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Cephalopods in the anthropocene: Multiple challenges in a changing ocean

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Cephalopods in the anthropocene: Multiple challenges in a changing ocean

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Editorial: Cephalopods in the Anthropocene: multiple challenges in a changing ocean

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Editorial on the Research Topic
Cephalopods in the Anthropocene: multiple challenges in a changing ocean

The Anthropocene describes the new geological epoch driven by humankind (Lewis and Maslin, 2015). Overfishing, pollution, and climate change are some of the unquestionable human-driven threats to ocean biodiversity (Pauly et al., 1998; Poloczanska et al., 2013; Steneck and Pauly, 2019; Sampaio et al., 2021) and within the notion of winners and losers of global change, there is evidence that some cephalopod populations may be benefiting from this changing ocean (Doubleday et al., 2016; Oesterwind et al., 2022). Within this context, this Research Topic (RT) aimed to compile the latest advances in cephalopod research, covering a wide range of disciplines, and encompassing different levels of biological organization (from molecules to ecosystems). Authors who contributed to the triennial Cephalopod International Advisory Council (CIAC) Meeting held in Sesimbra (Portugal), in April 2022, were especially encouraged to submit their findings here. CIAC 2022 provided a forum to discuss global issues related to human impacts while presenting the latest advances in cephalopod research. The meeting encompassed 90 oral presentations and 145 posters, grouped into eight topic sessions (Figure 1A), with 166 participants in person and 109 participants online, from 33 countries (Figure 1B).

This RT consists of 14 contributions, comprising 7 original research, 3 reviews, 2 brief research reports, and 2 perspectives (plus 1 correction), covering several different subjects, including life history, genomics, behaviour, physiology, biogeography, culture, climate change and other anthropogenic pressures. Regarding life cycles, all cephalopods have direct development, with no larval phase or metamorphosis. There are, however, two developmental modes: small planktonic paralarvae or large hatchlings as juveniles, among many other key traits. To build consensus towards a standard terminology regarding cephalopod ontogeny and life cycle patterns, Vidal and Shea review provides explicit definitions of the life phases and stages and cephalopod life cycle patterns as: Holopelagic, Holobenthic, Meropelagic, and Merobenthic. By doing so, the authors also

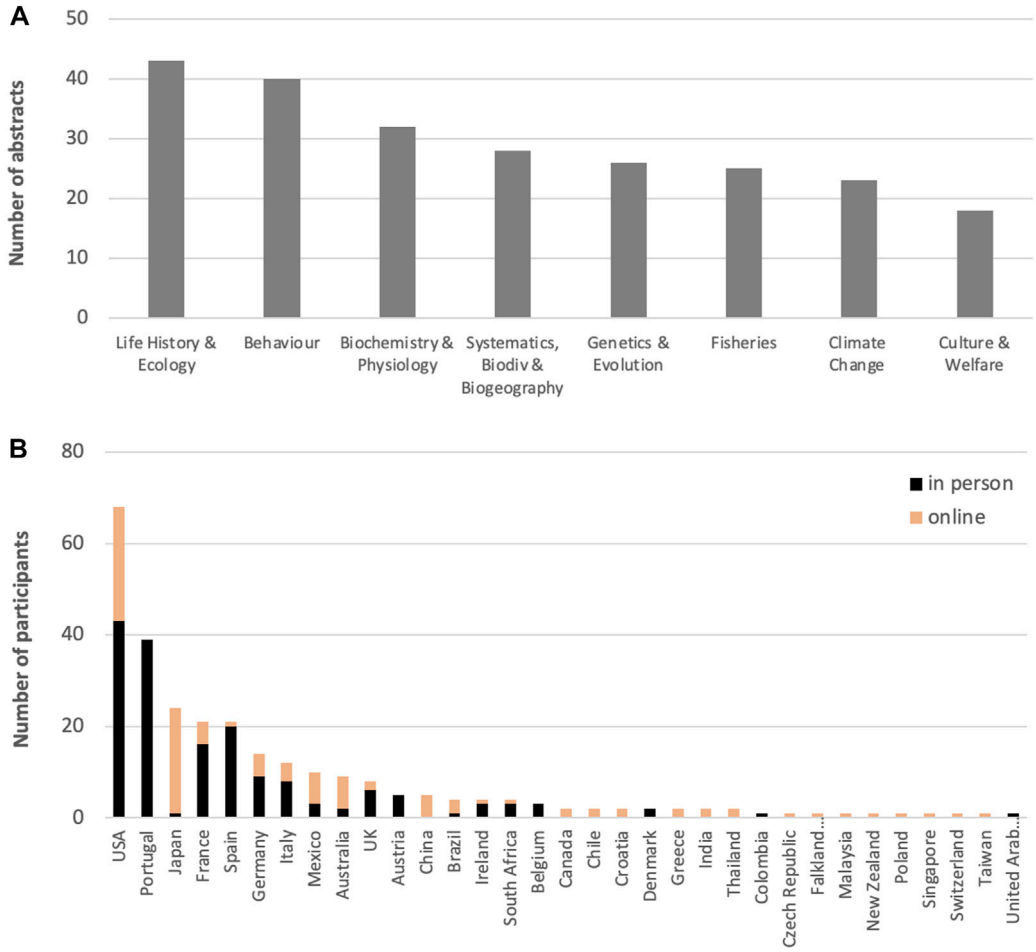

FIGURE 1
(A) Number of abstracts per session and **(B)** number of participants per country that participated in CIAC 2022 (Sesimbra, Portugal).

provide a unifying framework for future ecological and evolutionary research on cephalopods. In terms of genomics, studies describing cephalopod genomes have recently boomed. Yet, Vecchione et al. points out that: i) many studies do not provide suitable information to determine the source locality (for the genomic sequence), and ii) there is potential for taxonomic errors where the sampling area is very distant from the species' type locality. Last, they recommend that the genomic sample to be from the same biogeographic province (or "Large Marine Ecosystem") as the type locality, and that relevant information (e.g., museum catalogue number) should be included in resulting publications.

Regarding diversity and biogeography, Maloney et al. confirmed the presence of *Octopus insularis* in the Florida Keys, United States, by visual identification (body patterns and components) and through genetic analysis (COI, COIII, and 16S). Guarneros-Narváez et al. also used morphological and DNA barcoding (COI) tools to analyze species composition of Loliginidae paralarvae, respective abundance distribution (by size class and season), and genetic structure, on the Yucatan Shelf (Southeastern Gulf of Mexico). *Doryteuthis pleii* was the only loliginid recorded at the surface during three oceanographic cruises. High haplotype and nucleotide diversity, without population structure, suggest continuous gene flow throughout the studied region. Alongside, Zhu et al. showed that different-size forms of the purple flying squid (*Sthenoteuthis oualaniensis*) can be accurately distinguished in the South China Sea using gladius morphometrics. Such finding shows the valuable application of the gladius to study squid stock structure and population dynamics in a relatively cost-effective manner.

Concerning culture, Jolly et al. described a multi-generational laboratory system for two emerging cephalopod models, namely the hummingbird squid (*Euprymna berryi*), and Morse's bobtail squid (*Euprymna morsei*). Besides the description of the life cycles of these two *Euprymna* species, the authors discuss the general challenges of cephalopod culture and how these two species can help to build a bridge and establish cephalopods as model organisms. Behavioral ecotoxicology research is growing, and Gouveneaux et al. discusses the relevance of European common cuttlefish (*Sepia officinalis*) as a toxicological model. More specifically, they argue that the quantitative measurement of color change could be developed as a powerful endpoint for toxicological risk assessment. Regarding behaviour, the mirror self-recognition test (MSR) is commonly used as a means of testing self-awareness, but evidence of MSR in non-primates remains controversial. Here, Amodio and Fiorito provided

preliminary (baseline) data that can encourage further testing of MSR or similar behavioral tests in the *Octopus* (and other cephalopods).

Regarding climate change and other anthropogenic pressures, Borges et al. applied species distribution models to investigate potential changes in habitat suitability and geographical distribution of the *O. vulgaris* species complex (OVSC) in the future (2050 and 2100). Differential responses were observed in the OVSC species analyzed, namely: i) both *Octopus vulgaris* and *Octopus tetricus* showed a severe loss in distribution across their predicted range, ii) *Octopus americanus* exhibited projected removal close to the equator, with limited expansion towards the poles; iii) *Octopus aff. vulgaris* was projected to lose half of its current distribution; iv) *Octopus sinensis* exhibited moderate losses, with projected increases in northern areas; and v) *Octopus djinda* exhibited limited losses to its distribution. Alongside, Peinado et al. studied the predatory behaviour of *Sepioteuthis australis* under different thermal scenarios. They showed that squid efforts to capture prey were more persistent under warming conditions, presumably due to the associated higher energetic costs. However, the decrease in capture efficiency and increased prey handling time under warming suggest that important trade-offs need to be carefully explored. Both studies (Borges et al.; Peinado et al.) highlight the looming threat of ocean warming to cephalopods. Ocean acidification also has the potential to considerably impact cephalopod metabolism (Rosa and Seibel, 2008), suggesting some cephalopod species may not fare well under the increasingly changing conditions of the Anthropocene. Yet, Trueblood et al. showed that exposure to hypercapnia (1800 µatm) in the bathyal octopus *Muusoctopus leioderma* did not lead to changes in metabolic rates, critical partial pressure, and oxygen supply capacity. The ability to maintain aerobic physiology under these high CO_2 conditions is discussed and considered against phylogeny and life history. Last, Putland et al. examined potential effects of sound exposure (under laboratory conditions) on the hummingbird squid (*E. berryi*). They found that this species had significantly decreased hearing sensitivity following sound exposure, however such sensitivity was recovered within 2 hours. Because anthropogenic sounds have become more persistent, the authors argue that there may be limited time to recover from vessel sound exposure.

Workshops

Four workshops were held in Sesimbra before the CIAC conference (2–3 April 2022). Workshop 1—"*Cephalopod macroecology and biogeography,*" led by Christian Ibáñez and Rui Rosa, aimed to update the current knowledge on large-scale diversity and body size patterns in cephalopods (using Rosa et al., 2019, as a steppingstone) and discuss different biogeographic and macroecological hypotheses. Within the framework of this workshop, Otjacques et al. reviewed: i) the taxonomic diversity of luminous cephalopods and morphological features, ii) the respective large-scale biogeographic patterns, and iii) the research trends over the last 50 years on cephalopod bioluminescence.

Workshop 2—"*Research Topic, handling and care of cephalopod eggs and egg masses,*" led by Roger Villanueva, Anne-Sophie Darmaillacq, Michael J. Kuba, aimed to provide an overview in: i) methods to stimulate and increase spawning in laboratories, parental effects on embryo and hatchling quality; ii) natural and artificial oocyte fertilization; iii) egg Research Topic and/or monitoring egg masses from the wild; iv) environmental factors influencing embryonic development; v) incubation of eggs with and without maternal care; vi) artificial incubation of eggs in laboratory; vi) egg pathologies; vii) welfare, anaesthetics and humane killing of advanced embryos and hatchling, among others.

Workshop 3—"*The role of cephalopods as predators and prey: the relevance of cephalopod beaks in ecological studies,*" was led by José Xavier, Yves Cherel, Alexey Golikov, José Queirós, Catalina Perales-Raya, Rigoberto Rosas-Luis. In the resulting review paper, Xavier et al. discuss recent scientific developments in this field and identify future challenges, particularly in relation to taxonomy, age, growth, composition (i.e., DNA, proteomics, stable isotopes, trace elements) and physical (i.e., structural) analyses. New techniques (e.g., 3D geometric morphometrics) for identifying cephalopods from their beaks were also highlighted.

Workshop 4—"*Cephalopod genomics and evolution,*" led by Oleg Simakov and Caroline Albertin, aimed to consolidate, and solidify exchanges of protocols in the fields of cephalopod sequencing and evolutionary analyses, including but not limited to phylogenomics, (single cell) transcriptomics, regulatory genomics, etc. The feasibility of combining those approaches to obtain a measure of how much (little) is known about cephalopod gene regulation was discussed. Common problems faced by all cephalopod sequencing-related projects, and integration among different cephalopod systems were also examined.

Author contributions

RR, Lead writing; ZD, co-writing, revision and editing; MK, co-writing, revision, and editing; JS, co-writing, revision, and editing; EV, co-writing, revision, and editing; RV, co-writing, revision and editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Loliginid paralarvae from the Southeastern Gulf of Mexico: Abundance, distribution, and genetic structure

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Global commercial catches of squid have increased greatly in the last few years. However, approximately a quarter of the squid catches are still unidentified. In the southeastern Gulf of Mexico (SGoM), the squid catches are not recorded most of the time and are unidentified. This lack of knowledge limits the evaluation of the populations and prevents the establishment of conservation strategies. In this study, we used morphological and molecular (DNA barcoding– cytochrome c oxidase subunit I gene) identification tools to analyze the species composition of the family Loliginidae based on their paralarvae (PL), their abundance distribution by size class, and their genetic structure on the Yucatan Shelf, SGoM. A total of 134 PL were also collected from three oceanographic cruises held in 2015, 2016, and 2018. A total of 56 adults were collected from three ports of Yucatan. Both adults and PL were identified as *Doryteuthis pleii* (Blainville, 1823). The highest abundances of PL were detected from the West and the Central zones of the Yucatan Shelf at 50- and 15-m depth isobaths at 163 and 21 km from the coastline, respectively. The abundance was higher (316 PL•1,000 m^{-3}) in the early summer (June 2018), medium (213 PL•1,000 m^{-3}) at the end of summer and early autumn, and very low (24 PL•1,000 m^{-3}) in late autumn. A high haplotype and nucleotide diversity (*Hd*= 0.77; π= 0.002) with no structured population (F_{st} < 0) was also found, suggesting a continuous gene flow throughout the Yucatan Shelf. This information establishes the basis for a future comprehensive understanding of their biological cycle and population dynamics.

KEYWORDS

squids, *Doryteuthis pleii*, hatchlings, DNA barcoding, Yucatan Shelf

Introduction

Squids are relevant species for several fisheries around the world (FAO, 2020). Over the past two decades, the commercial catch and trade of squid have remained at the relatively high levels that have marked its almost continuous growth worldwide, while many coastal human populations obtain a significant proportion of their protein intake from locally caught cephalopod species (Arkhipkin et al., 2015; FAO, 2020).

The bulk of the global squid catch comprises species from two families, the Ommastrephidae and Loliginidae (Arkhipkin et al., 2015). However, approximately a quarter of the squid catches are still unidentified and/or are usually reported as common squid "Loliginidae" and/or "various squids" categories (Rodhouse, 2005). This is also true for the southeastern Gulf of Mexico (SGoM), where the loliginid squid fishery is mostly obtained incidentally as a by-catch from the shrimp and sardine fisheries, while catches per unit of effort are not recorded most of the time (Solís-Ramírez et al., 1998; Comisión Nacional de Pesca (CONAPESCA), 2018). The lack of reliable information limits the evaluation of the stocks and impedes the establishment of local management and/or conservation strategies (Ward, 2000; Olmos-Pérez et al., 2018).

Loliginid squids are demersal species, usually occupying coastal marine waters in tropical and temperate regions worldwide (Jereb and Roper, 2010). Like other cephalopods, loliginids have a zooplanktonic larval stage known as paralarvae (PL) (Young and Harmnan, 1988). Most PL are distributed in the first 200 m, so their catch is more efficient than adults and they are good indicators of the species richness of a region (De Silva-Dávila et al., 2018).

The taxonomy and systematics of the adults of the Loliginidae family are complicated due to the lack of taxonomic stability (subfamily to subgenus levels), inconsistent identification diagnosis, the existence of cryptic species, and natural hybridization (Brakoniecki, 1996; Vecchione et al., 2005; Jereb and Roper, 2010; Granados-Amores et al., 2013). Moreover, there is a lack of PL descriptions for tropical species (Sweeney et al., 1992; Vecchione et al., 2005), and there are important morphological differences between PL and juveniles, which do not allow morphological identification through their ontogenetic development (De Silva-Dávila et al., 2013; Kim et al., 2019). These constraints prevent unraveling loliginid life histories, particularly in tropical areas of the western Atlantic region (Rodhouse, 2015), such as the Gulf of Mexico (GoM).

At least five loliginid species inhabit the GoM, that is, *Doryteuthis* (*Amerigo*) *pealeii* (Lesueur, 1821), *D.* (*Doryteuthis*) *pleii* (Blainville, 1823), *Lolliguncula* (*Lolliguncula*) *brevis* (Blainville, 1823), *D.* (*Doryteuthis*) *roperi* (Cohen, 1976), and *Sepioteuthis sepioidea* (Blainville, 1823) (Judkins et al., 2009; Jereb and Roper, 2010; Judkins et al., 2010; Judkins et al., 2017). Ecological information available relies on adult collections created for taxonomic identification (Barrientos and García-Cubas, 1997; Díaz-Santana-Iturrios et al., 2019; Bravo-Muñoz, 2020), to describe their distribution, abundance, fishing prospects (Judkins et al., 2009; Díaz-Santana-Iturrios et al., 2019), and on fishing reports aimed to analyze the exploitation state of squid populations (Arreguín-Sánchez and Arcos-Huitrón, 2011). Although there are descriptions of loliginid hatchlings, the information on their presence in the GoM is scanty (Sweeney et al., 1992).

Molecular tools have facilitated the identification of cephalopod species at any developmental stage (Allcock et al., 2015; Díaz-Santana-Iturrios et al., 2019; Castillo-Estrada et al., 2020; Santana-Cisneros et al., 2021). This can be done using molecular markers such as the cytochrome *c* oxidase subunit I (COI) gene, which serves as a universal DNA barcode for organisms (Hebert et al., 2003). This gene can also provide information about many ecological traits, such as genetic diversity, population structure, genetic connectivity, phylogeographic analysis, and gene flow (Cowen and Sponaugle, 2009; Hellberg, 2009; Bucklin et al., 2011). This information is fundamental for the studies of population ecology and fisheries management (Ibáñez and Poulin, 2014; Sales et al., 2017; Roura et al., 2019).

Based on the above, in this study, we identified loliginid PL collected in the SGoM and compared them with the adult species reported in the fishing landings in the Yucatan Peninsula to know if they are the same species/populations. This information will aid in the understanding of the species composition and genetic structure of loliginids in the SGoM useful to support further fishing programs and/or management of this fishery.

Materials and methods

Study area

The SGoM is dominated by a large continental shelf that extends 200 km to the north offshore the Yucatan Peninsula and is 600 km long in an East–West direction. The study area (Figure 1) comprises the complete length and breadth of the Yucatan Shelf (YS), also known as the Campeche Bank, where the main fisheries of the region take place (Botello et al., 2010). In this area, there are three climatic seasons: the rainy season (June–October)—when tropical depressions occur, the dry season (March–May), and the anticyclonic ("Nortes") season (November–February), with cold fronts in which the water column is mixed, homogenizing environmental conditions (Lara-Lara et al., 2008; Enriquez et al., 2013).

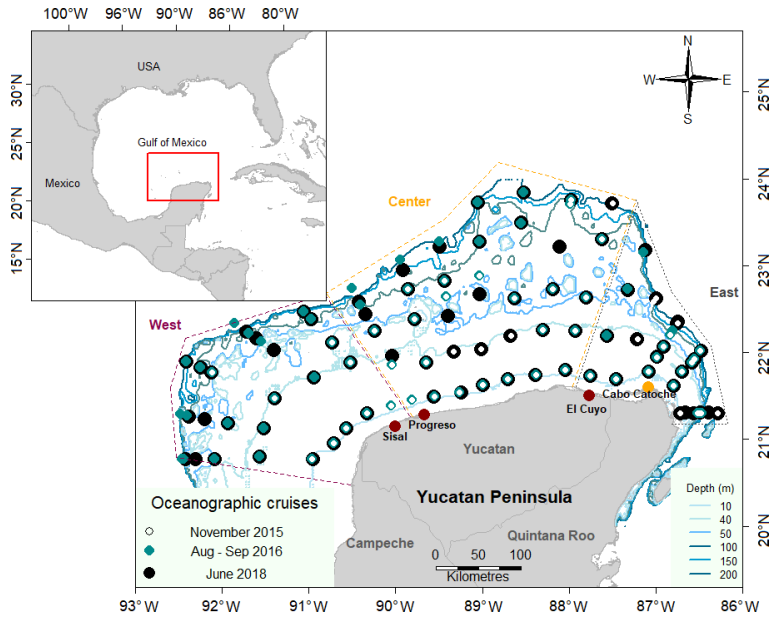

FIGURE 1
Study area and zooplankton sampling stations in the Yucatan Shelf, Southeastern Gulf of Mexico (SGoM). Red dots represent the ports where adult organisms were sampled. The dotted lines represent the three zones: Western (— purple), Central (—orange), and Eastern (—gray).

Field sample collection

Zooplankton samples were collected during three oceanographic cruises held from 2nd to 20th November 2015 (GOMEX-4, G4), 25th August to 8th September 2016 (GOMEX-5, G5) both onboard of the R/V "Justo Sierra" of the Universidad Nacional Autónoma de México, and from 5th to 18th July 2018 (GOMEX-6, G6) onboard the O/V Alpha Helix of the Centro de Investigación Científica y de Educación Superior de Ensenada. A permit for the mentioned collections (PPF/DGOPA-070/16) was issued by "Comisión Nacional de Acuacultura y Pesca."

The sampling design consisted of 18 transects perpendicular to the Yucatan coast, separated horizontally from each other by 35 km. Each transect comprised five sampling stations, and these were located to follow five different isobaths: 15, 50, 100, 150, and 200 m. The study area was also divided into three zones according to the oceanographic conditions on the shelf: West (influenced mainly by the Campeche Canyon Current, CCC), Central (as a transition zone and influenced by submarine groundwater discharges near the coast), and East, influenced by the Cabo Catoche upwelling (Enriquez et al., 2013) (Figure 1).

In each sampling station, surface trawls (10-m depth) were carried out at a constant speed of 2.5 knots using a standard Bongo structure (0.6-m diameter, with 333-μm mesh nets), and equipped with mechanically calibrated flowmeters (Sea Gear), used to estimate the volume of filtered water (Sameoto et al., 2000).

The samples of the G4 and G6 cruises were fixed in 96% ethanol with a complete replacement of the fixative after 24 h. The G5 samples were fixed in a 7% buffered formaldehyde solution. The G5 samples were not considered for molecular identification but were deposited at the collection of the Zooplankton Laboratory of the Centro de Investigación y de Estudios Avanzados of the IPN Unit Mérida. In addition, 34% (26 PL) of the G6 samples were included in the biological repository.

Adult loliginid specimens from the Yucatán coast were sampled in September and November 2019 in Sisal, Progreso, and El Cuyo ports where the by-catch fauna of the local artisanal sardine fishery is landed. Moreover, these ports are in the West, Central, and East zones of the studied area, respectively. Adults were sampled in only one vessel per port, and in each case, a tissue sample of ~1 cm^3 was dissected from the third left arm, fixed in 96% ethanol for molecular identification, and tagged.

Morphological and molecular identification

Loliginid PL were extracted from the entire zooplankton samples without fractionation. Some of the best-preserved specimens were photographed in dorsal and ventral views to keep a visual record of their main taxonomic characteristics (dorsal head and mantle chromatophore patterns) and were

identified to the most precise taxonomic level according to the criteria of Hanlon et al. (1992) and Vecchione et al. (2001). Adults from the landings were identified according to Jereb and Roper (2010) and Young et al. (2019).

The full body of G4 and G6 loliginid PL, and the adult samples (<1 g) were used for genomic DNA (gDNA) extraction using the Quick-DNA™ Universal kit (Zymo Research©) according to the manufacturer's protocol for total DNA isolation from solid tissues. The DNA concentration and quality were checked in a UV-visible Spectrophotometer QQIAxpert System de Qiagen. The genetic barcode identification was done with the mitochondrial cytochrome *c* oxidase (COI) gene, amplified using the universal metazoan primers LCO1490 (5'-GGTCAA CAA ATC ATA AAG ATA TTG G-3 ') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AATCA-3') (Folmer et al., 1994). Polymerase chain reactions (PCRs) were carried out using 0.25 µl of a mix of the two primers (10 µM), 12.5 µl of DreamTaq Green PCR Master Mix (Thermo Scientific©), 1 µl of gDNA (5–300 ng μl^{-1}), and the remainder of nuclease-free water to obtain a final volume of 25 µl. Amplification conditions were: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min, with a final elongation at 72°C for 5 min in a C1000 Touch Thermal Cycler (BIORAD™). Amplicons were sequenced in both senses using the Sanger et al. (1977) method by Macrogen Inc., Seoul, South Korea. DNA sequences were aligned with MUSCLE (Edgar, 2004) in MEGA7 software (Kumar et al., 2016), and aligned sequences were manually curated. The filtered sequences were compared with the Barcode of Life Data (BOLD) using their Identification System for COI-accepted sequences (https://boldsystems.org).

To corroborate the identity of the DNA sequences, we used published sequences of each loliginid species whose geographical distribution is within the study area (Table S1): *D. pealeii*, *L. brevis*, *S. sepioidea*, and sequences of *D. pleii* for North America (northern GoM and the northwestern Atlantic Ocean), western GoM—Tamaulipas, and for Brazil. The sequence of *Dosidicus gigas* was used as an outgroup. These sequences, together with the new sequences obtained from our analysis, were collapsed with CD-HIT-EST (t=0, c=0.98, n=5) to eliminate redundancies (Niu et al., 2010; Fu et al., 2012). The collapsed sequences were aligned with MUSCLE (Edgar, 2004) and formatted to nexus with Seqmagick (https://github.com/fhcrc/seqmagick).

The best evolutionary model was determined using the jModelTest2 (Darriba et al., 2012), using the Bayesian information criterion (BIC) for the Bayesian inference analyses. According to jModelTest2, we selected the GTR + I + G model. The phylogenetic tree was inferred using Mr. Bayes v. 3.2 (Ronquist et al., 2012). The tree was based on MCMC (Markov chain Monte Carlo) sampling, with 3,000,000 generations, a sampling frequency of 1,000, a consensus rule of 50%, and four chains (one cold and three hot), until achieving an average standard deviation of split frequencies >0.01. The 25% of the first trees sampled in the MCMC run were discarded as burn-in, and a consensus tree was obtained with the remainder. The resulting tree was converted to the Newick format with AfterPhylo.pl and was exported to the Interactive Tree of Life (iTol) (Ciccarelli et al., 2006 https://itol.embl.de/) for its visualization.

Size structure and abundance distribution of loliginid paralarvae

The dorsal mantle length (ML ± 0.01 mm) of 123 PL was measured, except for 11 PL that had no mantle and were not considered for this analysis, using the NEO PiWeb reporting plus Basic software, imaging ZEN and AxioVision connected to a digital camera AxioCam ERC Rev.2 under a binocular microscope ZEISS SteREO Discovery V8. To estimate the fresh ML based on the preserved PL (PPL) sizes, a shrinkage correction factor (SCF) of 25.8% was added to the computed values (Villanueva et al., 2016) because the size of fresh PL decreases with fixation and preservation (Goto, 2005).

Since the abundance of PL at the species-specific hatching size is a precise indicator of a recent hatching event (Yatsu et al., 1999; Zeidberg et al., 2012), we used the ML of fresh and of PPL for size-frequency analysis. Considering that the hatching size reported for *D. pleii* is 1.5-mm ML (McConathy et al., 1980) and that the general hatching size interval of 0.1–2.5 mm is likely for the newly hatched in several cephalopods (Villanueva et al., 2016), those PL from 2.6- to 4.0-mm ML were considered older than recently hatched. The total abundance per sampling station and the abundances by size interval by the sampling station were standardized as the number of PL in 1,000 m^3 of filtered seawater (PL•1,000 m^{-3}) (Smith and Richardson, 1977; Diekmann et al., 2006). The abundance distribution of PL at the sampling stations per cruise was plotted using the R programming language (R Core Team, 2019).

Genetic analysis

Genetic diversity indices [haplotype number (h), haplotype diversity (Hd), and nucleotide diversity (π)] (Nei, 1987) were estimated using the DnaSP v5 software (Librado and Rozas, 2009). To test the genetic population differences between zones, we performed a genetic structure analysis (F_{st}) and the analysis of molecular variance (AMOVA) with 10,000 permutations using Arlequin v. 3.5.2 (Excoffier and Lischer, 2010). Finally, a haplotype network was generated to visualize the relationships among haplotypes using the median-joining method implemented in Network Software V 10.1.0.0 (Bandelt et al., 1999).

Results

During the three oceanographic surveys, we collected a total of 134 loliginid PL, whereas 56 adult specimens were sampled from the fishing landings in the three ports of Yucatán (Table 1).

Morphological and molecular identification

Using the morphological identification approach, we identified 70 PL at the species level, 38 at the genus level (*Doryteuthis* spp.), and 26 at the family level (Loliginidae). All the adults were identified to species level. *D. pleii* was the only species found for both PL (Figure 2A) and adults (Figure 2B) according to the following morphological traits.

PL—(Figures 2A–C) Body form as bullet-shaped with well-developed terminal fins; well-developed ventral arms (arms IV > I) and tentacle clubs with four rows of suckers on the manus (Figure 2D). The chromatophore arrangement in the ventral head (in a roughly diamond-shaped quadrangle posterior to each eye) was initially observed but later faded with the fixation. Chromatophore pattern in the dorsal head, in well-preserved specimens, is 2 + 2 + 2, forming a hexagon and two central chromatophores in the lower dorsal mantle (McConathy et al., 1980; Hanlon et al., 1992).

Adult—Body long, fusiform. Mantle long, slender, cylindrical, the posterior end acutely pointed (Figure 2E), mature males present a striped pattern along the ventral surface of the mantle (Figure 2F). Fins are rhomboidal, longer than broad, usually 60% ML, and their sides fairly straight. Edge of vane straight (often slightly curved in females), thick, and ribbed or rod-like (especially in mature males). Suckers on ventral buccal lappets. Eyes not large; diameter of the externally visible eyeball 14%–19% ML, diameter of dissected lens 2%–7% ML. Left ventral arm hectocotylized in mature males by a modification of distal half to a fourth of arm that extends to arm tip; one-half to three-fourths (42–82) of suckers in a dorsal row much smaller than half the size of their ventral counterparts; modified (small) suckers on small, narrow, triangular pedicels (Figure 2G) (Hanlon et al., 1992; Vecchione & Young, 2010; Díaz-Santana-Iturrios et al., 2019; Migkuavacca and Simone, 2020).

We extracted DNA from 45 PL previously identified morphologically at the species level, two identified at the genus level, and eight identified at the family level. All adult samples were used for the molecular identification. DNA sequences were successfully obtained from 39 PL (71% of the DNA extractions: 30 from morphologically identified PL at the species level, 1 at the genus level, and 8 at the family level) and 56 adults (100%). In the remaining 16 PL, we did not obtain DNA samples of good quality, probably due to poor conservation of the samples. Thus, their identification was only based on morphological characteristics, such as the 26 PL of the G6 samples selected for the biological collection and the 53 PL of the G5 samples fixed in formalin (Table 1).

The BLAST analysis revealed that the sequences from the PL (39) and the adults (56) had 99.76%–100% similarity (99%–100% coverage) with the sequences of *D. pleii* (Table S2). The sequences obtained in this study were phylogenetically clustered with the *D. pleii* sequences from North America and western GoM but were separated from the Brazil sequences (Figure 3). The number of PL and adults and their final identification, either morphologically or molecularly, are shown in Table 1. Therefore, with both identification approaches, *D. pleii* was the only loliginid species found for the SGoM during November 2015, August–September 2016, and July 2018 in surface-collected zooplankton samples (Table 1, Figure 2).

Size structure and abundance distribution of loliginid paralarvae

In G4 (November 2015), the total loliginid PL collected accounted for 24 PL•1,000 m^{-3}, during G5 (August–September

TABLE 1 Number of paralarvae and adults of loliginids from the southeastern Gulf of Mexico (SGoM) and level of identification by morphological and DNA barcoding approach.

Specimen	Survey	Number	Morphology Identification			*DNA Barcode*
			Family	*Genus*	*Species*	*Species*
Paralarvae	G4	4	0	1	1	2
	G5	53	15	36	2	0
	G6	77	3	0	37	37
Total		134	18	37	40	39
Adults	Sisal	25	0	0	0	25
	Progreso	15	0	0	1	15
	El Cuyo	16	0	0	0	16
Total		56	0	0	0	55

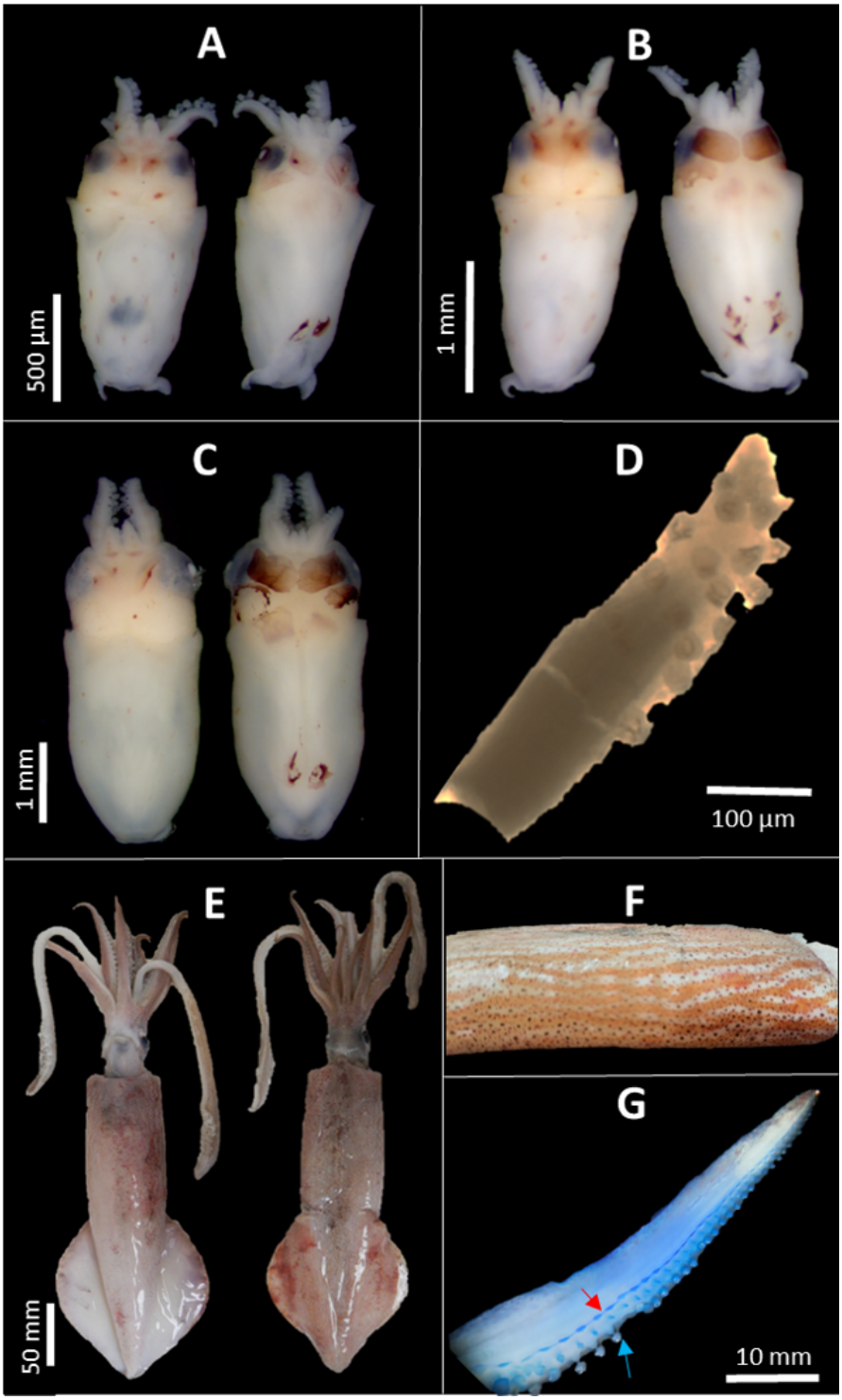

FIGURE 2
Doryteuthis pleii paralarvae collected in the Yucatan Shelf. Ventral and dorsal views of paralarvae of **(A)** 0.8 mm **(B)** 1.4 mm, **(C)** 3 mm of mantle length. **(D)** Tentacle club with four rows of suckers on manus of a 3 mm paralarvae. **(E)** Ventral and dorsal (265 mm ML), **(F)** hectocotylus (red arrow indicating modified suckers and blue arrow the regular ones) and **(G)** ventral mantle with the striped pattern along the surface of a male adult from Progreso port of Yucatan.

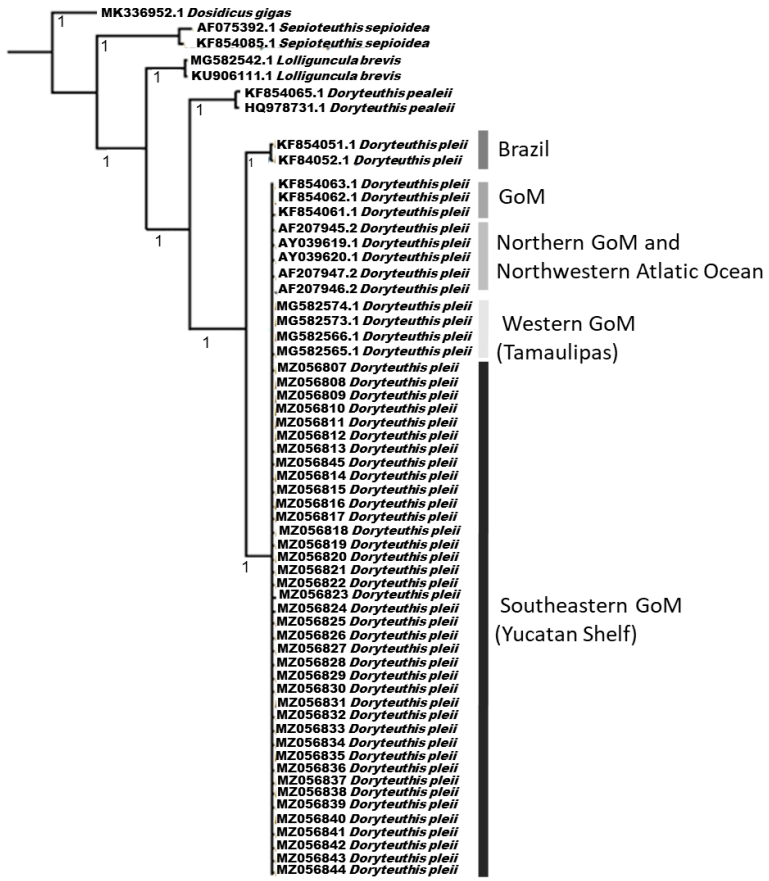

FIGURE 3
D. pleii phylogenetic tree comparing representative sequences of the species in other geographic regions and the sequences of other three loliginid species that occur in the same distributional area. *Dosidicus gigas* is used as an outgroup. The posterior probability values are plotted on each resolved branch.

