life of an individual, as seen for example in cuttlefish (Darmaillacq et al., 2008). Nevertheless, most of the species adapt well to the laboratory and the diet may be narrowed to a few selected items (see Fiorito et al., 2015).

Live Food and Prepared Diets

The provision of live food (mostly commonly crustaceans, mussels, or small fish) to captive cephalopods is the most common practice. It is normally based on freshly caught (when applicable) items, that are not subjected to nutritional decay (e.g., oxidation in frozen food) as it may affect the growth of the cephalopod (Correia et al., 2008a,b). Live food greatly increases husbandry requirements as most cephalopods require crustaceans which produce higher levels of waste material (i.e., exoskeleton material containing uneaten soft tissues), thus potentially affecting water quality in tanks; this puts an additional burden on filtration systems and cleaning logistics. Providing live food also raises ethical issues as the death of the prey may not be humane, especially if it has the *potential* to suffer as fish are assumed to (Sneddon, 2015), and decapod crustaceans (Barr et al., 2008) may do.

Cephalopods are likely to have been exposed to parasites or toxins from their diet in the wild, however concern in the use of live food for laboratory housed animals may be also taken into account because of the hypothetical risk posed by introduction through the food given.

Contrarily, the use of live food is simply neither practical nor economical in production scale aquaculture so there is a considerable research effort into the development of prepared diets (Iglesias et al., 2014; Martínez et al., 2014; Cerezo Valverde and García, 2017). If successful, these alternative feeds would also possibly become a standard in the laboratory and public aquaria.

Although prepared diets may be cheaper, are likely to be more convenient and have a lower potential infection risk than live food, there is an argument that the provision (possibly intermittently) of live or frozen prey as food can be considered as "enrichment" for the cephalopod and hence beneficial for overall welfare (Cooke and Tonkins, 2015; see review in Fiorito et al., 2015). The importance of environmental ("tank") enrichment in animal experimentation (Bayne and Würbel, 2014; Yasumuro and Ikeda, 2016) and aquaculture (Ashley, 2007; Martins et al., 2012; Näslund and Johnsson, 2016) is being increasingly appreciated.

Feeding Frequency and the Amount of Food Provided

In the wild, the frequency of food intake in a cephalopod is considered to be influenced by multiple factors including: the availability of prey; the ability of the animal to locate and catch the prey (this will be affected by its health status); the time taken to ingest the prey and any pre-absorptive satiety signals (e.g., crop or stomach distension via mechanosensitive afferents if they exist in cephalopods); the time taken to digest the prey, absorb the nutrients and eliminate waste (all likely to be affected by any digestive tract pathology; see below) together with any post-absorptive satiety signals; the size, amount and nutritional value of the prey; the metabolic rate of the animal; activity level; environmental temperature and seasonality; life stage and sexual maturity.

Food availability and seawater temperature are considered to be the major factors influencing growth of cephalopods (Semmens et al., 2004). However, even if these parameters are optimal with respect to animal needs, if the digestive tract is in a pathophysiological state, food utilization will be affected with negative impact on bodily maintenance, growth and overall health.

In the laboratory or other captive settings, feeding frequency and amount of food provided may also considered within the framework of the "Five Freedoms" principle outlining minimal welfare standards (http://ec.europa.eu/food/animals/welfare_en; Huntingford, 2008). One of the "Freedoms" states that animals should be "free from hunger" (www.rspca.co.uk/education). It is not known if cephalopods experience a sensation of hunger, or a functionally equivalent sensation, comparable to that in humans. Tracking the behavioral changes such as latency and time to attack, following a large meal ("satiety") to the time when the meal has been fully digested (presumed presence of "hunger") will provide insights into the frequency with which food should be provided to minimize the possibility of unpleasant sensations arising from lack of food (i.e., hunger). Below we will focus on two modalities of providing food to animals: *ad libitum* and intermittent.

Ad libitum feeding is usually taken to mean that the animal has access to an "excess" amount of food at all times so that in principle it can eat to satiety (or potentially over feed) whenever it requires. However, few studies define what they mean by *ad libitum*. Even when the frequency of food provision is stated the amount provided is not, making comparison of regimes difficult. For example, an *ad libitum* regime in juvenile *S. officinalis* is provided in some settings by live grass shrimp (*Palaemonetes varians*) given twice daily although the amount given is not specified (Gonçalves et al., 2012). Examples of species in which feeding described as *ad libitum* feeding has been used include: for sepiolid, *Euprymna tasmanica,* (live mysids, Moltschaniwskyj and Johnston, 2006); for cuttlefish, *S. officinalis*, (live and frozen grass shrimp, Sykes et al., 2013); for octopus, *O. vulgaris*, live crabs (e.g., Agnisola et al., 1996); for *O. maya* (frozen crabs, Linares et al., 2015). *Ad libitum* feeding is achieved by placing food in the tank to allow the animals free access, with sufficient food provided to last until replenished.

An alternative may be to re-examine the utility of automated food dispensers previously investigated for example for feeding *O. vulgaris* (Nixon, 1969). Such automated methods could easily be applied to pelleted diets (artificial or synthetic) and would allow the feeding pattern to be studied which will be essential for investigating the neural and hormonal mechanisms controlling food intake and digestion. With a food dispenser there is a possibility that the animal will access, but not eat food, increasing wastage and tank contamination. Additionally, animals housed in groups (e.g., *O. vulgaris* in some aquaculture settings) may compete for access to the dispenser. There is also a possibility that the animal (particularly octopuses) will damage the food dispenser or may damage themselves on the food dispenser.

For aquaculture, *ad libitum* feeding will be necessary to ensure fastest growth rates although the health and welfare consequences (if any) of such regimes require study. However, it is reported that it is impossible to over feed a cephalopod and that they will reject excess food, but the evidential basis for this appears unclear (Boucaud-Camou and Boucher-Rodoni, 1983) although it is consistent with the experience of one of the authors of this review (AS).

Intermittent feeding applies to conditions where a given amount of food is provided to the animal which it will consume in one "meal" and it is most commonly applied to providing food either daily (e.g., grass shrimp for *Abdopus aculeatus*, Alupay et al., 2014; pieces of fish for *O. vulgaris*, Matzner et al., 2000; García García et al., 2011), more frequently (e.g., frozen squid twice daily for *O. vulgaris*, Garcia-Garrido et al., 2010) or less frequently (e.g., a crab every second day in *O. vulgaris*, Boucher-Rodoni and Mangold, 1985; shore crab twice weekly in *S. officinalis*, Wearmouth et al., 2013). Food is not available at all times but obviously if the amount of food provided is greater than can be ingested in a single meal then in effect food is provided *ad libitum*. For example, in *Octopus maya* animals given one small crab/50 g body weight daily ate all the crab, but if two crabs/50 g body weight were provided at the same time animals did not ingest all the crab (Walker et al., 1970). The literature also has examples of feeding an individual "meal," but providing food on multiple occasions during the day. For example, Yacob et al. (2011) report feeding *S. officinalis* on frozen shrimp and live zebra fish twice daily and in another cuttlefish study animals were fed frozen krill and silversides by hand 3–4 times a day (Tressler et al., 2014).

A further example of issues relating to the description of feeding regimes is provided by a study of *O. vulgaris* which comments, "octopuses were fed once per day to satiation with filet of frozen bogue (*Boops boops*)" (García García et al., 2011, p. 162). It is presumed that satiety was indicated by the refusal to take further food when offered but it would not necessarily be known if the animals would eat again if offered food prior to the next day. If the term "satiety" is used it would be helpful to have an indication of the criteria on which the assessment was made.

It is evident that the Methods sections of publications do not always give sufficient information either for the feeding regime to be replicated or to enable valid inter-study comparison. In view of this we would recommend that as a minimum the Authors should specify: the type of food provided (e.g., live, freshly killed,

previously frozen, processed/prepared); the amount provided (weight, including for live food; see Methods in Lamarre et al., 2012, for an example); the frequency of feeding and the time at which the food was provided.

For example, Garcia-Garrido et al. (2010) report feeding *O. vulgaris* on frozen squid (*Loligo gahi*) given at two times (9 a.m. and 3 p.m.), with the total amount of food provided daily being 5% of the body weight of the octopus. Ideally the energy density and chemical composition of the diet should also be given (for an example see Estefanell et al., 2011) or a reference provided to previous studies where similar food has been provided.

Food Amount

Despite the diverse feeding regimes and paucity of detail in much of the literature, a number of publications have undertaken detailed studies of the feeding requirements of several species (e.g., *O. vulgaris*, Estefanell et al., 2011; *Octopus tetricus*, Joll, 1977; *S. officinalis*, Domingues et al., 2001). Feeding requirements of a particular species, at a particular life stage and temperature are usually given as the weight of food as a percentage of body weight. The latter assumes the energy density of the food is sufficient to meet all the calorie and nutrient requirements and micronutrients. However, not all diets are equivalent. For example, in adult *O. vulgaris* growth rate is lower when they are fed *ad libitum* exclusively on sardines compared with squid or crab (Quintana et al., 2015). One reason to explain this difference could be the high neutral lipid content of sardines. Cephalopods seem to have a low capability to digest neutral lipids due to the absence of lipid emulsifiers in their digestive tracts (Vonk, 1962; O'dor et al., 1984; Morillo-Velarde et al., 2015).

For a particular diet, under well-defined environmental conditions (particularly temperature) it should be possible to give guidance on the amount of food required daily for a given species and life stage. This approach is illustrated by the study of Garcia-Garrido et al. (2010) in which *O. vulgaris* juveniles were fed 5% of their body weight daily on a diet of frozen squid or by the studies of Castro et al. (1993), Castro and Lee (1994), and Domingues et al. (2008), where *S. officinalis* were fed ≈8% of their body weight daily on shrimp species (either *Palaemon* or *Palaemonetes*).

As publications adopt a more systematic and detailed approach to describing diets in the methods sections it will be possible to undertake meta-analyses of the dietary data and provide stronger evidence based guidance on the amount of a particular food to provide.

Studies of feeding regimes are currently driven mainly by the aquaculture potential of cephalopods and the requirement to either develop artificial diets (Iglesias et al., 2014; Vidal et al., 2014) or to optimize natural diet formulations (e.g., ratio of fish to crab for *O. vulgaris*; García-García and Cerezo-Valverde, 2006). It is essential that the diet provided in the laboratory, aquaculture or public aquaria fulfills all the nutritional requirements of the animal, including micronutrients (Navarro et al., 2014). In this context, studies comparing metabolism of animals fed natural and artificial diets are of particular relevance (e.g., *O. maya,* Rosas et al., 2007). Studies of food intake and growth can be supplemented by measurements of haemolymph protein, amino acids and long chain polyunsaturated fatty acids (Linares et al., 2015), digestive enzymes (e.g., Villanueva et al., 2002) or body composition to provide an objective assessment of metabolic status (Garcia-Garrido et al., 2010; Navarro et al., 2014; Linares et al., 2015).

Particular Challenges in Feeding Hatchlings and Paralarvae

Cephalopods do not undergo metamorphosis like fish larvae (Young and Harman, 1988) but several species have a first life stage that starts with hatching and lasts until all its systems mature, including the digestive tract. Nonetheless, as in fish (Zambonino-Infante and Cahu, 2001), this period of maturation is normally a period of a month, depending on seawater temperature (Moguel et al., 2010; Sykes et al., 2013; Iglesias and Fuentes, 2014), and has been identified as a recurrent bottleneck for the development of cephalopod aquaculture (Sykes et al., 2006; Villanueva et al., 2014). Despite the multitude of reproductive strategies that cephalopods display (Rocha et al., 2001), the first life stage after hatching is usually termed "paralarvae" for species that undergo indirect embryonic development, and "hatchlings" for those which display direct development. These differences in development correspond to differences in the number and size of eggs laid by females (higher in indirect development species, e.g., *O. vulgaris*) and the relative "degree" of development at hatching (higher in direct development species, e.g., *S. officinalis, O. maya*; Boletzky, 1981, 1986). This is the key point leading to particular challenges regarding welfare of paralarvae and hatchlings in both the laboratory and aquaculture. In both cases (either paralarvae and hatchlings), cephalopods kept under captivity hatch with inner yolk reserves (Boletzky, 1994) that may last for days or weeks depending on temperature and proper food availability, as they display a mixed embryonic and postembryonic nutrition (Boletzky and Villanueva, 2014). Despite the differences in size at hatching, neither paralarvae nor the hatchlings display a prey ingestion problem related to the size of the "mouth," as that reported for finfish (Yúfera and Darias, 2007). However, the use of any feeds, other than live prey, during this first life stage (Sykes et al., 2014) limits growth and development, and eventually will impact welfare, (Navarro and Villanueva, 2000; Sykes et al., 2013).