2016), they increased to 428 PL•1,000 m^{-3}, and abundance during G6 (July 2018) reached a maximum of 974 PL•1,000 m^{-3}. However, only those PL measured (in good morphological conditions) and their standardized abundance were included for size structure analysis. The loliginid PL from the three cruises recorded a size range between 0.88 and 4.15 mm ML (2.04 ± 0.74, n= 123) after applying the SCF (Figure 4A). The total abundance of hatchlings (≤2.5-mm ML) (for the entire period of study) was 380 PL•1,000 m^{-3}, representing 75% of total abundance (this percentage increases by 16% if the SCF is not applied).

The loliginids collected in November 2015 had a range size of 1.76–2.26-mm ML (2.05 ± 0.21, n= 4) and all were hatchlings (Figures 4A, B). In this cruise (November 2015), the lowest PL abundance recorded was 24 PL•1,000 m^{-3}, from which 4 PL•1,000 m^{-3} were *Doryteuthis* spp. and 20 PL•1,000 m^{-3} were identified as *D. pleii* occurring mainly at the Central zone at 20–50 m deep and between 24 and 120 km from the coastline (Figure 5A, B, 6A, B).

In August–September 2016 (G5), the size range was 1.25–3.52 mm ML (1.91 ± 0.48, n= 51) (Figure 4A) of which 88% were hatchlings, but this percentage dropped by 8% in the PPL (Figure 4B). Total PL abundance in this cruise was 213 PL•1,000 m^{-3}, of which 63 PL•1,000 m^{-3} were identified only at the family level, 141 PL•1,000 m^{-3} as *Doryteuthis* spp., and 9 PL•1,000 m^{-3} as *D. pleii* (Figures 6C, D). Hatchlings represented 42% of the total abundance occurring more in the West zone, over the isobaths of 20–50 m (Figure 6C). The zones with the highest abundance are in the range of 20–150 km from the coastline (Figure 5B).

In July 2018 (G6), the largest size range for loliginid PL was registered. The PL measured from 0.88- to 4.15-mm ML (2.14 ± 0.9, n= 68) (Figure 4A) of which 65% were hatchlings (Figure 4B). The highest abundance (316 PL•1,000 m^{-3}) was also recorded on this cruise. From these, 13 PL•1,000 m^{-3} were identified as Loliginidae, and 303 PL•1,000 m^{-3} as *D. pleii* (Figures 6E, F). Hatchlings representing 56% of the total abundance occurred with the highest abundance at the West

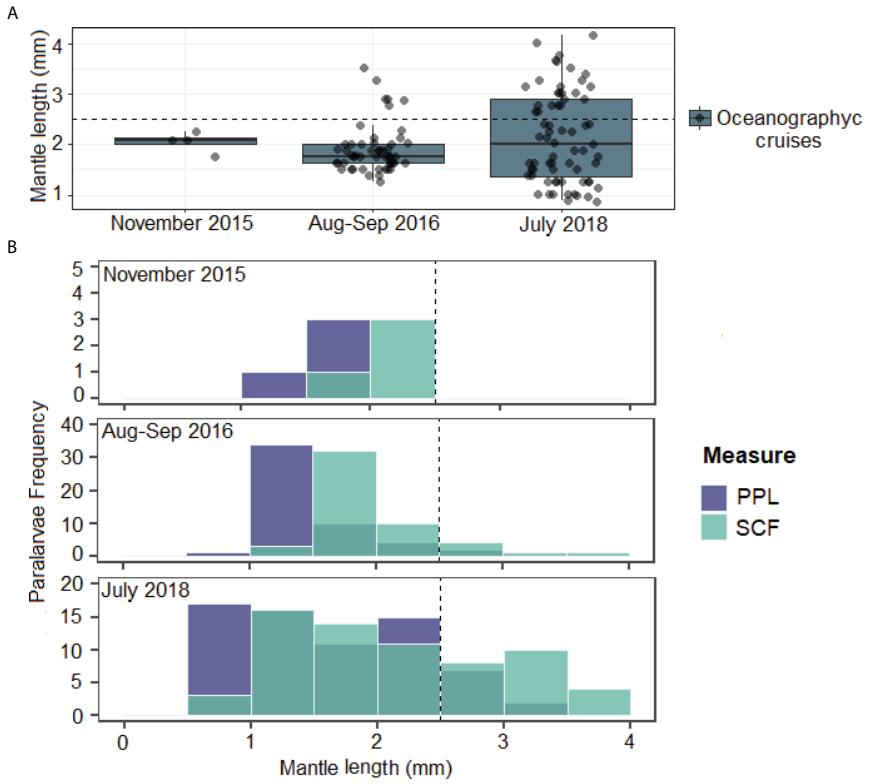

FIGURE 4
Loliginidae paralarvae. **(A)** Mantle length (ML) data dispersion by cruise carried out at the southeastern Gulf of Mexico. **(B)** Size structure of paralarvae during November 2015, August-September 2016, and July 2018. Frequency of preserved paralarvae (PPL) and frequency of fresh specimens were calculated with the shrinkage correction factor (SCF). The percentage of newly hatched organisms (<2.5 mm ML) with the SCF is shown.

zone between the isobath 20–50 m and 44 m of depth and 162 km away from the coastline (Figure 5, 6E).

D. pleii paralarvae genetic structure

All the 91 COI sequences analyzed (35 PL and 56 adults) corresponded to *D. pleii*, distributed in 39 haplotypes (Table S2). Based on the values calculated by Goodall-Copestake et al. (2012), there was a high haplotype diversity (*Hd*= 0.77) and nucleotide diversity (π = 0.002). These values varied slightly among sampling zones, with a general high haplotypic and nucleotide diversity (Table 2). The AMOVA with 1,023 permutations showed no differences among populations (Table 3), the fixation index among the three zones had a negative value (F_{st}= -0.0035, *p*= 0.67) which should be taken as zero and indicates that there is no population structuring. This is consistent with the haplotype network results, where we did not find any formed haplogroup, with the presence of a central haplotype has the highest frequency (*n*= 44), shared by the PL and adults from the three zones (Figure 7).

Discussion

Morphological and DNA barcoding identification

In the GoM, there are five loliginid species reported (*D. pealeii, D. pleii, L. brevis, D. roperi*, and *S. sepioidea*) (Judkins et al., 2009, 2010; Jereb and Roper, 2010; Judkins et al., 2017). However, based on the morphological characteristics and molecular analysis of PL and adults reported herein, *D. pleii* (Blainville, 1823) was the only species of the Loliginidae family recorded during our surface sampling. In the Northwest Atlantic, *D. pealeii* (the most closely related species to *D. pleii*) migrate to shallow waters to reproduce in spring (Jereb and Roper, 2010). As this season was not sampled in our study, it is reasonable that we could not register this species. Other factors to consider in explaining the absence of the other loliginid species are the differences in PL distribution and egg size. In *L. brevis*, PL are most abundant near the bottom in inshore coastal waters with a salinity of approximately 26 UPS and can already tolerate very low oxygen concentrations. *Sepioteuthis sepioidea* (the Caribbean reef squid) has a distribution mainly limited to

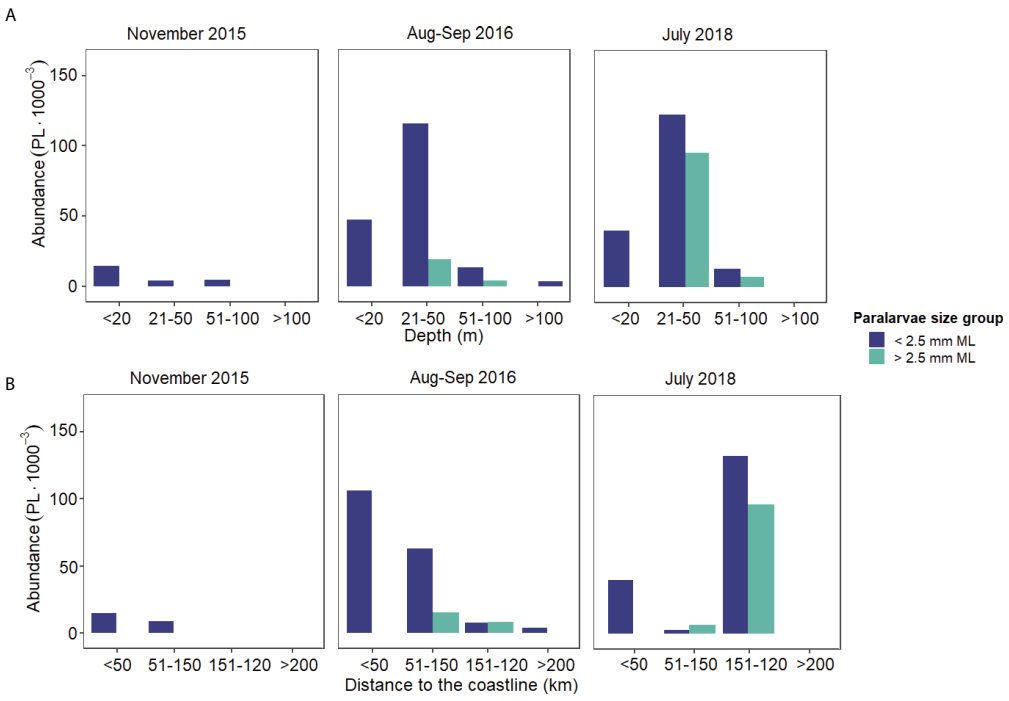

FIGURE 5
Horizontal distribution of Loliginidae PL abundance by size class (0.1–2.5 mm ML and 2.6–4.15-mm ML), **(A)** depth range and **(B)** distance to coastline per survey performed at the Yucatan Shelf, Southeastern Gulf of Mexico.

coral reefs, and turtle-grass flats (*Thalassia testudinum*), where they laid small clusters of three-to-four eggs in large, gelatinous capsules under rocks or in conch shells (Jereb and Roper, 2010). *Doyteuthis roperi* has been described as associated to islands (Vecchione and Young, 2010); nevertheless, Diaz-Santana-Iturrios et al. (2019) considered that this species could be synonymous to *D. pleii* since they are very similar morphologically and the morphometric differences are the result of intraspecific variability.

Furthermore, in the phylogenetic analysis with the new sequences from the present study, we found evidence supporting Sales et al. (2013; 2017) that *D. pleii* from the northwestern Atlantic and Gulf of Mexico represents a genetically distinct species from *D. pleii* in the southwestern Atlantic.

Abundance distribution of loliginid paralarvae according to ML

The hatching size in loliginids varies according to species (Guerra et al., 2001). The hatching size reported for *D. pleii* was 1.5-mm ML in a range of temperatures between 21°C and 23°C (Mc Conathy *et al.*, 1980), being one of the loliginid species with the smallest reported hatching size. In the YS, we found PL with less than 1-mm ML size at a temperature range of 21°C–29.8°C. The smaller PL with MLs <1.0 mm representing a low proportion of the PL collected probably reflects the effect of the temperature since squid are known to display different growth strategies depending on temperature (Jackson and Forsythe, 2002; Rosa et al., 2012), with warmer temperature resulting in smaller hatchlings (Vidal et al., 2002; Villanueva et al., 2003). In addition, the small size could be affected by the shrinkage that occurred during the fixation and preservation of the PL; the use of the SCF determined by Villanueva et al. (2016) provides a better approximation of the probable size of our collected PL previous to the fixative solution application. When applying the SCF adjustment, the percentage of PL smaller than 2.5-mm ML dropped to 16%. Similarly, Martínez-Soler et al. (2021) analyzed the PL community in the mouth of the Gulf of California, Mexico, and observed an overall decrease (11%) in the percentage of PL recently hatched after applying the same SCF. However, the high proportion of specimens with sizes <2.5-mm ML in our study, suggests that hatching occurs over the north YS mainly in the Central and Western zones. Due to the limited information about the shrinkage percentage that occurred by fixation in loliginids, the SCF we used here must be considered with caution as it was estimated using five cephalopod species of families Octopodidae, Eledonidae, Megaleledonidae, and Sepiolidae (Villanueva et al., 2016), which are very different from our PL from YS.

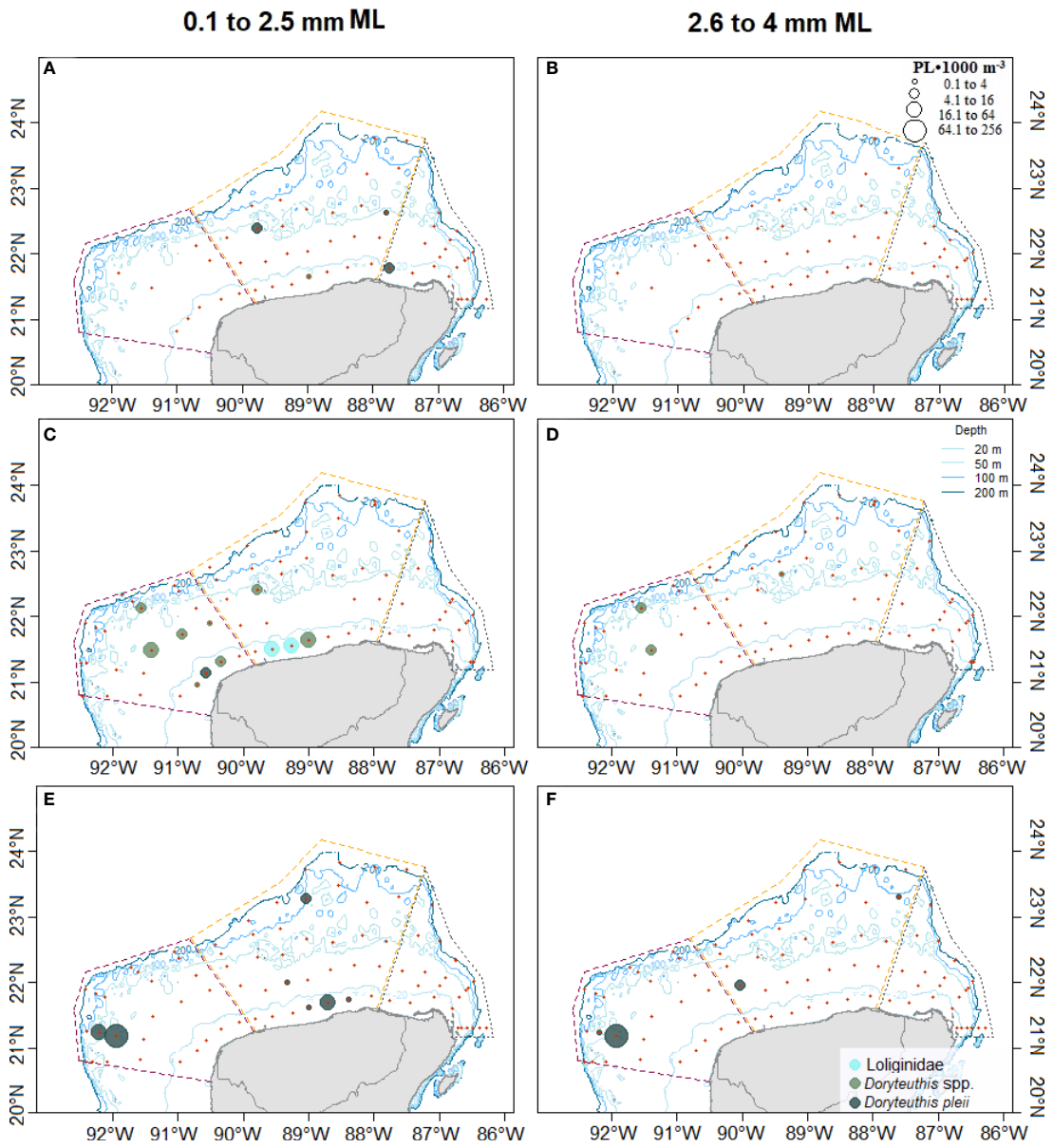

FIGURE 6
Distribution of the abundance of loliginids by cruise and size group in the Yucatan Shelf, Southeastern Gulf of Mexico **(A, B)** November 2015, **(C, D)** August-September 2016, and **(E, F)** July 2018. The dotted lines represent the three zones: Western (— purple), Central (—orange), and Eastern (—gray). Red crosses represent sampling stations.

TABLE 2 *Doryteuthis pleii* haplotype diversity from the Yucatan Shelf, SGoM.

Location	N	h	Hd ± SD		π ± SD	
All localities	91	39	0.77	0.002	0.0023	0.0003
West	49	23	0.78	0.004	0.0023	0.0004
Central	26	13	0.76	0.008	0.0021	0.0004
East	16	9	0.767	0.013	0.0029	0.0008

N, number of sequences; h, number of haplotypes; Hd, haplotype diversity; SD, standard deviation; and π, nucleotide diversity.

TABLE 3 Analysis of molecular variance of the cytochrome *c* oxidase subunit I region of *D. pleii* mtDNA, from the Yucatan Shelf, SGoM.

Source of variation	df	SS	Variance components	Percentage of variation	*p*	Fst
Among populations	2	1.265	-0.00244	-0.35	0.67058	-0.0035
Within populations	88	61.504	0.69891	100.35	–	–
Total	90	62.769	0.69647	–	–	–

df, degrees of freedom; SS, sum of squares; Fst, fixation index.

The PL abundance (with sizes close to their species-specific hatching size) has been used as an indicator of the reproductive activity of adults (Martínez-Soler et al., 2021). In the YS, the PL abundance showed an important variability, with higher abundance in early summer (June 2018), medium at the end of summer and early autumn (August–September 2016), and low in late autumn (November 2015). This PL abundance pattern could be related to the maturity season of the species.

The maturity and lifespan of *D. pleii* vary by region due to environmental factors (Jackson and Forsythe, 2002; Perez et al., 2006). Mature squids have been found year-round with two seasonal peaks: spring and autumn in the Caribbean Sea (during late spring to early summer and autumn in northeastern Venezuela), and in summer and winter in the southern Brazilian waters (Perez et al., 2002; Jereb and Roper, 2010). The life span of *D. pleii* in the northwestern GM encompasses 6 months (Jackson and Forsythe, 2002) and an annual cycle in the southern Brazilian waters (Perez et al., 2006). In the SGoM, it has not been determined yet, but considering the temporal abundance of hatchlings of *D. pleii*, and using the *D. pealeii* egg size, spatial distribution, and hatching time between 15 and 20 days (Summers, 1983) as the closest species reference, together with the results of this study, we infer that the spawning event for *D. pleii* may occur during the spring season (March–May) and that the hatching may take place during the summer and early autumn (June–October) were the highest abundance took place in the Central-Western region. The East zone is mainly influenced by the Cabo Catoche upwelling and the Yucatan Current. Thus, the small number of PL (11 PL•1,000 m^{-3}) recorded in this area suggests that hatching could be transported to the Central and West zones or contrastingly advected far from the shallow YS to the northern Gulf of Mexico, favoring the connectivity of the species.

D. pleii genetic structure

Dispersion plays a fundamental role in structuring populations (Weersing and Toonen, 2009). The biological and ecological characteristics of organisms affect their dispersal ability and the gene flow among their populations (Ibáñez and

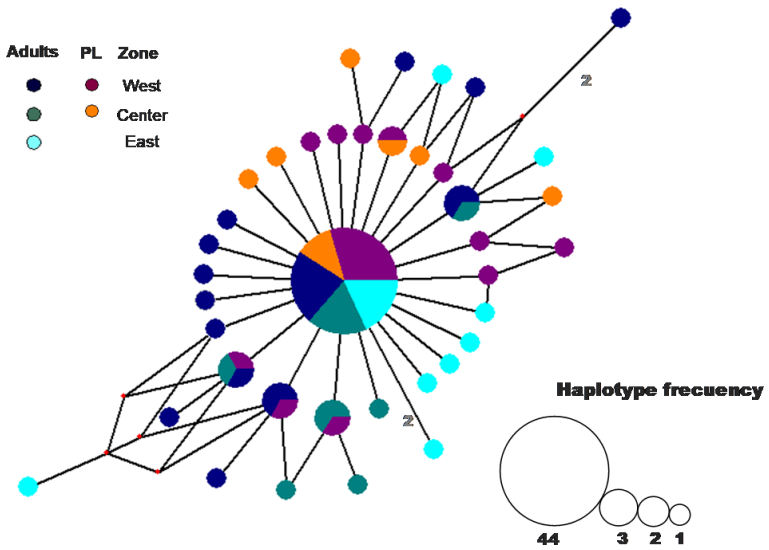

FIGURE 7
D. pleii haplotype network of PL and adult specimens from Southeastern Gulf of Mexico. Circles represent a unique allele. Red dots are the calculated average vectors.

Poulin, 2014). The loliginids PL exhibit a "merobenthic" dispersal (Boletzky, 2003), which can affect their horizontal distribution along the continental shelf (Roura et al., 2016). However, they could travel hundreds of kilometers (Roberts, 2005).

The period that *D. pleii* remains as a planktonic PL is still unknown, but loliginids remain between 2 and 3 months as part of the plankton (García-Mayoral et al., 2020). In addition, the smaller the hatchling, the broader the latitudinal distributional range of the species (Villanueva et al., 2016). As a result of its high dispersal capacity, loliginids should keep genetic homogeneity through an increased gene flow (Weersing and Toonen, 2009; Ibáñez and Poulin, 2014; Roura et al., 2019). In the present study, we observed that *D. pleii* had a high haplotype and nucleotide diversity, according to the values proposed by Goodall-Copestake et al. (2012), with no population structuring. The haplotype network presented a star shape also found in this species (Sales et al., 2017) and other loliginid species (Olmos-Pérez et al., 2018; Roura et al., 2019; García-Mayoral et al., 2020) where many unique haplotypes radiate from few common haplotypes. This pattern indicates high gene flow, which suggests that there are no barriers preventing the spread of this species along and across the YS. In contrast, Sales et al. (2017) reported a subpopulation of this species in Campeche (Southern GoM), which has a low haplotypic diversity (*Hd*= 0.396) and does not share haplotypes with the other populations throughout the distribution of the species (North Northwestern Atlantic, Northern Gulf of Mexico, SGoM–Campeche, Southwestern Atlantic). Our study does not clarify whether the YS population shares haplotypes with the Campeche population or with the rest of the populations around, but it is consistent with the high haplotype diversity of the species and differs from the low haplotypic diversity of Campeche specimens reported for Sales J.B. de et al. (2017).

Based on our results, we encourage further surveys to explore different seasons in which the presence of other species in the area might be revealed and to relate the oceanographic influence on the PL distribution and their dispersal capacity, to explain the genetic structure of *D. pleii* on the YS. It is also the key to analyze the relation of the greatest upwelling pulse, which occurs mainly in the eastern region of the YS, since it is reported that loliginid PL is positively related to them (Moreno et al., 2009; Roura et al., 2016; Ruvalcaba-Aroche et al., 2018; Roura et al., 2019).

Conclusions

This study reports for the first time the species composition of PL of the family Loliginidae, their abundance distribution by size class, and the genetic structure of *D. pleii* on the Yucatan Shelf, SGoM. Based on morphological and molecular identification, the only species of loliginid identified was *D. pleii*. The highest abundance of PL reported here was found in the West and Central zones of the YS. We suggest that the spawning event of *D. pleii* may occur from May to September. Genetic analysis indicates that there is a high gene flow throughout the Yucatan Shelf, SGoM. This knowledge establishes the basis for a future comprehensive understanding of their biological cycle and population dynamics for the design of strategies for management and conservation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The approval was not necessary since the organisms sampled were obtained from the plankton samples and the adult organisms came from the commercial catches of the fishermen.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by PG-N, RS-D, JZ-B, and MA-P. Funding acquisitions, project administration, resources, and software were performed by RR-C, AS, UO, and IV-A. The first draft of the manuscript was written by PG-N, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.941908/full#supplementary-material

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A preliminary attempt to investigate mirror self-recognition in *Octopus vulgaris*

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Mirror self-recognition (MSR) is a potential indicator of self-awareness. This capability has been widely investigated among vertebrates, yet it remains largely unstudied in invertebrates. Here we report preliminary data about behavioural responses exhibited by common octopuses (*Octopus vulgaris*) toward reflected images of themselves and explore a procedure for marking octopus' skin in order to conduct the Mark test. Octopuses (n = 8) received four familiarization trials with a mirror and four familiarization trials with a control stimulus: a non-reflective panel (*Panel* group, n = 4) or the sight of a conspecific housed in an adjacent tank (*Social* group, n = 4). Subsequently, octopuses were marked with non-toxic nail polish in the area where the Frontal White Spots are usually expressed, and they received one test trial with the mirror and one control trial with no mirror. We found that octopuses in the *Panel* group tended to exhibit a stronger exploratory response toward the mirror than the non-reflective panel, but performed agonistic responses only in the presence of the mirror. In contrast, octopuses in the *Social* group exhibited comparable exploratory and agonistic behaviours toward the mirror and the sight of the conspecific. In the Mark test, octopuses frequently explored the mark *via* their arms. However, mark-directed behaviours were also observed in the absence of the mirror and in sham-marked individuals, thus suggesting that proprioceptive stimuli drove these responses. Despite the limitations associated with our marking procedure, the baseline data collected in this pilot study may facilitate the further testing of MSR in the octopus and other cephalopods.

KEYWORDS

mirror, self-recognition, octopus, cephalopod, mark test

Introduction

Mirrors are popular tools in the field of animal behaviour and cognition. Classically used to investigate agonistic behaviour (e.g., Lissmann, 1932; Tinbergen, 1951), reflective surfaces have acquired a prominent role in the study of self-awareness since Gallup (1970) devised the so-called "Mark test" to probe mirror self-recognition (MSR) in chimpanzees. In short, individuals are confronted with their reflected image after a dye has been applied

on a part of the body that can only been seen by the animal itself through the mirror. It is assumed that passing the Mark test—i.e., exhibiting mark-directed behaviours only in front of the mirror and towards visible marks, but not sham marks—is evidence that the individual can recognize its reflection. Consequently, this is considered an indicator of self-awareness (Gallup, 1970). Note, however, that alternative accounts have challenged the relationship between successful performances in the Mark test, MSR, and self-awareness (e.g., Heyes, 1994; Mitchell, 1997) or evoked a more gradualist approach to studying self-awareness and interpreting the performance of non-human animals in the Mark test (de Waal, 2019; Wittek et al., 2021).

Studies testing MSR involve a preliminary phase of familiarization in which unmarked individuals are allowed to freely interact with a mirror. Those that pass the test typically exhibit different kinds of response in a progressive fashion during the familiarization phase. In particular, 1) social responses such as agonistic displays, and 2) physical exploration of the mirror and the area behind precede 3) contingency checking, i.e., unusual repetitive body movements; finally, 4) self-exploratory behaviours (e.g., inspection of body parts that can only be seen in a mirror) are exhibited (Plotnik et al., 2006; Brecht et al., 2020). Therefore, performance in the familiarization phase may provide important indications about whether mirror reflections are interpreted as the image of a conspecific or the individual itself (Anderson and Gallup, 2015).

More than 50 years after Gallup's (1970) study, MSR has been widely investigated in vertebrates, from primates to fish. In addition to the great apes (Gallup, 1970; Suarez and Gallup, 1981; Anderson and Gallup, 2015), only a few species have been claimed to have passed the Mark test, most notably dolphins (Reiss and Marino, 2001), elephants (Plotnik et al., 2006), magpies (Prior et al., 2008), and cleaner wrasses (Kohda et al., 2019, 2022). However, evidence of MSR in non-primates remains controversial, typically due to the difficulties in interpreting the performance in the Mark test by animals whose morphologies constrain clear attempts to remove the mark or methodological issues with the marking procedure and design (Gallup and Anderson, 2018, 2020). For instance, Prior et al. (2008) used stickers to mark magpies, a procedure that might have provided tactile stimuli to the birds, therefore confounding the results (Soler et al., 2014). A more cautious interpretation of MSR capabilities in magpies is further justifed by the negative results obtained in the same species (Soler et al., 2020) and in other members of the corvid family (e.g., carrion crows, Brecht et al., 2020; Vanhooland et al., 2020; for reviews in corvids see Baciadonna et al., 2021; Brecht and Nieder, 2020). In contrast to the foregoing studies in vertebrates, MSR remains largely unexplored in invertebrates (but see Riojas-Schnier and Toth, 2022 for a recent experiment in wasps). Coleoid cephalopods (cuttlefish, squid and octopus) represent a valuable candidate invertebrate group for addressing this gap in a systematic fashion. First, these molluscs exhibit complex nervous systems (Young, 1971, 1991; Shigeno et al., 2018) and flexible behavioural repertoires (reviewed in: Amodio et al., 2019b, 2019a; Hanlon and Messenger, 2018), two features that may indicate a certain degree of cognitive sophistication (for a critical discussion see: Amodio, 2019; Schnell et al., 2021; Ponte et al., 2022). Second, coleoids evolved not only acute vision (reviewed in: Budelmann, 1995), but also visual systems that are specialized for detection of transient skin markings such as stripes and spots, to mention but a few. Adaptations in coleoid visual systems clearly evolved, at least in part, for the perception of dynamic body patterning exhibited through the skin which afforded the rich repertoires of bodily appearance used as signals to communicate with conspecifics and heterospecifics (for review see: Borrelli et al., 2006; Hanlon and Messenger, 2018). Third, coleoids are equipped with sets of flexible appendages that can be employed to physically explore their own bodies as well as the external environment. Octopuses regularly groom their heads and mantles through their arms (Mather, 1998). Importantly, experimental evidence also indicates that octopuses can guide the movement of one arm toward a specific location using visual information (Gutnick et al., 2011). Therefore, the anatomical, behavioural, and sensory adaptations of coleoids seem particularly well suited to the detection of skin marks and the expression of mark-directed responses, both of which are crucial requirements for passing the Mark test. Nevertheless, the investigation of MSR in coleoids poses unique—albeit not intractable—challenges. The lack of fur, feathers, or scales on the bodies of these animals, together with their colour changing ability, make the marking procedure more complicated than in the case of other species.

Despite the promise of coleoids as good candidates for the study of MSR, little is known about how these molluscs perceive and respond to reflected images of themselves; nevertheless, some possible indications of MSR have been collected in the context of visual communication. Examining the role of polarization vision in intraspecific interactions, Shashar et al. (1996) found that short exposures (e.g., up to 30 s) to mirrors induce common cuttlefish (*Sepia officinalis*) to retreat from images of themselves. In the same species, Palmer et al. (2006) observed a previously undescribed body pattern (termed "*Splotch*") displayed only by female cuttlefish in the presence of mirrors and same-sex conspecifics, thus suggesting that reflected self images may have been perceived as another individual of the same sex. In addition, a few preliminary investigations by Ikeda and colleagues have employed mirrors to explore MSR in coleoids (for a review see Ikeda, 2009). In one experiment, Ikeda and Matsumoto (2007) presented a group of squid (*Sepioteuthis lessoniana*) with a mirror and a wood board. They found that the former induced some individuals of the school to approach and physically explore the stimulus, whereas the latter did not alter the schooling behaviour relative to when no stimulus was present. In a subsequent study, Ikeda and

Matsumura (2008) extended testing of mirror-induced response to seven additional species, including a cuttlefish (*Sepia pharaonis*) and several octopus species (e.g., *Octopus laqueus*, *Hapalochlaena lunulata*, *Abdopus aculeatus*). The authors reported that squid and cuttlefish showed, respectively, strong and moderate interest towards reflected images of self (e.g., physical exploration, agonistic response), while octopuses did not react to the stimulus (Ikeda, 2009). Finally, a preliminary Mark test experiment in *S. lessoniana* by Ikeda (2007) indicated that individuals with visible marks showed a stronger tendency to observe and physically interact with the mirror, relative to sham-marked individuals (Ikeda, 2009). Unfortunately, the latter two studies have only been published as conference abstracts, so no detail is provided regarding methods and results.

Here, we report a pilot study exploring MSR in the common octopus (*Octopus vulgaris*). In particular, we aimed to: 1) acquire preliminary data about the behavioural response exhibited by octopuses toward a mirror as well as that in response to two control stimuli, namely, a non-reflective panel and a conspecific; and 2) test a procedure for marking octopus' skin in order to conduct the Mark test.

Methods

Subjects and housing

Eight common octopuses (*Octopus vulgaris*) of both sexes (5 M, 3 F) were included in the study (body weight range: 154–406 g). The animals were caught by artisanal fishermen in the Gulf of Naples (Mediterranean Sea, Italy) and transferred to the Stazione Zoologica Anton Dohrn, within a few hours of capture. Octopuses were housed in standardized tanks (60 × 100 × 50 cm) comprised of dark grey PVC, with the front side consisting of a glass panel (45 × 35 cm) to permit video recording of experiments. Following Fiorito and co-workers (e.g., Fiorito and Scotto, 1992; Amodio and Fiorito, 2013; Borrelli et al., 2020), adjacent tanks were separated by a clear lateral wall made of glass which could allow visual—but not physical—interaction between two individually-housed octopuses. This clear lateral wall could be obscured via a removable dark grey PVC panel to prevent visual interaction between pairs of octopuses outside a specific testing condition (see Procedures). A thin layer of sand covered the bottom of the tanks and two bricks, arranged as a den, were placed in the back corner of each tank, opposite the adjacent tanks (Borrelli et al., 2020). Circulating seawater (a semi-open system) was pumped directly from the Gulf of Napoli through the Stazione Zoologica life support systems. Lamps (Neodymlite dichroic halogen MR16, Oy Airam AB, Finland) were positioned at 1.40 m above the tanks and programmed to reproduce the appropriate seasonal dark-light daily cycle at the local latitude (for details see: Borrelli et al., 2020). Animals were fed every other day with a live crab (*Carcinus mediterraneus*). The present study was conducted in November 2012.

Acclimatization

Octopuses were acclimatized to the laboratory prior to the start of the experiment. In accordance with previous studies (e.g., Amodio et al., 2014; Borrelli et al., 2020), we used octopus' predatory response as a proxy to evaluate the level of acclimatization. Following the day of arrival in the laboratory, animals were presented each morning with a live crab attached to a cotton thread. The crab generally started to move spontaneously after reaching the bottom of the tank, but if necessary (i.e., in case the crab remained still or exhibited freezing behaviour), the experimenter induced movements by the crab through gentle pulling of the thread. The prey was promptly pulled out of the tank just before the octopus could seize it. We measured the *Latency to attack*—e.g., "the time elapsed from the appearance of the crab at the water surface to just before the octopus' final pounce on the prey" (Borrelli, 2007, p. 82). Octopuses recovered their predatory response and readily attacked the crab (*Latency of attack* < 10 s) within approximately 1 week. As the predatory response is also considered a measure of the overall motivation and wellbeing of the octopus (Fiorito et al., 2015), we continued to monitor the *Latency of attack* in acclimatized animals daily throughout the study.

Experimental procedure

The experiment encompassed a familiarization phase followed by the Mark test. During the familiarization phase, octopuses received four trials in which they were exposed to a glass mirror (80 × 50 cm) and four trials in which they were exposed to a control stimulus. The animals were randomly assigned to two groups that differed in the type of control stimulus. The *Panel* group (n = 4) was presented with a non-reflective dark grey PVC panel (80 cm × 50 cm), whereas the *Social* group (n = 4) was presented with the sight of a conspecific of the same sex and comparable size (e.g., body weight difference <35 g) that was housed in the adjacent tank. This allowed only visual—but not physical—interactions between animals. Octopuses in the *Social* group were exposed to the same individual throughout the familiarization phase.

On each trial, the experimenter waited for the octopus to be inside or in close proximity to the den before presenting the stimulus. The mirror and non-reflective panel were introduced at a distance of approximately 30 cm from the entrance of the den and placed such that the stimulus leaned on the opposite long side of the tank, creating an angle of approximately 20°. This ensured that the octopus could see the stimulus from the den and

TABLE 1 Definitions of the behavioural variables that were scored in the study.

Category	Behavioural variable	Definition	References
Exploration	*Latency to Touch*	Time elapsed from the moment when the stimulus contacted the surface of the water (or when the partition separating the adjacent tanks was being raised) to the moment of the first contact between the octopus and the stimulus (or the glass side separating the adjacent tanks)	
	Physical Contact	Duration of time spent in physical contact (via one or multiple arms) with the mirror, non-reflective panel, or glass side separating the two adjacent tanks. See also Figure 1A	
	Behind Stimulus	The octopus explores the area of tank hidden behind the mirror or non-reflective panel. See also Figure 1B	
Agonistic	*Attack*	The "octopus launches itself directly towards the . . . stimulus, swimming by the propulsion of water from its funnel . . . and without touching the bottom" (p. 39). See also Figure 1C	Packard, (1963)
	Active Avoidance	The octopus maximises the distance between its body and the stimulus, while typically moving away from it and displaying a uniform dark brown coloration. See also Figure 1D	
	Bishop	The octopus exhibits curved arms with interbrachial web maximally spread and mantle rounded, often pointed upwards. The animal is dark brown with typically paling arms. See also Figure 1E	Borrelli et al., (2006)
Self-directed	*Grooming*	The octopus "uses one to two arms and bends its arms tubes vertically and laterally so the distal halves extend over its head and mantle and even inside the mantle cavity, generally moving laterally and unevenly in a wormlike motion" (p. 313)	Mather, (1998)
	Cleaning Manoeuvre	The movement consists of a "rapid twirling of the arms while they are held in close to the sides of the body starting at the base and continuing with increasing speed to the tips. The keratinuos linings to the suckers are shed in the process and subsequently blown away from the animal by jets from the funnel" (p. 785)	Packard and Sanders, (1971)
Other	*Unilateral*	The animal "may ... be in different phase on one side of the body from that on the other: one side dark, the other light" (p. 93). See also Figure 1F	Packard and Sanders, (1969)
	Passing Cloud	A localized "dark flush (lasting less than a second) that passes outwards from the head over [the] dorsal region of arms and web" (p. 785). See also Supplementary Video S1	Packard and Sanders, (1971)

could explore the area behind the stimulus. To allow visual interaction between animals in the *Social* group, the experimenter lifted the opaque partition obscuring the glass wall between the two tanks, thereby allowing the octopus to see the conspecific housed in the adjacent tank. After 20 min following the presentation of the stimulus, the experimenter introduced a crab attached to a thread into the tank and tested the octopus' response toward the prey following the same procedure described earlier. In light of limited interaction with mirrors reported for other octopods (Ikeda and Matsumura, 2008; Ikeda, 2009), this aspect of our experimental design was intended to induce the octopus to leave the den and thus increase the chances that the animals would interact with the stimulus. Finally, 30 min after the start of trial, the stimulus was removed and the initial conditions restored.

The familiarization phase was conducted during two consecutive days. On each day, octopuses participated in two sessions (two trials per session), one in the morning and one in the afternoon, approximately 1.5 h after the first session. The order of presentation of stimuli was kept constant such that within each session, octopuses performed the trial with the control stimulus (i.e., non-reflective panel: *Panel* group; conspecific: *Social* group) before performing the trial with the mirror. The inter-trial interval within each session was set to 30 min.