PARASITES AND TOXIN LOAD OF THE CEPHALOPOD DIGESTIVE TRACT: POSSIBLE IMPACT ON ANIMAL WELFARE AND CONFOUNDING VARIABLES IN EXPERIMENTAL DESIGN

The wild remains the main source for cephalopods used in research so there is a realistic possibility that animals in the laboratory may have parasites in the digestive tract. Parasites are of concern for several reasons including, the impact on the overall health and welfare of the animal (Sykes and Gestal, 2014), as a source of infection for other animals (including humans unless precautions are taken, see Fiorito et al., 2015), and as a confounding factor in an experiment.

The digestive tract parasite *Aggregata octopiana* (Protozoa: Apicomplexa) is considered to be one of the main epizootic agent in both wild and reared *O. vulgaris* and exemplifies some of the issues. The intermediate host for this coccidian are crustaceans that form part of the normal diet for *O. vulgaris*. *Aggregata* is reported to cause loss of epithelial cells, mucosal atrophy and inflammation in the caecum and intestine resulting in impaired body growth, proposed to all be due to intestinal malabsorption syndrome (Gestal et al., 2002a,b). The prevalence of *A. octopiana* is high and reported to reach 98% (Gestal et al., 2002a) in the local population (Vigo, Spain). Although it is possible to count the number of sporocysts to assess the presence and magnitude of infection (Castellanos-Martinez et al., 2014) this requires death of the animal and removal of gut tissue, so it is not currently possible to assess the presence of *Aggregata* at the time animals are allocated to an experimental group. Whilst random allocation should reduce the potential for inadvertently assigning animals with high and low levels of infection to "control" and "test" groups for an experiment, it is possible that including groups with some infected animals may increase the variability within the group, potentially leading to a false negative result from statistical testing. In addition, the variability will affect the validity of any power calculation used to estimate group size in experimental design if the estimated effect size is influenced by the parasitic infection.

In theory, it should be possible to identify animals infected with *Aggregata* by looking for sporocysts in the feces of living animals prior to allocation to study groups, but this possibility has not been explored in published accounts, to the best of our knowledge. In *O. vulgaris*, it may also be possible to inspect the terminal intestine for sporocysts by gentle retraction of the ventral mantle or by endoscopy. Until techniques are available for *in vivo* assessment of *Aggregata* status or for its eradication prior to experiment it is probably wise to assume a high prevalence of infection in animals caught from the wild, unless there is evidence to the contrary in a local population used by a particular laboratory. We recommend that as a minimum the digestive tract is carefully examined macroscopically *post mortem* for the presence of sporocysts, particularly in the caecum and intestine but they may also be visible in the crop. Ideally the number of *Aggegata* sporocysts should be counted so that the magnitude of any infection can be investigated for correlation with experimental parameters to build up a profile of its biological effects. Once the spectrum of effects of *Aggregata* infection is known it will then be possible to make an informed assessment about its role as a confounding factor in experiments.

Whilst we have focused on *Aggregata* as a potential confounding factor in research we should not overlook the fact that animals with any infection may have a lower health status than those without and this raises a broader question of whether animals with *Aggregata* should ever be used in research studies other than those studying the effects of *Aggregata* itself.

We have used the infection of the digestive tract by *Aggregata* to illustrate one potential problem with using wild caught animals but we should also consider the digestive tract flora. The digestive tract microbiome and its role in health and disease has been the subject of detailed investigation in mammals but studies of the digestive tract flora are required in cephalopods. We mention the digestive tract microbiome here in relation to welfare as when animals are transferred from the wild to the laboratory, possibly involving a change of diet, it is highly likely that the flora in the digestive tract will change with as yet unknown effects on the functionality of the digestive tract and overall animal health. In addition, the impact of feeding prepared diets on the digestive tract flora of either wild caught or laboratory bred animals is not known.

A final factor to consider is the impact of any toxins that the animal may have been exposed to in the wild and which gain access to the body via the digestive tract. To illustrate this point we will use as examples shellfish toxins produced by phytoplankton species, ingested by bivalves which are subsequently ingested by benthic cephalopods. Domoic acid is responsible for amnesic shellfish poisoning in mammals (Pulido, 2008; Lefebvre and Robertson, 2010) and several compounds (e.g., saxitoxin, neosaxitoxin, gonyautoxins) are responsible for paralytic shellfish poisoning (Lopes et al., 2013). In mammals, these shellfish toxins have acute onset (<24 h) neurological (e.g., amnesia and locomotor paralysis) and gastrointestinal (nausea, vomiting and diarrhea) effects (for reviews see Lefebvre and Robertson, 2010; Visciano et al., 2016).

In cephalopods, although there are reports of mass stranding of Humboldt squid (*Dosidicus gigas*; Lopes et al., 2013) attributed to paralytic shellfish toxins (PST), we were unable to find any reports of acute onset effects of PSTs. In a study of PSTs in *O. vulgaris*, Lopes et al. (2014) comment (p. 210), "Despite of the remarkably high levels of toxins detected no apparent harm neither signs of behavioral changes were observed."

For the amnesic shellfish toxin domoic acid reports of acute effects are also lacking, an observation that is particularly intriguing as glutamate is known to be a neurotransmitter in both central and peripheral neural tissues in cephalopods (Messenger, 1996). Both domoic acid and PSTs have been shown to accumulate in the digestive gland of *O. vulgaris* (Costa and Pereira, 2010; Lopes et al., 2014) but the effects of these toxins on digestive gland function or other tissues is not known. Animals caught from the wild and utilized in research studies are likely to have different digestive gland concentrations of amnesic and paralytic shellfish toxins and these may vary seasonally depending upon algal blooms. The impact of the "toxin load" on health of the animal is unknown and differences in toxin concentration are a possible contributor to experimental variability; research is required to characterize the acute (e.g., brain and digestive tract function) and chronic (e.g., digestive gland metabolism, animal growth, and ability to withstand infection) physiological effects of amnesic and paralytic shellfish toxins on the cephalopods commonly studied in the laboratory.

FOOD DEPRIVATION AS A COMPONENT OF RESEARCH INVESTIGATIONS

There are several situations in research when it may be considered necessary to deliberately deprive the animal of food. Here we consider the justification for food deprivation in

different contexts and also the duration of such deprivation within the framework of regulated procedures under Directive 2010/63/EU (European Parliament and Council of the European Union, 2010).

Transportation

Accumulation of toxic ammonia from renal excretion arising primarily from protein metabolism (García García et al., 2011) and fecal contamination are a risk when transporting cephalopods in non-circulating sea water resulting in acidification. Depending upon the transport distance, species and size of the animal food deprivation should be considered and may be combined with transport in water at a temperature below ambient to reduce metabolic rate and carbon dioxide production (Fiorito et al., 2015).

There are no specific recommendations for the duration of food deprivation for each species as this will depend upon the normal feeding frequency, diet and digestive tract transit time. It is unlikely that in most cases more than 1–2 days, food deprivation is necessary to void the digestive tract of food and digesta, with the longer time being more appropriate for species held at lower temperatures where transit time may be slower (Boucaud-Camou and Boucher-Rodoni, 1983; Mangold and Bidder, 1989). It is essential that the duration of any food deprivation prior to transport is scientifically justified and is minimized to avoid compromising health (see below) during what is likely to be a stressful event.

Surgery and Anesthesia

In humans, deprivation of food prior to anesthesia and surgery is justified because of the risk of aspiration of vomit during induction, before airway intubation and this is also normal practice prior to veterinary or experimental surgery in larger mammals. The justification for routinely depriving cephalopods of food prior to anesthesia and surgery is not established and obviously inspiration of any regurgitated digestive tract contents into the mantle does not pose the same risk to a cephalopod as aspiration of vomit in a mammal. However, there is an argument for food deprivation if the surgical procedure involves the digestive tract itself. A full stomach (e.g., *L. vulgaris*) or crop (e.g., *O. vulgaris*) may increase the risk of accidental damage by obscuring other structures (e.g., crop branch of the dorsal aorta) or limiting the plane of dissection, so food deprivation may also be justified although this would only need to be omission of one meal or about 24 h (Fiorito et al., 2015).

Metabolic Studies

Laboratory investigation of metabolism can require animals to be deprived of food or may require a reduction in the amount of food provided at each meal. The question arises at what point food deprivation itself, as part of a research study, falls within the definition of a *procedure* which would be regulated under Directive 2010/63/EU and hence should be included in protocols submitted as part of a project application to the National Competent Authority.

Under Directive 2010/63/EU the threshold for regulation is "any use of an animal covered by the Directive for experimental or other scientific or educational purposes, which may cause the animal pain, suffering, distress or lasting harm equivalent to or higher than that caused by the introduction of a needle in accordance with good veterinary practice." It is clearly not easy to translate this definition into the period for which a cephalopod of any particular species (for example, a highly active *L. vulgaris* vs. relatively slow moving *N. pompilius*) or life stage (e.g., *O. vulgaris* paralarvae vs. egg bearing female) can be deprived of food before it exceeds the threshold for regulation. Periods of food deprivation of 24–48 h have been used prior to investigating the effect of handling on attack latency (≈24 h in *O. vulgaris*; Agnisola et al., 1996), before a feeding experiment (48 h in *E. tasmanica*; Moltschaniwskyj and Johnston, 2006) or prior to a study of anesthesia (24 h in *S. officinalis*; Gonçalves et al., 2012) based largely upon measures of oro-anal transit time for the species under study and also taking into account normal feeding frequency for the species (see above). In *N. pompilius* a period of 5 days food deprivation was used to stimulate appetite prior to feeding barium sulfate labeled shrimp to measure digestive tract transit (Westermann et al., 2002).

A number of studies have investigated the effects of various periods of food deprivation in *S. officinalis*, *Loligo forbesi,* and *O. vulgaris* on metabolism and these are used to make proposals regarding the regulatory threshold and also humane end points (see below).

The physiological response to food deprivation in both vertebrates and invertebrates has three phases (Lamarre et al., 2016): *Phase I* - basal metabolism is sustained following a few days food derivation utilizing dietary constituents; *Phase II* - with continued food deprivation mobilization of stored lipids occurs and for cephalopods these are primarily located in the digestive gland. A recent study shows that their role in enabling cephalopods to survive prolonged food deprivation may have been underestimated (see Speers-Roesch et al., 2016); *Phase III* - characterized by protein catabolism, it is considered to be the real indication of starvation as opposed to food deprivation Phases I and II.

Juvenile and adult animals are most likely to be used for research studies requiring food deprivation and for these life stages we propose the following regarding the threshold for regulation; periods of food deprivation that do not exceed the metabolic effects of Phase I should be considered to be below the threshold for regulation as defined by Directive 2010/63/EU (European Parliament and Council of the European Union, 2010) whilst procedures that induce the metabolic changes characteristic of Phases II and III fall within the threshold for regulation and would fall into the mild and moderate prospective severity classes respectively (European Commission, 2013). The work by Lamarre et al. (2012) and Speers-Roesch et al. (2016) did not report any mortality in the prolonged food deprivation studies. However, in a group of *O. vulgaris* (body weight 1618.3 ± 175.5 g) deprived of food there was an overall 10% mortality with individuals dying by an undetermined cause at 16, 20, and 23 days (Garcia-Garrido et al., 2010). The overall weight loss in the animals that survived the entire 27 days food deprivation was 35%. The possibility that death may

occur in a food deprivation study would be likely to make the severity grading severe and such studies would require detailed justification of the scientific or other benefits vs. the harms to the animal.

The behavior of animals following prolonged food deprivation is not described in detail but in both O. *vulgaris* (Wells et al., 1983) and *S. officinalis* (Lamarre et al., 2016) reduced levels of activity are reported and in the latter there are also problems with buoyancy.

Data from the above metabolic studies can provide a guide to the durations of food deprivation exceeding the threshold for regulation under Directive 2010/63/EU and their severity classification. **Table 1** proposes how the physiological consequences of food deprivation could be linked to severity classification. Research will be needed to match the relatively well-defined metabolic changes to behavioral (or other) objective indices of "pain, suffering, distress, and lasting harm."

TABLE 1 | The prospective severity classification of experimental procedures as defined in Directive 2010/63/EU together with proposals for how this could relate to periods of food deprivation in an adult *O. vulgaris* or *S. officinalis* in good health at the beginning of the deprivation.