The Mark test was conducted on the third day of the study. In the morning, the octopus was transferred to a bucket filled with a mild anaesthetic solution: seawater with 1.5% $MgCl_2$ (Grimaldi et al., 2007). After the animal was sedated, it was marked in correspondence to the Frontal White Spots (Packard and Sanders, 1971; review in Borrelli et al., 2006): two oval spots that are transiently expressed approximately 1 cm below the eyes. This area was selected because it cannot be seen directly without a mirror and because the Frontal White Spots are a salient component of different body patterns (Packard and Sanders, 1971) that might also play a role in intraspecific communication. As a mark, we first applied a drop of Histoacryl® (Aesculap AG)—a soft tissue adhesive—and then, on top of this, a drop of non-toxic nail polish (Frais Monde ®). Five animals were marked with red polish, whereas the remaining three animals received a sham mark (i.e., transparent polish). After the marking

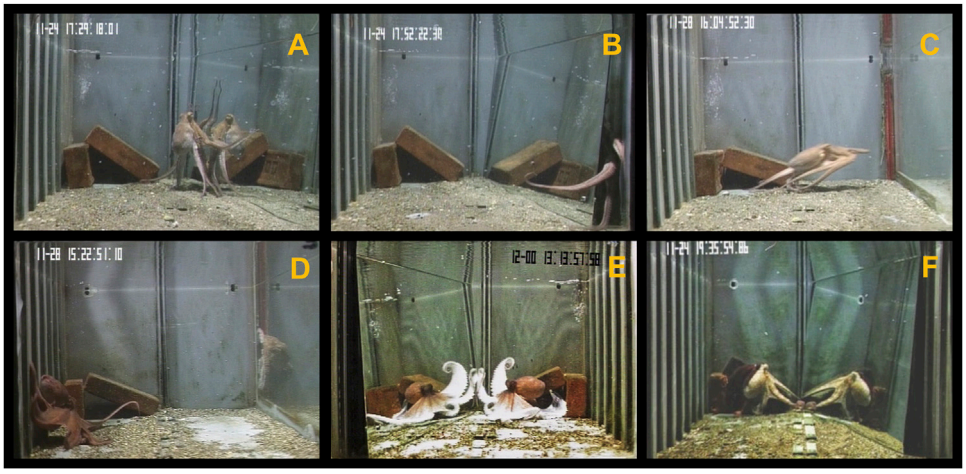

FIGURE 1
Octopus' response toward the mirror **(A,B,E,F)** and the conspecific **(C,D)**. Behaviors recognized: **(A)** *Physical contact*; **(B)** *Behind stimulus*; **(C)** *Attack*; **(D)** *Active avoidance*; **(E)** *Bishop*; **(F)** *Unilateral*.

procedure was completed, the octopus was moved back to the tank and given one hour to recover. Finally, the animal received two test trials. In the first trial, octopuses in the *Panel* group were presented with the non-reflective panel, whereas octopuses in the *Social* group were presented with no stimulus. In the second trial, all octopuses were presented with the mirror. Test trials were conducted following the same procedure described earlier for the familiarization phase. Inter-trial interval was again set to 30 min. All trials were video-recorded and subsequently analysed.

Data analysis

In the familiarization phase, octopus' response towards the stimuli was characterized in terms of exploratory, agonistic, and self-directed behaviours. We scored eight variables belonging to these three categories (see Table 1; Figure 1). Additionally, we focused on two body patterns that are exhibited in a variety of contexts, namely *Unilateral* and *Passing cloud* (Table 1). It was not possible for the coder to be blind to the conditions during the analysis of video recordings because the stimuli presented to the octopus (i.e., mirror, non-reflective panel, conspecific) were visible in the video recordings.

For each variable, we calculated individual mean duration (for *Latency to touch* and *Physical contact*; see Table 1) or frequencies (for all other variables; Table 1) by averaging the response towards each stimulus in the four familiarization trials. Subsequently, we used paired Wilcoxon signed-rank tests to compare the two conditions within each group. Alpha was set to 0.05. In the test, we scored the number of touches to marks performed in each trial. These data were reported descriptively. In addition, as in the case of the familiarization trials, we scored the behavioural variables described in Table 1. These data were not analysed statistically, but rather were collected and described as potentially informative for future studies. All statistical analyses were performed in R (v. 4.0.2) using the RStudio (v. 1.4.1103) wrapper (RStudio Team, 2018).

Results

Familiarization: *Panel* group

In the *Panel* group, no significant difference was detected in the comparisons of exploratory behaviours between conditions (Wilcoxon signed-ranked test: *Latency to touch*, $n = 4$, $W = 0$, $p = 1$; *Physical contact*, $n = 4$, $W = 8$, $p = 0.250$; *Behind stimulus*, $n = 4$, $W = 6$, $p = 0.375$). However, octopuses exhibited longer *Physical contact* and more frequent *Behind stimulus* behaviours toward the mirror than toward the non-reflective panel (Figure 2). This trend was also evident at the individual level for all octopuses except one (i.e., Animal 12/123). Agonistic behaviours were exhibited exclusively in the presence of the mirror (Figure 2), therefore no statistical test was conducted to compare the two conditions. Self-directed behaviours were observed at relatively low frequency in the presence of both stimuli (Figure 2). No statistical difference was found in the comparisons of self-directed behaviours between conditions (Wilcoxon signed-ranked test: *Grooming*, $n = 4$, $W = -4$, $p = 0.414$; *Cleaning Manoeuvre*, $n = 4$, $W = -2$, $p = 0.772$). Octopuses also exhibited the *Unilateral* body pattern. Despite the fact that discernible behaviours were observed more frequently in trials with the mirror than in trials with the non-reflective panel (Figure 2), comparison of the two conditions yielded no significant

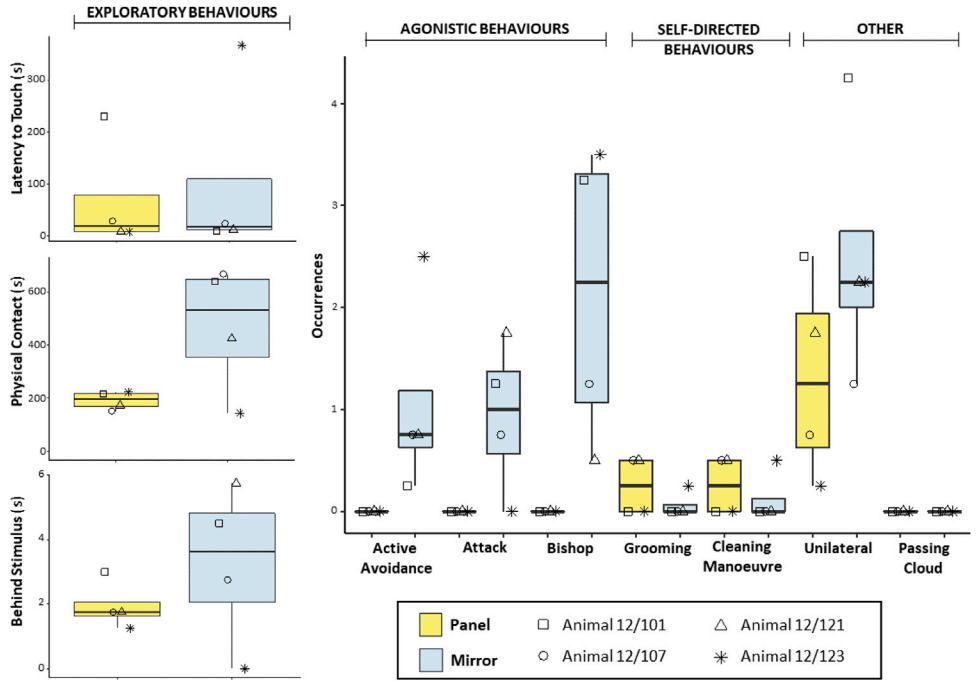

FIGURE 2
Box and whisker plot of the responses of *O. vulgaris* (*Panel* group) to the mirror (light blue) and the non-reflective panel (yellow) during the familiarization phase.

difference (Wilcoxon signed-ranked test: $n = 4$, W = 10, $p = 0.097$). Finally, the *Passing cloud* display was never observed in either condition.

Familiarization: *Social* group

The video footage of one trial was lost (octopus 12/111, trial 2, Social condition) before it could be analysed. For this individual, mean values for the Social condition were therefore calculated by averaging the performance across the three available trials.

Octopuses in the *Social* group exhibited comparable *Latency to touch* in the presence of the mirror and the conspecific, whereas they performed seemingly longer *Physical contact* in trials with the conspecific than in those with the mirror (Figure 3). However, no significant difference between conditions was detected for either exploratory behaviour (Wilcoxon signed-ranked test: *Latency to touch*, $n = 4$, W = −6, $p = 0.375$; *Physical Contact*, $n = 4$, W = −4, $p = 0.625$). Agonistic behaviours were recorded in both conditions, yielding comparable frequencies (Wilcoxon signed-ranked test: *Attack*, $n = 4$, W = −1, $p = 1$; *Active avoidance*, $n = 4$, W = −3, $p = 0.370$; *Bishop*, $n = 4$, W = 1, $p = 1$; Figure 3). Self-directed behaviours were again observed in both conditions, yielding comparable frequencies (Wilcoxon signed-rank test: *Grooming*, $n = 4$, W = −4, $p = 0.410$; *Cleaning manoeuvre*, $n = 4$, W = −1, $p = 1$; Figure 3). The *Unilateral* body pattern was exhibited in the presence of the mirror and the conspecific, yielding comparable frequencies (Wilcoxon signed-rank test: $n = 4$, W = 4, $p = 0.420$; Figure 3). Two individuals performed *Passing cloud* in the social condition and one individual also did so in the presence of the mirror (Supplementary Video S1). No statistical difference was found in the comparison between conditions (Wilcoxon signed-rank test: *Passing cloud*, $n = 4$, W = −3, $p = 0.370$).

Mark test

Seven out of eight octopuses (including the three sham-marked individuals) groomed their mark and attempted to remove it using their suckers (Table 2). This was typically achieved via a single arm, sometimes following the physical exploration of an area close to the mark, such as the head or the proximal part of one of the first pair of arms (Supplementary Video S2). Notably, in 30 out of 42 instances, mark-directed behaviours were observed in control trials, and thus when the mirror was not present in the tank (Table 2). In only one of the eight paired trials did the octopus (individual 12/109) touch the mark more often in the mirror trial than in the control trial.

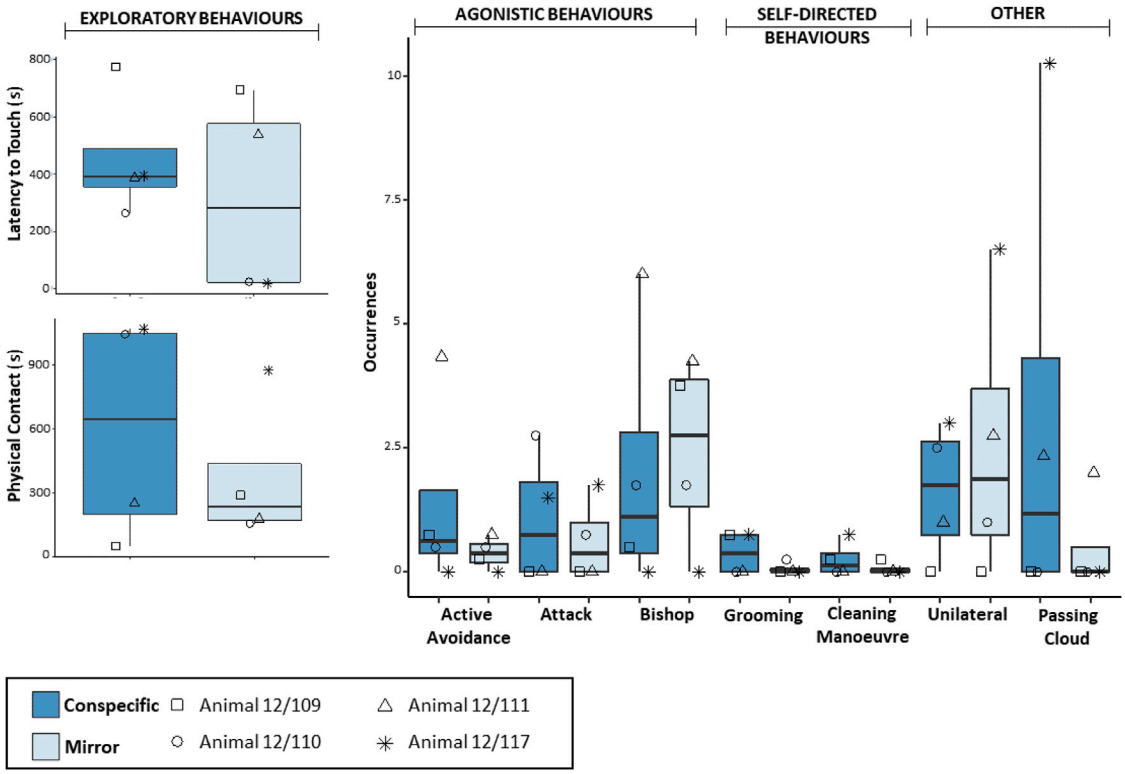

FIGURE 3
Box and whisker plot of the responses of *O. vulgaris* (*Social* group) to the mirror (light blue) and the conspecific (dark-blue) during the familiarization phase.

TABLE 2 Frequency of touches to marks observed in the test.

Individual	Mark	Control Trial	Mirror Trial
12/101	Sham	5	0
12/121	Sham	7	6
12/123	Sham	7	0
12/107	Red	0	0
12/109	Red	0	3
12/110	Red	6	1
12/111	Red	4	2
12/117	Red	1	0

Discussion

Familiarization phase

The first aim of this pilot study was to characterize the response triggered by reflected images of self in the common octopus. To this end, we exposed animals to four familiarization trials with a mirror and four familiarization trials with a control stimulus—namely, a non-reflective panel (*Panel* group) or the sight of a conspecific of the same sex and a similar weight (*Social* group). In contrast to the findings in other octopods (Ikeda and Matsumura, 2008; as reported in Ikeda, 2009), our preliminary results indicate that mirror-induced response is variable in the common octopus. We observed frequent physical investigations of the reflective surface and the area behind the surface, as well as attacks, avoidance behaviours, and aggressive displays directed towards the mirror. In the presence of the non-reflective panel, agonistic behaviours were completely absent, whereas exploratory behaviours were overall weaker (e.g., shorter *Physical contact*, less frequent *Behind stimulus*). An exception to the latter result was detected in the performance of one individual. Octopus 12/123 exhibited relatively long *Latency to touch*, minimal *Physical contact*, and never explored the area behind the mirror (*Behind stimulus*). At first blush, these data might be interpreted as a weak response or little interest directed toward the mirror. Yet, when the agonistic response of the animal is taken into account, it seems more likely that the observed limited exploration of the mirror could have been due to a fearful reaction. In fact, high frequencies of *Active avoidance* and *Bishop*, but no *Attack* were scored for octopus 12/123. Given the limited sample size (n = 4), it is possible that the performance of this

individual—which behaved in differently when compared with others—had a strong influence on the statistical analysis, thus affecting the outcomes and resulting in the non-significant findings in cases where apparent differences between conditions could be detected (i.e., *Physical contact* and *Behind stimulus*; Figure 2).

On the other hand, exploratory and agonistic responses in the *Social* group were comparable between conditions and, in some cases, also consistent at the individual level. For instance, two individuals (e.g., 12/109 and 12/111) never directed attacks toward their reflected image or a conspecific, whereas the remaining two individuals (i.e., 12/110 and 12/117) did so consistently in both conditions. The latter two octopuses were also observed exhibiting shorter *Latency to touch* and longer explorations of one or both stimuli (Figure 3). These data raise the possibility that within each pair, animals established a kind of dominant-subordinate relationship. Such a possibility is consistent with the fact that octopuses 12/110 and 12/117 were never exposed to each other, but rather to individuals 12/111 and 12/109, respectively. Note that, despite being non-gregarious creatures, octopuses have been reported to live at high densities in some sites (e.g., Guerra et al., 2014; Scheel et al., 2016, 2017), form social hierarchies in captivity (Cigliano, 1993), and distinguish between familiar and unfamiliar conspecifics (Tricarico et al., 2011, 2014).

With regard to self-directed behaviours, no clear trend was detected: grooming and cleaning manoeuvres tended to be expressed in low frequencies regardless of whether the animals were being exposed to the mirror, non-reflective panel, or conspecific. It is possible that our data simply represent the normal rates of expression of grooming and cleaning manoeuvres, such that the stimuli used in this study may not have played a key role as triggers. Notably though, the cleaning manoeuvre was displayed in the presence of the mirror only by octopuses 12/123 and 12/109, two individuals that showed an inclination to avoid self-reflected images relative to others. Considering that the cleaning manoeuvre could also function as a displacement activity in common octopus (Packard, 1963), the possibility cannot be excluded that some instances of this behaviour were in fact expressed in response to the stimuli used in the study, particularly if these were perceived as a source of distress. A relevant parallel here may be provided by self-scratching in primates, a displacement activity which is recognized as an indicator of anxiety (Dell'Anna et al., 2022; Troisi, 2002) and which has been observed in response to reflected images of self (Anderson and Gallup, 2015). Ultimately, investigating baseline rates for cleaning manoeuvres in the common octopus could provide insights regarding the interpretation of this behaviour in the presence of a mirror.

Overall, the kinds of mirror-induced responses that we observed in the common octopus match those reported in other coleoids—the physical exploration of the mirror in squid (Ikeda and Matsumoto, 2007) and social displays in decapods (Palmer et al., 2006; Ikeda and Matsumura, 2008; Ikeda, 2009)—and further expand upon them. Thus, our study seems to challenge the idea that the complexity of mirror-induced behaviours correlates with the degree of gregariousness in coleoids, in decreasing order from squids to cuttlefish to octopuses (Ikeda and Matsumura, 2008; Ikeda, 2009). More generally, the repertoire of exploratory, agonistic, and (potentially) self-directed behaviour, as well as inter-individual variability, also resemble those frequently reported among the vertebrates (Plotnik et al., 2006; Anderson and Gallup, 2015; Kohda et al., 2019; Brecht et al., 2020)—at least in the initial phase of exposure to the mirror. However, species that pass the mark test also tend both to perform unusual and repetitive body movements (contingency checking) and use the mirror to inspect otherwise non-visible body parts (self-exploratory behaviour). Throughout the familiarization phase, we could detect no self-exploratory response, though we did notice two unusual and repetitive responses. The first behaviour (here termed "*mantle bobbing*") resembles a social display described in *Abdopus aculeatus* (i.e., Mantle Bounce Display, Huffard, 2007) and comprised a slow and rhythmic up-and-down movement of the mantle (Supplementary Video S3). The second behaviour (here termed '*sweeping*') comprised a quick, repetitive, and worm-like movement of the distal part of one arm over the reflective surface (Supplementary Video S4). This differed from the more common physical exploration of the stimuli, in which a large part of the ventral surface of the body was kept in contact with mirror. Constant visual contact with the self-reflected image was maintained while the two behaviours were being performed. At this stage, it is not clear whether the *mantle bobbing* and the *sweeping* behaviours constitute true instances of contingency checking. Yet, these behaviours might provide insights about how octopus perceive their reflection, and as such, should be further investigated. To this end, longer familiarization with the mirror may be important, considering that in our experiment the exposure to the stimulus was short (i.e., 120 min) relative to other studies where it lasted several days (e.g., Povinelli et al., 1993; Kohda et al., 2019; Brecht et al., 2020).

Mark test

The second aim of the present study was to test a procedure for performing the Mark test in the octopus. To this end, sedated animals were marked with a thin layer of soft tissue adhesive overlaid by a layer of non-toxic nail polish. In the test trials, we observed grooming and attempts at removal of the marks both in the absence of the mirror and by sham-marked individuals. Therefore, it is likely that proprioceptive stimuli, rather than visual stimuli, triggered mark-directed behaviour in our experiment. This idea, together with a progressive reduction of mark-directed response, perhaps due to habituation, would

also explain the result that a frequency of touches to the mark greater than 70% was observed when the mirror was not present in the tank, namely in the first test trial (Table 2). Alternatively, more complex visual stimuli (e.g., reflected images of self) could have diverted octopus' attention from any proprioceptive stimulus induced by the marks. Future research should therefore explore alternative marking procedures to test MSR in cephalopods. The use of elastomers as marks might provide a valuable option, given that these subcutaneous tags have been successfully employed to monitor octopus populations in the wild (Brewer and Norcross, 2012), as well as to conduct the Mark test in fish (Kohda et al., 2019).

In conclusion, the present study shows that common octopuses display a distinct and varied repertoire of mirror-induced responses and, we believe, provides preliminary baseline data for further exploration of MSR in this species. Despite the shortcomings of the marking procedure we used here, results obtained in our test trials demonstrate that octopuses are capable of performing mark-directed responses and that the Mark test offers a suitable paradigm for investigating MSR in these animals. However, because many of the responses we observed were not visually mediated, it would be important for future research to include additional controls in the Mark test, including measurement of the frequency of physical exploration of an unmarked area of the body. In addition, another critical next step would be to provide longer exposure to the mirror during the familiarization phase. This would both provide an opportunity to explore variability of mirror-induced behaviour over time and afford further insights into how octopus perceive their own reflections.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the animal study because the Directive 2010/63/EU, which regulates the use of live animals (including cephalopods) for research purposes, entered into force on 1 January 2013 and was introduced into Italian national legislation in March 2014. The experiments included in this study were carried out in November 2012, i.e., before the Directive was in place. The present study was reviewed and approved by the Animal Welfare Body of the SZN after it was completed (Ethical Clearance, case SZ 2/2022/ec).

Author contributions

PA: data collection and analysis, production of figures, writing of original draft, review and editing of iterations of the manuscript. GF: conceptualization, supervision, review, and editing of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.951808/full#supplementary-material

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Form identification of purple flying squid (*Sthenoteuthis oualaniensis*) based on gladius morphology

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Stock structure information is an important part of bases for understanding the dynamics of cephalopod populations. Purpleback flying squid *Sthenoteuthis oualaniensis* is an abundant and highly productive species in tropical and subtropical waters of the Indo-Pacific region. However, it is characterized by a complex stock structure, and the stock discrimination is an urgent priority to understand basic biology and for stock assessment and management purposes. Here, we used gladius morphology to identify and discriminate the dwarf without a dorsal photophore and middle-sized typical forms of *S. oualaniensis* in the South China Sea. Results showed that both forms had double axes on the gladius and females consistently had a larger gladius than males. Multivariate analyses using the gladius morphometric characteristics indicated that individuals of dwarf form without a dorsal photophore were distinguished from those of middle-sized typical form, which was evidenced by the obvious groups and significant dissimilarity of gladii of these two forms. The discrimination based on the gladius morphometric characteristics showed a high rate of accuracy, in which a global discrimination accuracy was estimated to be 92.36% for both forms without regarding sexes and 65.82% for the discrimination to form and sex. In combination, these lines of evidence indicated that individuals of dwarf without a dorsal photophore and middle-sized typical forms of *S. oualaniensis* can be accurately distinguished using the gladius morphometric characteristics, and these results will warrant the application of the gladius to study the stock structure of *S. oualaniensis* and other squid else.

KEYWORDS

***Sthenoteuthis oualaniensis*, gladius, form discrimination, hard structure, South China Sea**

Introduction

Purpleback flying squid *Sthenoteuthis oualaniensis* is an abundant and highly productive species that inhabits open waters of the Indo-Pacific region, including tropical and subtropical waters (Jereb and Roper, 2010). This species is characterized by fast growth, short lifespan, and semelparous reproduction (Liu et al., 2016). *S. oualaniensis* plays a critical role in epipelagic to mesopelagic waters, preying on a wide spectrum of food organisms from mesozooplankton to myctophids and supporting diverse marine predators including squids, fishes, sharks, whales, and seabirds (Jereb and Roper, 2010). *S. oualaniensis* is also becoming an important fishery species (Zhang et al., 2014). In the central South China Sea (SCS), for example, *S. oualaniensis* is a major target for the small-scale jigging fishery (Chen et al., 2008) as well as for the large-scale light falling-net fishery (Yang, 2002; Zhang et al., 2013). Similar to other Ommastrephidae squid such as *Dosidicus gigas* and *Illex argentinus* (Keyl et al., 2011; Rodhouse et al., 2013), however, stock discrimination is still uncertain and an important research priority (Chen et al., 2012), particularly for the case of increasing fishing pressure in the SCS (Zhang et al., 2014).

Stock structure information provides a basis for understanding the dynamics of cephalopod populations. Each stock may have unique demographic properties and responses to exploitation (Boyle and Rodhouse, 2005). Morphometric characteristics form the basis for one of the simplest, and most often used, tools to identify and characterize squid stocks, i.e., in determining population assemblages (Rodhouse et al., 2013) and assigning individuals to stock (Fang et al., 2014). Morphometric characteristics can also be used to identify traits with evolutionary significance (e.g., Wanninger and Wollesen, 2018). Therefore, information on how, and the extent to which, a species or one stock within a species evolves specific morphometric relationships can potentially contribute to better management and conservation (Clavel and Morlon, 2017), as well as leading to a better understanding of species evolution, ecology, and ultimately stock assessment (Laptikhovsky et al., 2017; Wright et al., 2018).

S. oualaniensis exhibits a complicated intraspecific stock structure, with multiple morphological (Nesis, 1993), geographic (Chen et al., 2007; Yan et al., 2015), molecular (Staaf et al., 2010), and/or even spawning forms (Liu et al., 2008). In the SCS, available information indicates that there are probably two stocks of purpleback flying squid—the middle-sized (with dorsal photophore) and dwarf (without dorsal photophore) forms (Zhu et al., 2016; Wang et al., 2017; Li et al., 2019). These two stocks are, however, unconfirmed, presumably owing to a significant geographic overlap in morphology (Li et al., 2019). According to the different types of gladius, middle-sized individuals are divided into two forms: the middle-sized, typical form with double axes and the middle-sized not typical form with single axes (Jereb and Roper, 2010). Hard structures such as gladius, statoliths, and beaks are increasingly applied for stock discrimination of cephalopods (e.g., Liu et al., 2008; Fang et al., 2014; Fan et al., 2015) and show greater promise than the mantle and other body organs (Zhu et al., 2016; Wang et al., 2019). The gladius grows continuously throughout the lifetime of the species. The morphological characteristics and chemical composition of the gladius among species are obviously different, which is an important basis for the classification of cephalopods (Liu and Chen, 2010; Chen and Qiu, 2014). Little attention has been paid to morphological variations in gladius between different forms of the same species; it still needs relative information for classification (Gong et al., 2018).

Here, we used "form" to refer to different stocks of *S. oualaniensis*, following Jereb and Roper (2010). We analyzed the morphometric characteristics of the gladius of the middle-sized form (with dorsal photophore) and dwarf without a dorsal photophore form of *S. oualaniensis* and used multivariate statistics to analyze the differences in morphometric characteristics of the gladius between the two forms, with the ultimate aim of acquiring novel insights into stock identification. Specifically, we examined the following questions: 1) how different are the gladius morphologic characteristics between forms and sexes of *S. oualaniensis*? and 2) can gladius morphologic characteristics be used to distinguish these forms and/or sexes? These results will help put forward our understanding of the structure of this species and also warrant consideration of using the work as a framework to be applied in other commercially exploited squid species.

Material and methods

Sample collection

S. oualaniensis was sampled at 20 stations in the SCS (9.80° N–17.25°N, 110.25°E–115.02°E) by the Chinese lighting falling-net vessel Gui Beiyu 61999, from May to June in 2017 and 2018 (Figure 1). Four hundred thirty-eight specimens were randomly caught and collected and immediately frozen (–18°C) on board for further laboratory analysis.

After being defrosted at room temperature in the laboratory, *S. oualaniensis* was categorized into the dwarf without a dorsal photophore and middle-sized forms based on the absence or presence of the dorsal photophore and the apparatus' length (Zhu et al., 2016). The middle-sized forms were categorized into the typical and not typical forms based on the double axes or single axis of the gladius (Jereb and Roper, 2010). The dorsal mantle lengths (ML) were measured to the nearest 1 mm and body mass (BW) weighted to the nearest 1 g. Thereafter, a subsample of 275 specimens (130 females, 145 males) were used

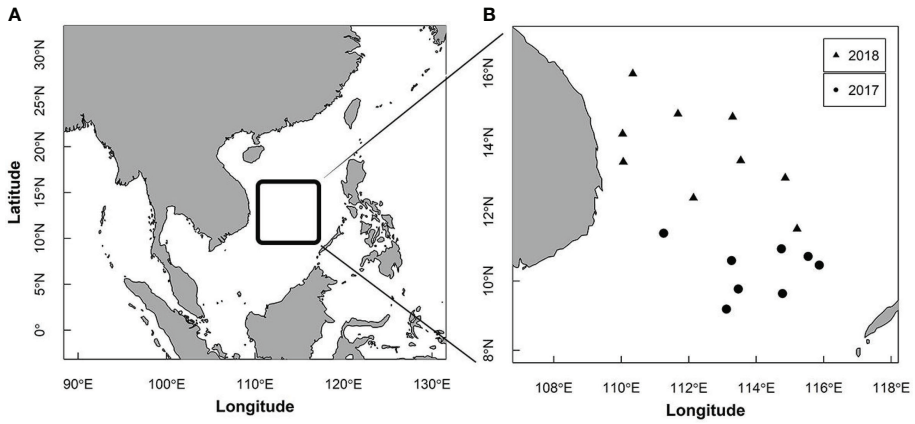

FIGURE 1
Study region **(A)** and stations **(B)** for *Sthenoteuthis oualaniensis* sampled in the South China Sea.

for gladius morphology analysis, whereas the gladius of other samples had been abandoned due to damage. The subsampled *S. oualaniensis* were dissected, sexed, and assigned a maturity stage on a macro scale following Lin (2015): I immature, II developing, III physiologically maturing, IV–V physiologically mature, VI functionally mature, VII spawning, and VIII spent.

Gladius morphometric measurement

The gladii of each subsampled *S. oualaniensis* were removed and measurements made of the following morphometric characteristics (Figure 2): total gladius length (GL), cone length (CL), greatest width of the cone (GWC), proostracum length (PL), length of the vane (VL), greatest width of the vane (GWV), the length of greatest width of the proostracum (GWPL), and greatest width of the proostracum (GWP). All these characteristics were measured accurately to be 0.01 mm.

Statistical analysis

Each morphometric measurement of the gladius was checked for normal distribution with the one-sample Kolmogorov–Smirnoff test as well as for homogeneity of the variances with the Levene's test (Zar, 1999). One-way ANOVA was used to test the difference between different forms. When significant differences were achieved, a Tukey's *post-hoc* test was applied to determine where the differences occurred (Zar, 1999). When either of normal distribution or homoscedasticity was not

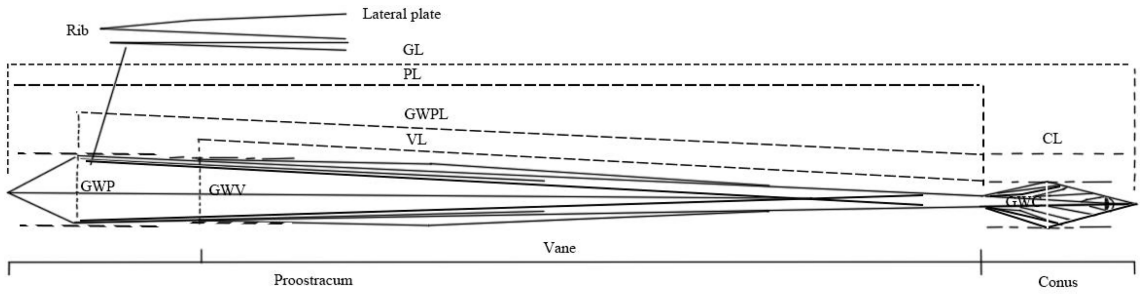

FIGURE 2
Scheme of gladius morphometric measurements for *Sthenoteuthis oualaniensis*. GL, total gladius length; CL, length of the cone; GWC, greatest width of the cone; PL, proostracum length; VL, length of the vane; GWV, greatest width of the vane; GWPL, length of the greatest width of the proostracum; GWP, greatest width of the proostracum.

achieved, data were subjected to a Kruskal–Wallis non-parametric one-way ANOVA test and a Games–Howell *post-hoc* test was performed (Zar, 1999).

Non-metric multidimensional scaling (nMDS) analysis and analysis of similarity (ANOSIM) employing the Bray–Curtis dissimilarity measure were used to access the dissimilarities of gladii between forms. This allowed for the potential identification of individuals that belong to the dwarf form without a dorsal photophore or middle-sized form. Furthermore, a stepwise discriminant analysis was performed to identify the gladius morphological characteristics that significantly classified individuals from the dwarf without a dorsal photophore and middle-sized forms (Rencher, 2002). The leave-one-out cross-validation was used to determine the rate of correct classification for different groups. Prior to nMDS, ANOSIM, and stepwise discriminant analyses, each morphometric measurement of the gladius was standardized by dividing by mantle length to eliminate the possible effects of allometric growth of animals, and then square root transformation was performed to all the standardized gladius morphometrics (Zar, 1999). The standardized morphometric measurements are represented by a subscript "s", i.e., GL_s, CL_s, GWC_s, PL_s, VL_s, GWV_s, $GWPL_s$, and GWP_s.

Statistical analyses were obtained using OriginPro version 2015 (OriginLab Corporation 2015) and R version 3.5.0 (R Core Team, 2018). In all statistical tests used, significant differences were considered only when $P < 0.05$.

Results

The dwarf form without a dorsal photophore of *S. oualaniensis* was 80–124-mm mantle length (ML, mean 88 ± 6 mm) for females and 76–95 mm (mean 78 ± 5 mm) for males. Individuals of the middle-sized form were larger with mantle lengths of 132–173 mm (mean 135 ± 15 mm) for females and 116–158 mm (mean 118 ± 12 mm) for males.

Gladius morphology of dwarf without a dorsal photophore and middle-sized forms

The gladius of both dwarf and middle-sized forms had a pair of double ribs at the edge of the medial plate of the proostracum (Figure 2); it was reasonable to expect that the individuals of middle-sized form were affiliated to the middle-sized typical form. The total gladius length (GL) of the dwarf-form individuals, however, was significantly shorter than that of the middle-sized typical-form individuals (Kruskal–Wallis test, $\chi^2 = 224.41$, $P < 0.05$) (Table 1). Similar findings were obtained with the analyses of other morphological characteristics, namely, the cone length (CL, Kruskal–Wallis test, $\chi^2 = 199.45$, $P < 0.05$), greatest width of the cone (GWC, Kruskal–Wallis test, $\chi^2 = 213.13$, $P < 0.05$), proostracum length (PL, Kruskal–Wallis test, $\chi^2 = 223.17$, $P < 0.05$), length of the vane (VL, Kruskal–Wallis test, $\chi^2 = 222.27$, $P < 0.05$), greatest width of the vane (GWV, Kruskal–Wallis test, $\chi^2 = 220.13$, $P < 0.05$), the length of greatest width of the proostracum (GWPL, Kruskal–Wallis test, $\chi^2 = 223.27$, $P < 0.05$), and greatest width of the proostracum (GWP, Kruskal–Wallis test, $\chi^2 = 225.07$, $P < 0.05$). The gladius for female individuals of dwarf form was consistently larger than that for males, and there were significant differences in the morphological characteristics of the gladius between males and females, except for cone length (CL) (Table 1). Similarly, females of the middle-sized typical form had a larger gladius than males. Consequently, each gladius characteristic differed significantly between females and males of the middle-sized typical form, with females being consistently larger (Table 1).

Meanwhile, univariate statistics (ANOVA) showed that standardized gladius morphometric characteristics were

TABLE 1 Measurements of gladius morphometric characteristics (mean ± SD, mm) for dwarf without a dorsal photophore and middle-sized typical form *Sthenoteuthis oualaniensis* in the South China Sea.

Terms	Dwarf form without a dorsal photophore		Middle-sized typical form	
	Male	Female	Male	Female
GWC	2.22 ± 0.31^a (1.46-3.27)	2.49 ± 0.28^b (1.87-3.83)	4.03 ± 0.63^c (2.34-5.54)	4.92 ± 0.97^d (2.88-8.15)
GWV	3.27 ± 0.28^a (2.44-4.25)	3.70 ± 0.38^b (2.8-5.05)	5.69 ± 0.73^c (3.33-7.79)	6.70 ± 1.13^d (4.45-11.63)
GWP	3.83 ± 0.32^a (3.18-4.97)	4.43 ± 0.48^b (3.36-5.58)	6.77 ± 0.79^c (4.07-8.98)	7.97 ± 1.20^d (5.27-11.94)
CL	11.49 ± 1.38^a (8.38-15.73)	12.21 ± 1.90^a (7.96-20.08)	17.41 ± 2.76^b (10.76-24.63)	21.22 ± 3.64^c (13.71-30.96)
VL	57.25 ± 3.02^a (51.13-64.05)	62.3 ± 4.66^b (48.6-82.9)	84.28 ± 7.41^c (63.97-105.4)	92.84 ± 10.68^d (73.37-138.61)
GWPL	68.43 ± 3.57^a (60.8-77.25)	74.53 ± 5.42^b (58.79-99.79)	100.8 ± 8.76^c (75.53-127.89)	110.82 ± 12.65^d (87.95-165.28)
PL	72.29 ± 3.83^a (64.52-81.75)	78.85 ± 5.66^b (62.23-104.16)	106.89 ± 9.40^c (79.68-134.14)	117.55 ± 13.46^d (91.91-175.17)
GL	83.74 ± 4.07^a (76.53-94.71)	91.04 ± 6.83^b (71.52-124.28)	124.13 ± 10.74^c (91.74-158.13)	138.56 ± 15.85^d (105.74-203.93)

The range values presented in parenthesis under the mean ± SD values. GWC, greatest width of the cone; GWV, greatest width of the vane; GWP, greatest width of the proostracum; CL, cone length; VL, vane length; GWPL, the length of greatest width of the proostracum; PL, proostracum length; GL, gladius length. Different subscript letters within rows indicate significant differences ($P < 0.05$).

significantly different between the dwarf and middle-sized typical forms (P <0.05), with the exception of GL/ML (P > 0.05). Within a single form, female and male individuals differed in seven of nine standardized gladius morphometric characteristics, dwarf (except GWC/ML, GWV/ML), and middle-sized typical forms (except GWV/ML, GWP/ML).

Dissimilarity analysis of the gladius between forms

In non-metric multidimensional scaling (nMDS) analysis, ordination of all graphs with stress values equal to or below 0.1 was considered fair, which could reflect the actual distribution characteristics (Figure 3). Regardless of sexes, nMDS analysis revealed that most of the gladii of dwarf without a dorsal photophore and middle-sized typical forms grouped together separately (Figure 3A) and the dissimilarity between them was significant, with an ANOSIM statistical R value of 0.24 (P = 0.001) (Table 2). For female and male individuals of both forms, the gladii were distinctly separate, with the exception of male dwarf and female middle-sized typical forms (Figures 3B–E). The difference between females and/or males of both forms was significant, and a higher dissimilarity value was obtained between female dwarf and female middle-sized typical forms (ANOSIM-R = 0.30) and between male dwarf and female middle-sized typical forms (ANOSIM-R = 0.37) (Table 2).

Discriminant analysis of gladius morphology between forms

Stepwise discriminant analyses indicated that the difference in the gladius morphology between the dwarf without a dorsal photophore and middle-sized typical forms could be explained using four gladius morphometric characteristics, namely, GWC_s, GWV_s, GWP_s, and GL_s. Accordingly, the accuracy of the discriminant analyses was estimated to be 88.71% for the middle-sized typical form, 95.36% for the dwarf form, and the global accuracy and cross-validation accuracy were the same, being 92.36% (Table 3).