	Below threshold	**Mild**	**Moderate**	**Severe**
Definition of severity from EU 2010* and EC 2013*	Does not reach the regulatory threshold of causing pain, suffering, distress or lasting harm equivalent to, or higher than that caused by the insertion of a hypodermic needle in accordance with good veterinary practice (EU 2010)	Likely to cause the animals to experience short- term mild pain, suffering or distress as well as procedures with no significant impairment of the well-being or general condition of the animals (EC 2013)	Likely to cause the animals to experience short- term moderate pain, suffering or distress as well as procedures that are likely to cause moderate impairment of the well-being or general condition of the animals (EC 2013)	Likely to cause the animals to experience severe pain, suffering or distress or long-lasting moderate pain, suffering or distress as well as procedures that are likely to cause severe impairment of the well-being or general condition of the animals (EC 2013)
Duration of food deprivation	0–48 h (0–2d)**	48–120 h (2–5 d)$^{+}$	120–288 h (5–12 d)$^{+}$	>288 h (>12 d)
Anticipated body weight loss (%) (based on Garcia-Garrido et al., 2010)	0 ≤ 5%	<10%	∼10–15%	>15%
Anticipated digestive gland weight loss (%) (based on Garcia-Garrido et al., 2010)	0–30%	30–50%	50–60%	>60%
Metabolic phase	Early Phase I	Late Phase I-early Phase II (Speers-Roesch et al., 2016, "middle phase")	Late Phase II-Early Phase III (Speers-Roesch et al., 2016, "middle phase"–"final phase")	Phase III- metabolic collapse (Speers-Roesch et al., 2016, "final phase")
Metabolic status (Based on Speers-Roesch et al., 2016; Table 1)	Normal metabolism from mixed biochemical sources but by 2–3d signs of a shift from anabolic to catabolic metabolism will emerge	Progressive utilization of lipids from the digestive gland and clear shift from anabolic to catabolic metabolism in the digestive gland.	Lipid dominant metabolism supplemented by tissue protein catabolism. No glucose utilization.	Dependence on amino acid metabolism with no glucose use and depleted lipid stores
Anticipated behavioral and other possible effects	No negative effects anticipated but possible increase in food seeking behavior; experience of hunger sensation?	Progressive reduction in activity. Adaptive changes in the digestive tract such as upregulation of epithelial transport mechanisms.	Progressive reduction in activity. Suppression of digestive tract motility.	Overall decline in health; increased susceptibility to infection and skin lesions); potentially irreversible damage to critical tissues (e.g., gills). Death with prolonged deprivation$^{\#}$.

The exact boundaries may change depending on temperature and life stage (see text for details). Based largely on studies of O. vulgaris (Garcia-Garrido et al., 2010) and S.officinalis (Speers-Roesch et al., 2016) we also indicate the underlying biochemical changes. The behavioural or other externally visible consequences of food deprivation are not well defined as indicated by the paucity of specific information in the last row (see text for details). Note that this table illustrates the principles and National Competent Authorities may have different views on severity and the above proposed classification requires validation.

**"EU 2010" and "EC 2013" are abbreviations of European Parliament and Council of the European Union (2010) and European Commission (2013), respectively.*

***For comparison in an adult salmonid 48 food deprivation would be considered to be below the regulatory threshold but it is also noted that as in cephalopods there is considerable inter-species variation (Hawkins et al., 2011).*

$^{+}$For comparison in adult rats, food deprivation of <24 h would be classified as mild and deprivation for 48 h as moderate (European Parliament and Council of the European Union, 2010).

$^{\#}$In O. vulgaris Garcia-Garrido et al. (2010) reported death at 16, 20, and 23 days of food deprivation.

If food deprivation is required for whatever reason, a clear justification should be made both in the application to the National Competent Authority (if the work is covered by Directive 2010/63/EU) and reiterated in any publication. The period of food deprivation should be kept to the minimum compatible with achieving the stated objectives and the impact on all aspects of the animals' health should be carefully assessed (see ARRIVE reporting guidelines; https://www.nc3rs.org.uk/sites/default/files/documents/Guidelines/NC3Rs%20ARRIVE%20Guidelines%202013.pdf). In the case of prolonged food deprivation (e.g., Lamarre et al., 2012), the fate of the animal at the end of the period of deprivation will need to be considered and specified in the application to the National Competent Authority (NCA) in research performed under Directive 2010/63/EU (European Parliament and Council of the European Union, 2010). Whilst it is possible that after an extended period of food deprivation the animals may regain body weight when sufficient food is available, the welfare of the animal during this recovery period must be carefully assessed and there should be some assurance that the period of deprivation did not have irreversible effects such as structural protein or lipid loss in critical tissues (brain, heart, and gills) that may cause persistent suffering to the animal.

THE DIGESTIVE TRACT AS A ROUTE FOR SUBSTANCE ADMINISTRATION

Drug administration by gavage has been used in squid (Berk et al., 2009) and could be used in cuttlefish and octopus although we are not aware of any publications. If gavage is used, care must be taken to taken to avoid damage to the brain as the gavage tube passes from the beak into the esophagus and then into the crop or stomach depending on species. The tube to be utilized should be semi-rigid and round ended, to prevent mucosal damage; measurements should be made on cadavers to estimate the length to which the tube should be inserted unless this is done using ultrasound assisted *in vivo* imaging.

Food deprivation is probably advisable to facilitate rapid absorption of any drug and prevent drug/food interaction, but the volume of substance administered should be well within the normal volumes found in the crop or stomach to minimize chances of damage or activation of visceral nociceptors (should they exist in cephalopods). Careful consideration should be given to the drug vehicle to avoid substances likely to damage the mucosa or which themselves have effects on mucosal functions (e.g., changes in pH that will alter the pH optimum of digestive enzymes and mucolytics will impair epithelial barrier function).

An alternative to gavage is to include the drug in the food. This route was used for the administration of titanium dioxide nanoparticles to *O. vulgaris* by feeding the animals on mussels that had been exposed to the nanoparticles (Grimaldi et al., 2013). Inclusion of drugs in the food (e.g., injected into a crab, a mussel, a piece of fish or incorporated into a piece of artificial diet just prior to feeding) provides a non-invasive approach to administration but there are several issues to be considered if a study using this approach is to have a meaningful outcome: (1) Although the total dose of drug in the food is known it may be difficult to know the precise amount ingested by the animal unless there is assurance that no drug has leaked into the water during food capture or ingestion and that the animal has eaten all the food. Microencapsulation of the drug will reduce leakage compared to injecting the drug into the food in solution or mixing as a powder but assessing the ingested does remains problematic; (2) The secretions of the salivary glands injected into the prey during capture and prior to ingestion contains powerful enzymes and a wide array of other bioactive substances (Cornet et al., 2014; Mancuso et al., 2014) so some consideration should be given to whether the drug is likely to be affected by the salivary secretions and rendered ineffective. Degradation of the drug by the salivary secretions could lead to false negative conclusion about drug efficacy in cephalopods; (3) As the food with the drug is likely to come into contact with the suckers, particularly in the peri–oral region, there is a possibility that the animal may reject the food based upon "taste" although micro-encapsulation could be used to prevent direct contact.

In cuttlefish, Darmaillacq et al. (2004) showed induction of a learned aversion to food (crabs) painted with quinine, which is bitter tasting to humans, although we do not know what sensation (if any) it may evoke in cephalopods. If the experimental drug evokes rejection and a learned aversion to that food in a similar manner to quinine (many alkaloids are bitter tasting and are also drugs, e.g., atropine and scopolamine), then it may not be ingested and may induce an aversion to that food if encountered again. A learned aversion leading to avoidance of the food on a subsequent occasion can also be induced if the ingested food causes the animal to be ill. In humans the analogous situation would be a food that induced nausea and vomiting (Andrews and Sanger, 2014).

As it may not be possible to predict if a drug will have an adverse effect on the animal it should not be given in the food most commonly used, otherwise if the animal does develop an aversion maintenance of the normal feeding regime may not be possible.

RESEARCH ON DIGESTIVE PHYSIOLOGY AND DIETS FOR CEPHALOPODS: IMPACT OF DIRECTIVE 2010/63/EU AND OTHER REGULATIONS AND POLICIES

Directive 2010/63/EU and related mandated minimal recommendations on the accommodation and care of animals, include species specific sections that are relatively detailed for vertebrates, but no equivalent indications are provided for cephalopods. However, the "general section" of the Directive (European Parliament and Council of the European Union, 2010) applies to all species and includes some general principles of feeding (Annex III, 3.4) which we outline here as they are relevant to the subsequent discussion of cephalopod diets.

The key elements included under the heading "Feeding" in the Directive are:

- Form, content and presentation of the diet should meet the nutritional and behavioral needs of the animal;
- Diet should be palatable and non-contaminated and precautions taken to minimize chemical, physical and microbiological contamination;
- Individual animals should be able to access food, with sufficient space provided to limit competition.

These principles embodied in the Directive and national transposition legislation, are reflected in the Guidelines on the Care and Welfare of Cephalopods (Fiorito et al., 2015), and also included in a simplified version, in the Code of Practice on Housing and Care published by the United Kingdom Home Office (see section on Cephalopods; https://www.gov.uk/government/publications/code-of-practice-for-the-housing-and-care-of-animals-bred-supplied-or-used-for-scientific-purposes).

Directive 2010/63/EU applies to the protection of animals used for scientific purposes but as many aspects of this review also apply to animals in an aquaculture setting we draw attention to the following regulations: Council Directive 98/58/EC on the protection of animals kept for farming purposes (Council of the European Union, 1998); Regulation (EC) N° 882/2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules (European Parliament and Council of the European Union, 2004); and Council Regulation (EC) N° 1099/2009 on the protection of animals at the time of killing (Council of the European Union, 2009).

The identification of optimal dietary formulations (natural or synthetic) for use in cephalopods in the laboratory and aquaculture, at all life stages, requires research into their effects on growth and metabolism (Sykes et al., 2006; Navarro et al., 2014). There is debate about whether research into different diets falls within the remit of Directive 2010/63/EU (see Sykes et al., 2012) so the issues of concern are discussed here. Firstly, investigation of the effects of different diets is clearly "research" as the outcome is not known and an experimental approach is required to obtain the answer so does not come under the exempt category of "non-experimental agricultural practices" (Article 1, 5a). Secondly, the response of the animal to a novel experimental diet may not be immediately apparent so there is a potential that the animal may experience suffering, distress or lasting harm that exceeds the threshold for regulation. Until the novel diet is studied its effects on the animal cannot be fully assessed. Third, many studies are directed at identification of diets or environmental factors to improve survival of paralarvae in species such as *O. vulgaris* where mortality in aquaculture conditions from hatchling to the benthic phase is ≈100% in most of the studies and has changed little over 20 years (Villanueva, 1995). Note that the survival rate in the wild is not known so comparison with survival rates in laboratory condition is not possible. In studies of diet and environmental changes, mortality/survival rates are often used as one of the outcome measures. The aim of many paralarvae studies is to modify the diet, light or temperature to improve survival rates so mortality is a valid outcome measure. However, the severity classification of procedures would classify as "severe" toxicity testing where death is an end point or where there is a severe impairment of well-being or general condition (e.g., see paper by Feyjoo et al., 2011). The effect of a novel diet on survival cannot be predicted, so it is possible that the mortality may be either higher or lower than a current feeding regime. Whilst the degree of suffering which may be experienced by a paralarvae before death is not known, this discussion illustrates the regulatory challenges raised by the inclusion in Directive 2010/63/EU of cephalopods from the time of hatching.

THE POTENTIAL IMPACT OF EXPERIMENTAL PROCEDURES ON THE CEPHALOPOD DIGESTIVE TRACT: ISSUES TO CONSIDER

The potential impact of any experimental procedure on functionality of the digestive tract should be assessed as part of experimental planning. For investigators whose research is regulated by Directive 2010/63/EU (see below) a full assessment of the effects of an experimental investigation and assessment of the anticipated harms (to the animal) and potential benefits (of the answer to the research question) is an essential component of the application for authorization to the National Competent Authority. There have not been any formal studies on the effect of common procedures on the cephalopod digestive tract but below we comment on the most likely areas of concern to stimulate discussion and raise awareness of the issues. As for other issues discussed in this is overview the specific effects on the digestive tract of any intervention are likely to be affected by life stage and species.

Anesthesia and Surgery

A number of authors have commented that cephalopods recover rapidly from brief periods of general anesthesia, not usually involving surgery, with reports of food ingestion within 10–15 min of recovery anesthesia (Joll, 1977; Gonçalves et al., 2012). Food ingestion indicates an unimpaired attack response but does not necessarily imply that the digestive tract itself is unaffected by anesthesia, although it is suggestive. Whilst an anaesthetic may have an effect on the digestive tract by acting on the brain regions regulating the extrinsic innervation (visceral and sympathetic nerves; Young, 1967, 1971) a direct action on the digestive tract enteric nerves and muscle is also possible. For example, magnesium chloride is commonly used as an anesthetic agent in cephalopods (Fiorito et al., 2015) and in the isolated stomach and rectum of *Loligo pealii* it has an inhibitory effect on contractions and tone (Bacq, 1934). This inhibitory effect on the digestive tract is also consistent with the bradycardia and reduced stroke volume produced by magnesium chloride on the isolated systemic heart of *O. vulgaris* (Pugliese et al., 2016). For both the heart and digestive tract, the most likely mechanism by which magnesium chloride has its inhibitory effects is by interfering with calcium fluxes, but this requires confirmation by experiment. If digestive tract motility is inhibited for the duration

of anesthesia it is likely that transit time will be prolonged until motility is normalized.

Surgery of the digestive tract and in particular lesions affecting the innervation (e.g., sympathetic nerves, gastric ganglia) will not surprisingly affect digestive tract function (e.g., Best and Wells, 1983) and hence impact the overall welfare of the animal. The impact of surgery outside the digestive tract on digestive tract functionality is not known, but if nociceptors are activated by the surgery (and analgesics are not provided) then neural and endocrine pathways regulating digestive tract function may be affected. Particular care needs to be taken to consider the likely effects of brain lesions on the ability of the animal to recognize, capture and ingest food and hence maintain normal metabolism. For example, *O. vulgaris* with lesioned anterior and posterior basal lobes are unable to feed themselves (Wells, 1978) so if there is a need to study these animals for more than a few days consideration will need to be given to how to ensure adequate nutrition to maintain health.