With respect to sexes, stepwise discriminant analyses revealed that individuals from female middle-sized typical forms, male middle-sized typical forms, female dwarf forms, and male dwarf forms could be successfully discriminated by five gladius morphometric characteristics, namely, GWP_s, PL_s, GWC_s, GWV_s, and GL_s. The accuracy of discrimination ranged from 58.18% to 74.44% for form and sex, respectively. The global accuracy and cross-validation accuracy were 65.82% and 63.64%, respectively.

Discussion

Gladius morphology

Because the gladius of cephalopods has the function of supporting its body, it grows continuously throughout the lifetime of the species. The morphological characteristics of the gladius among species are obviously different, which is an important basis for the classification of cephalopods. Our findings showed that the gladius had double axes at the edge of the medial plate of the proostracum for both dwarf forms without a dorsal photophore and middle-sized forms in the SCS. For the middle-sized form, this observation was in agreement with previous research that the feature of double axes on the gladius was typical for the middle-sized form purpleback flying squid (see Jereb and Roper, 2010). However, it was noteworthy that the gladius morphology of the middle-sized form appeared to vary with oceanic regions. For example, individuals of the middle-sized form in the Indian and Pacific Oceans had double axes on the gladius like those from the SCS, whereas the median form animals from the Red and Arabian Seas had a single axis on the gladius (Bizikov, 1995). The presence of double axes on the gladius was typical for middle-sized *S. oualaniensis*, which was the most abundant and widely distributed form within their distribution range (Jereb and Roper, 2010). In contrast, the middle-sized not typical forms with a single axis on the gladius are narrowly distributed in the Red and Arabian Seas and the Gulf of Aden (Jereb and Roper, 2010).

The gladius of middle-sized typical forms in the SCS was relatively smaller than those from the Indian Ocean and Hawaiian waters (Indian Ocean, Chen et al., 2007; Hawaiian water, Harman et al., 1989). This may be related to smaller mantle size for the animals in the SCS given that the gladius size was related closely to animal size, which in turn may be attributed to food availability (Seibel et al., 2000). Low primary productivity was well documented for the SCS (Shen et al., 2008), which could lead to slower growth rates and smaller body sizes, presumably owing to their "live for today" life history strategy (Pecl and Moltschaniwskyj, 2006). On the other hand, individuals of the middle-sized typical form with larger sizes in the SCS have been found migrating to areas such as the Taiwan waters (Tung et al., 1973), which would also be expected to lead to smaller middle-sized typical form animals in the SCS. However, it is not possible to confirm any hypothesis at present and further research is needed.

In contrast, little information appeared to be available on the gladius morphology for the dwarf form of *S. oualaniensis*, although an assumption of more than one type is expected based on Bizikov (1995). We found that dwarf form individuals without a dorsal photophore in the SCS had only one type of

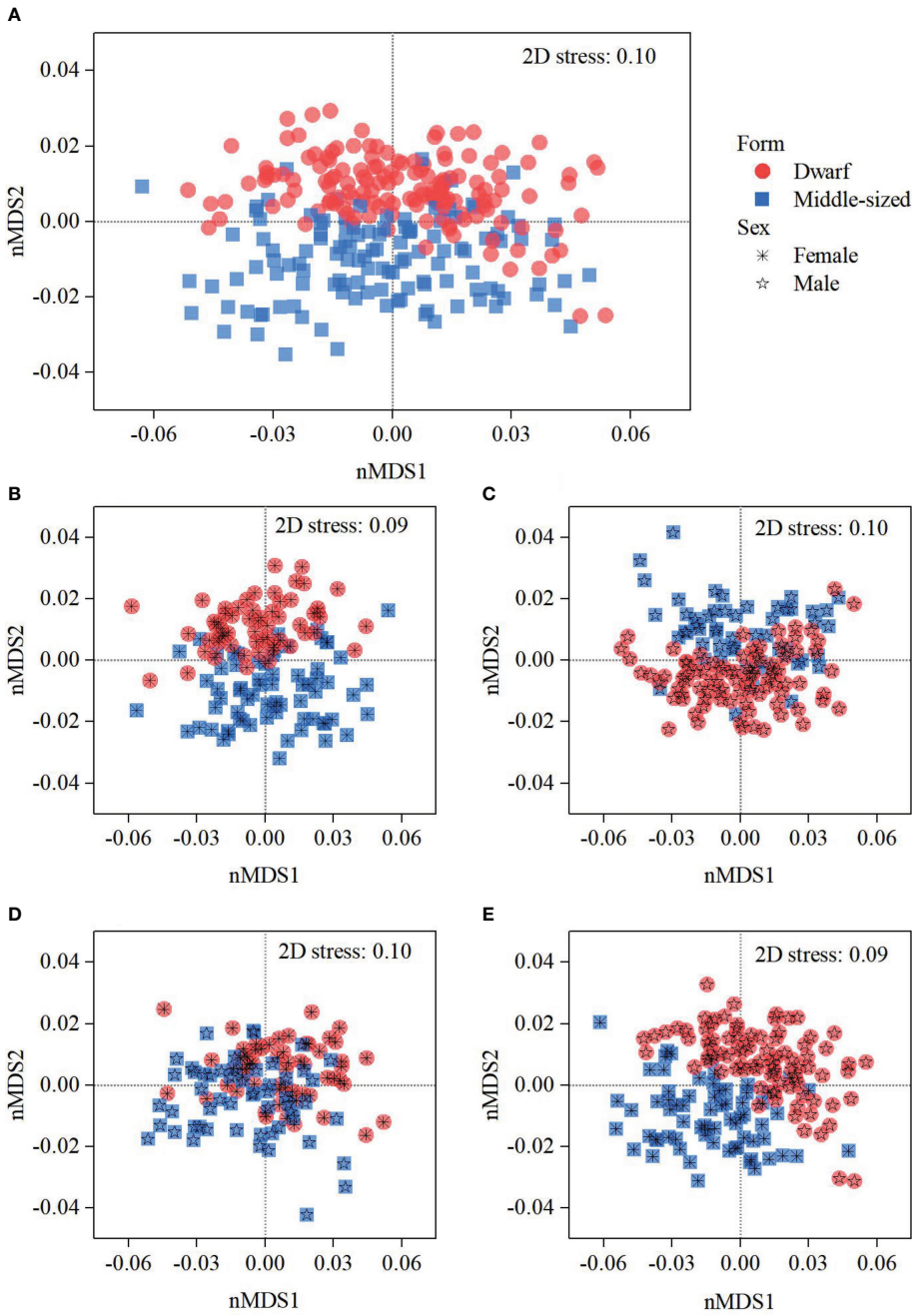

FIGURE 3
Non-metric multidimensional scaling (nMDS) ordination of gladius morphometric characteristics for dwarf without a dorsal photophore and middle-sized typical forms of *Sthenoteuthis oualaniensis* in the South China Sea. **(A)**, nMDS plots for the analysis between forms without regarding sexes; **(B)** nMDS plots for the analysis between females of both forms; **(C)** nMDS plots for the analysis between males of both forms; **(D)** nMDS plots for the analysis between female dwarf without a dorsal photophore and male middle-sized typical forms; **(E)** nMDS plots for the analysis between male dwarf and female middle-sized typical forms.

gladius morphology, which has double axes. This finding was in contrast to the assumption that the morphology of the gladius was variable for individuals from different types of dwarf form without a dorsal photophore (Zuyev et al., 2002). Thus, given that the gladius morphology is reflective of different forms among purpleback flying squid (Jereb and Roper, 2010), it is reasonable to expect only one type of dwarf form without a dorsal photophore of purpleback flying squid in the SCS.

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Gladius morphological characteristics in form identification

The size of the gladius of dwarf forms without a dorsal photophore was significantly smaller than that of middle-sized typical forms (Table 1). This could be associated with the individual's body size, as it is obvious that dwarf forms had a smaller mantle length than middle-sized typical forms. Within forms, furthermore, there was a significant difference among sexes, in which the gladius of females was larger than that of males (Table 1). This observation could be explained by the sexual dimorphism (Snÿder, 1998) as well as faster growth for female individuals (Liu et al., 2016). Accordingly, the gladius morphometric is confident to identify individuals between dwarf and middle-sized typical forms, evidenced by the obvious groups of the gladii of dwarf and middle-sized typical forms (Figure 3A). Such evidence was further provided by the significant dissimilarity detected in the gladius morphometric characteristics between dwarf and middle-sized typical forms (ANOSIM-R = 0.24, $P = 0.001$). In comparison with female and male individuals, nMDS and ANOSIM analyses of gladius morphometric characteristics further highlighted that dwarf form individuals were distinguishable from middle-sized typical form ones (Figures 3B–E; Table 2).

Furthermore, the stepwise discriminate analyses revealed that the gladius morphometric characteristics were ideal to distinguish the dwarf and middle-sized typical form *S. oualaniensis* in the SCS, in which the individuals from these two forms can be accurately classified by four characteristics, namely, GWC_s, GWV_s, GWP_s, and GL_s (Table 3). In comparison with other methods such as body morphometric characteristics, the accuracy of discrimination for these two forms is relatively high, e.g., 93% in this study vs. 85% in Zhu et al. (2016) who used body morphometric characteristics. In addition, Liu et al. (2015) have reported that beak morphological characteristics achieved a discriminant accuracy around 79% for classifying the geographical forms of *D. gigas*. Therefore, using gladius morphological characteristics to identify forms of *S. oualaniensis* would be expected to achieve more accurate results, probably also for other cephalopods.

Regarding the sexes, individuals of dwarf form were successfully distinguished from middle-sized typical forms using gladius morphometric characteristics. The difference could be explained by five gladius morphometric characteristics, namely, GWP_s, PL_s, GWC_s, GWV_s, and GL_s, even though the discrimination accuracy was relatively low (Table 4). The low discrimination accuracy could be partly attributed to the lack of dimorphism in gladius morphology

TABLE 2 Results of analysis of similarities (ANOSIM) for gladius morphometric characteristics of the dwarf without a dorsal photophore and middle-sized typical forms *Sthenoteuthis oualaniensis* in the South China Sea.

Terms	N	R-value	Significance
Pooled	275	0.24	0.001
Dwarf vs. middle-sized females	130	0.30	0.001
Dwarf vs. middle-sized males	145	0.19	0.001
Female dwarf vs. male middle-sized	116	0.22	0.001
Male dwarf vs. female middle-sized	159	0.37	0.001

TABLE 3 The results of stepwise discriminant analyses for the gladius morphometric characteristics of the dwarf without a dorsal photophore and middle-sized typical forms *Sthenoteuthis oualaniensis* in the South China Sea.

Stepwise discriminant analysis

Step	Terms	F to enter	Wilks' λ	df1	df2	P value
1	GWP_s	323.44	0.46	1	273	<0.01
2	GWC_s	234.45	0.37	2	272	<0.01
3	GL_s	185.42	0.33	3	271	<0.01
4	GWV_s	149.62	0.31	4	270	<0.01

Accuracy estimation

Group	Classification sample			Cross-validation (%)
	Middle-sized	Dwarf	Original (%)	
Middle-sized	110	14	88.71	88.71
Dwarf	7	144	95.36	95.36

The abbreviation of morphometric characteristic as in Table 1.

TABLE 4 The result of stepwise discriminant analyses for gladius morphometric characteristics of female and male dwarf without a dorsal photophore and middle-sized typical forms *Sthenoteuthis oualaniensis* in the South China Sea.

Stepwise discriminant analysis

Step	Terms	F to enter	Wilks' λ	df1	df2	P value
1	GWP_s	112.23	0.45	3	271	<0.01
2	PL_s	67.89	0.33	6	540	<0.01
3	GWC_s	52.41	0.27	8	655	<0.01
4	GL_s	42.03	0.24	12	709	<0.01
5	GWV_s	34.94	0.23	15	737	<0.01

Accuracy estimation

Group	Classification sample					Cross-validation (%)
	Female middle-sized	Male middle-sized	Female dwarf	Male dwarf	Original (%)	
Female middle-sized	44	19	5	1	63.77	63.77
Male middle-sized	15	32	7	1	58.18	54.55
Female dwarf	1	2	38	20	62.30	59.02
Male dwarf	0	4	19	67	74.44	72.22

The abbreviation of morphometric characteristic as in Table 1.

between sexes within forms. This observation is very similar to the findings based on beak and statolith morphometric characteristics for other Ommastrephids. For example, it has been reported that female and male *Ommastrephes bartramii* can be somewhat separated using beak morphology, with a discrimination value of around 56% (Fang et al., 2014). Similar findings were found in the analyses using statolith morphology and beak morphology for *Illex argentinus* (Fang et al., 2012).

In conclusion, the dwarf without a dorsal photophore and middle-sized typical forms of *S. oualaniensis* in the SCS exhibited double axes on the gladius. The size of the gladius of dwarf forms was significantly smaller than that of middle-sized typical forms. Individuals of dwarf form were obviously identified from the middle-sized typical form using the gladius morphometric characteristics, evidenced by the obviously separate groups of gladii of these two forms. Significant differences in the morphometric characteristics between dwarf and middle-sized typical forms were confirmed by the significant dissimilarity values calculated by ANOSIM analysis. Furthermore, stepwise discriminant analyses revealed that there was a high rate of discriminant accuracy for distinguishing the dwarf and middle-sized typical forms using gladius morphometric characteristics. Because the gladius is easier to collect and measure, it is an ideal hard structure to identify forms of *S. oualaniensis*. Although there are still few studies using the gladius for stock/form identification in squids, the successful distinction of dwarf and middle-sized typical forms of *S. oualaniensis* here could warrant consideration of the gladius morphology and its morphometric characteristics to study the complicated population structure of squids.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the animal study because the samples we studied were all from frozen squid caught in commercial fishing activities.

Author contributions

KZ: conception and design of this study, acquisition of data, visualization, software, writing—original draft. DL: Writing—review and editing. XC: investigation, resources. KX: Writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The significance of cephalopod beaks as a research tool: An update

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The use of cephalopod beaks in ecological and population dynamics studies has allowed major advances of our knowledge on the role of cephalopods in marine ecosystems in the last 60 years. Since the 1960's, with the pioneering research by Malcolm Clarke and colleagues, cephalopod beaks (also named jaws or

mandibles) have been described to species level and their measurements have been shown to be related to cephalopod body size and mass, which permitted important information to be obtained on numerous biological and ecological aspects of cephalopods in marine ecosystems. In the last decade, a range of new techniques has been applied to cephalopod beaks, permitting new kinds of insight into cephalopod biology and ecology. The workshop on cephalopod beaks of the Cephalopod International Advisory Council Conference (Sesimbra, Portugal) in 2022 aimed to review the most recent scientific developments in this field and to identify future challenges, particularly in relation to taxonomy, age, growth, chemical composition (i.e., DNA, proteomics, stable isotopes, trace elements) and physical (i.e., structural) analyses. In terms of taxonomy, new techniques (e.g., 3D geometric morphometrics) for identifying cephalopods from their beaks are being developed with promising results, although the need for experts and reference collections of cephalopod beaks will continue. The use of beak microstructure for age and growth studies has been validated. Stable isotope analyses on beaks have proven to be an excellent technique to get valuable information on the ecology of cephalopods (namely habitat and trophic position). Trace element analyses is also possible using beaks, where concentrations are significantly lower than in other tissues (e.g., muscle, digestive gland, gills). Extracting DNA from beaks was only possible in one study so far. Protein analyses can also be made using cephalopod beaks. Future challenges in research using cephalopod beaks are also discussed.

KEYWORDS

cephalopod ecology, beak taxonomy/composition/morphology/microstructure/paleontology, cephalopod trophic dynamics, cephalopod population dynamics, cephalopod ecotoxicology

Introduction

The important role of cephalopods (Mollusca: Cephalopoda) in many marine ecosystems has been widely acknowledged (Boyle and Rodhouse, 2005). They are commercially exploited around the World (Rodhouse et al., 2014; Arkhipkin et al., 2015; Doubleday et al., 2016; Arkhipkin et al., 2021; Sauer et al., 2021), are predators on numerous prey and are preyed by predators (Santos et al., 2001; Boyle and Rodhouse, 2005; Bello et al., 2011; Hoving et al., 2014; Xavier and Cherel, 2021), whose predator-prey interactions has been helping the development of a conservation framework for some of these predators (Luna et al., 2021). As cephalopod flesh gets quickly digested in predator stomachs, cephalopod beaks (synonym of jaws and mandibles) can resist digestion for as long as several months (Xavier et al., 2005; Barrett et al., 2007). Malcolm Clarke revolutionised the way cephalopod beaks could be used in ecological research, by providing evidence that many of them have unique shapes at species level (Clarke, 1962; Clarke, 1980; Clarke, 1986). Clarke and colleagues also developed the currently used terminology for different parts of the upper and lower beaks (Clarke, 1986) (Figure 1). Such initial efforts helped many other colleagues to develop beak identification guides (Imber, 1978; Pérez-Gándaras, 1983; Wolff, 1984; Kubodera and Furuhashi, 1987; Lu and Ickeringill, 2002; Xavier and Cherel, 2021; Pedà et al., 2022) and supported studies to understand the importance of cephalopods in the diet of different predator taxa (Clarke, 1996; Croxall and Prince, 1996; Klages, 1996; Smale, 1996; Cherel and Klages, 1998; Ménard et al., 2013; Abreu et al., 2019; Romanov et al., 2020; Queirós et al., 2021b; Cherel, 2021; Guímaro et al., 2021). This is particularly important as much information cannot be obtained by other means (e.g., scientific nets are too slow to catch faster cephalopods and catch far fewer species and narrower range of sampled sizes) (Clarke, 1977; Santos et al., 2001; Staudinger et al., 2013; Hoving et al., 2014; Cherel, 2020). Cephalopod beaks can also provide considerable information on a wide range of physiological, biological and ecological traits, including cephalopod availability, consumption of cephalopods, migrations, competition between cephalopod predators, levels of cephalopod scavenging by predators, distribution, age, growth, cohorts, life-events, stress, thermal changes, reproduction, feeding ecology, behavior, spawning areas, post-spawning mortality and sexual dimorphism [e.g., see review in Xavier et al. (2016) and Arkhipkin et al. (2018); Table 1]. More recently, new emergent techniques for work on beaks (e.g., stable isotope and trace elements analyses, geometric morphometrics and microstructure analysis) have provided further information on habitat and trophic position, composition, contamination, response of cephalopods to climate variability at individual and/or population levels,

embryonic morphogenesis, paralarval ontogeny, ecology and age estimation (Cherel and Hobson, 2005; Perales-Raya et al., 2014b; Franco-Santos and Vidal, 2014; Xavier et al., 2016; Perales-Raya et al., 2018; Queirós et al., 2018; Golikov et al., 2019a; Golikov et al., 2019b; Northern et al., 2019; Abreu et al., 2020; Queirós et al., 2020a; Armelloni et al., 2020; Queirós et al., 2020b; Franco-Santos and Vidal, 2020; Golikov et al., 2020; Perales-Raya et al., 2020; Fang et al., 2021b; Lishchenko and Jones, 2021). Consequently, the importance of cephalopod beaks in ecological studies continues to attract attention and recognition, with various workshops being organised (Clarke, 1986; Xavier et al., 2007a; Jackson et al., 2007; Xavier et al., 2015). A workshop on cephalopod hard structures including beaks was held in Florida (United States), prior to the 2018 Cephalopod International Advisory Council (CIAC) Conference, results of which should be published in the near future. Most recently, a workshop focused on cephalopod beaks was held in Sesimbra (Portugal) at the 2022 CIAC Conference (Figures A1, A2) in order to review the latest scientific advances on the use of cephalopod beaks in marine ecological studies and discuss future challenges in this and other related research fields. The individual sections below concern key topics in cephalopod research based on the beak analysis discussed during this 2022 CIAC workshop.

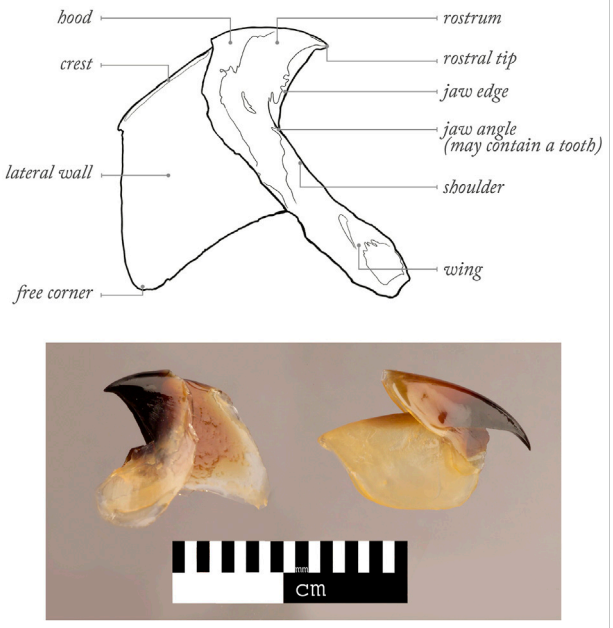

FIGURE 1
Examples of profiles of lower (on the left) and upper (on the right) beaks of cephalopods and the principal terms used to characterize decapod beaks (Xavier and Cherel (2021) following Clarke (1986)).

Advances in taxonomy, beak morphology, microstructure and paleontology

Taxonomy and beak morphology

Taxonomic identification is a critical issue of every investigation using accumulated cephalopod beaks from food samples (Table 1). Erroneous identifications can propagate along the studies through the years spreading and proliferating information, not only on predator-prey relationships but also on every subsequent analysis on beaks, regardless the nature of the analyses (e.g., species occurrence/distribution, stable isotopes, trace elements, growth increments) (Cherel, 2020, 2021; Xavier and Cherel, 2021). Substantial efforts have been directed to facilitate the identification of these hard structures through drawings, photographs (from different angles), 3-D videos, measurements, and by materials being accessible through regular publications or on the internet [e.g., Tree of Life web project–http://tolweb.org/articles/?article_id=5274 (Young, 2009); https://www.kahaku.go.jp/research/db/zoology/Beak-E/intro.htm (Kubodera et al., 2005)] (Clarke, 1986; Cherel, 2020; Xavier and Cherel, 2021). Nevertheless, the need for more research experts (e.g., boost/support a new generation of early career scientists in this field) on cephalopod beaks, as well as updated collections and more comprehensive guides will continue to be a necessity in the future (Xavier et al., 2007a; Xavier et al., 2015; Cherel, 2020; Xavier and Cherel, 2021).

Several methods applicable to cephalopod beak shape analysis were developed to date (Fang et al., 2018; Lishchenko and Jones, 2021). Nowadays, the group of methods which can be called "traditional morphometrics" (measurements of the linear distances, or indices, based on these measurements), is criticised for leading to a significant loss of information due to the complexity of studied structures and the multicollinearity between measurements (Adams et al., 2004; Volpedo and Vaz-dos-Santos, 2015). To some extent, this criticism is justified, although these analyses allowed the development of the first steps of beak shape analysis relevant for identification (Mangold and Fioroni, 1966; Wolff, 1982b; Wolff, 1984; Ogden et al., 1998; Fang et al., 2018). Moreover, they are the least laborious among morphometric methods and the richest in terms of available data for comparison.

An alternative approach is represented by two groups of geometric morphometric methods. The first group is based on obtaining Cartesian coordinates of biologically definable points, also called landmarks (Bookstein, 1991; Cadrin, 2014). In addition, sliding semi-landmarks, which are defined by equidistant points between two landmarks are also used to represent curves and surfaces of structures (Bookstein, 1997; Gunz et al., 2005; Gunz and Mitteroecker, 2013). These landmarks and semi-landmarks

TABLE 1 A non-exhaustive overview of the use of cephalopod beaks in ecological studies and related research.

Research field	Taxa	References
Taxonomy (including beaks ID) and biogeography	Cephalopoda	Clarke (1962), Akimushkin (1965), Mangold and Fioroni (1966), Iverson and Pinkas (1971), Pinkas et al. (1971), Hotta (1973), Imber (1978), Wolff and Wormuth (1979), Clarke (1980), Wolff (1982a), Wolff (1982b), Pérez-Gándaras (1983), Wolff (1984), Clarke (1986), Kubodera and Furuhashi (1987), Lu and Ickeringill (2002), Franco-Santos and Vidal (2014), Fang et al. (2018), Cherel (2020), Acuña-Perales et al. (2020), Pacheco-Ovando et al. (2021), Xavier and Cherel (2021)
Distribution	Cephalopoda	Clarke (1962), Akimushkin (1965), Mangold and Fioroni (1966), Iverson and Pinkas (1971), Pinkas et al. (1971), Hotta (1973), Clarke (1980), Pérez-Gándaras (1983), Clarke (1986), Kubodera and Furuhashi (1987), Lu and Ickeringill (2002), Xavier et al. (2002b), Xavier et al. (2003a), Cherel et al. (2004), Xavier et al. (2005), Xavier et al. (2006), Xavier and Croxall (2007), Cherel et al. (2009b), Cherel et al. (2011), Xavier et al. (2014), Seco et al. (2016), Pereira et al. (2017), Queirós et al. (2019), Cherel (2020), Guímaro et al. (2021), Queirós et al. (2021a), Xavier and Cherel (2021)
	Squid	Wolff and Wormuth (1979), Wolff (1982a), Wolff (1982b), Wolff (1984), Xavier et al. (2002a), Xavier and Croxall (2007), Cherel et al. (2008), Xavier et al. (2013), Guerreiro et al. (2015), Liu et al. (2015), Queirós et al. (2018), Abreu et al. (2019), Abreu et al. (2020), Cherel (2020), Woods et al. (2022)
	Octopods	Queirós et al. (2020b)
	Bobtail squid	Golikov et al. (2020)
	Vampyroteuthis	Golikov et al. (2019a)
Predators vs nets catch composition	Cephalopoda	Clarke (1977), Cherel (2020)
Allometric equations	Cephalopoda	Clarke (1962), Clarke (1980), Clarke (1986), Roeleveld (2000), Lu and Ickeringill (2002), Açik and Salman (2010), Xavier and Cherel (2021)
	Squid	Wolff (1982a), Wolff (1982b), Wolff (1984), Ivanovic and Brunetti (1997), Golikov et al. (2018)
	Octopods	Smale et al. (1993), Golikov et al. (2022)
	Vampyroteuthis	Golikov et al. (2019a)
Top predators: in general	Cephalopoda	Clarke (1962), Clarke (1980), Clarke (1986), Santos et al. (2001), Clarke et al. (2002), Ménard et al. (2013), Staudinger et al. (2013), Cherel (2020), Xavier and Cherel (2021)
	Squid	Woods et al. (2022)
Top predators: marine mammals	Cephalopoda	Akimushkin (1955), Clarke (1962), Akimushkin (1965), Clarke (1980), Clarke (1986), Klages (1996), Santos et al. (2001), Pedà et al. (2015), Cherel (2021)
	Squid	Cherel et al. (2008), Abreu et al. (2019)
Top predators: seabirds	Cephalopoda	Pinkas et al. (1971), Furness et al. (1984), Croxall and Prince 1996, Cherel and Klages 1998, Xavier et al. (2003a), Xavier et al. (2005), Xavier et al. (2006), Xavier et al. (2007b), Xavier et al. (2014), Seco et al. (2016), Queirós et al. (2019), Guímaro et al. (2021), Xavier and Cherel (2021)
	Squid	Cherel and Weimerskirch (1999), Xavier et al. (2002a), Xavier and Croxall (2007), Xavier et al. (2013), Guerreiro et al. (2015), Cherel et al. (2017)
Top predators: sharks and other fishes	Cephalopoda	Pinkas et al. (1971), Clarke and Stevens (1974), Smale (1996), Xavier et al. (2002b), Peristeraki et al. (2005), Lansdell and Young (2007), Romeo et al. (2011), Kousteni et al. (2018), Ménard et al. (2013), Romanov et al. (2020), Queirós et al. (2021a)
Global cephalopod biomass estimation	Cephalopoda	Clarke (1977), Clarke et al. (2002)
Community trophic structure	Cephalopoda	Cherel and Hobson (2005), Jackson et al. (2007), Cherel et al. (2009b), Cherel et al. (2011), Guímaro et al. (2021), Queirós et al. (2021a), Queirós et al. (2021b)
	Squid	Guerreiro et al. (2015), Cherel et al. (2019), Abreu et al. (2020), Woods et al. (2022)
	Octopods	Matias et al. (2019), Fang et al. (2021a)
	Bobtail squid	Golikov et al. (2020)

(Continued on following page)

TABLE 1 (*Continued*) A non-exhaustive overview of the use of cephalopod beaks in ecological studies and related research.

Research field	Taxa	References
Long-term changes in community structure	Cephalopoda	Guímaro et al. (2021)
	Squid	Abreu et al. (2020)
Trophic ecology of single species	Squid	Castro and Hernández-Garcia (1995), Ruiz-Cooley et al. (2006), Guerra et al. (2010), Fang et al. (2016a), Gong et al. (2018), Liu et al. (2018), Queirós et al. (2018), Trasviña-Carrillo et al. (2018), Hu et al. (2019), Liu et al. (2019a), Liu et al. (2019b), Queirós et al. (2019), Gong et al. (2020), Wang et al. (2022)
	Octopods	Franco-Santos et al. (2014), Queirós et al. (2020b)
	Bobtail squid	Golikov et al. (2019b)
	Vampyroteuthis	Golikov et al. (2019a)
Marine trace metal pollution	Squid	Xavier et al. (2016), Northern et al. (2019), Queirós et al. (2020a)
	Octopods	Matias et al. (2020)
Physical and chemical properties of beak material	Cephalopoda	Dilly and Nixon (1976), Uyeno and Kier (2005)
	Squid	Miserez et al. (2007), Miserez et al. (2008), Miserez et al. (2010), Queirós et al. (2018)
	Octopods	Matias et al. (2019)
Migrations	Squid	Cherel and Weimerskirch (1999), Guerra et al. (2010), Liu et al. (2019a), Liu et al. (2019b), Queirós et al. (2019), Queirós et al. (2021a)
Inter- and intraspecific competition	Squid	Gong et al. (2018), Gong et al. (2020)
	Octopods	Matias et al. (2019), Fang et al. (2021a)
	Bobtail squid	Golikov et al. (2020)
Age and growth	Cephalopoda	Bello (1991), Clarke (1993), Arkhipkin et al. (2018)
	Squid	Clarke (1965), Jarre et al. (1991), Liu et al. (2015), Fang et al. (2016a), Hu et al. (2016), Liu et al. (2017), Jin et al. (2019), Perales-Raya et al. (2020)
	Octopods	Perales-Raya and Hernández-González (1998), Hernández-López et al. (2001), Perales-Raya et al. (2010), Rodríguez-Domínguez et al. (2013), Perales-Raya et al. (2014a), Franco-Santos et al. (2016), Garrido et al. (2016), Perales-Raya et al. (2018), García-Fernández et al. (2019), Schwarz (2019), Armelloni et al. (2020), Schwarz et al. (2020)
	Vampyroteuthis	Schwarz et al. (2020)
Stock assessment	Squid	Fang et al. (2016a)
Population dynamics	Squid	Liu et al. (2015), Hu et al. (2016), Fang et al. (2021b)
Reproduction	Squid	Hernández-Garcia et al. (1998b), Cherel and Weimerskirch (1999), Xavier and Croxall (2007)
Sexual dimorphism	Squid	Jackson (1995), Bolstad (2006), Cherel et al. (2009a)
Fisheries management	Cephalopoda	Xavier et al. (2007b)
Paleontology	Cephalopoda	Clarke and Maddock (1988)

may be two- or three-dimensional, and generally are discrete and homologous (Zelditch et al., 2004). The coordinates obtained are modified using Procrustes analysis to avoid impact of the position and size of the studied object.

The second group of methods includes those methods which describe the structure's outline as a whole. The most applied methods in this group are Fourier transform (where outlines are expressed as a function of equally spaced radii or of the tangent

angle to the outline or of the curvilinear abscissa) and wavelet transform (with outlines expressed by a set of functions representing the dilations and translations of a single unique function). Application of both approaches to analyse the shape of cephalopod beaks have benefits and limitations (Lishchenko and Jones, 2021). Specifically, landmark-based methods allow selecting the points of interest which presumably have some biological or taxonomic meaning (e.g., rostral tip and wing fold, whose position reflects the length and curvature of the rostrum). On another hand, landmark selection inevitably leads to the loss of information, which may be crucial if the points of interest were chosen incorrectly. Additionally, process of landmark selection is particularly laborious, time consuming and demands a certain level of qualification of the researcher. The outline-based methods of the shape analysis do not have these drawbacks and allow detailed description of a beak's contour on the image. However, the latter methods are associated with a very specific issue, which is called a "pixel noise" by some authors (Haines and Crampton, 2000; Harbitz and Albert, 2015) (i.e., "pixel noise" stands for the excessive and meaningless set of information which hampers the analysis of the structure's shape).

Despite these limitations, the potential of these approaches is substantial, supported by the results of recent studies. Most commonly, geometric morphometrics methods were successfully applied for taxonomic classification, species identification or stocks (Neige and Dommergues, 2002; Crespi-Abril et al., 2010; Tanabe et al., 2015b; Fang et al., 2017; Jin et al., 2017; Fang et al., 2018; Pacheco-Ovando et al., 2021; Díaz-Santana-Iturrios et al., 2022). At the beginning of the millennium, the application of these approaches to a wide diversity of species was scarce (Neige and Dommergues, 2002; Tanabe et al., 2015b) and provided limited resolution of identification. Their findings suggested that the 2-D lateral shapes of beaks (lateral wall, hood and wing contours) are clustered at high taxonomic levels (orders, suborders) and that the upper and lower parts of the beak carry a slightly different information. By showing that to be true, quantitative analysis of beak shape might assist identification at high taxonomic levels (at least, to the level of family). However, in the last decade, geometric morphometric methods began to flourish. Different authors applied either 2D landmark-based methods (Fang et al., 2017; Fang et al., 2018; Pacheco-Ovando et al., 2021; Díaz-Santana-Iturrios et al., 2022) or outline-based methods (Jin et al., 2017). Both approaches showed high level of identification accuracy, up to 100% in classification of the genera (Pacheco-Ovando et al., 2021), up to 93% in species identification (Fang et al., 2018), and up to 70% correct classifications of stock units (Fang et al., 2017). These results point to the potential to engineer automated identification programs.

Several studies revealed the potential of beak shape analysis in ecological studies (Fernández-Álvarez et al., 2020; Pacheco-Ovando et al., 2021; Roscian et al., 2022). Specifically, Fernández-Álvarez et al. (2020) studied impacts of developmental malformations of the buccal mass on the trophic position of *Eledone cirrhosa* and found that the habitat and the trophic position were not significantly affected by the malformations. Other authors found significant differences in the beak shapes of pelagic and benthic species in relation to their trophic levels (Roscian et al., 2022), between species living in coastal and oceanic habitats (Pacheco-Ovando et al., 2021) and between species/populations living in different feeding habitats (Pacheco-Ovando et al., 2021). New phylogenomic techniques applied to cephalopods (Anderson and Lindgren, 2021; Sanchez et al., 2021; Fernández-Álvarez et al., 2022) may help to assess which morphological characters of the beaks are determined by phylogeny and which are explained by other drivers.

Geometric morphometric studies of cephalopod beaks have the greatest potential in the field of species identification, as part of both the routine monitoring process and as high-end studies. Application of outline-based methods allows it even without additional efforts. At present, an automated similar system has been used for the identification of fish using otolith contours (Lombarte et al., 2006). This approach shows that this system may allow accurate identification of animals even when there is only very basic information available about the subject of research and it is probably the least time-consuming method of them all. Development of such a system is a long-term process that needs close validation checks, but even at the early stage of development, it could substantially expand our knowledge on cephalopods.

Beak morphological changes during the early life

Beaks of embryos and paralarvae are quite distinct from those of juveniles and adults (Franco-Santos et al., 2014; Franco-Santos and Vidal, 2014; Franco-Santos et al., 2016; Armelloni et al., 2020; Franco-Santos and Vidal, 2020). The beaks of hatchlings and smaller squid and octopus paralarvae studied so far, are very fragile and slightly pigmented, being nearly transparent. Growth rings might or not be clearly visible in the lateral wall of both the upper and lower beaks (Franco-Santos and Vidal, 2014), and they might be visible in the anterior pigmented region of upper beaks that corresponds to the rostrum (Perales-Raya et al., 2014b; Franco-Santos et al., 2016; Arkhipkin et al., 2018; Perales-Raya et al., 2018). Paralarval beaks in several cephalopod families (E.g., Family Ommastrephidae, family Octopodidae) have teeth that might be present in both beaks or only in the lower one (Boletzky, 1971; Wakabayashi et al., 2002; Uchikawa et al., 2009; Franco-Santos et al., 2014; Franco-Santos and Vidal, 2014; Franco-Santos and Vidal, 2020). In the upper beak of many species, the rostrum has not yet protruded, and has a typical sagittal slit between the two-halves that could be slightly or very pronounced (Figure 2). In addition, conspicuous features of the beaks, such as the hood and lateral walls might not be developed

yet in paralarvae of some families, such as Ommastrephidae (Franco-Santos and Vidal, 2020), giving the beak a more rounded shape. A study with late embryonic hatching stages of *Octopus vulgaris* embryos has shown that the upper beak is rudimentary and lacks the hood, but the teeth are already visible and these stages represent the beginning of the pigmentation process. The hood and shoulder develop along with the exposure of the dentition on the rostrum just prior to hatching (Armelloni et al., 2020), suggesting that beak development is intensified afterwards during the paralarval phase (Franco-Santos and Vidal, 2014; Franco-Santos and Vidal, 2020).

The beak morphology of smaller paralarvae suggests a weak bite force. Thus, at this stage the beak has not yet assumed the functions of biting flesh and masticating the hard exoskeleton of crustaceans (Franco-Santos and Vidal, 2014). Indeed, beaks of smaller paralarvae seem to be adapted for a specialised feeding mode that involves external pre-digestion and suction of body fluids of crustacean prey (Franco-Santos et al., 2014; Franco-Santos and Vidal, 2014; Franco-Santos and Vidal, 2020). This is supported by observations of the feeding behaviour of *O. vulgaris* and loliginid squid paralarvae (Hernández-García et al., 2000; Franco-Santos and Vidal, 2014).

As paralarvae grow, the teeth are eroded and disappear, and the hood and lateral walls grow rapidly as they are important sites for buccal musculature attachment (Uyeno and Kier, 2007). The sagittal slit progressively closes and gives way to the rostrum. The pigmented area increases through the deposition of sclerotized layers (i.e., not chitin during the darkening process), particularly in the rostrum, which protrudes very fast in both beaks (Franco-Santos and Vidal, 2014; Franco-Santos and Vidal, 2020). Beak darkening is a continuous process, through which beaks become harder and more robust (Hernández-García et al., 1998a, b; Miserez et al., 2007). Fully pigmented beaks were considered to be "mature beaks" by Clarke (1986).