Currently, no analgesics have been used as part of the anesthesia protocol in cephalopods (Fiorito et al., 2015) but if potential substances are identified their pharmacological effects on the digestive tract should be ascertained. For example, opioid receptor agonists (e.g., morphine and synthetic derivatives) are used as an analgesic in mammals and are associated with constipation as a side effect, as amongst other actions they modulate transmission in the enteric nervous system to reduce transit (De Giorgio et al., 2007). Delta-opioid receptors have been identified in the digestive tract (Sha et al., 2012) and kappa-opioid receptors in *O. vulgaris* (Zarrella et al., 2015) but functional effects of their activation have not been investigated.

Nociception and Stress

In mammals, activation of somatic and visceral nociceptors inhibits gastric and intestinal motility contributing to an overall increase in oro-anal transit time via reflex and endocrine mechanisms. The nociceptors activate spinal and supra-spinal pathways modulating the sympathetic outflow to the digestive tract and the secretion of adrenaline from the adrenal medulla (Janig, 2013; Janig and Mclachlan, 2013).

Although mechano-nociceptors have been described in cephalopods (Crook et al., 2011, 2013, 2014; Alupay et al., 2014) in locations equivalent to somatic nociceptors in vertebrates their central projections are not known so we are unable to speculate if information from these nociceptors can influence the innervation to the digestive tract (visceral nerves and "sympathetic" nerves; Young, 1967). The existence of visceral nociceptors has not been investigated in cephalopods but there is structural and behavioral evidence for the existence of visceral afferents (Young, 1960, 1967). Nothing is known about the responses of the cephalopod digestive tract or the cardiovascular system (also modulated by the visceral nerves) to noxious stimuli but it would be surprising if neither system was affected. Non-painful, but unpleasant stressful stimuli such as those produced by restraint or hypoxia are also likely to affect digestive tract function via the innervation or secretion of "stress" hormones. For example, removal of *E. cirrhosa* from water for 5 min results in elevation of haemolymph dopamine levels (Malham et al., 2002), which could act on the crop where *in vitro* studies in *O. vulgaris* have shown dopamine to increase tone (Andrews and Tansey, 1983b) or alternatively the neurones in the gastric ganglion could be the target as there is histochemical evidence for its presence (Juorio, 1971) and hence dopamine receptors are likely to be present.

Both noxious and non-noxious but stressful external stimuli may also have both acute and chronic effects on the digestive tract via up or down regulation of genes in critical control locations such as the gastric ganglion. A diverse range of genes has been implicated in the response of octopus to environmental stressors (reviewed in Di Cosmo and Polese, 2016) but the biological effects of the gene products on the physiology of the digestive tract and its control requires investigation.

Drug Administration

A drug given systemically and being used as part of a research project to investigate one system (e.g., brain neurochemistry and effects on behavior) may also have unintended effects on control of food intake or the functioning of the digestive tract. For example, catecholamines are present in the brain (Tansey, 1980) and in neurones in the digestive tract and cardiovascular system of cephalopods (Andrews and Tansey, 1983a; Versen et al., 1999). Drugs used to investigate the role of catecholamines in brain functioning (e.g., learning and memory) either by depleting nor-adrenaline (e.g., reserpine; Tansey, 1980) or acting as competitive antagonists of adrenergic receptors (e.g., phentolamine; Versen et al., 1999) could affect digestive tract motility as nor-adrenaline stimulates contractile activity in the crop, stomach and intestine of *O. vulgaris* (Andrews and Tansey, 1983b). The potential problems are further illustrated by scopolamine (a non-selective muscarinic acetylcholine receptor antagonist) used to investigate memory recall in *O. vulgaris* (Fiorito et al., 1998) but as acetylcholine (receptor not characterized) has inhibitory effects on digestive tract motility (Andrews and Tansey, 1983b) this effect may be lost in animals treated with scopolamine, allowing excitatory effects to predominate. In neither paper using reserpine (Tansey, 1980) or scopolamine (Fiorito et al., 1998) were overt effects on the digestive tract reported but neither were they investigated explicitly and such effects may be subtle.

Developmental Changes

The publication of the genome of *O. bimaculoides* (Albertin et al., 2015) will be a major stimulus to cephalopod research. The intended and potential unintended effects of gene expression modifications (e.g., gene knock out, gene over expression) will need to be considered during project design and plans put in place to monitor adverse (or even beneficial) effects. Of particular concern would be modification to genes which may affect the development of the digestive tract including the neural control mechanisms. Exposure of recently spawned eggs to abnormal environmental condition can also produce developmental changes as demonstrated by an increase in malformations, early hatching and mortality in squid eggs exposed to sea water at 2°C above ambient (Rosa et al., 2012) or eggs of *O. vulgaris* exposed to an increase of 3°C (Repolho et al., 2014).

REDUCED FOOD INTAKE AS A HUMANE END-POINT IN PROCEDURES: WHAT ARE THE LIMITS?

A humane end point is the earliest point at which a specific intervention must be made to end an animal's suffering and for each procedure under Directive 20110/63/EU. Humane end-points are specified in the application to the National Competent Authority and describe the type of suffering that may occur, its magnitude and duration and these judgements contribute to the "prospective assessment of severity" of a procedure which is also indicated in application to the NCA (see below).

Typical interventions as the humane end point is reached could include: (i) removing the animal from the study; (ii) providing analgesia to alleviate pain or by treating other symptoms unrelated to the experimental outcomes but this is reliant on validated treatment being available and knowledge that they will not affect the primary experimental outcomes; (iii) humanely killing the animal and/or terminating the study.

There are three different types of study where the consequences and limits of reduced food intake need to be considered and these are discussed below to illustrate the issues. For completeness, we also include the special case (see below) of research involving senescent cephalopods.

Investigation of the Effects of Food Deprivation

There are a number of studies aimed at investigating the effects of different periods of food deprivation on a cephalopod (e.g., studies of *O. vulgaris*, Garcia-Garrido et al., 2010; *S. officinalis* studies of Lamarre et al., 2012, 2016; Speers-Roesch et al., 2016). In such studies the duration of food deprivation is pre-determined and justified in terms of the anticipated harms to the animals vs. the potential scientific (or other) benefits of the study. Weight loss is an expected outcome of these studies, but if the protocol in the NCA submission has set a limit for the degree of weight loss (e.g., 20% over 14 days in adult *O. vulgaris*) then if this is exceeded, the animal would be removed from the study immediately. Humane end-points indicating that the food deprivation is having additional deleterious effects could include loss of the ability to maintain normal position in the water column, autophagy, skin lesions or an excessive reduction in the size of the digestive gland (e.g., measured by ultrasound; for a description of welfare monitoring parameters see Table 5 in Fiorito et al., 2015). If these signs were used as humane end-points then, if observed, that animal would be removed from the study.

Interventions Intended to Modify Food Intake

There are a number of studies in which an intervention (e.g., an agonist with potential anorexic effects, administration of a pathogen or a nerve lesion such as gastric ganglion ablation) is intended to, or is likely to, modify food intake. In this type of study the issue is what limits should be placed on the reduced food intake? For example, for how many days should an animal be allowed to go without food before intervention? The duration will be related to the scientific outcomes; if the aim is to show that an intervention (e.g., investigating modulation of food intake by members of FMRFamide family; Walker et al., 2009) affects food intake then a period of 24 h may be sufficient to show an acute effect, but if there is a need to know the duration of any effect then longer may be needed. The duration of the study will be the minimum compatible with the scientific objectives assuming that the duration can be justified in the project application. Decisions about how long an animal should go without food or have reduced food intake before intervention should take into account metabolic studies (e.g., Garcia-Garrido et al., 2010; Lamarre et al., 2012, 2016; Speers-Roesch et al., 2016) but until more detailed information is available about the effects of food intake on physiology and behavior of the animals we would advise adoption of the precautionary principle in studies where food intake is likely to be affected.

Procedures Affecting Food Intake as An Unintended Outcome

In some cases experiments include a procedure that may affect food intake although this is not either the primary intent of the lesion or topic of study. In this case the reduced food intake is viewed as an "adverse" or "side" effect of the procedure. This type of study differs from food deprivation described above in that the effect on food intake is unintended rather than being a primary objective of the study but in both cases the issues relating to the duration of reduced food intake are the same.

Senescence

Markedly reduced or absent food intake is a well-known feature of senescent cephalopods occurring in post-mating adults and in females during egg-brooding (Anderson et al., 2002). Although this is a normal terminal life-stage in the wild, if the animal is being kept in the laboratory to study the physiology of senescence (e.g., how is appetitive drive inhibited?) then as the animal is likely to experience some suffering it arguably comes within the scope of Directive 2010/63/EU (see Smith et al., 2013, for discussion). As the reduction in food intake progresses the animals will rapidly pass through metabolic phases I, II described above in animals deprived of food and will probably spend the longest time in phase III until death ensues (Moltschaniwskyj and Carter, 2013). Studying senescence is further complicated because animals not only stop eating but may develop other symptoms such as cataracts, skin lesions and increased, but uncoordinated, locomotor activity (Anderson et al., 2002; Sykes et al., 2012; Sykes and Gestal, 2014) none of which can be alleviated. If studies of the digestive changes accompanying reproduction and senescence are to be investigated a critical question will be whether it is necessary to study the animal until death to answer the scientific question posed? We would argue that understanding the mechanism of food intake suppression only requires a relatively short period of study (e.g., a week) but if the topic of interest is whether the enteric nervous system shows signs of degeneration with age, as occurs in mammals (Hetz et al., 2014; Saffrey, 2014), then longer periods of study may be required.

RECOMMENDATIONS AND RESEARCH QUESTIONS TO ENHANCE CEPHALOPOD HEALTH AND WELFARE

This review has drawn attention to the numerous close relationships between the normal physiological functioning of the digestive tract in cephalopods and their health and welfare. Although the relationships between normal digestive tract function, health and welfare may appear obvious, conforming with regulatory requirements (particularly Directive 2010/63/EU) necessitates developing a more evidence based approach to ensuring adequate nutrition and identification of biomarkers of health and welfare.

The text below summarizes some of the key points and areas which we consider require research.

(i) Reporting feeding regimes in publications is variable so a consistent approach should be adopted to include as a minimum: type of food; amount of food (weight or as a % body weight); feeding frequency and time. This will facilitate meta-analysis of diets.

(ii) Cephalopods for research are frequently taken from the wild and may have been exposed to ingested toxins (e.g., domoic acid) and parasites (e.g., Aggregata); these are considered as potential confounding factors in research which may be unidentified prior to allocation to experimental groups. The impact of parasites and toxins on health and welfare requires research.

(iii) Techniques for non-invasive investigation of digestive tract physiology are required to enhance routine health and welfare assessment (for review see Ponte et al., 2017), to improve understanding of the effects of experimental interventions and also to develop humane end-point criteria for regulated procedures.

(iv) Little is known of relationships between behavior and digestive tract functionality in cephalopods. For example: would digestive tract obstruction result in behavioral change due to visceral nociceptor activation? What behavioral changes accompany food deprivation of different durations?

(v) Periods of food deprivation may be required for various types of research and we considered this in relation to knowledge of cephalopod metabolism and the regulatory threshold and prospective severity assessment; deprivation >2d is proposed to reach the threshold for regulation under Directive 2010/63/EU in the "mild" category and >12d would be "severe."

(vi) The potential negative impact of experimental interventions such as food deprivation, anesthesia (e.g., inhibition of smooth muscle contraction by magnesium chloride) and surgery, (e.g., brain lesion), pain (e.g., activation of nociceptors) and stress (e.g., weighing in air), drug administration (e.g., cholinergic and adrenergic receptor antagonist impacting digestive tract innervation) and genetic modification on the functioning of the digestive tract are considered from the perspective of animal welfare.

(vii) Research on the digestive tract physiology is needed to understand constraints of *O. vulgaris* aquaculture and in particular the maturation of function and control mechanisms (e.g., gastric ganglion) and changes occurring between the paralarvae and settlement phases where dietary changes occur and there are likely to be changes in the digestive tract microbiome.

(viii) Improved understanding of all aspects of the physiology of the digestive tract (food intake regulation, motility, secretion, absorption, neural, and endocrine control) in a diverse range of species will play a key role in ensuring optimal health and welfare of cephalopods in the laboratory, aquaculture and display aquaria.

Good animal welfare and good science are inextricably linked (see Hubrecht, 2014, for review). We hope that this paper will stimulate research on the digestive system which in addition to providing novel insights into the physiology will also enhance the welfare of cephalopods in both research and aquaculture.

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AVS and EA are equal first authors. All authors contributed to the manuscript and drafted, edited and approved the final version of the manuscript.

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