These studies have shown that the underdeveloped beaks of paralarvae are rapidly transformed, giving way to the prominent structures present in juvenile and adult beaks. Such studies have provided the foundation for inferences about rostrum functionality and its relationship with feeding strategy and prey selection during the first stages of the life cycle, about which little is known (Franco-Santos and Vidal, 2014; Nande et al., 2017; Vidal and Salvador, 2019; Franco-Santos and Vidal, 2020). In addition, beaks of *O. vulgaris* paralarvae have been used to validate daily growth increments in the early stages, for comparison of wild and cultured specimens to understand massive mortalities previous to the juvenile phase (Garrido et al., 2016; García-Fernández et al., 2019) and to solidify our understanding of the significant influence of temperature on increment deposition (Perales-Raya et al., 2018). The accuracy of age estimation inferred from growth marks in the upper beak rostrum has been also confirmed in late-stage embryos (Armelloni et al., 2020). In addition, it was suggested that these growth marks might be used as biomarkers for stress during rearing of *O. vulgaris* paralarvae (Franco-Santos et al., 2016).

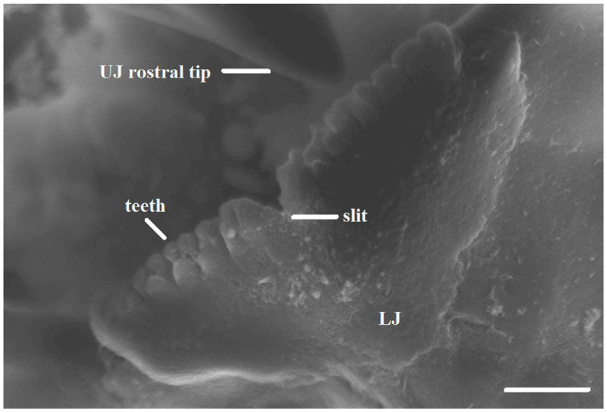

FIGURE 2
Close-up of a scanning electron microscope image indicating (lines) the teeth and slit in the lower beak (LJ) and the rostral tip in the upper beak (UJ) of an *Illex argentinus* paralarva of 4.0 mm ML. Scale bar 10 μm ((Franco-Santos and Vidal, 2020); copyright permission obtained).

Microstructure: Age, growth and record of life extreme events

Although statoliths are the most frequently used material for age determination in cephalopods, it has been suggested that beaks could provide additional/complementary data, especially when it is impossible to obtain access to age data from statoliths (Liu et al., 2014). This is the case for octopods, which lack visible increments in the microstructure of their statoliths (Clarke, 1978). In addition, statoliths of adult cephalopods need to be ground on both sides, which is time-consuming and labour-intensive, whereas beaks do not need to be ground on both sides (Arkhipkin et al., 2018). Beaks are present throughout the life cycle of all extant cephalopod species, and can be easily extracted and preserved (Clarke, 1986). Beak growth process takes place along the posterior border, where the most recent chitinized and hydrated material is deposited, so that the oldest and most pigmented material is found in the anterior tip (Miserez et al., 2008; Perales-Raya et al., 2014a). Moreover, growth increments have been observed and validated along several parts of cephalopod beaks [e.g., lateral wall surfaces, rostrum sagittal sections (Perales-Raya et al., 2010)]. Perales-Raya et al. (2010) recommended counting growth increments in the lateral wall surface of beaks of *Octopus vulgaris* as fewer increments were detected in rostrum sagittal sections, probably due to erosion of the rostral tip when the animal is feeding. In some cephalopods (including *O. vulgaris*) these increments have been validated as being deposited daily (see below for further discussion). Thus, counts of growth increments on these structures can potentially provide absolute age estimates and growth data in any ontogenetic phase (Figure 3). Comparison with other

TABLE 2 Detailed information on cephalopod species that have been attempted using beak increment analysis.

Species	Beak part	Validated	Study
Octopoda and Vampyromorpha			
Octopus vulgaris	Rostrum sagittal section of upper beak	No	Perales-Raya and Hernández-González, (1998)
	Lateral wall of upper beak	Yes (paralarvae)	Hernández-López et al. (2001)
	Rostrum of upper beak	Partially (5 specimens marked)	Oosthuizen, (2003)
	Rostrum sagittal section of upper and lower beak; lateral wall of upper beak	No	Perales-Raya et al. (2010)
	Lateral wall of upper beak	Yes	Canali et al. (2011)
	Lateral wall of upper beak	No	Castanhari and Tomás, (2012)
	Lateral wall of upper beak	No	Cuccu et al. (2013)
	Lateral wall and rostrum sagittal section of upper beak	No	Perales-Raya et al. (2014b)
	Lateral wall and rostrum sagittal section of upper beak	Yes	Perales-Raya et al. (2014a)
Octopus maya	Rostrum sagittal section of upper beak	Yes	Bárcenas et al. (2014)
	Lateral wall of upper beak	Yes	Rodríguez-Domínguez et al. (2013)
Octopus huttoni	Lateral wall of upper beak	No	Donlon et al. (2019)
Pareledone aequipapillae, Pareledone charcoti, Megaleledone setebos, Muusoctopus rigbyae, Adelieledone polymorpha, Pareledone aurata, Pareledone felix, Pareledone turqueti	Lateral wall of upper beak	No	Schwarz et al. (2019)
Japetella diaphana, Vampyroteuthis infernalis	Lateral wall of upper beak	No	Schwarz et al. (2020)
Cuttlefish			
Sepia apama	Lateral wall of upper beak, rostrum of upper beak	No	Hall, (2002)
Squid			
Illex argentinus	Lateral wall of upper beak	Yes (paralarvae)	Sakai et al. (2007)
	Rostrum sagittal section of upper beak	No (cross-verification with statoliths)	Liu et al. (2015)
Ommastrephes caroli	Lateral wall of upper beak	Yes (paralarvae)	Sakai et al. (2007)
	Rostrum sagittal section of upper beak	No (cross-verification with statoliths)	Liu et al. (2015)
Dosidicus gigas	Lateral wall of upper beak	Yes (paralarvae)	Sakai et al. (2007)
	Rostrum sagittal section of upper beak	No (cross-verification with statoliths)	Liu et al. (2015)
Sthenoteuthis oualaniensis	Lateral wall of upper beak	Yes (paralarvae)	Sakai et al. (2007)
	Rostrum sagittal section of upper beak	No (cross-verification with statoliths)	Liu et al. (2015)

(Continued on following page)

TABLE 2 (*Continued*) Detailed information on cephalopod species that have been attempted using beak increment analysis.

Species	Beak part	Validated	Study
Todarodes pacificus	Lateral wall of upper beak	Yes (paralarvae)	Sakai et al. (2007)
Architeuthis dux	Rostrum sagittal section of lower beak	No	Perales-Raya et al. (2020)
Uroteuthis chinensis	Rostrum sagittal section of upper beak	No	Jin et al. (2019)
Uroteuthis edulis	Rostrum sagittal section of upper beak	No	Lin et al. (2019)
Histioteuthis bonnellii	Lateral wall of upper beak	No	Mereu et al. (2011)

structures in which deposition of growth increments is thought to be daily, as it has been validated for statoliths in several species of squid (E.g., *Illex illecebrosus, Loliolus noctiluca, Loligo chinensis, Loligo vulgaris reynaudii*) and cuttlefish (E.g., *Sepia officinalis*) (Hurley et al., 1985; Jackson, 1990; Lipinski et al., 1998; Bettencourt and Guerra, 2001), can be used to infer the periodicity of increment deposition in the beaks of newly studied species or when validation experiments are not feasible. Nevertheless, validation experiments involving mark-recapture or captive rearing of known-age or chemically-marked specimens are needed to ensure absolute age determination in the species (Campana, 2001). Even then, and considering that a circadian rhythm has been proved in many species (Cobb et al., 1995; Meisel et al., 2003), the fact that growth increments are shown to be deposited daily does not prove that this will always be the case to all cephalopod species/populations as increment deposition may depend on various factors (e.g., food availability, water temperature) (Bettencourt and Guerra, 2000; Zumholz et al., 2006; Canali et al., 2011).

Since growth increments in beaks were first reported in the 1960s for the squid *Moroteuthopsis longimana* (misidentified as *Moroteuthis ingens* by Clarke (Clarke, 1965; Cherel, 2020), many attempts have been made to use these structures to estimate cephalopod age (Tables 1, 2). Indeed, the growth increments in cephalopod beaks, initially observed by Clarke (1965), in the surface of lateral walls did not show a suitable sequence of increments to estimate the age in the species examined. The use of beak microstructure for age estimation was re-assessed in the 1990's in octopuses (Perales-Raya and Hernández-González, 1998), due to the absence of evident increments in their statoliths. Perales-Raya and Hernández-González (1998) observed a sequence of thin increments in sagittal sections of the rostrum of *O. vulgaris* beaks, and suggested that their deposition should be related to an individual's age. Successful analysis of lateral wall surfaces of *O. vulgaris* beaks was then performed by Hernández-López et al. (2001) and daily deposition was confirmed in paralarvae. Then, both techniques were compared and improved by Perales-Raya *et al.* (2010). Validation studies

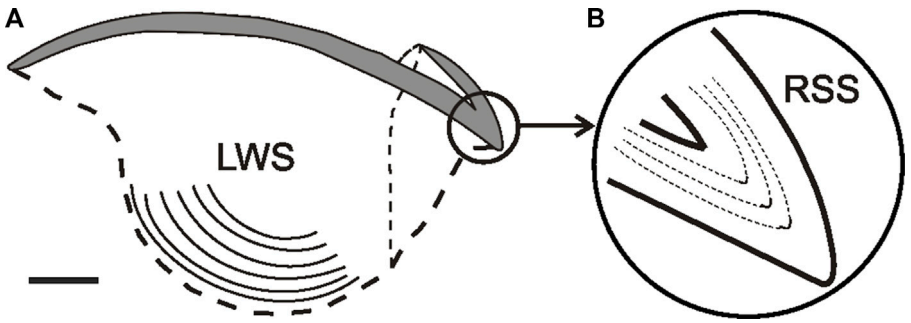

FIGURE 3
Lateral view of the upper beak in *Octopus vulgaris* **(A)** Sagittal section showing the inner lateral wall surface (LWS) bearing increments (lines). **(B)** Rostrum sagittal section magnified and showing the daily increments. From Perales-Raya et al. (2014a); copyright permission obtained. Bar: approx. 2 mm.

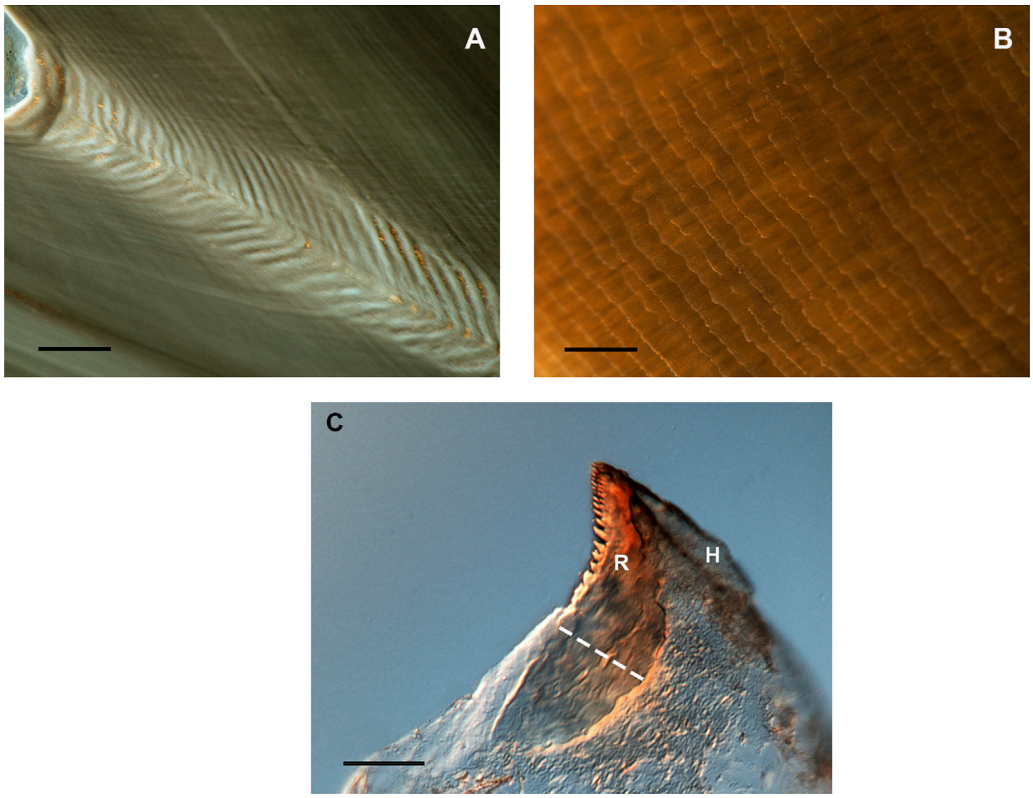

FIGURE 4
Microstructure of beaks showing growth increments in cephalopod beaks (*Octopus vulgaris*). **(A)** Increments in the rostrum sagittal sections (RSS). Bar: 100 μm. **(B)** Increments in the inner lateral wall surface of the upper beak. Bar: 200 μm. **(C)** Increments in the rostrum surface of the upper beak in early stages. R = rostrum; H = hood; dotted white line = reading area. From Arkhipkin et al. (2018) and Perales-Raya et al. (2018); copyright permission obtained). Bar: 50 μm.

were subsequently performed to confirm daily deposition also in octopuses (E.g., *Octopus vulgaris*, *Octopus maya*) (Canali et al., 2011; Rodríguez-Domínguez et al., 2013; Bárcenas et al., 2014). Finally, daily deposition was validated in the full ontogenetic range for *O. vulgaris* on both the rostrum sagittal sections and the lateral wall surfaces by Perales-Raya *et al.* (2014a). These authors used the recording of specific events in beaks of *O. vulgaris* taken into captivity (e.g., capture, temperature changes) as dated marks to validate the temporal deposition of increments. Consequently, the analysis of beaks as life event recorders has been applied in wild populations to understand the effects of environmental variations, biological events, stress of capture (Perales-Raya et al., 2014b), and to improve the welfare of early stages in reared populations (Franco-Santos et al., 2016).

Since the daily deposition was validated in *O. vulgaris* (Figure 4), the beak microstructure has been used in age and growth studies of several species of squid, cuttlefish and octopods, providing key information on their life history and population dynamics (Cuccu et al., 2013; Fang et al., 2016a; Liu et al., 2017; Batista et al., 2021). Validation studies have been performed in species such as *Sepia officinalis* (Lishchenko, unpublished data) and *Octopus insularis*, based on the growth increments in the rostrum sagittal sections or lateral wall surface of the beak (see references in Table 2).

In relation to using beaks for age determination in cephalopods, it was found that the tip erosion during the feeding process may bias increment counts in the anterior region of the beak but counting the oldest increments in the dorsal area of the section prevents age underestimation (Arkhipkin et al., 2018). A simple method has been recently developed to quantify the tip erosion using the number of increments in the dorsal non-eroded region and the width of increment of the central reading region (Perales-Raya et al., 2020). Moreover, the beak microstructure of emblematic deep-sea species such as the giant squid *Architeuthis dux* (Perales-Raya et al., 2020) (Figure 5) provided age data and a maximum lifespan estimation of around 3 years, based on rostrum sagittal sections from the lower beaks. The same technique was used in the beaks of the warty squid *M. longimana* from the stomach contents of Antarctic toothfish to estimate the age of this Southern Ocean species (Queirós, Bartolomé, Xavier, Perales-Raya, unpublished data). In both studies, the authors tested the lateral wall surface of

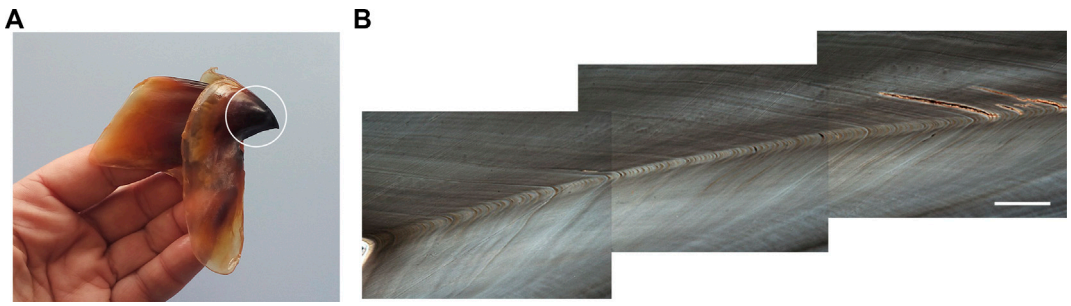

FIGURE 5
Lower beak of the giant squid *Architeuthis dux*. A white circle highlights the rostrum **(A)**. Image composition of the sagittal section of the lower beak rostrum showing growth increments **(B)**. From Perales-Raya et al. (2020); copyright permission obtained). Bar: 200 μm.

upper beaks and the rostrum sagittal sections of upper and lower beaks but only the latter showed a suitable sequence of increments for age estimation. On the contrary, the lateral wall surface has been the only beak region explored in Antarctic incirrate octopods from the families Megaleledonidae and Enteroctopodidae (Schwarz, 2019; Schwarz et al., 2019). These authors suggested lifespans exceeding 3 years and the possibility that deposition of growth increments in beaks of Antarctic octopods is not daily.

Growth increments (usually a few dozen) are also known on the surface of ammonite aptychi (calcitic coverings of the lower beak). The major growth increments were hypothesised to represent fortnightly tidal cycles or months, and the minor increments to correspond to days or semi-diurnal tidal cycles. If this assumption is correct, the ammonite life span would last between 1 and 6 years and the growth would be sigmoidal, with slowing down at maturity (Hewitt et al., 1993; Machalski, 2021).

Palaeontology: Fossil beaks

The nautiloid and ammonoid beaks are the cephalopod structures that fossilise most effectively after the shells. They are most often preserved in 2D but 3D fossils occasionally occur (Nixon, 2015). Ammonoid lower beaks are the most abundant in the fossil record, particularly from the Lower Jurassic (Toarcian) where a bivalve calcareous covering appears on the lower beak (Tanabe et al., 2015a). Similarly, the rostrum of the upper beaks of nautiluses, consisting of a hard, pointed calcareous tip (rhyncholite), is quite common in the fossil record from the Triassic onwards. Entirely organic beaks such as those of coleoids are very rare in the fossil record but are nevertheless known from exceptionally well-preserved deposits, the so-called *Lägersttätten*. Numerous examples are found in ammonoids dating back to the Devonian (Tanabe et al., 2015a; Klug et al., 2016). They are rarer in the Nautilida, but a few specimens are usable for shape analysis (Klug et al., 2021a). In Coleoids, beaks are found from the end of the Lower Jurassic (Nixon, 2015; Klug et al., 2021b). Fairly well-preserved 3D specimens have been related to Sepiolida (Harzhauser, 1999), Oegopsida (Tanabe et al., 2006), Cirrata (Tanabe et al., 2008), Vampyromorphida (Tanabe and Hikida, 2010), or undetermined teuthid taxa (Tanabe et al., 2015b).

All these fossils are still underused because of the shortage of comparative analyses between current and fossil beaks. The identification of beak shape adaptations to habitats or prey selection in modern species could inform our understanding of fossil shapes, thus complementing the classical paleoecological inferences from shells, gladius, soft tissues or fossil deposits (Fuchs and Iba, 2015; Fuchs et al., 2016). For example, investigating habitat shifts from shallow neritic to mesopelagic or bathyal environments (Hoving et al., 2014; Košťák et al., 2021) or *vice versa* (Arkhipkin et al., 2012) is of importance for understanding the evolution of modern coleoid lineages and for interpreting radiation events during cephalopod evolutionary history.

Currently, the data support a significant diversification event in Octopodiformes and Decapodiformes in relation to the so-called Marine Mesozoic Revolution, characterised by a major remodelling of shallow ecosystems, the rise of durophagous predators, and the resulting ecological “arms race” between predators and prey (Salamon et al., 2012). In this context, the diversification of coleoid cephalopods (stem Decabrachia, Spirulida) would have been driven by competition with predatory fish (Packard, 1972; Tanner et al., 2017; López-Córdova et al., 2022). The lineages of modern coleoid species are thought to have originated from one or more radiation pulses before the Cretaceous-Paleogene extinction (K-Pg crisis) (Fuchs and Lukeneder, 2014; López-Córdova et al., 2022). After the K-Pg crisis, a radiation pulse was stimulated by the disappearance of many predators and the availability of ecological space. Finally, another radiation pulse occurred during the mid-Cenozoic, controlled by climate change and low predator pressure (Fuchs and Lukeneder, 2014; López-Córdova et al., 2022). New data

contributing to the inference of habitat, trophic level or predatory abilities of fossil species will be useful to assess the proposed evolutionary scenarios. To achieve this goal, thorough analysis of the relationship between beak shape and species ecology in current cephalopods, followed by integration of the results with the fossil species would be an excellent step forward.

Advances in analysis if the composition of cephalopods beaks relevant to ecological studies

Cephalopod beaks are secreted by a single layer of cells in the buccal tissue, the beccublast cells, which are tall columnar epithelial cells (Dilly and Nixon, 1976; Tan et al., 2015). Beaks grow without replacement throughout the life of cephalopods (Perales-Raya et al., 2014a). Therefore, performing chemical analyses on entire beaks yields an average value for the entire life of the individual, whereas dividing the beak into different sections allows us to study different periods within the life cycle (Cherel and Hobson, 2005; Cherel et al., 2009a; Guerra et al., 2010; Xavier et al., 2016; Queirós et al., 2020a).

Beak chemical composition as a challenge to the application of chemical analyses

Beaks are composed of chitin-protein complexes (Miserez et al., 2007). There are differences in the composition among cephalopod taxa, with beaks of octopods being composed exclusively of α chitin, whereas those from squid, while mainly composed by α chitin, also contain β chitin (Miserez et al., 2007; Matias et al., 2019). However, these variations are not expected to result in significant differences when performing chemical analyses on these structures. In contrast, the ratio between chitin and protein varies along the beak, which can have significant influences on the analyses (e.g., lowering of $\delta^{15}N$ values when the amount of chitin is higher) (Miserez et al., 2008; Tan et al., 2015). These differences in the chitin: protein ratio can be easily observed in the pigmentation, with the untanned parts of the beak having a higher content of chitin than the fully tanned portion, which has a higher protein content (Miserez et al., 2008).

Stable isotopes

Stable isotopes, particularly of $\delta^{13}C$ and $\delta^{15}N$, are widely used in ecological studies (Peterson and Fry, 1987; Bearhop et al., 2004; Newsome et al., 2007). Using $\delta^{13}C$ values, it is possible to determine the carbon source at the base of the food chain and, ultimately, the feeding habitat of individuals (Cherel and Hobson, 2005). In marine systems, $\delta^{13}C$ values are known to vary with latitude (lower towards the poles), with inshore–offshore gradient (lower values towards offshore waters) and between benthic and pelagic environments (lower in pelagic organisms) (Cherel and Hobson, 2005; Newsome et al., 2007; Magozzi et al., 2017). In addition, anthropogenic CO_2 in the atmosphere has resulted in a decrease in $\delta^{13}C$ (as well as in ^{14}C) in both the atmosphere and the oceans, due to fossil fuels being relatively depleted in heavier carbon isotopes, the so-called Suess effect (Keeling, 1979; Gruber et al., 1999; Sonnerup et al., 1999). Regarding $\delta^{15}N$ values, these are used to study the trophic position of the individuals, based on the principle that predators are enriched in ^{15}N in relation to their prey (Peterson and Fry, 1987).

Bulk stable isotopic analyses (SIA) on beaks have been routinely perfomed, with $\delta^{13}C$ and $\delta^{15}N$ measured in cephalopods from all ocean basins (Hobson and Cherel, 2006; Cherel et al., 2009b; Guerra et al., 2010; Navarro et al., 2013; Golikov et al., 2019a; Queirós et al., 2020b; Fang et al., 2021a). They have been used: 1) in ecological and biogeographical studies to determine the foraging habitat and the role of cephalopods in food webs (Ruiz-Cooley et al., 2006; Guerra et al., 2010; Golikov et al., 2018; Staudinger et al., 2019; Fang et al., 2021a), 2) in fisheries to study stocks' distribution, contributing also to the implementation of ecosystem-based management (Fang et al., 2016b; Queirós et al., 2019), and 3) to study impacts of climate change and environmental fluctuations in these organisms (Golikov et al., 2019a; Hu et al., 2019; Abreu et al., 2020). Furthermore, SIA on beaks can also be used to study the foraging ecology of their predators (Guerreiro et al., 2015; Abreu et al., 2019; Guímaro et al., 2021). SIA of the entire beak gives us an average value for the life of the individual, and when applied to different sections of the beak (using the methodology specifically applied for SIA analyses (Guerra et al., 2010; Queirós et al., 2018)), it enables the study of ontogenetic changes throughout the lifespan (Cherel and Hobson, 2005; Guerra et al., 2010; Queirós et al., 2018; Wang et al., 2022). More recently, compound specific stable isotopes on amino acids (CSIA-AA) have also been studied in cephalopod beaks, mainly to delete the chitin effect that lowers the bulk $\delta^{15}N$ values of beaks when compared to other tissues (Cherel et al., 2019; Woods et al., 2022) (see below).

The SIA of beaks is a well-established technique with several advantages when compared to the use of other tissues. Cephalopod beaks recovered from predators' stomachs have comparable stable isotopic compositions regardless the time they were subjected to digestive processes and independently of the time-period during which they have been kept in collections (Cherel and Hobson, 2005; Abreu et al., 2020). The preservation method (e.g., dried, frozen, in ethanol, in formalin) also does not affect SI values/ratios, enabling its application to museum beak collections and retrospective investigations (Ruiz-Cooley et al., 2011). However, when using SIA, there are some caveats to be aware of (see those listed below). Some of these can be overcome using CSIA-AA but due to the higher costs of this

technique, its wider use has been so far limited. In addition, there are common problems (e.g., amount of sample required, knowledge of the trophic enrichment factor) associated with both CSIA-AA and bulk SIA. Below, we summarise the main problems associated with SIA, how to detect them and possible solutions. First and foremost, the amount of sample (usually ~0.35 mg but it is dependent of the equipment/methodology/analyzer used) that is necessary for both SIA and CSIA-AA (and other analyses) can be a limitation, especially if the study aims for a sequential analysis along the beak or concerns smaller species/specimens whose entire beaks do not make up the necessary mass for the analyses. In this situation, a possible solution is to pool the minimum number of different beaks necessary to perform the analyses. However, it must be guaranteed that beaks belong to similar individuals (i.e., similar size, collected in the same location and at the same time) to ensure they belong to the same school/cohort (e.g., Guímaro et al., 2021; Queirós et al., 2021a). Interpretation of SIA results from cephalopod beaks (and other tissues) is dependent on baseline stable isotopic values (i.e., to determine the habitat and trophic level). It is advisable to determine the δ^{13}C values of Particulate Organic Matter (POM) from the region and/or the δ^{15}N values of an organism with a known trophic position (e.g., δ^{15}N value of a filter-feeder species that it is in the second trophic level). To overcome the necessity of analysing POM stable isotopic values, it is possible to use values obtained from previous studies, or studies that modelled isotopic variation around the world's oceans (Somes et al., 2010; Farmer et al., 2021; St John Glew et al., 2021). However, when using values from previous studies or when comparing values obtained in beaks collected in different periods, it is necessary to account for the temporal variation of isotopes in the environment. To solve this, the obtained δ^{13}C values need to be corrected considering the Suess effect when values span across decades or consider modelled past δ^{13}C values (Farmer et al., 2021). For the δ^{15}N, the use of values obtained by previous modelling studies is so far the best option (Somes et al., 2010; Farmer et al., 2021; Verwega et al., 2021). If the capture location of the individual is known, the isotopic value of the near-death beak segment [i.e., the end of the hood and crest in the upper beak and the end of the hood and crest and wing in the lower beak (Queirós et al., 2018)], can be related to the local isotopic values. This may not be appropriate when using beaks sampled from predators' stomachs, since it needs to also take into consideration the daily (and long-time scale) movements of the predator and the time the beak spent in the stomach and these will vary depending on predator and cephalopod species involved (Xavier et al., 2003b; Xavier and Cherel, 2021). Nevertheless, for bulk SIA, without measuring baselines at the time of collection, it is impossible to estimate the trophic position of the individual. In contrast, a baseline is not necessary when determining the trophic position using CSIA-AA because the use of so-called "source amino acids" (e.g., phenylalanine) provides a baseline for the cephalopod beak (Cherel et al., 2019). However, using bulk SIA it is still possible, by comparing different beaks or different sections of the beaks (Queirós et al., 2018), to identify changes in habitat or trophic position. One limitation when studying changes in habitat is that changes in δ^{13}C values can be related to latitudinal, inshore-offshore, or benthic-pelagic changes (Cherel and Hobson, 2007; Newsome et al., 2007); thus results should be considered carefully and conclusions should be supported by previous knowledge of the ecology of the species. Furthermore, it is important to note that δ^{13}C also increases by around ~1‰ per trophic level through the food web, so that a significant correlation between δ^{13}C and δ^{15}N values could be due to a habitat change or diet change. Determining the slope of a regression between δ^{13}C and δ^{15}N values may however enable these two possibilities to be distinguished.

Regarding trophic position changes, when comparing δ^{15}N values, it is important to know the relevant trophic enrichment factor (TEF) (trophic discrimination factor (TDF) in CSIA-AA) (i.e., the differences between the δ^{15}N values of predator and its diet). Previous studies using *S. officinalis* raised in captivity showed that for beaks, this value is ~3.4‰ (Hobson and Cherel, 2006). However, recent studies showed that the TEF tends to decrease with increasing trophic position, requiring the use of the "scale δ^{15}N framework" (Hussey et al., 2014a; b). This δ^{15}N framework also needs to be region specific (Hussey et al., 2014a; b). In CSIA-AA, the δ^{15}N framework is not applicable and a TDF of ~7.6‰ is used universally (O'Connell, 2017; Whiteman et al., 2019), although no cephalopod-specific studies exist to date. Also, CSIA-AA requires information on the difference between δ^{15}N values of «trophic» and «source amino acids » in producers (trophic level 1), commonly referred to as β, to estimate consumer's trophic position. A value of β of −3.4‰ is universally used in aquatic ecosystems (O'Connell, 2017; Whiteman et al., 2019). It should be borne in mind that δ^{15}N values do not change only with the trophic position or prey species, but can be related to other changes in the diet of the predators such as the proportions of different prey that are eaten (Bearhop et al., 2004) and to changes in the diet of the prey species.

Currently neither bulk nor compound-specific SIA allows the identification of specific prey species. Even if the measurement of δ^{13}C and δ^{15}N values in a specific target prey species is performed, it is difficult to confirm that the tested species was eaten rather than another species with similar diet and trophic level (Fogel and Tuross, 2003; Naito et al., 2013; Pinkerton et al., 2014; Golikov et al., 2020). So-called mixed models are commonly used to infer predator diet composition from bulk SIA data. They require information on the δ^{13}C and δ^{15}N values of all putative prey but, even then, the natural variation around average values needs to be accounted for and, fundamentally, using values of only two variables (δ^{13}C and δ^{15}N values) to determine the values of multiple model parameters (the dietary importance of N putative prey types) is difficult (Phillips et al., 2014). In this sense, CSIA-AA offers a way forward since δ^{13}C and δ^{15}N values are potentially available for multiple

trophic amino acids, greatly increasing the theoretical discriminatory power of the data to allow diet composition to be determined.

The $\delta^{13}C$ and $\delta^{15}N$ values obtained from bulk SIA and CSIA-AA are not directly comparable, and even if using the estimated trophic position allows the two types of $\delta^{15}N$ measurement to be compared indirectly, there is currently no such method for $\delta^{13}C$. One key limitation when applying SIA to beaks is their chitin content. Chitin is a polymer of N-acetyl-glucosamine (i.e., a complex sugar that contains N atoms that are impoverished in ^{15}N when compared to amino acids). Thus, $\delta^{15}N$ values are not only lower in beaks than in other tissues, but also vary between different sections of the same beak due to the varying chitin: protein ratio (as mentioned above). It is necessary to carefully evaluate the C:N mass ratios obtained in the results, with higher ratios suggesting a higher amount of chitin (Cherel et al., 2009a). It may be possible to define a C:N ratio beyond which SIA results should be discarded, or to calculate a correction factor based on previous studies [e.g., Post et al. (2007)]. Results are usually considered unusable if C:N ratio values are above 4.0 but it may be appropriate to use results from part of the beak (i.e., the more heavily pigmented part) or to relax the rule if the study is on a rare species. This is particularly important when analysing beaks of juvenile cephalopods for which much of the structure is transparent, thus with higher chitin concentration than in the fully tanned beaks of adults (Clarke, 1986; Cherel et al., 2009a). This limitation can be overcome by removing the transparent part of the beak (Matias et al., 2019; Staudinger et al., 2019) or by using CSIA-AA which is not dependent on the amount of chitin in the beaks (Cherel et al., 2019; Woods et al., 2022). Regarding the use of different sections of the beaks for SIA, it should be noted that values obtained from the tip of the rostrum, sometimes considered as a proxy for juvenile life, result from a mixture of beak material deposited in early life stages and new beak material deposited over the life of the individual (Queirós et al., 2018). This suggests that in species that migrate and increase their trophic position throughout their life, $\delta^{13}C$ values are higher or lower depending on the habitat occupied by the adult, and $\delta^{15}N$ values are higher due to the higher trophic position in the later life-stage (Queirós et al., 2018).

Trace elements

Trace elements occur naturally in the environment, yet their concentrations are increasing due to anthropogenic activities (Sen and Peucker-Ehrenbrink, 2012). These elements can be essential (e.g., copper, iron, zinc) or non-essential (e.g., cadmium, mercury, lead), with both being potentially toxic at a given concentration (Jakimska et al., 2011). They may bio-accumulate throughout the life of individuals and some of them can bio-magnify through the food webs, with the main uptake being by prey ingestion (Szynkowska et al., 2018). Because of their importance in the food web, trace element concentrations have been extensively studied in cephalopods (Bustamante et al., 2000; Seixas et al., 2005; Pierce et al., 2008; Lischka et al., 2020; Seco et al., 2020). However, they have only recently been measured in cephalopod beaks (Fang et al., 2019; Lin et al., 2019; Northern et al., 2019). While numerous studies measured the concentration of mercury in beaks (Xavier et al., 2016; Matias et al., 2019; Queirós et al., 2020a), studies analyzing the concentration of other trace elements are rarer. Northern et al. (2019) measured the trace element concentrations in three different sections of *Moroteuthopsis ingens* lower beaks using both solution based inductively coupled plasma mass spectrometry (SB-ICP-MS) and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) methodologies. Both techniques were able to measure the concentrations of at least 23 elements, though LA-ICP-MS was able to detect three additional elements (i.e., Be, Y and Zr), that SB-ICP-MS did not detect, and also found more variability between beak sections (Northern et al., 2019). More studies are needed to understand whether this inequality is related to the methodology or to differences between the studied specimens.

For trace elements analyses, in contrast to SIA, the tip of the rostrum cannot be considered a proxy for the juvenile life phase as results obtained in this section are similar to those obtained in the end of the hood (Queirós et al., 2020a). Here, the anterior section of the hood is the most appropriate to study this life phase (Queirós et al., 2020a). Analyses of trace elements on beaks are useful for: 1) ecotoxicological studies (Xavier et al., 2016; Queirós et al., 2020a), 2) biogeographical studies to determine the distribution and migration of individuals (Fang et al., 2019; Northern et al., 2019), 3) ecological studies to evaluate the individuals trophic ecology and diet changes during ontogeny (Matias et al., 2019), and 4) the identification of cryptic species (Fang et al., 2021c). As cephalopods are usually widely distributed all around the world, measuring the concentrations of the different trace elements in beaks can also be used for biomonitoring the variability of contamination across ocean basins and, by using beaks preserved in collections, it may allow an evaluation of how concentrations of these elements have changed over time. Moreover, as performed in other taxa, such as bivalves and fish (Campana et al., 1994; Gomes et al., 2016), the analysis of trace elements over the life of individuals in beaks can be used to study the connectivity between different areas to help in the conservation of species and management of cephalopod fisheries.

It is worth noting that, when analysing trace element concentrations in cephalopod beaks, the resulting concentrations are significantly lower than those in other tissues such as muscle, digestive gland, or gills (Xavier et al., 2016; Matias et al., 2019; Matias et al., 2020). Although a previous study found a relationship between mercury concentrations in beaks and muscle in one Antarctic octopod species, which would suggest the former as a potential proxy for the mercury concentration of the flesh, no such relationship was found in other species (Matias et al., 2020). However, the analyses of trace element concentrations in different sections of the beaks may

allow to determine differences (i.e., ratios) of elements concentrations in different life stages (Queirós et al., 2020a). For example, mercury concentrations on beaks suggest that adults of *M. longimana* have twice as more mercury than juveniles, which also may happen in the muscle (Queirós et al., 2020a). Ultimately, element concentrations measured in beaks can be used to estimate the concentration of the element that might be transferred from the prey to the predator.

Measuring trace element concentrations in cephalopod beaks does have some limitations. As beaks tend to have lower concentrations of the target elements, the detection limit of the techniques can be a problem, especially when looking for minor elements and when analysing subsections of the beaks for trace metal analyses. This should not be a problem when analysing entire beaks (except if they are from small species or very young individuals). When a specific equipment is available for an element, e.g., Advanced mercury analyser to mercury, its use should be prioritised as it is more sensitive to the element and enables the measurement of lower concentrations. Otherwise, LA-ICP-MS is probably the best option as it has a lower detection limit than other techniques (Fang et al., 2019; Northern et al., 2019; Fang et al., 2021c), and is thus suitable to determine concentrations of minor elements or to measure the concentrations of trace elements in small sections of the beaks. The amount of sample needed to perform some trace element analyses can also be a problem for both entire small beaks and/or beak sections. Here, as for SIA, pooling beaks with similar size, origin and time of sampling is a possible solution (Queirós et al., 2020a; Guímaro et al., 2021). These limitations also apply to the emerging compound-specific trace element analyses (Weiss et al., 2008; Wiederhold, 2015). Emerging compound-specific trace element analyses, such as mercury stable isotopes, can offer a great opportunity to delineate the vertical habitat of cephalopods, as already shown in sharks (Le Croizier G. et al., 2020; Le Croizier G.l. et al., 2020; Besnard et al., 2021) and seabirds (Renedo et al., 2018). Indeed, when using trace elements to study individual migrations, it is important to know if there is any ontogenetic change in the diet or trophic position, because as trace elements are mostly taken up by diet (Szynkowska et al., 2018), we need to be sure to be sure that changes in trace element concentrations are in fact related to changes of habitat and not with the trophic position of cephalopods.

Beak composition should always be considered in any of the analyses mentioned here. As some trace elements have a higher affinity for proteins, e.g., mercury (Bustamante et al., 2006), the changing protein:chitin ratio along the beak can potentially influence the result. Hence, it is important to be careful when analysing the results, especially when comparing different beak sections or beaks from different species for trace elements, sizes and maturation states (Queirós et al., 2020a). As with SIA, this limitation can be overcome with the compound specific trace elements analysis (Renedo et al., 2018; Cherel et al., 2019; Whiteman et al., 2019; Besnard et al., 2021).

DNA analyses

Genetic analysis plays an important role in the study of cephalopod systematics and evolution (Boyle and Rodhouse, 2005; Strugnell et al., 2011; Allcock et al., 2015; Bolstad et al., 2018). It has also been used to identify cephalopod flesh found in the stomachs of predators as well to study the role of cephalopods as predators (Deagle et al., 2005; Braley et al., 2010; Hoving et al., 2014; Olmos-Pérez et al., 2017; Fernández-Álvarez et al., 2018; Queirós et al., 2021b). These previous studies used the flesh, both muscle and buccal mass, of individuals from collections, or that were captured or washed up on the shore (Bolstad et al., 2018; Queirós et al., 2020b). As some cephalopods, in particular oceanic squids, tend to easily avoid capture and many predators prey/scavenge on them, extracting DNA from the beaks would be an important tool as it would facilitate the identification of some species whose beaks are very similar [e.g., *Histioteuthis eltaninae* and *H. atlantica* (Xavier and Cherel, 2021)], the identification of beaks belonging to undescribed species [e.g., Oegopsida sp. A, *Taonius* sp. (Clarke), *Onychoteuthis* sp. B (Imber) (Cherel et al., 2004; Cherel et al., 2011; Cherel, 2020)], and to study the phylogeny and taxonomy of these hard-to-catch species.

As far as we know, only one study was able to extract DNA from cephalopod beaks (Vecchione et al., 2009). These authors extracted DNA from a beak of *Muusoctopus thielei* collected from a whole specimen. Another successful tentative trial was made in the beaks of *Architeuthis dux* but the amount of extracted DNA was not enough to follow up with further research at the time (Tom Gilbert, personal communication). In contrast, Xavier et al. (2016) did not succeed in extracting DNA from beaks of *M. longimana* and *Filippovia knipovitchi*. When comparing the studies of both Vecchione et al. (2009) and Xavier et al. (2016) to understand what could influence DNA extraction, one primary difference is that the former study used fresh beaks retrieved from the individual, while the latter used non-fresh beaks from predators' diet. After spending time in the stomach, the beaks lost their transparent part which is the area closer to the beccublast cells that synthesise the beak proteins (Tan et al., 2015). Further differences can also be found in the methodology used in both studies (Vecchione et al., 2009; Xavier et al., 2016). We believe that it is worthwhile to continue to attempt the extraction of DNA from cephalopod beaks, although we suggest that studies should focus on the transparent parts of the beaks, rather than the entire, fully sclerotized, beak. Nevertheless, the use of the transparent part of the beak could be a limitation for these methodologies in smaller species in which the transparent part is reduced, as well as in beaks from predators' stomachs since the transparent part disappears with the action of the gastric acids (Clarke, 1986; Duffy and Jackson, 1986). Furthermore, we suggest trying different methodologies, including approaches that have been used to successfully extracted DNA from other difficult-to-handle issues e.g.,

bones or fossils (Vecchione et al., 2009; Xavier et al., 2016; Campos and Gilbert, 2019; Modi et al., 2021).

Structural analysis

The rostrum of cephalopod beaks is among the hardest and stiffest fully organic materials on Earth, while the lateral walls and wing areas of these beaks are generally soft and flexible (Miserez et al., 2008). Indeed, the beak rostrum can be harder than engineering polymers and present an intermediate response to blunt abrasion (Miserez et al., 2007). Because of these characteristics, beaks are seen as an inspiration for new engineering protein-based and environmentally load-bearing polymers (i.e., polymers that can support great amounts of weight) that can replicate properties of living organisms (Miserez et al., 2010; Linder, 2015; Sun et al., 2020). Furthermore, there is also an interest in chitosan, a biopolymer obtained from chitin (Miserez et al., 2008), which has applications in the food industry, pharmaceuticals, textiles and biotechnology (Morin-Crini et al., 2019). However, for such compounds to be replicated, it is important to understand how they are formed and what gives the beaks their unique properties since they do not contain metal ions, minerals, or halogens, which typically confer hardness and stiffness to biomaterials [e.g., Zinc (Zn) ions on polychaete jaws (Miserez et al., 2007; Linder, 2015)]. The determination of beak characteristics can also be used in the study the trophic ecology (by providing insights into what kind of prey could be eaten) (Matias et al., 2019).

Several studies analysed the structure and mechanical properties of cephalopod beaks, most of them using those of the jumbo squid *Dosidicus gigas* (Miserez et al., 2007; Miserez et al., 2008; Miserez et al., 2010; Tan et al., 2015). To our knowledge, apart from these studies, only Matias et al. (2019) have studied the mechanical properties of cephalopod beaks, using two Antarctic octopod species, *Pareledone turqueti* and *Adelieledone polymorpha*. The methodology used by all these studies was very similar [i.e., optical and scanning electron microscopy and high-resolution microcomputed tomography to determine the microstructure and structural features, x-ray diffraction to study the density, nanoindentation test to determine the beak mechanical properties and single-edge notched tension (SENT) to determine the fracture toughness]. In contrast to previous methodologies, only one limitation was found, and it was related to the size of the beak and its suitability for some tests (e.g., SENT test). This is why the authors decided to use very large *D. gigas* beaks (Miserez et al., 2007). The similarity between the techniques used to date suggests that future studies could use the same approach to study the structural properties of beaks, facilitating comparisons, providing insights into the ecology of the species and inspiring the engineering of new materials. Indeed, different results obtained for three species showed different hardness in the following sequence, from hardest to least hard: *D. gigas* > *P. turqueti* > *A. polymorpha* (Miserez et al., 2007; Matias et al., 2019).

Proteomics

The study of an organisms' proteome can assume a major role in understanding how species will react to climate change, pollutants and other environmental stressors (Nunn and Timperman, 2007; Braconi et al., 2011; Tomanek, 2014). Indeed, previous studies showed that temperature, toxic trace elements, food limitation, or hormones can all affect the proteins in molluscs, as well as their amino acid pool (Veldhoen et al., 2012; Clark et al., 2017). Furthermore, an organisms' proteome can help in species' identification (Mazzeo and Siciliano, 2016). A proteomic approach has been used in cephalopods to understand their colours, toxins, host-parasite relationships and their immune system (Gestal and Castellanos-Martínez, 2015; Roumbedakis et al., 2018; Albertin and Simakov, 2020; Gonçalves and Costa, 2021). These studies used tissues such as skin (Crookes et al., 2004), slime (Caruana et al., 2016), saliva (Cornet et al., 2014), and in cuttlefish cuttlebones (Pabic et al., 2017).

In a pioneering study, Miserez et al. (2008) carried out the first analysis of the proteins in cephalopod beaks. Using beaks of the jumbo squid *D. gigas,* they found differences in the amino acids composition between the tanned and untanned areas (Miserez et al., 2008) and showed that the stiffness of the beaks was linked to the amount of certain proteins, identifying l-3,4-dihydroxyphenylalanine-histidine (dopa-His) as providing mechanical strength to the beak material. The authors subsequently found many cross-links were actually based on (His)-4-methylcatechol and not dopa-His (Miserez et al., 2008; Miserez et al., 2010). Following these studies, Tan et al. (2015) combined different transcriptomic (using mRNA from beccublast) and proteomic techniques to study the proteins in cephalopod beaks. They found the presence of two major families of proteins: the chitin-binding proteins (DgCBPs) and the histidine-rich beak proteins (DgHBPs), the former new to science (Tan et al., 2015). However, the precise distribution of each protein in the beak remains unknown, and only estimations using its wet mass are available (Tan et al., 2015). Despite this major step, it is still true that very little is known about the proteome of cephalopod beaks, especially because these studies focused only on one species and, as proteins are related to stiffness and beaks of different species have different stiffness structural analyses (Miserez et al., 2008), it is important to know if these proteins are the same for all species. Additionally, the presence of stress marks during the formation of beaks suggests changes during their formation that can be related to proteins (Perales-Raya et al., 2014a).

The study of cephalopod beak proteins is important to protein engineering as they can be a model for liquid-liquid

phase separation (Sun et al., 2020). Another advantage of studying beak proteins, as with other techniques, is their availability in collections and the possibility of using predators as biological samplers. This suggests that cephalopod beaks can be used to study environmental changes through time but also be useful to study impacts of environmental stressors in species that are challenging to sample. As proteomics can also be used in evolutionary and ecological studies (Diz et al., 2012), it also has the potential to help in the study of cephalopod evolution. However, there are known limitations to studying beak proteins, and major uncertainties since this is a very new field of study. The major limitation found by Tan et al. (2015) is that classic protein extraction protocols do not work on beaks. They overcame this limitation by using non-enzymatic reagents that cleave peptide bonds, releasing them from the beak structure (Tan et al., 2015). However, other strategies are needed to extract beak proteins without destroying them. Furthermore, it is still unknown whether different methods of preservation have different effects on proteins and whether proteins are still present in beaks from museum collections.

Future challenges in research in cephalopods beaks

Regarding the use of beaks for taxonomy, some relevant key features, limitations and perspectives are outlined below. Most previous investigations used lower beaks only (Clarke, 1986; Xavier et al., 2007a; Xavier et al., 2015). However, discarding upper beaks is a potential loss of information that was highlighted in subsequent investigations (Cherel et al., 2000; Cherel et al., 2017). Hence, both lower and uppers beaks should be included in future studies (Xavier et al., 2011). Also, care is needed when using names of species assigned to beaks in older publications, due to a combination of past misidentifications and subsequent improvements in both beak identification and cephalopod taxonomy over the last few decades (Cherel, 2020, 2021). Also, beaks are routinely used in the studies of trophic ecology of predatory species to estimate prey length and mass. However, regression formulas of relationships between beak size and length and mass of cephalopods are lacking for the majority of species. Extensive collection and routine publishing of such data is an essential task in cephalopod research in the future. Another challenge is that, despite the recent global revision of some families (e.g., Onychoteuthidae) (Bolstad, 2010; Bolstad et al., 2018), cephalopod taxonomy is still problematic: the chaotic state of some taxa precludes identifying beaks to the species level with confidence in many cases (e.g., Brachioteuthidae, Chiroteuthidae). Improvement in beak identification requires that both taxonomic revision and the description of new species include drawings and/or photos of the lower and upper beaks, ideally from early stages to mature adults. It also requires exploring new methods to extract DNA from biological samples in poor condition. In most cases, as reported above, conventional procedures fail to extract DNA from partly digested cephalopod flesh, thus preventing the use of buccal masses from food samples to confirm/inform and thus improve cephalopod identification based on the corresponding beaks. This loss of information is unfortunate because most oceanic squids are notably difficult to catch using traditional means, while some species form a significant part of predators' diet. In a few cases, the reverse is true, with beaks helping to solve systematic issues. For example, conventional examination of squid morphology and anatomy failed to find differences between *Histioteuthis bonnellii bonnellii* and *H. b. corpuscula* (Voss et al., 1998) [presently considered synonym: *H. bonnellii* (MolluscaBase, 2022)], while both beak morphology and size clearly indicate that they belong to different taxa (Clarke, 1980), a finding that merits further genetic investigation using the new generation of efficient DNA tools. Finally, identifying beaks from their morphology is time-consuming and needs expertise. We thus recommend getting expert advice before attributing a species name to a beak (Xavier and Cherel, 2021). Unfortunately, the most important bottleneck of the method now is the low and decreasing number of experts, meaning that efforts must be made to train early career researchers to identify cephalopod beaks from their morphology. The value of conventional photographic guides of cephalopods beaks for training and species identification should be recognized. Existing beak identification guides have covered only minor part of cephalopods diversity and their regional morphological variability. Therefore, the development of new extended guides or web-based solutions (see examples above) are still essential. Additionally, simultaneous collaborative efforts focused on collection of beaks, their photographs and DNA samples deposited as public web-based resource may also serve as important reference database for cephalopod identification in the future, as it already occurs with fish (i.e., AFORO as an example for collaborative otoliths database (http://aforo.cmima.csic.es/) (Lombarte et al., 2006).

A promising alternative or supplement to identification of cephalopods based on the morphology of their beaks is the development of a software for geometric morphometric-based automatic identification of the beaks. However, this approach also has some limitations. The first issue to solve is the development of efficient methods of acquisition of images for processing. At the moment, there is no agreement about the equipment to use, from which angles to record images, and how to take photos of the beaks. Some authors used 2-D images of the beak's lateral view (Fang et al., 2017; Jin et al., 2017; Tan et al., 2021), others used a complex system of mirrors to obtain combined 2-D images of frontal and lateral views (Crespi-Abril et al., 2010), and others used a combination of underwater photogrammetry and CT scanning to obtain 3-D images of beaks (Roscian et al., 2022). As results of studies based on 2-D images suggest, this is enough for routine identification of abundant species (Tan et al., 2021), although in-depth ecological, paleontological, or taxonomic studies may demand more

complex method (Roscian et al., 2022). We argue that the use of beaks for identification purposes demands a simple approach, which can be adopted both in the field and in the laboratory. From this point of view, acquisition of 2-D images of the beak's lateral view seems more promising (Fang et al., 2017; Tan et al., 2021), but further studies on the loss of information and accuracy, when in using images of only the lateral view, are still needed.

Another issue is the selection of methods for analysis of images. Landmark-based methods of analysis have plenty of advantages in this regard, specifically when it comes to preserving ecologically or taxonomically meaningful information, but approach requires experienced users. On another hand, outline-based methods more suitable for automation of the process, since they do not require the user to select the points of interest or otherwise fine-tune the analysis. Thus, the software for automatic identification of the cephalopods based on the shape of their beaks may be based on Fourier or wavelet transformation, similar to the system developed for fish identification (Lombarte et al., 2006). It should be noted that accuracy of such an approach to identification may be improved if supplemented by the analysis of beak's pigmentation (Fang et al., 2017), or if only the pigmented part is analysed (Lishchenko and Jones, 2021).

Further studies on a large number of taxa, testing the phylogenetic signal carried by beak shape will be necessary to establish the level of accuracy that could be achieved in identification. Moreover, as 3-D reconstruction is time-consuming and more complex than 2-D analyses, recent work using 3-D geometric morphometrics, which allows the complexity of beak shape to be better captured, has shown that the phylogenetic signal of upper and lower beaks is significant but moderate and that it cannot explain all the morphological variation in beaks on its own (Roscian et al., 2022). On the other hand, cephalopod beaks remain complex to image in three dimensions without damaging them. The use and improvement of advanced imaging technologies such as X-ray tomography and underwater photogrammetry on small objects will allow most species to be digitized (Roscian et al., 2021; Ziegler and Sagorny, 2021; Roscian et al., 2022). Nevertheless, the digitization of specimens is a long task and the accumulation and accessibility of the data to the community is a major challenge in achieving a comprehensive sampling of cephalopod 3-D model beaks. These 3-D models could indeed allow us to thoroughly renew our understanding of the signal carried by the shape of the beaks and be very complementary to 2-D analyses. Recently, Roscian et al. (2022) showed, using 3-D geometric morphometrics, that there is a likely link between ecological parameters such as habitat and trophic level and beak shape. These outcomes are in accordance with those obtained for squid and octopod paralarvae indicating a relationship between beak shape variation and diet shifts (Franco-Santos et al., 2014; Franco-Santos and Vidal, 2014; Franco-Santos and Vidal, 2020).

The study of developmental features of the beak during the early ontogeny of cephalopods is a nearly unexplored field of study. Much remains to be investigated in relation to the developmental pattern, shape and chemical composition of the beak and its relationship with the feeding ecology during early life. These studies encourage further research into the analysis of beak shape in relation to the ecology of the taxa, to test whether adaptive traits can be identified in these structures at any phase of the life cycle. To achieve this goal, it will be necessary not only to have many 3-D models but also to collect more data on the diets, habitats and trophic positions of species for which information remains scarce or poorly known, such as some deep-sea octopods or squids (i.e., some members of the genus *Opisthoteuthis*, the pelagic *Vitreledonella richardi* or *Asperoteuthis lui*). This approach will open new opportunities to study the evolutionary history of cephalopods, as it will be possible to integrate fossil forms. Although the contribution of fossil beaks to the reconstruction of the evolutionary history of coleoids is challenging due to the deformations of the fossil record and their rarity, methods are now available to overcome these problems (Hughes and Jell, 1992; Schlager et al., 2018; Demuth et al., 2022). Quantitative comparative analyses of modern and fossil beaks are now achievable and should produce significant advances in paleoecological interpretation of selected fossil forms.

Regarding using beaks in age and growth studies, some key relevant features, limitations and perspectives of the field are outlined below. When working on age determination of a species for the first time, exploration of both lateral wall surfaces and rostrum sagittal sections is mandatory. Increments do not always present regular pattern in the lateral wall surfaces, but they usually do so in the rostrum sagittal sections. Moreover, both upper and lower beaks need also to be examined to select the most suitable for the species of interest. Some species exhibit high erosion and irregular increment sequences in lateral wall surfaces of upper beaks (e.g., *Architeuthis* and *Moroteuthopsis*), whereas in others (e.g., *Octopus vulgaris*) the upper beaks are the most suitable for age estimation. When using beaks from cephalopod predators (stomach contents), the loss of material from the external border of these beaks might be substantial, presenting problems if the lateral wall surfaces are used for age estimation, therefore the information on how long it takes for the transparent part of lateral walls to disappear would help to prevent age underestimations. On the other hand, it is necessary to know how long the beak has been in the stomach to have a proxy of the death day of the specimen. These issues are covered in depth further down since further research is desirable. Age validation experiments are scarce in beaks, but they are required to confirm the periodicity of deposition of the growth increments in the species of interest. Mark-recapture methods in wild populations are expensive but others such as captive experiments, using marking or known-age specimens, are suitable for age validation when aquaculture facilities are accessible and the species can live in

captivity for some time. It is also important to validate the age of the first increment, not only the temporal deposition of increments, to obtain reliable age estimations. When the species of interest is unsuitable for validation experiments (e.g., deep-water species), cross-verification by comparing with other validated structures (e.g., statoliths in squids or vestigial shells in octopuses), is an alternative.

The observation, counting and analysis of daily increments is time-consuming. The life-mode approach to performing semi-automated counts is advisable but not usually available in current image analysis systems. Artificial intelligence could provide a useful tool to save time and improve the detection and count of increments since it could "learn" from the images previously analysed by experienced readers. Finally, the cumulative width of the daily increments could be a potential tool to estimate growth in the wild, before capture (e.g., Perales-Raya et al. (2020) for *Architeuthis dux*; Queirós, Bartolomé, Xavier and Perales-Raya, unpublished for *M. longimana*). Preliminary results on reared *O. vulgaris* used correlations between cumulative widths and body mass to estimate the growth in the wild, before capture (Perales-Raya, Bartolomé, Márquez, Felipe and Almansa, unpublished data). Moreover, future research should also further evaluate beak growth under warming (e.g., climate change scenarios) under laboratory conditions, which is known to cause thermal stress in cephalopod beaks (E.g., in *Octopus vulgaris*) (Perales-Raya et al., 2014a) (Perales-Raya et al., 2014b), in order to validate the magnitude of such beak marks and their ecological implications.

The application of different chemical and structural analyses on cephalopod beaks to study different aspects of the species and individual life-cycle is increasing, though with some techniques being well-established in cephalopods (e.g., stable isotopic analyses), while others are still in development (e.g., proteomics). Nevertheless and independently of whether a method is "established," all methods present issues and challenges that should be addressed in the future. Stable isotopic analysis, as is the case for most of the techniques applied on beaks, is dependent on the chitin:protein ratio, which varies throughout the beak. Future studies should evaluate whether the variation of the chitin:protein ratio is similar across species. Furthermore, as a higher proportion of chitin results in lower δ^{15}N values, a correction factor for chitin, similar to those used in stable isotopic analyses on muscle for lipids (Hobson and Cherel, 2006), should be found to enable the direct comparison of δ^{15}N values between different sections of the beak and different life-stages and to compare with other tissues. Several studies applied SIA on beaks from predators' stomachs, with most of these determining the habitat using known gradients of δ^{13}C values in the environment (Cherel and Hobson, 2005; Guerreiro et al., 2015; Abreu et al., 2020), especially when using different sections of the beak (Guerra et al., 2010; Queirós et al., 2018). If the capture location is known, the δ^{13}C value of the last formed beak material could be used as a baseline that helps to determine the movement of the individual. However, to have an idea of the capture location if the sample came from stomach contents, it is important to know how long the beak remained in the stomach. Although there is some information on the amount of time a beak can stay in a predator stomach (Ashmole and Ashmole, 1967; Clarke, 1980; Jackson and Ryan, 1986; Gales and Cheal, 1992; Xavier et al., 2011), future experimental studies are needed to understand the average time a beak takes to be egested in relation to the type of predator and how long it takes for transparent parts of a beak to disappear, depending on both its own size and darkening stage as well as on the predator biology. Consequently, it is essential for diet studies to distinguish the beaks that are from recently eaten prey (i.e., beaks recently consumed by predators that still have flesh attached, beaks in buccal masses or from complete or partially digested specimens) from those which have been in the stomach a long time and may be eroded (i.e., beaks without transparent parts or flesh attached).

SIA is also dependent on a baseline value and, despite some previous studies determining POM isotopic values for the different areas or modelling these values, future studies should update these values, partly because they may change over time, as well as using beaks from individuals with known capture location to create an isoscape specifically for cephalopod beaks. Apart from the baselines, future studies should focus on establishing a specific trophic enrichment factor for cephalopod beaks (ideally for each cephalopod species in a given region) that enables the study of the trophic level of an individual using an enrichment factor adapted for these structures rather than general enrichment factors that are common for all marine organisms.

Regarding the trace elements analyses, further studies should investigate in detail whether the differences found between results from different techniques used in previous studies (i.e., SB-ICP-MS and LA-ICP-MS) (Northern et al., 2019), are a consequence of the technique or simply a function of which beaks were used. It is also important that future studies investigate the relationship between element concentrations in the beaks and those found in other tissues of the individual. Due to the different chitin:protein ratio along the beak and the different affinity that some elements have with proteins, it is important that future studies evaluate how the change in beak composition can affect the concentrations of the different elements, and look in detail at the rostrum as this part of the beak may be prone to accumulate high levels of some elements, not working as a proxy for the juvenile life-phase as it does in SIA (Queirós et al., 2020a). Additionally, it is important to evaluate the usefulness of beaks as proxy of environmental pollution, such as studying these trace elements in beaks related to pollution (e.g., increased mercury levels due to anthropogenic sources), to provide a proxy of pollution in cephalopods.

Concerning the most recent techniques applied to beak structure and protein composition, we suggest that the various available methodologies are tested in order to find which are the most efficient. Considering previous studies, we suggest that future research aiming to extract DNA from (fresh) beaks from predators' stomachs that still have their transparent parts, as well as the use of techniques that have been shown to recover DNA material from fossils or bones. Regarding structure analyses, we suggest that future studies should aim to develop an understanding of the interspecific variability in the beak structure and how it can influence, for example, the diet of each species. The study of the beak proteins is, as far as we know, one of the most recent techniques that has been applied to these structures. Based on its importance for beak composition and its influence on the ecology of the species, we suggest that new techniques should be explored: techniques used in the past able to retrieve proteins, though destroying the beaks, should be applied in other species to understand the inter-species variability. Future studies should also explore the effect of the different preservation methods on the beak proteins to evaluate whether beaks in museum collections can be used in these studies.

Author contributions

The workshop was planned and organized by JX, AG, JQ, CP-R, RR-L, and YC who produced the initial framework of the manuscript. The final structure of the manuscript and text was based on the contribution of all authors during and after the workshop. These contributions were initially gathered by JX, JQ, and CP-R and then reviewed and accepted by all authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Appendix

FIGURE A1
Group photo of the participants, in person, of the CIAC 2022 beak workshop (Sesimbra, Portugal).

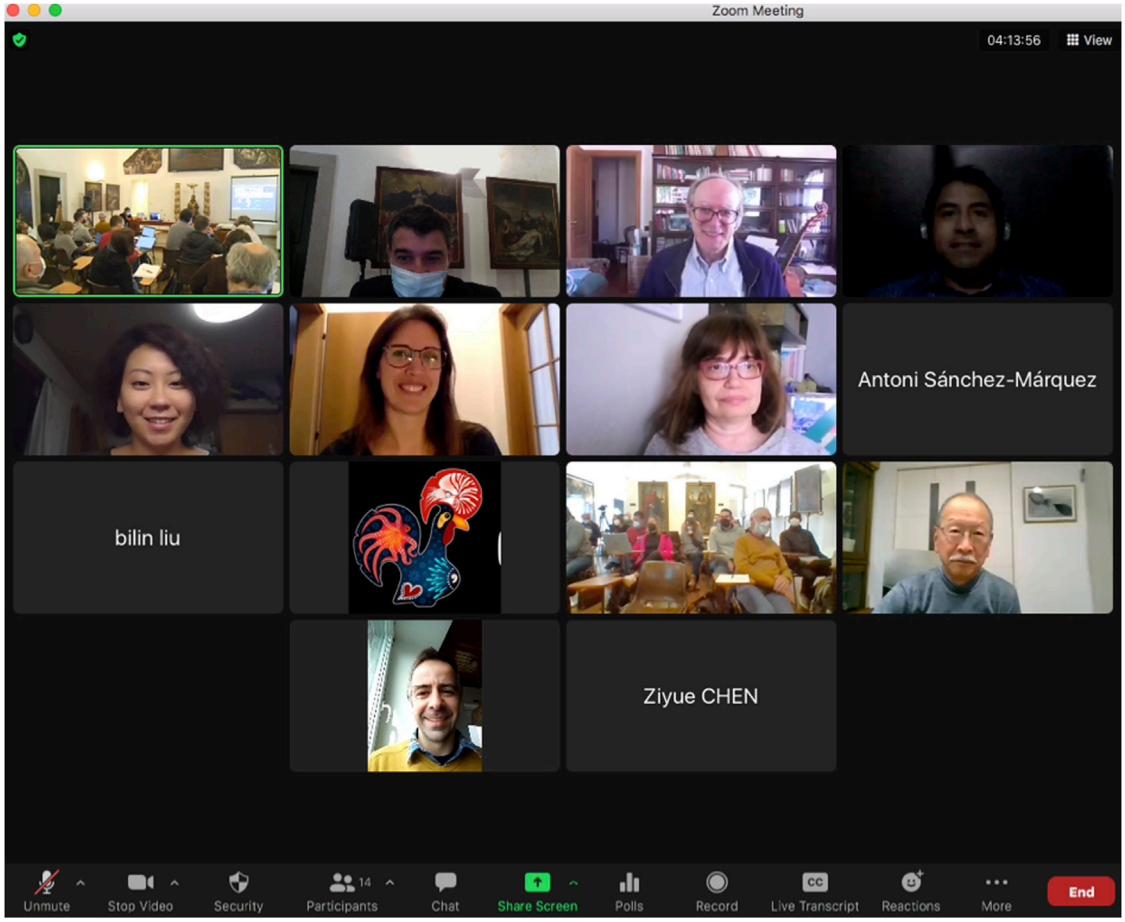

FIGURE A2
Group photo of the participants online of the CIAC 2022 beak workshop (Sesimbra, Portugal).

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Corrigendum: The significance of cephalopod beaks as a research tool: An update

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A Corrigendum on
The significance of cephalopod beaks as a research tool: An update

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In the published article, there was an error in the author list, and author Jorge Hernández-Urcera was erroneously excluded. The corrected author list appears below.

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In the published article, there was an error in affiliation 28. Instead of "Centre d'Etudes Biologiques de Chizé, UMR 7372 du CNRS-La Rochelle Université, La Rochelle, France", it should be "Centre d'Etudes Biologiques de Chizé, UMR 7372 du CNRS-La Rochelle Université, Villiers-en-Bois, France."

The authors apologize for these errors 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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Projecting future climate change impacts on the distribution of the '*Octopus vulgaris* species complex'

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Introduction: Historically considered to be a single cosmopolitan species, the so called *Octopus vulgaris* species complex (OVSC) is now recognized to be a group of (at least) six cryptic species: *O. americanus* (in the west Atlantic), *O. vulgaris* (in the northeast Atlantic and Mediterranean Sea), *O.* aff. *vulgaris* (in the region of South Africa), *O. tetricus* (southeastern Oceania), *O. sinensis* (northwestern Pacific), and *O. djinda* (western Australia). The potentially different environmental preferences of this highly cryptic species complex may result in distinct consequences under future environmental conditions.

Methods: The present study employed species distribution models (SDM) using MaxEnt to investigate potential changes in habitat suitability and geographical distribution of the OVSC in the future (*i.e.*, 2050, and 2100), across four representative concentration pathway scenarios (RCP-2.6, 4.5, 6.0, and 8.5, CMIP5).

Results: Differential responses were observed in the OVSC species analyzed. Specifically, *O. vulgaris* and *O. tetricus* exhibited a severe loss in distribution across their predicted range; *O. americanus* exhibited projected extirpation close to the equator, with limited expansion towards the poles; *O.* aff. *vulgaris* was projected to lose half of its current distribution; *O. sinensis* exhibited moderate losses, with projected increases in northern areas; and finally, *O. djinda* exhibited limited losses to its distribution. Except for *O. sinensis*, increasing RCP severity exacerbated changes in mean habitat suitability and projected distribution gains and losses.

Discussion: Ultimately, this study provides information on the potential biogeographical effects of marine climate change on a key worldwide ecological and economic resource to further disentangle the effects over each OVSC species, with the goal of assisting toward the sustainable management of octopus species at the global scale.

KEYWORDS

biogeography, climate change, species distribution models, species complex, common octopus

Introduction

Cryptic species complexes represent a particularly challenging topic in marine ecology. The term 'species complex' is employed when referring to a group of closely related species, historically classified as a single taxon, given the difficulty in their distinction through traditional morphology-based taxonomic methods (Knowlton, 1993; Bickford et al., 2007). Despite their typically high phenotypical proximity, which ultimately prevents their accurate discrimination, considerable genetic divergence can be observed (Espíndola et al., 2016). Cryptic diversity is relatively common in the Tree of Life, with cryptic species occurring almost homogenously among metazoans (Pfenninger and Schwenk, 2007) and protozoa (Bass et al., 2007). Moreover, cryptic species can be found in all major terrestrial and aquatic taxa, across the entire latitudinal profile (Beheregaray and Caccone, 2007). Over the past decades, techniques such as molecular sequencing, DNA barcoding, and mitochondrial DNA (mtDNA) analysis have enabled the disentangling of such instances, revealing a vast array of cryptic species complexes (Hebert et al., 2003; Bickford et al., 2007). The cephalopod class is no exception, with well-known species complexes, including those of the big-fin reef squid, *Sepiotheuthis* cf. *lessoniana* (Lesson 1930) (Cheng et al., 2014), the pharaoh cuttlefish, *Sepia pharaonis* (Ehrenberg, 1831) (Farhadi and Anderson, 2021), the squid *Lolliguncula* sp. (Steenstrup, 1881) (Sales et al., 2014; Costa et al., 2021), Ommastrephes squids (Fernández-Álvarez et al., 2020); and the common octopus, *Octopus vulgaris* (Cuvier 1797).

Historically, *O. vulgaris* was considered a cosmopolitan species, inhabiting shallow benthonic waters in disjunct populations spread around tropical waters worldwide (Robson, 1932; Roper et al., 1984). Initially described as a single species with a rather broad range (occurring along the eastern North Atlantic and the Mediterranean Sea, in the Americas and the eastern South Atlantic, Oceania, and the Northwest Pacific Ocean), recent genetic and molecular analyzes have enabled researchers to discriminate genetically distinct species within the *O. vulgaris* complex (Söller et al., 2000; Leite et al., 2008; Amor et al., 2015; Gleadall, 2016). At the same time, the existence of geographically and genetically isolated populations also contributed to the inclusion of 'Type' species in the complex (Norman et al., 2014). To this day, the OVSC is known to be comprised of (at least) six *Octopus* species, including: i) *O. vulgaris* - previously named *O. vulgaris sensu strictu* and which occurs in the North Atlantic Ocean, from North Africa to the English Channel, and in the Mediterranean Sea (Mangold and Hochberg, 1991; Mangold, 1998; Rosa et al., in press); ii) *Octopus americanus* - occurring along the Atlantic continental shelves of the Americas (Avendaño et al., 2020); iii) *Octopus* aff. *Vulgaris* – occurring in the coastlines of South Africa and Madagascar (Oosthuizen and Smale, 2003; Oosthuizen et al., 2004); iv) *Octopus sinensis*, occurring in the north-western Pacific Ocean, in the region of Japan and the Eastern China Sea (Reid and Wilson, 2015; Gleadall, 2016); v) *Octopus tetricus*, occurring in the south-eastern Australian coastline and northern New Zealand (Guzik et al., 2005; Amor et al., 2017); and vi) *Octopus djinda* Amor, - formerly *Octopus cf. tetricus*, occurring in the south-western coastline of Australia (Amor and Hart, 2021). This species complex is highly valued worldwide, representing one of the world's most important cephalopod fisheries (Balguerías et al., 2000; Sauer et al., 2021). Along with its high value, extensive exploitation is poised to negatively impact this group, with some populations exhibiting signs of depletion (Quetglas et al., 2015). Indeed, octopus catches have increased in the past decades (FAO, 2021), despite global cephalopod fisheries declining since 2014 (FAO, 2020). This increase in fishery pressures, together with poor taxonomic resolution and catch under-reporting at regional and even species level could potentially lead to overexploitation of octopus populations (Norman et al., 2014; Sauer et al., 2021), as has been the case for certain species of the complex [e.g., *O. sinensis* (Gleadall, 2016)]. Likewise, small scale coastal fisheries are also becoming unsustainable in the long term, for example in Southern Europe, due to the growing interest in *Octopus*

fisheries allied to depleting finfish stocks and rising prices (Pita et al., 2021). The difficulty implicit to species discrimination within the complex leads to increased difficulty when compiling data on each of these species. At the same time, population identification and stock discrimination preclude an accurate assessment of the status of the OVSC around the world (Sauer et al., 2021), raising concerns over the real impact of anthropogenic pressures on each of the complex's species. Additionally, within such pressures and given the potentially distinct habitat requirements between species, there is a particularly pressing need to disentangle the potential effects of anthropogenic climate change over these species.

Rising carbon dioxide (CO_2) concentrations in the atmosphere over the past centuries (Shukla et al., 2019; Masson-Delmotte et al., 2021) are responsible for significant changes to the earth's climate and the global ocean system (Pecl et al., 2017). Indeed, oceanic waters have been warming over the past decades due to the ocean's ability to absorb 90% of excess atmospheric heat trapped by greenhouse gases (Zanna et al., 2019). At the same time, increased absorption of approximately 30% of the excess atmospheric CO_2 has also been changing ocean chemistry, leading to decreasing pH levels (Gobler and Baumann, 2016). These changes in ocean chemistry and temperature have further contributed to increased ocean stratification and slowed and disrupted current patterns, leading to the expansion of areas with low oxygen (Gobler and Baumann, 2016). These phenomena are both consequences and drivers of ocean climate change and are set to yield a vast array of negative impacts on marine ecosystems worldwide (Sampaio et al., 2021). Among cephalopods, some lines of evidence indicate that this group may benefit from the impending shift in ocean conditions (Doubleday et al., 2016), although their responses are likely to be complex (Pecl and Jackson, 2008; Rodhouse et al., 2014). Their high phenotypic flexibility and consequent environmental plasticity (Liscovitch-Brauer et al., 2017), together with their 'live fast and die young' lifestyle (O'Dor and Webber, 1991) likely represent an advantage over other marine taxa when confronted with the various challenges of a changing climate. Indeed, the synergistic effects of warming and the overfishing of their predators and competitors, have been linked to the increased fitness of cephalopod species (Rodhouse et al., 2014; Doubleday et al., 2016). On the other hand, several experimental studies have shown potential deleterious effects of climate change for a wide array of cephalopod species, including octopuses (Repolho et al., 2014; Rosa et al., 2019), squid (Rosa and Seibel, 2008; Rosa et al., 2012; Rosa et al., 2014), and cuttlefishes (Rosa et al., 2013; Moura et al., 2019; Otjacques et al., 2020). At the same time, distribution shifts associated with climate forcing have already been observed for several octopuses (Ramos et al., 2014; Arreguín-Sánchez, 2019; Ponce-Márquez et al., 2020), and other cephalopod groups (Golikov et al., 2013; Alabia et al., 2020; Oesterwind et al., 2022), suggesting a broad redistribution of cephalopod species over the coming decades as they seek suitable environmental conditions (Xavier et al., 2016; Rosa et al., 2019).

Given the relatively high potential for future climate change to induce differential changes to marine species, there is a need to accurately describe a species' ecological and geographical distribution. With the combination of biogeographic knowledge and modelling in climate change impact studies, it is possible to attempt the prediction of the future impacts on biodiversity and ecosystem health (Hannah et al., 2002). Species distribution models (SDMs) present a very useful tool in this regard. Indeed, its development and use have increased steeply over the past decades (Zimmermann et al., 2010), since these models offer a good framework for predicting changes in species distributions, across vast geographical spaces and regarding large species assemblages (Elith et al., 2006). These models take geo-referenced occurrence data and environmental predictors for a defined geographical extent and establish the relationship between a species' occurrence and environmental conditions, defining a species' ecological niche and allowing researchers to perform projections on the potential changes in a species distribution ranges across time and space (Miller, 2010). While these models are bounded by a set of assumptions and limitations that must be taken into consideration during interpretation (Araújo et al., 2005; Araújo and Guisan, 2006; Heikkinen et al., 2006; Fitzpatrick and Hargrove, 2009), SDMs incorporating future climate change predictions are considered a very effective way to address some of the questions regarding climate change effects on biodiversity (Sinclair et al., 2010).

To this day, a growing body of literature has employed SDM frameworks with cephalopod species (Puerta et al., 2015; Alabia et al., 2016; Xavier et al., 2016; Boavida-Portugal et al., 2022). However, research employing this modelling approach to project potential biogeographical impacts of marine climate change on octopuses remains very scarce (Hermosilla et al., 2011; Lima et al., 2020; Ángeles-González et al., 2021; Schickele et al., 2021; Boavida-Portugal et al., 2022), with few studies focusing on species of the OVSC and at this scale. In this context, the present study aims to evaluate the potential biogeographical impacts of future oceanic climate change on the distribution of the six OVSC species, by implementing an SDM workflow using MaxEnt modelling to predict present-time habitat suitability and species occurrence distribution and project these into two future periods (*i.e.*, 2040/2050 and 2090/2100), across four Representative Concentration Pathway scenarios (RCP; RCP-2.6, 4.5, 6.0, and 8.5, CMIP5). With this approach, the present study also aims to aid the correct management of octopus fisheries worldwide, particularly with regards to the six species studied. By identifying the most threatened areas of each species' distribution, it is possible to better inform policy makers and economic agencies to prevent the collapse of local or regional populations.

Material and methods

Collection and curation of occurrence data

To obtain the necessary geo-referenced occurrence data on the species of interest, the Global Biodiversity Information Facility (GBIF) [GBIF.org (24 May 2022)] (GBIF, 2022) and the Ocean Biodiversity Information Facility (OBIS) [OBIS.org (24 May 2022)] databases were surveyed. As a way of limiting the data retrieved in each database to only valid occurrences, a specific set of filters were used, positively selecting for those occurrences obtained through 'Human Observation' and 'Preserved Specimen', and filtering out duplicate observations, improper datum conversion points, missing (NA) values in either Latitude or Longitude, as well as rounded latitude/longitude coordinates. When dealing with species complexes, there is the risk of misidentification of specimens with the original morphospecies. Online databases such as GBIF and OBIS, contain significant contributions from citizen-science databases (*e.g.*, iNaturalist) and non-directed scientific surveys, which undergo different degrees of scientific validation (iNaturalist, 2022), but are still susceptible to frequent cases of species misidentification, mainly when dealing with highly cryptic species complexes. In this sense, since most retrieved occurrences were labeled as *Octopus vulgaris*, one of the most recent illustrations of the potential distribution of each species of the complex (Avendaño et al., 2020) was used to re-label each occurrence point. In the case of *Octopus djinda*, occurrence data from Amor and Hart (2021) was also included in the species' dataset, due to the relative lack of geo-referenced occurrences in either online database. For the present analysis, occurrences of *O. sinensis* from the Kermadec islands (Reid and Wilson, 2015) were excluded, and the extent of *O. sinensis was* limited to the Northwestern Pacific Ocean.

The compiled dataset was then curated, restricting all occurrences to the continental shelf area [*i.e.*, from the surface to a depth of 200 m; (Laruelle et al., 2018)] and removing potential occurrences referenced on land. Since GBIF and OBIS do not include comprehensive records for the depth of each occurrence in most species, the occurrence data was converted into a spatial polygon object which was then used to extract a vector of the depth values at each occurrence's coordinates (using the function 'extract' from the package 'raster'), from a bathymetry raster layer obtained from Ocean Climate Layers for Marine Spatial Ecology (MARSPEC) (Sbrocco and Barber, 2013). The spatial polygon object was then converted back into a data frame and merged with the depth vector, and each species data frame was subset to exclude depths greater than 200 m. To remove data occurring on land, a second clipping of the occurrence data was performed by erasing all points outside a shapefile of the world's ocean bodies (downloaded from Natural Earth Data, https://www.naturalearthdata.com/). These restriction procedures were performed because SDMs must ideally restrict model calibration to accessible areas (Peterson and Soberón, 2012). The curated occurrence dataset, together with the plotted occurrences for each species and the script used for data curation are supplied in the Supplementary Material (see Curated_species_datasets.zip). Dataset curation and the following analyses were all performed in R studio software (v.4.1.2) (R Studio Team, 2022). All scripts are available in the Supplementary Material.

Predictor variables

The predictor variables used in this study included one topographic variable – *i.e.*, bathymetry – and three oceanographic variables: temperature, salinity, and current velocity. Concerning the oceanographic variables, both surface and maximum depth (benthic) layers were chosen, since octopuses are primarily benthonic, but can still be influenced by surface conditions. The choice of the oceanographic variables was primarily based on the availability of environmental predictors projected for the future periods (*i.e.*, 2040-2050 and 2090-2100), and Representative Concentration Pathway scenarios (*i.e.*, RCP2.6, 4.5, 6.0, and 8.5). The mean, max, min, and range for each predictor were obtained from Bio-ORACLE, which offers global geophysical, biotic, and climate layers at a common spatial resolution (5 arcmins) and a uniform landmask (Tyberghein et al., 2012; Assis et al., 2018). Bathymetry, in turn, was retrieved from MARSPEC at a resolution of 5 arcmins (Sbrocco and Barber, 2013) and used as a predictor variable, to incorporate the water column height into the spatial analysis.

Modelling

The species distribution models were built for MaxEnt modelling, using the 'megaSDM' package (Shipley et al., 2022). For this purpose, each predictor variable layer had to be reprojected to an equal-area projection (*i.e.*, specifically the cylindrical equal-area projection – "+proj=cea +lat_ts=0 +lon_0 = 0 +x_0 = 0 +y_0 = 0 +datum=WGS84 +no_defs"), because conventional non-equal area projections have grids which vary in their area the further away from the equator, and MaxEnt randomly samples cells from the available geographic space, assuming cells of equal area in the entire extent of each predictor (Elith et al., 2011). To detect and prevent collinearity in the predictor variable list (*i.e.*, a strong correlation between two or more variables), the function 'vifcor' from the package 'usdm' was used (Naimi, 2015). Specifically, the function tests the variables against each other until it finds a pair of variables with a maximum linear correlation greater than a previously specified threshold [*i.e.*, in this case, 0.7 (Cohen et al., 2003)],

excluding the variable with a greater variance inflation factor value, and repeating this process for all variable combinations. This was performed in two separate analyses, *i.e.*, first to the surface predictors, and then to those from the benthos, to prevent instability in parameter estimation. The results from the 'vifcor' function with the remaining variable list for the surface and benthonic predictor stacks are present in the Supplementary material (see collinearity.rar), together with the correlation plots obtained using the 'ENMTools' package (Warren et al., 2021).

The SDM analysis followed the workflow described in the 'megaSDM' package documentation (Shipley et al., 2022). In short, the training area, where the occurrence and background points are located, and the study area, where the model will be projected, were defined using the functions 'TrainStudyEnv' and 'PredictEnv', which take specific raster stacks and manipulates them to standardize the present and future period's environmental data input, re-projecting, clipping, and resampling raster predictors when necessary (Shipley et al., 2022). The package then takes and manipulates the occurrence data and employs a series of measures to mitigate the inherent bias, typical of collected or downloaded occurrence data (Phillips et al., 2009; Boakes et al., 2010) and which decreases the overall accuracy of SDMs (Phillips et al., 2009; Beck et al., 2013; Varela et al., 2014). To mitigate environmental and spatial biases within the occurrence data, the package performs environmental filtering of the occurrence data by dividing the environmental values at each point into a pre-determined number of bins (n = 25 in the present study), and then selecting one point from each unique combination of bins, obtaining a subset of occurrence points that is filtered by the environment (Varela et al., 2014; Castellanos et al., 2019; Shipley et al., 2022). This method allows the removal of clustered or oversampled records, while still maintaining the range of environments in which a species was found (Varela et al., 2014). The number of occurrence records remaining after the environmental filtering process is present in Table 1.

Since the species occurrence data frames only feature presence data, there was the need to generate background points for each species. Background points are artificially created species occurrence points, which describe the environmental conditions of the training area, and allow the incorporation of true data or pseudo-absence data into the models (Peterson and Soberón, 2012). The 'megaSDM' package uses a 'combined' method to generate the background points, which employs both random and spatially constrained sampling, each weighted by a user-defined threshold (*i.e.*, 50% each for the present study) (Shipley et al., 2022). As such, half of the user-set number of background points (*i.e.*, n = 1000 per species) was randomly sampled from the entire study area (Barbet-Massin et al., 2012), while the remaining half were sampled from within shapefiles buffered around each true occurrence point (with the radius of each buffer being proportional to the 95% quantile of the distance to the nearest neighbor). Using a combined method to generate background points reduces potential model overestimation of environmental suitability that typically arises in regions of greater occurrence point densities, usually in more easily sampled areas (*i.e.*, spatial bias), and which random background point generation by itself does not consider (Lobo et al., 2010; Kramer-Schadt et al., 2013). At the same time, this method also reduces the susceptibility of extreme extrapolation errors and overfitting induced by spatially constrained methods, by assigning a percentage of the background points to be sampled randomly from the entire environmental extent (Radosavljevic and Anderson, 2014). During generation, the background points were also environmentally filtered, similar to the occurrence data, creating an even spread across available environmental space, while retaining its spatial weighting (Shipley et al., 2022).

The present habitat suitability and distribution of each species were estimated using the MaxEnt modelling technique, which employs maximum entropy methods and is particularly resilient when dealing with presence-only species records (Elith et al., 2010). This method also has a predictive performance that is consistently competitive with other high-performing methods (Elith et al., 2006; Feng et al., 2019). The 'megaSDM' package employs MaxEnt modelling using replication and the subsequent ensemble of the replicate model predictions – in the present case, a replicate number of 5 was used per species, with each model run using a different subset of occurrence points (Shipley et al., 2022). The area under the curve (AUC) value was employed to evaluate each model replicate and the final ensemble model. This was performed by taking the AUC value of each replicate and comparing it to the AUC of a null model, where multiple replicates of occurrence points were placed at random in the

TABLE 1 Number of valid entries per species pre- and post-curation, and post-environmental filtering.

Species name	Pre-curation	Post-curation	Post-environmental filtering
Octopus vulgaris	5276	1797	836
Octopus americanus	1360	703	438
Octopus aff. *vulgaris*	245	104	72
Octopus djinda	74	35	19
Octopus sinensis	60	34	28
Octopus tetricus	872	280	88

training area (Raes and ter Steege, 2007; Shipley et al., 2022). The 'MaxentProj' function removed all models with validation AUC values lower than a specified threshold (*i.e.*, in this case, 0.70). The same function then projected all models onto the specified future environments, across all RCP scenarios, and created the ensemble of all replicate maps using the median value of each pixel, thus reducing the effect of outliers (Araújo and New, 2007; Shipley et al., 2022). The evaluation plots and tables for each species' models are supplied in the supplementary material (see Evaluation.zip). From the ensembles, the habitat suitability maps were obtained. To produce binary maps of probability of occurrence (0 or 1), a threshold value was applied to the continuous habitat suitability maps. This threshold consisted of the mean model TSS criteria of model evaluation, also named 'maximum test sensitivity and specificity' logistic threshold, which maximizes the specificity and sensitivity of the receiver operating curve (*i.e.*, the ROC curve), and is particularly effective in presence-only data (Liu et al., 2005). All the model projections, including the habitat suitability ensembles and the binary maps, are supplied in the supplementary material (see Projections.zip).

Post-analysis

The post-analysis followed a similar framework to the one previously described by Borges et al. (2022). Also, since the OVSC species do not exhibit a global distribution, instead having their areas of accepted occurrence in specific continents or regions of the globe, the post-analysis was performed in subsets of the total extent of the model predictions, encompassing only the currently accepted areas of distribution. First, changes to each species latitudinal distribution (within their currently accepted range) were assessed by plotting each species latitudinal centroid (i.e., the arithmetic mean latitude for the species-occupied pixels) for each time and RCP scenario. Second, the latitudinal trends in mean habitat suitability were obtained, by converting each ensemble into matrix form and calculating each row's mean value. To obtain the projected changes in the latitudinal mean habitat suitability, the resulting present-day vector was subtracted to the 2050 and 2100 vectors, and the respective outputs were plotted along the latitudinal (y) axis. Afterwards, the binary maps of probability of occurrence were used to create a visualization of unidirectional range shifts (i.e., expansion and/or contraction) and transitory fluctuations (*i.e.*, range contraction followed by expansion, and vice-versa), per scenario, per species (Early and Sax, 2011) using the function 'createTimeMaps' (Shipley et al., 2022). The Time Maps for each species and RCP scenario are supplied in the Supplementary Material (see Projections.zip). Data regarding this analysis is present in the supplementary material (see Post_analysis.zip).

Results

Variable contribution

The variable contribution for each species' ensemble model is presented in Table 2 (see Supplementary material for further details). Overall, Temperature (either benthic or surface levels) contributed the most for the determination of habitat suitability and species occurrence. Except for *O. vulgaris* – where benthic salinity maximum provided the highest contribution - all OVSC species exhibited a temperature-related environmental layer as their most contributing variable. In the top four most contributing variables, temperature layers dominated, with bathymetry and salinity occurred isolated (salinity mainly in *O. americanus*). Variables associated with Current Velocity, in general, were the least contributing variables (apart from *O.*

TABLE 2 Ensemble model variable contribution. The top four most contributing variables per species are presented below.

Species name	#1	#2	#3	#4
Octopus vulgaris	Salinity Maximum Benthic (80.9%)	Temperature Mean Surface (9.9%)	Temperature Mean Benthic (3.8%)	Bathymetry (3.6%)
Octopus americanus	Temperature Mean Benthic (39.7%)	Salinity Range Benthic (25.4%)	Salinity Max Benthic (13.7%)	Temperature Range Surface (5.9%)
Octopus aff. *vulgaris*	Temperature Mean Benthic (24.8%)	Temperature Range Surface (19.1%)	Salinity Range Benthic (18.1%)	Temperature Mean Surface (11.7%)
Octopus djinda	Temperature Range Surface (33.3%)	Bathymetry (22.8%)	Temperature Mean Surface (15.1%)	Temperature Mean Benthic (11.5%)
Octopus sinensis	Temperature Range Surface (20.4%)	Temperature Range Benthic (17.4%)	Salinity Maximum Benthic (13.3%)	Current Velocity Maximum (13.1%)
Octopus tetricus	Temperature Range Benthic (41.5%)	Salinity Range (27.1%)	Temperature Mean Benthic (16.2%)	Temperature Mean Surface (9%)

sinensis, where maximum surface current velocity was the fourth most contributing variable).

General patterns of habitat suitability

All six species of the OVSC exhibited a shift in their centroid of latitudinal distribution over time (Figure 1), despite relatively different responses between species and scenarios. Indeed, while *O. vulgaris* did not exhibit a clear trend over time and RCP severity – with the centroid barely moving until 2050 and with either a shift north (RCP-4.5 and RCP-6.0) or south (RCP-8.5) depending on the scenario (Figure 1A) - the other species exhibited fairly clear trends. Specifically, the centroid of distribution for *O. americanus* exhibited a considerable northward shift for all RCP scenarios, with the magnitude of this shift increasing along RCP severity, particularly between 2050 and 2100 (Figure 1B). For *O.* aff. *vulgaris* (Figure 1C), there is a clear southward shift that is exacerbated over time in RCP-6.0 and RCP-8.5, while for the two less severe scenarios the centroid expansion contracts until the end of the century, relative to 2050. Akin to *O. americanus*, *O. sinensis* also exhibited a clear northward shift of its centroid until the end of the century (Figure 1D), in a trend that is in general exacerbated with RCP scenario severity – except for RCP-6.0, where the northward shift is smaller when compared to RCP-4.5. Similar to *O.* aff. *vulgaris*, *O. tetricus* exhibited the same southward shift in its centroid, with a considerable southward shift until 2050 which then contracts northward in RCP-2.6 and RCP-4.5 - although not returning to the present-day latitude – and the successive shift southward for the more severe RCP scenarios (Figure 1E). The *O. djinda* (Figure 1F) also exhibited a similar pattern with a shift southward.

Latitudinal trends in habitat suitability and occurrence distribution

In this section, the results of mean habitat suitability change across latitude and the plotted difference in projected occurrence distribution regarding present-day are presented in a species-by-species fashion. In terms of global trends, *O. vulgaris* was projected to undergo considerable reductions in habitat suitability and consequently local extirpations in its projected future distribution, which were exacerbated with the increasing severity of the RCP scenario. Specifically, mean latitudinal habitat suitability is projected to decrease over most of its latitudinal distribution (in the northern hemisphere) until the end of the century (Figure 2), mainly along the North African and Mediterranean range (Figure 3).

Changes in habitat suitability for this species are relatively similar across RCP scenarios until 2050 - the largest decreases in suitability occur generally over the same areas but are larger for the Mediterranean and North Sea latitudes (Figure 2, left). For the year 2100, there is a considerable decrease in latitudinal suitability for most of the species' range (Figure 2, right), with the most extreme scenarios (i.e., RCP-6.0 and RCP-8.5) featuring the largest declines. Gains in mean habitat suitability were projected for a small latitudinal band in the northernmost latitudes, which increased between 2050 and 2100 for RCPs 4.5 to 8.5, moving northward in the two most severe scenarios (Figure 2). The changes in mean habitat suitability induced the

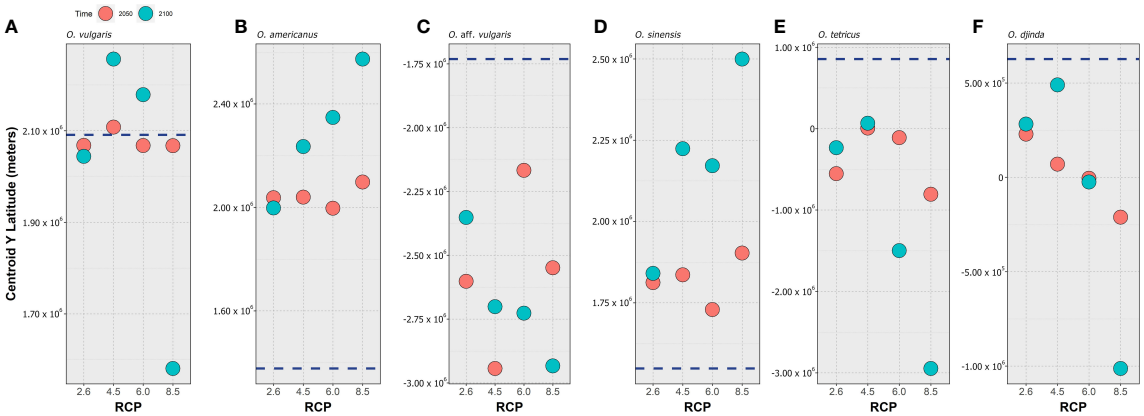

FIGURE 1
Projected changes to the centroid of latitudinal distribution (i.e., the mean latitude of the occupied pixels) calculated for 2050 (red circles) and 2100 (blue circles) and Representative Concentration Pathway scenario (RCP-2.6, 4.5, 6.0, and 8.5; CMIP5), for **(A)** *Octopus vulgaris*, **(B)** *O. americanus*, **(C)** *O.* aff. *vulgaris*, **(D)** *O. sinensis*, **(E)** *O. tetricus*, and **(F)** *O. djinda*. The dark blue, dashed horizontal lines represents the centroid value for the present-day (2000–2014 environmental conditions based on monthly averages).

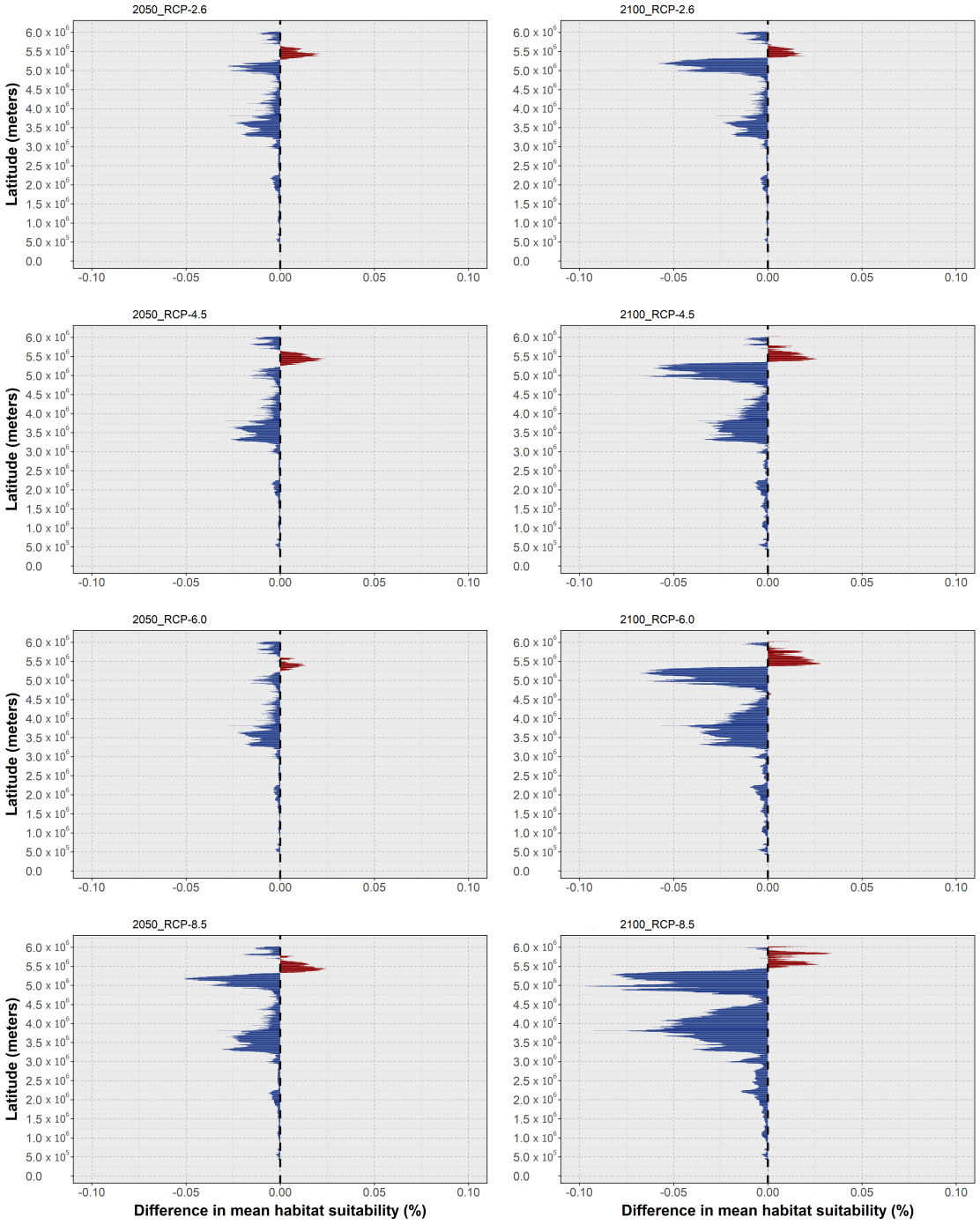

FIGURE 2
Changes in mean latitudinal habitat suitability for *Octopus vulgaris* between the present-day and 2050 (left side) and 2100 (right side), for each Representative Concentration Pathway scenario (RCP-2.6, 4.5, 6.0, 8.5; CMIP5), relative to the present-day baseline (i.e., the black dashed vertical line). Data to the right of the dashed line represents a relative increase in mean habitat suitability (red) and data to the left represents a relative decrease (blue).

projected extirpation of *O. vulgaris* from localized regions along the Mauritanian coastline until 2050, in certain regions of the southern and eastern Mediterranean Sea, and in the waters surrounding the United Kingdom (Figure 3). For RCP-2.6, gains in distribution were very sparse and restricted to small areas in the northernmost limits of the species' predicted distribution. Following the trends in mean habitat suitability, increasing severity of the RCP scenario led to a considerable

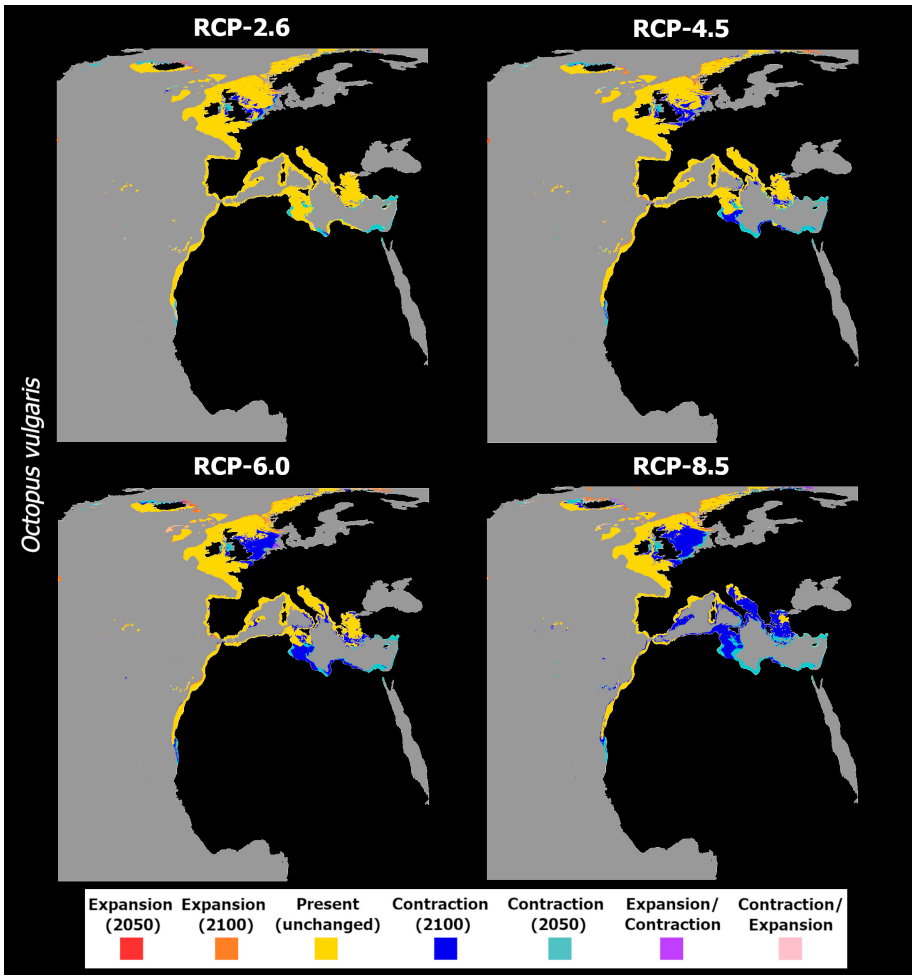

FIGURE 3
Time maps of the projected changes in species occurrence distribution for *Octopus vulgaris*, showing unidirectional range shifts in occurrence distribution [i.e., range expansion (in red and orange) or contraction (in dark and light blue)] as well as transitory fluctuations (i.e., range contraction followed by expansion (pink), and vice-versa (purple)] across time (i.e., 2050 and 2100) and RCP scenarios (RCP-2.6, 4.5, 6.0, 8.5; CMIP5).

decrease in the specie's areas of occurrence (Figure 3A). In RCP-4.5, mid-range latitudes exhibit greater losses between 2050 and 2100, compared to the previous scenario. In terms of projected distribution changes (Figure 3B), there is a considerable increase in the areas of species extirpation – again, mainly along the coastline of Mauritania, in the Mediterranean Sea, and in the North Sea. Projections in RCP-6.0 emphasize the areas of distribution loss, mainly in the Mediterranean and the North seas. In the former, extirpation of the species until the end of the century expands in the Adriatic Sea and around Italy, while also appearing in the Balearic Sea (Figure 3C). For the most extreme scenario, RCP-8.5, there was a severe increase in the areas of species distribution loss over most of Macaronesia and in the Mediterranean Sea (Figure 3D). Northward, the species is projected to lose considerable areas of its distribution in the Sea of Ireland, while some potential exists for expansion in the northernmost limits of its distribution.

In the case of *O. americanus*, the ensemble models predict an overall decrease in mean habitat suitability in tropical and subtemperate areas near the equator until 2050, with slight increases in the higher latitudes of both the Northern and Southern hemispheres (Figure 4). This pattern is exacerbated by the increasing severity of the RCP scenario. Specifically, for RCP-2.6, the ensemble model predicted decreasing mean habitat suitability until 2050 in lower latitudes (Figure 4A), while in higher latitudes there are predicted increases until the mid of the century as well, although with localized decreases between 2050 and 2100 in some latitudes (Figure 4B). In terms of distribution, this led to projected localized extirpation by 2050 in the tropical and subtropical areas between Cuba and the Yucatán Peninsula

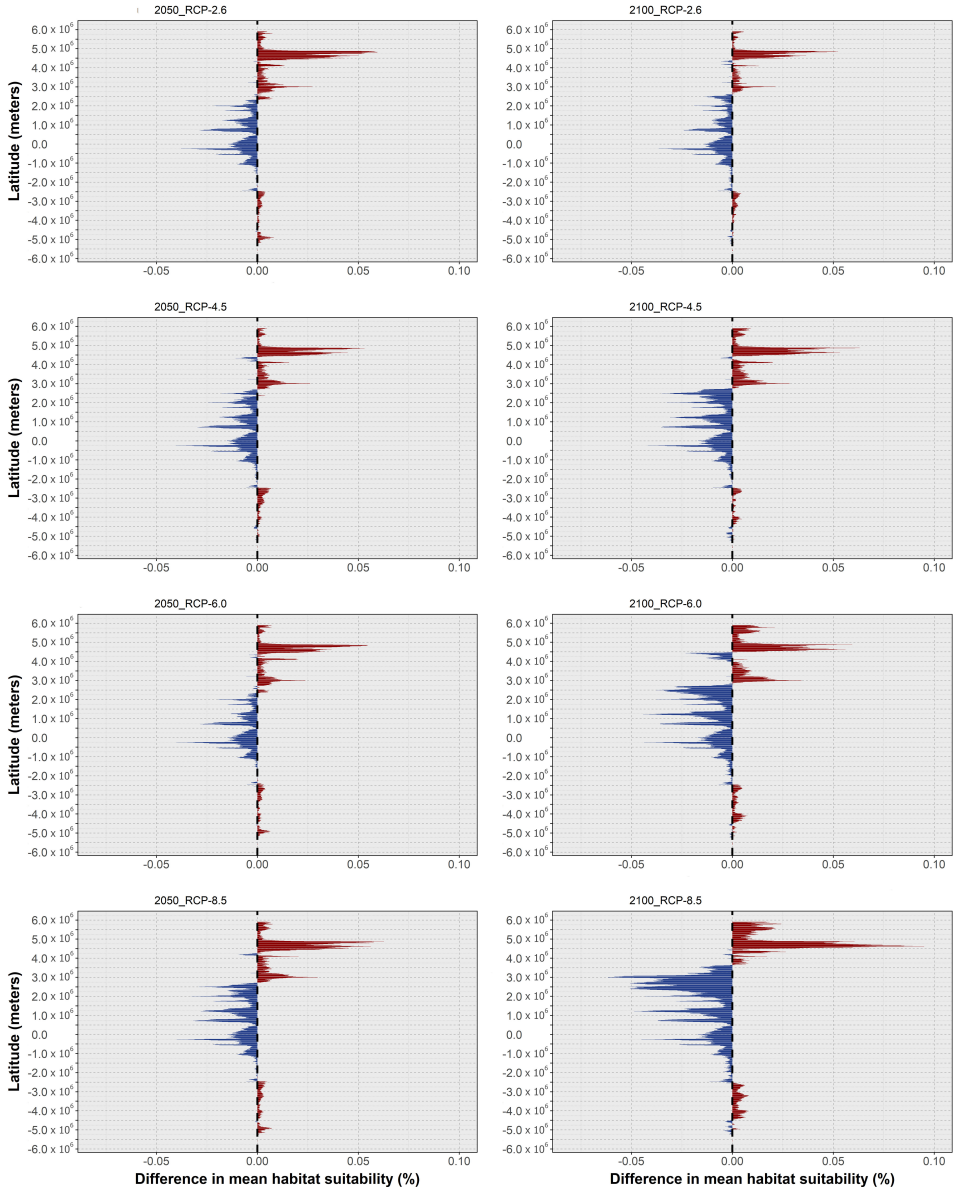

FIGURE 4
Changes in mean latitudinal habitat suitability for *Octopus americanus* between the present-day and 2050 (left side) and 2100 (right side), for each Representative Concentration Pathway scenario (RCP-2.6, 4.5, 6.0, 8.5; CMIP5), relative to the present-day baseline (i.e., the black dashed vertical line). Data to the right of the dashed line represents a relative increase in mean habitat suitability (red) and data to the left represents a relative decrease (blue).

in the Gulf of Mexico, southward over the coastline of Brazil (Figure 5A). North of the Yucatán Peninsula, there is some projected localized distribution loss by 2050, together with areas of transitory fluctuation of contraction by 2100 following expansion until 2050. In the northernmost regions of its distribution, *O. americanus* is also projected to undergo a considerable gain in distribution.

In RCP-4.5, the tropical and subtemperate latitudes exhibited an increased loss in mean habitat suitability by mid-century (Figure 4C), with some areas also showing further losses between the present-day and 2100 (Figure 4D). At the same time, mean habitat suitability until 2100 in the northernmost latitudes increases further than in the previous scenario. This trend translated into an expansion of the areas of extirpation by 2050 from the region Brazil to the seas between Cuba and the Bahamas (Figure 5B), and in the Yucatán Peninsula. In this area of the Gulf of Mexico, there is also projected distribution loss by the end of the century in the seas to the west of the Yucatán

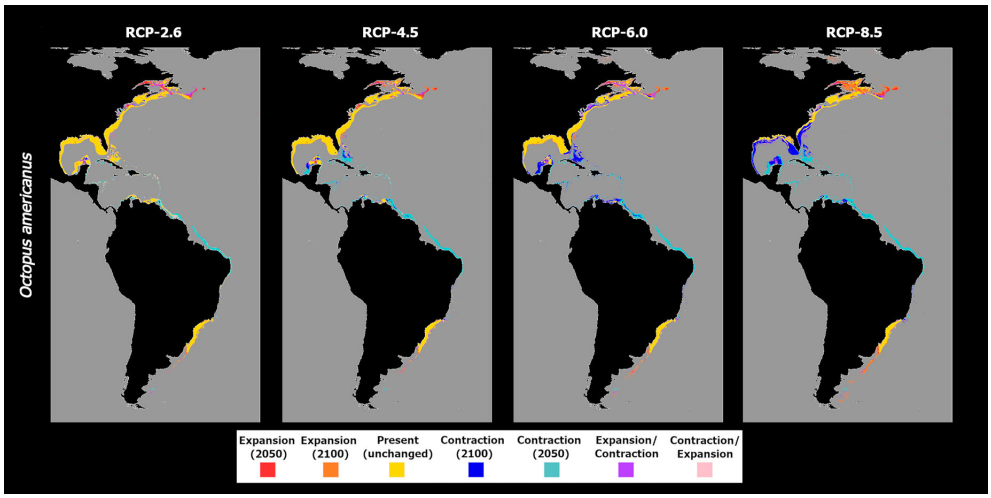

FIGURE 5
Time maps of the projected changes in species occurrence distribution for *Octopus americanus*, showing unidirectional range shifts in occurrence distribution [i.e., range expansion (in red and orange) or contraction (in dark and light blue)] as well as transitory fluctuations (i.e., range contraction followed by expansion (pink), and vice-versa (purple)] across time (i.e., 2050 and 2100) and RCP scenarios (RCP-2.6, 4.5, 6.0, 8.5; CMIP5).

Peninsula, and in the region of the Bahamas and the continental shelf near the United States. In RCP-6.0, there was a considerable relative increase in mean habitat suitability in the northernmost latitudes of this species' distribution (Figures 4E, F), together with some localized increases in higher latitudes in the southern hemisphere. However, most of the species distribution extent in the tropical and subtemperate bands is again considerably reduced. This led to an increased decline in *O. americanus* distribution in these areas, particularly in the Greater Antilles and in the Gulf of Mexico, where the models projected considerable losses between 2050 and 2100 (Figure 4C). In higher latitudes, the increase in mean habitat suitability leads to increased areas of potential expansion in the northern and southern limits of the species distribution. Finally, RCP-8.5, the changes in mean habitat suitability were particularly exacerbated regarding the previous scenarios (Figures 4G, H) leading to a potential expansion of the areas of distribution loss from the eastern Brazilian coastline, along the entire Central American shores by 2050, and northward along the coastline of most of the US. At the same time, expansion in the offshore areas of Canada, in the Gulf of Saint Lawrence and to the east of Newfoundland, and southward in Uruguay and Argentina, suggests an exacerbation of this shift towards the poles (Figure 5D).

Regarding *O.* aff. *vulgaris*, there was a considerable decrease in mean latitudinal habitat suitability relative to the present-day, projected for most scenarios (Figure 6). Indeed, until 2050, and save for localized suitability gains in the southernmost latitudes in RCP-2.6 and RCP-8.5 (both of a very small scale compared to the decreases), most of the latitudinal extent for this species is projected to undergo a considerable loss in suitability (Figure 6, left). Until 2100, however, RCP-2.6 and RCP-4.5 exhibit decreases in habitat suitability across most of the species' distribution which are relatively smaller compared to 2050. This relative increase in mean habitat suitability between 2050 and 2100 is also followed by a localized gain in suitability relative to the present-day in the southernmost latitudes, for both scenarios (Figure 6, right). For RCP-6.0 and RCP-8.5, however, the overall trend of decreasing habitat suitability regarding the present-day continued (Figure 6, right).

In terms of distribution projections (Figure 7), an oscillating decrease in mean habitat suitability in RCP-2.6 is observed, with a likely extirpation of this species from its northernmost areas of distribution - from the Quirimbas islands to the east of the Maputo Bay on the east-African shores, and sparsely along the western coastline (Figure 7A). Considerable loss by 2050 is also observed along northern Madagascan waters. In these regions, there are also large extensions of areas projected to undergo transitory fluctuations, disappearing in 2050 but re-emerging by 2100, due to the oscillating values of mean habitat suitability. For RCP-4.5, the ensemble model projects a very different situation between the southern Indian Ocean and southern Atlantic Ocean African shores (Figure 7B). Specifically, the models project some areas of fluctuation, with the species potentially disappearing in 2050 and remerging by 2100 in the northern region of Namibia. In the western areas of its distribution, offshore Namibia and South Africa, this kind of transitory fluctuations also occurs, alongside projected expansions into greater depths by the end of the century. In the southern Indian Ocean, the model projects *O.* aff. *vulgaris* to potentially

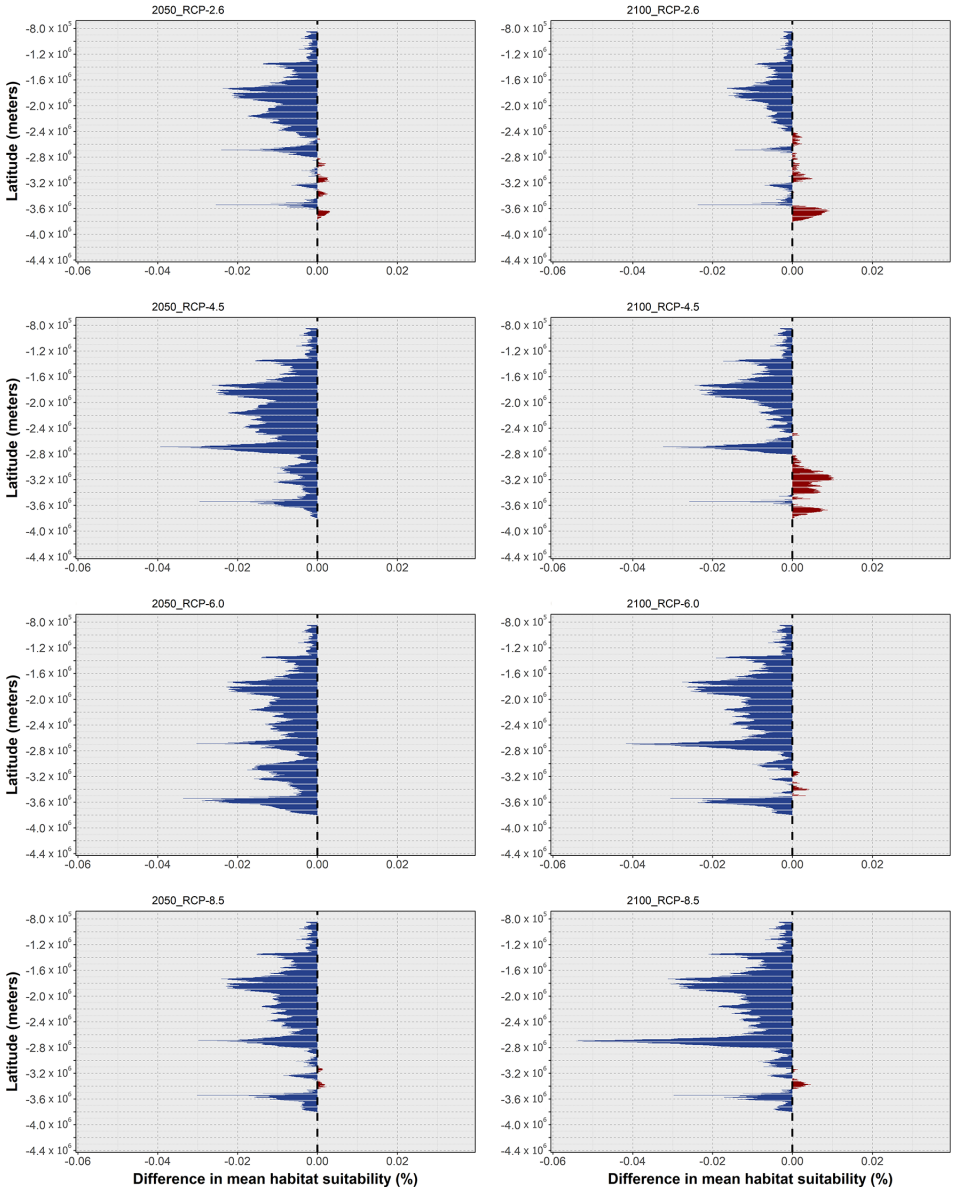

FIGURE 6
Changes in mean latitudinal habitat suitability for *Octopus* aff. *vulgaris* between the present-day and 2050 (left side) and 2100 (right side), for each Representative Concentration Pathway scenario (RCP-2.6, 4.5, 6.0, 8.5; CMIP5), relative to the present-day baseline (i.e., the black dashed vertical line). Data to the right of the dashed line represents a relative increase in mean habitat suitability (red) and data to the left represents a relative decrease (blue).

disappear by 2050 from most of the Mozambican shores and from Madagascar's northern and southwestern coastlines. In RCP-6.0, this pattern of loss becomes more evident. The progressive decrease in mean habitat suitability until the end of the century further exacerbated the projected loss in Madagascar and Mozambican waters (Figure 7C), with new areas of extirpation between 2050 and 2100 appearing in the southern parts of these regions. Despite some localized distribution restrictions, no considerable changes occur over the southern South African continental shelf. On the south Atlantic shores, there are sparse projected losses in the distribution in the Namibian coastline, and the area of projected fluctuation also seen in RCP-4.5 is projected to occur southward, in the western South African shelf. RCP-8.5 exhibited a more severe scenario, with the species being completely extirpated from the coastal areas of Madagascar (Figure 7D) - despite a small region in the southeast resisting until the mid of the century but eventually disappearing by 2100.

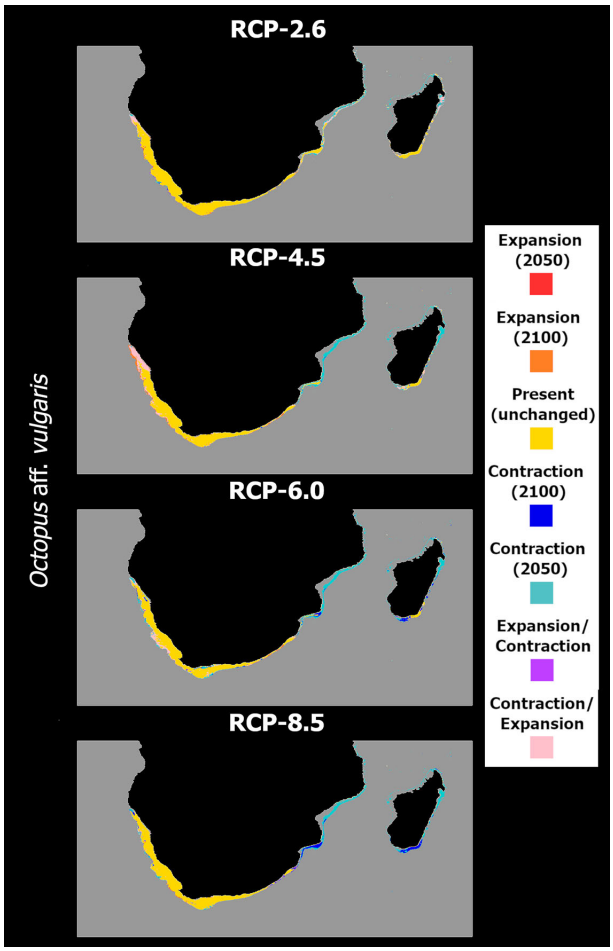

FIGURE 7
Time maps of the projected changes in species occurrence distribution for *Octopus* aff. *vulgaris*, showing unidirectional range shifts in occurrence distribution [i.e., range expansion (in red and orange) or contraction (in dark and light blue)] as well as transitory fluctuations (i.e., range contraction followed by expansion (pink), and vice-versa (purple)] across time (i.e., 2050 and 2100) and RCP scenarios (RCP-2.6, 4.5, 6.0, 8.5; CMIP5).

The situation is the same for the waters of Mozambique, but the species remains relatively undisturbed in South African and Namibian waters, despite localized losses in the latter region.

For *O. sinensis*, the models predict a different response of this species since no progressive exacerbation of the observed trends is seen with increasing RCP severity (Figure 8). In RCP-2.6, mean habitat suitability increases until the mid- and end of the century for most of its latitudinal distribution in the northwestern Pacific Ocean, with relatively small decreases being observed only at the lowest latitudes of its extent (Figures 8A, B). In this scenario, there are no considerable changes to the species distribution, with losses being virtually non-existent, and projected expansions localized to areas near the western coastlines of Korea in the Yellow Sea, and around Hokkaido and Sacalina islands in the Sea of Japan (Figure 9A). In contrast, RCP-4.5 exhibits a different trend, with mean habitat suitability decreasing considerably in the southern latitudes, where present-day suitability is predicted to be highest (Figures 8C, D). In the areas of higher suitability, a temporary increase in habitat suitability until 2050 is observed (Figure 8C) but quickly followed by a decrease below present-day values until the end of the century (Figure 8D). In terms of distribution, the projected map is dotted with areas of projected loss in distribution between 2050 and 2100, mainly in the outer areas of the predicted distribution in the East China Sea, in the Yellow Sea, and across the coastlines of Japan (Figure 9B).

Due to the oscillating habitat suitability in higher latitudes, areas of temporary emergence followed by extirpation are observed in the eastern regions of the Yellow Sea and dotted alongside the northern areas of the Sea of Japan. Also in these regions, there are instances of projected expansions, although restricted, localized in the Yellow Sea and mainly on Hokkaido Island. RCP-6.0 presents a very similar situation to the previous scenario in terms of habitat suitability and projected distribution

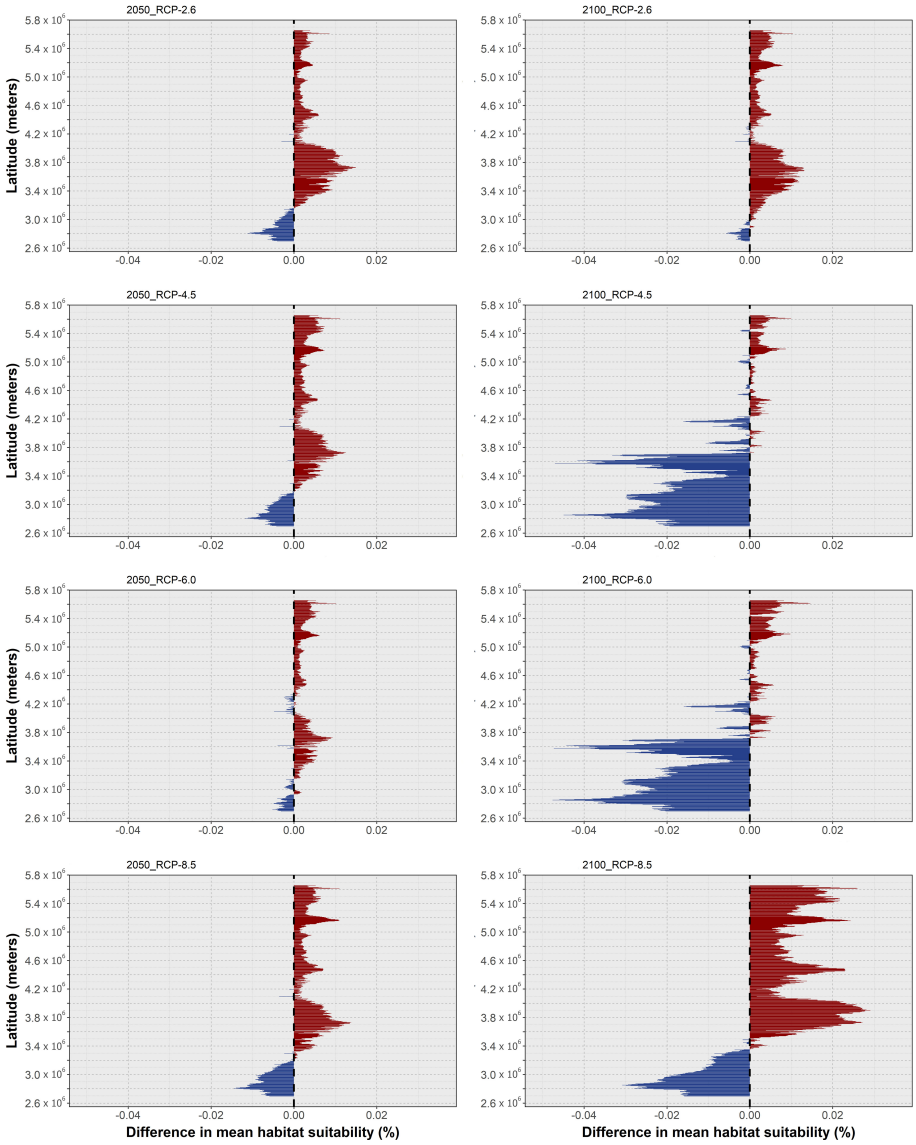

FIGURE 8
Changes in mean latitudinal habitat suitability for *Octopus sinensis* between the present-day and 2050 (left side) and 2100 (right side), for each Representative Concentration Pathway scenario (RCP-2.6, 4.5, 6.0, 8.5; CMIP5), relative to the present-day baseline (i.e., the black dashed vertical line). Data to the right of the dashed line represents a relative increase in mean habitat suitability (red) and data to the left represents a relative decrease (blue).

changes (Figures 8E, F, 9C). However, for RCP-8.5, the ensemble model predicted somewhat similar response to RCP-2.6. In this scenario, there is an exacerbation of the increase in mean habitat suitability until 2100 in the northern regions (Figures 8G, H). To the south, however, mean suitability decreases progressively until the end of the century. This leads to a relatively small but present restriction in the species' distribution at greater depths in the East China Sea (Figure 9D). However, considerable expansion is observed in the north and eastern region of the Yellow Sea, on the coastlines of Korea by 2050, and on the shores of China until the end of the century, as well as in the Sea of Japan.

Regarding *O. tetricus*, this species exhibited an overall trend of decreasing habitat suitability over its northern extent of distribution in Australia, with gains in its southernmost limits (Figure 10). This pattern was consistent across RCP scenarios, and the projected changes stayed relatively stable between 2050 and 2100, despite a slight overall increase between 2050 and 2100 for RCP-2.6, and an exacerbation of mean habitat suitability decrease for RCP-8.5 for the same period.

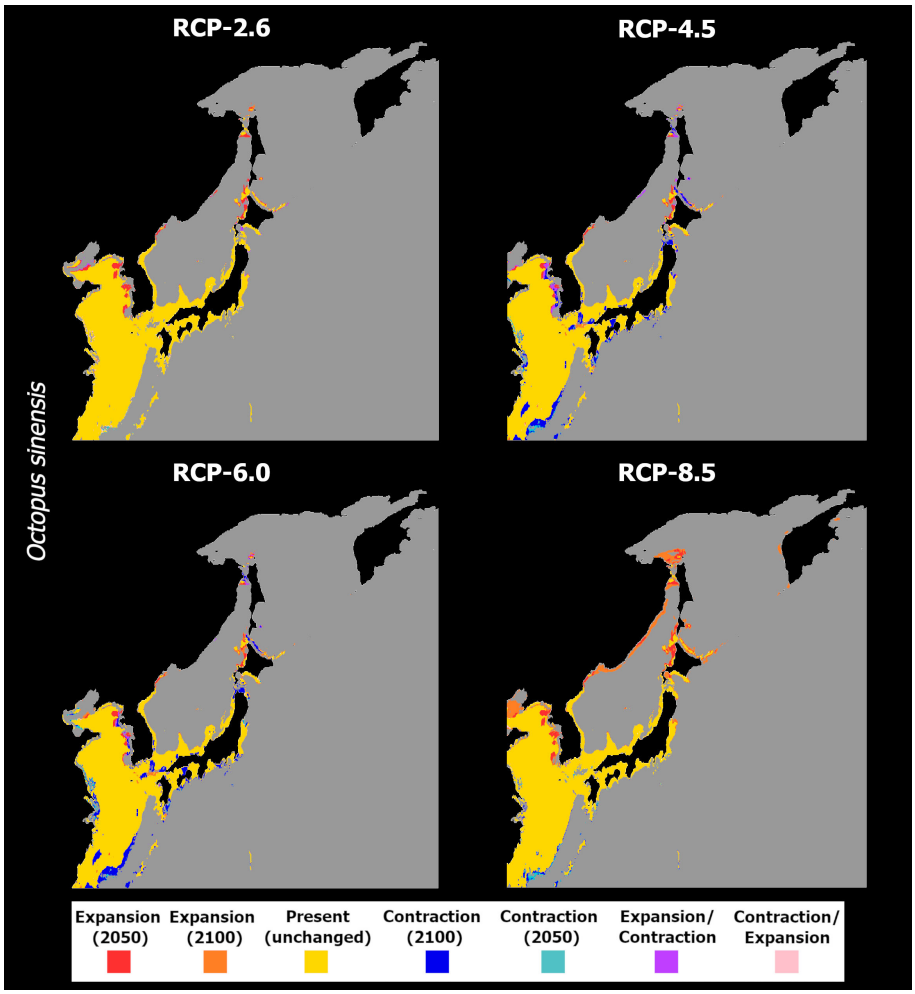

FIGURE 9
Time maps of the projected changes in species occurrence distribution for *Octopus sinensis*, showing unidirectional range shifts in occurrence distribution [i.e., range expansion (in red and orange) or contraction (in dark and light blue)] as well as transitory fluctuations (i.e., range contraction followed by expansion (pink), and vice-versa (purple)] across time (i.e., 2050 and 2100) and RCP scenarios (RCP-2.6, 4.5, 6.0, 8.5; CMIP5).

In terms of projected distribution, the oscillating pattern of mean habitat suitability in the north under RCP-2.6 (Figures 10A, B) led to extirpation until 2050 in the areas near Shark Bay in the west, and a temporary potential extirpation in eastern Australia, near the Coral Sea (Figure 11A), and permanent extirpation in the areas north of this region. In the South of Australia, slight increases in mean habitat suitability led to expanding distribution towards greater depths in the Great Australian Bay and Tasmania. In the westernmost area of this species occurrence, specifically in the Bass Strait, the models also predicted a fluctuating pattern of expansion in 2050, followed by extirpation in 2100, due to oscillating mean habitat suitability in this region. Under RCP-4.5, the deeper western and southern coastlines of Australia are projected to undergo localized extirpation (Figure 11B), as well as the higher latitudes of the species' distribution in both western and eastern Australian coastlines. Also, on the southern Australian coastline, there is an expansion of the transitory fluctuation patterns compared to RCP-2.6, evidencing an expansion followed by extirpation until the end of the century in this region. Lastly, in New Zealand, there is an overall expansion of the distribution towards greater depths all around its distribution. Projections for RCP-6.0 show a similar pattern as RCP-4.5 for both eastern and western Australian coastlines and for the Great Australian Bay, projecting losses in distribution until 2050 and 2100 for these regions (Figure 11C). However, increasing mean habitat suitability in the southern latitudes until 2100 (Figure 10F) led to a potential expansion of the species in the southwestern region of Australia (i.e., near the Bass Strait), while transitory fluctuations expand in New Zealand – in the Northwestern

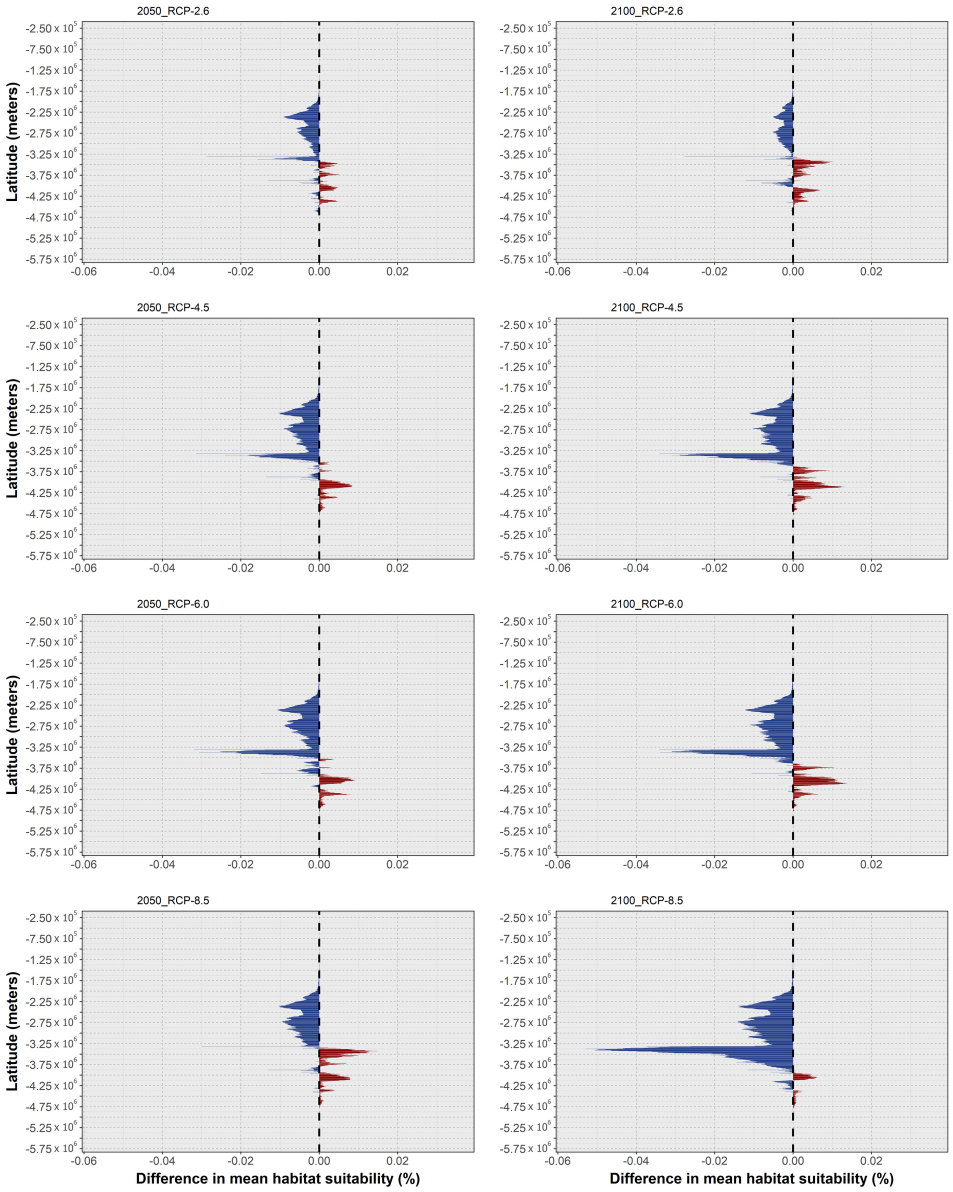

FIGURE 10
Changes in mean latitudinal habitat suitability for *Octopus tetricus* between the present-day and 2050 (left side) and 2100 (right side), for each Representative Concentration Pathway scenario (RCP-2.6, 4.5, 6.0, 8.5; CMIP5), relative to the present-day baseline (i.e., the black dashed vertical line). Data to the right of the dashed line represents a relative increase in mean habitat suitability (red) and data to the left represents a relative decrease (blue).

coastlines of New Zealand, fluctuations between contraction in 2050 and expansion until 2100 are projected from North Taranaki Bight until Auckland, while expansion followed by contraction is projected in greater depths in the Southeastern coastlines. RCP-8.5 was the scenario with the most exacerbated responses, following the considerable decrease in mean habitat suitability between the present-day and the year 2100 (Figures 10G, H). Specifically, the models projected a considerable loss in distribution along most of the eastern, western, and southern Australian coastlines (Figure 11D), apart from the region of Tasmania. The Great Australian Bay area also exhibited a considerable area of transitory fluctuation, with a temporary expansion in the area in 2050, followed by its extirpation until 2100.

Regarding *O. djinda*, the ensemble models projected decreasing mean habitat suitability across most of its

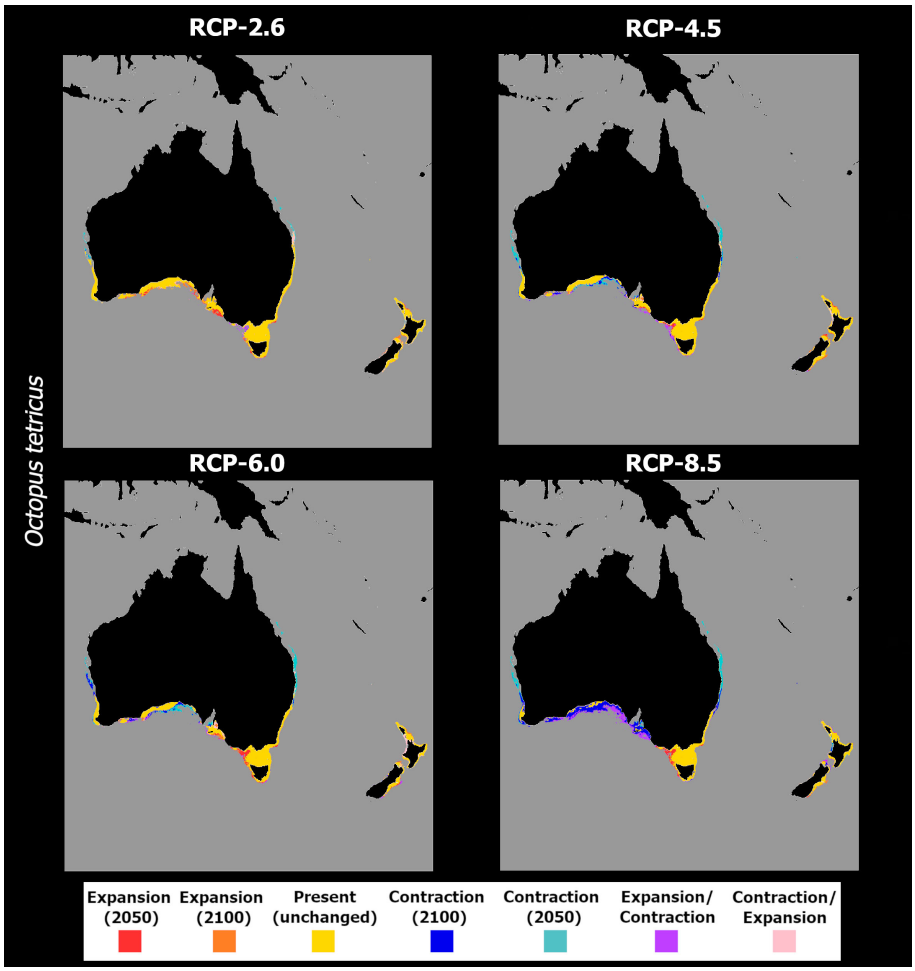

FIGURE 11
Time maps of the projected changes in species occurrence distribution for *Octopus tetricus*, showing unidirectional range shifts in occurrence distribution [i.e., range expansion (in red and orange) or contraction (in dark and light blue)] as well as transitory fluctuations (i.e., range contraction followed by expansion (pink), and vice-versa (purple)] across time (i.e., 2050 and 2100) and RCP scenarios (RCP-2.6, 4.5, 6.0, 8.5; CMIP5).

distribution (Figure 12), in a pattern that is exacerbated between 2050 and 2100 for most RCP scenarios. RCP-2.6 exhibited the smallest magnitude of habitat suitability loss, relative to the other RCP scenarios, and the smallest temporal change (Figures 12A, B). This scenario also exhibited a relative increase until 2050, followed by a decrease until the end of the century (albeit still above the present-day baseline). These patterns led to relatively small changes in the species distribution, with some projected losses until 2100 occurring in the northernmost range of its distribution – in the western Australian coastline (Figure 13A), and sparse gains together with a large span of momentary expansion followed by contraction along the southern coastline.

In RCP-4.5, the loss in mean habitat suitability increases until the end of the century (Figures 12C, D), while the southernmost latitudes exhibit a considerable increase in suitability until the end of the century. This led to a larger area of projected losses in distribution, relative to RCP-2.6, in the western Australian coastline, together with increased areas of expansion in the South (Figure 13B). For RCP-6.0, the projections are very similar to the previous scenario, although the projected extirpation in the western Australian coastline is greater between 2050 and 2100, with further expansion on the southern shores during the same period (Figure 13C), due to a relative exacerbation of the changes in mean habitat suitability relative to RCP-4.5 (Figures 12E, F). Finally, in RCP-8.5, despite projected habitat suitability loss being considerably exacerbated (Figures 12G, H) leading to extirpation across the entire western Australian coastline (Figure 13D), there is considerably lesser expansion projected for the southern areas of its distribution (*e.g.*, mainly localized around the Great Australian Bay).

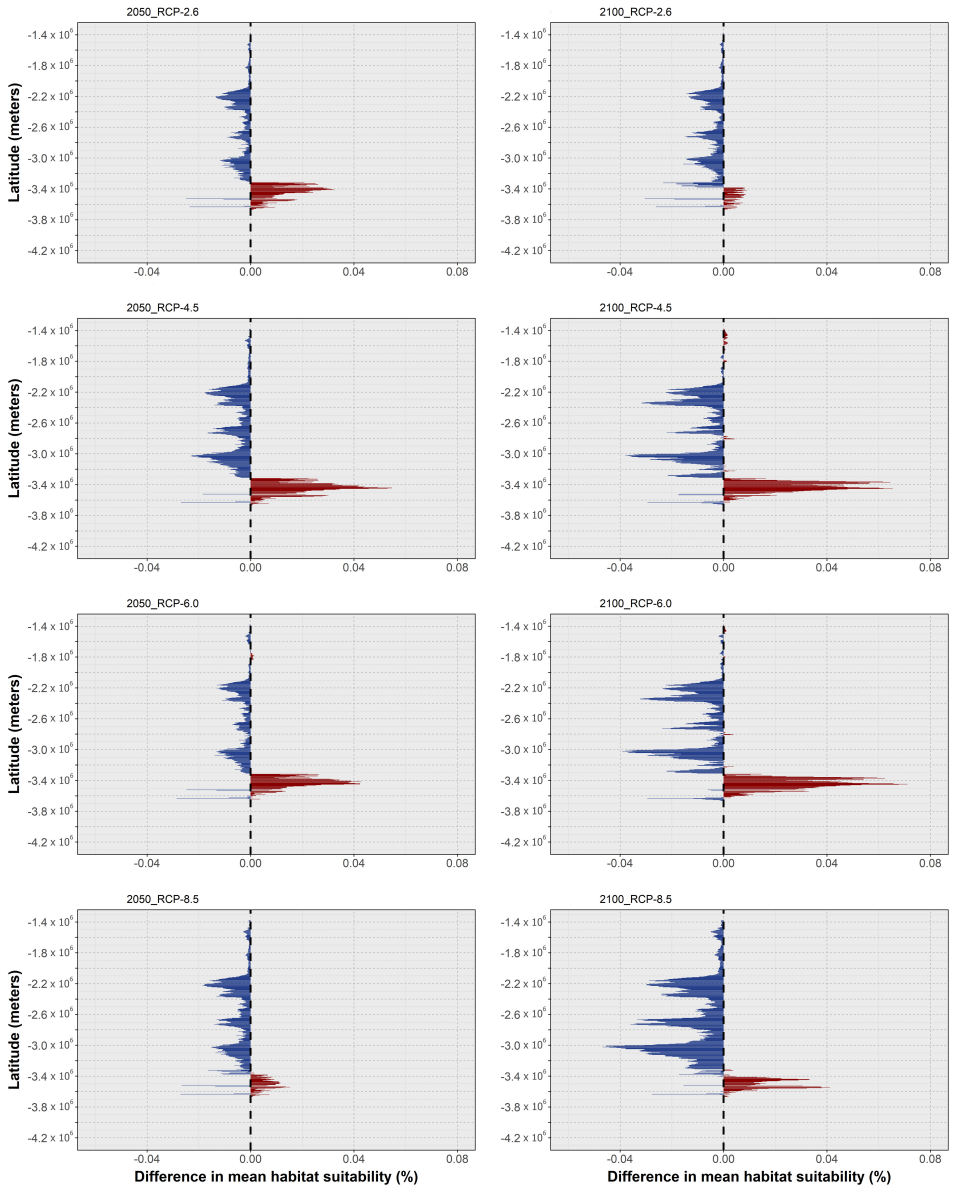

FIGURE 12
Changes in mean latitudinal habitat suitability for *Octopus djinda* between the present-day and 2050 (left side) and 2100 (right side), for each Representative Concentration Pathway scenario (RCP-2.6, 4.5, 6.0, 8.5; CMIP5), relative to the present-day baseline (i.e., the black dashed vertical line). Data to the right of the dashed line represents a relative increase in mean habitat suitability (red) and data to the left represents a relative decrease (blue).

Discussion

In terms of present-day distribution of each OVSC species, the ensemble models were able to make relatively accurate predictions, with small exceptions that are discussed below. Regarding future projections, the biogeographical response to climate change-associated changes in environmental variables was different across OVSC species, hinting at potentially different long-term consequences and management requirements for each species within the so-called OVSC and historically referred to as *Octopus vulgaris*. However, a general trend of decreasing mean habitat suitability with increasing RCP severity, with associated losses in the current distribution range, was observed across species.

For *O. vulgaris*, the ensemble models predicted a distribution very similar to that presented in Rosa et al. (in press), with the species occurring along west Africa and in the Macaronesia region (Adam, 1962; Gonçalves, 1991; Sánchez et al., 2015); in

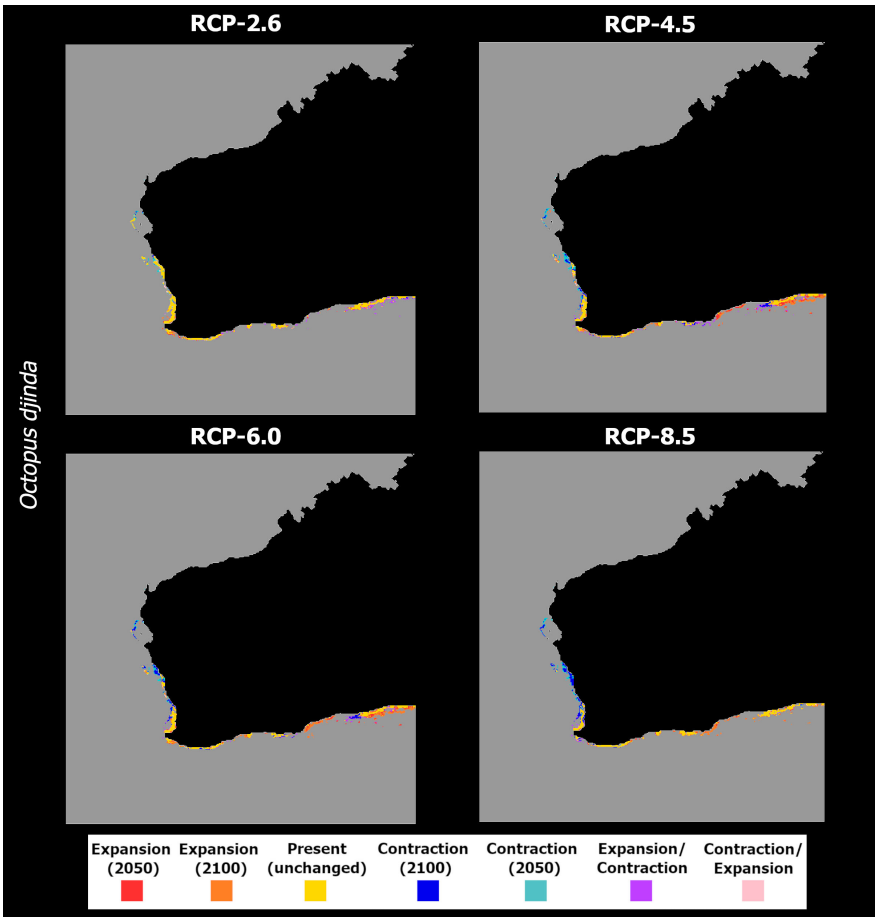

FIGURE 13
Time maps of the projected changes in species occurrence distribution for *Octopus djinda*, showing unidirectional range shifts in occurrence distribution [i.e., range expansion (in red and orange) or contraction (in dark and light blue)] as well as transitory fluctuations (i.e., range contraction followed by expansion (pink), and vice-versa (purple)] across time (i.e., 2050 and 2100) and RCP scenarios (RCP-2.6, 4.5, 6.0, 8.5; CMIP5).

the Mediterranean Sea and along the Iberian Peninsula (Rees, 1956; Sousa Reis, 1985) and northward into the Northeast Atlantic Ocean and the English Channel (Massy, 1928; Rees, 1950; De Luca et al., 2014). However, its occurrence was also predicted in Iceland and in the North Sea, where it currently does not occur (Goud et al., 2019; Oesterwind et al., 2022). Notwithstanding, previous SDM analyses performed on *O. vulgaris* in the same region, by Schickele et al. (2021), have led to similar predictions over the eastern North Sea, despite slightly different results. In terms of future changes, the present study projected a decrease in habitat suitability for the Mediterranean and the eastern Atlantic, exacerbated with scenario severity, but did not predict increasing habitat suitability and consequent northward distribution gains in the Baltic Sea (Schickele et al., 2021). These differences likely stem from the environmental predictors and geographical restrictions included in the analysis. Indeed, to avoid over-parametrization, Schickele et al. (2021) only employed sea surface salinity and mean annual sea bottom temperature (including mean, range, and variance), and in the case of *Octopus vulgaris*, the analysis excluded salinity altogether. The present study, however, employed bathymetry and current velocity as environmental predictors, but also used both max depth (*i.e.*, sea bottom) and surface layers for current velocity, temperature, and salinity. This approach aimed to accurately describe the octopuses' habitats, while still avoiding over-parametrization. Notwithstanding, both studies projected increasing environmental pressure in the southern areas of this species' distribution range, which could lead to severe impacts on populations at this trailing edge and potential socioeconomic repercussions, given the relative importance of this resource for regional fisheries (Sánchez et al., 2015; Sauer et al., 2021). Indeed, the present study projected severe contractions in the Mediterranean Sea and along the Iberian and French coastlines, with minimal expansion. Together, these results point to a

potential severe decrease in habitat suitability towards the end of the century for this species, with severity increasing alongside the emissions implicit to each RCP scenario.

Regarding *O. americanus*, its predicted distribution was in line with its current accepted distribution (Avendaño et al., 2020). Indeed, the ensemble model predicted its occurrence from the north of Argentina, along the continental shelf of Yucatan, and into the northwest coast of the United States and Canada. In terms of projections, this species exhibited a clear pattern of decreasing suitability in tropical and subtropical waters (mainly in the regions of the Yucatan Peninsula, northern Brazil, and towards Florida) and increases in higher latitudes (offshore Argentina and alongside the north-western US and Canada), both exacerbated with RCP scenario severity. This is a slightly different response to that projected for *O. vulgaris*, which saw its available environmental niche decrease considerably with time and RCP scenario. It is worth noting that other American octopuses have been analysed with an SDM framework, where similar trends were observed. For instance, *O. insularis* (Leite et al., 2008), which occurs in tropical and subtropical waters of the Central and South American continents, sharing most of its distribution with part of *O. americanus*', is projected to undergo an increase in suitable niche space in the tropical Atlantic, potentially expanding into the temperate northern Atlantic, temperate South America, and temperate South Africa (Lima et al., 2020). In the present-study, however, *O. americanus* is projected to undergo a severe range contraction in lower latitudes, in the tropical and subtropical Atlantic, suggesting that suitable niche space decreases in these regions. Alongside this severe range contraction at lower latitudes, the increase in habitat suitability at higher latitudes, particularly in the northern hemisphere, presents a potential poleward shift, suggesting the possibility of a split in the distribution of *O. americanus* between North and South America which could isolate both populations and lead to genetic divergence in the long term. In fact, *O. americanus* is mainly a subtropical and temperate species (O'Brien et al., 2021), linked to waters between the 18 and 25°C depending on the life stage (Bastos, 2018; Ángeles-González et al., 2020). Juveniles and adults of this species typically occur in cooler waters up to 200 m in depth (Avendaño et al., 2020), and shallow waters associated with upwelling systems (O'Brien et al., 2021), which limits their occurrence in tropical areas. Therefore, the prediction of *O. americanus* in warmer shallow waters in the tropics (e.g., in the Caribbean region) is likely an overprediction introduced by the occurrence points utilized. As will be discussed below, one limitation of the use of online databases is the potential for misidentification in highly morphologically similar and co-occurring species [e.g., as is the case between *O. americanus* and *O. insularis* (O'Brien et al., 2021)]. Nevertheless, the observed projected trend of reducing mean habitat suitability in equatorial and low-latitude tropical areas is likely to be accurate, with increasing oceanic temperatures leading further constraining the species in these areas. Yet, it is also important to note that one may also argue that adult individuals may also move into deeper, cooler waters, together with the projected regional displacement; alongside, the weakening of regional upwelling systems, such as in Brazil, associated with climate change could also lead to further contraction in coastal areas (de Souza et al., 2020).

For *O.* aff. *vulgaris*, the predicted present-day distribution was (again) quite similar to the accepted distribution range, with the species occurring in the coastlines of Namibia in the South-eastern Atlantic Ocean, and in the Indian Ocean shores of Mozambique and Madagascar (Oosthuizen and Smale, 2003; Oosthuizen et al., 2004). Future projections predict a severe pressure on the eastern range of its distribution, in the Indian Ocean, with decreasing habitat suitability leading to losses in northern latitudes. Indeed, over time and for all scenarios except RCP-2.6, this species is projected to potentially be extirpated from most of Madagascar and Mozambican shores. This could lead to a reshuffling in local food webs and a significant impact on subsistence and recreational fisheries in the eastern regions of South Africa, where most of this species' exploitation occurs (Robertson et al., 1997; Oosthuizen and Smale, 2003).

The *O. djinda* has only recently been shown to indeed be a distinct species than *O. tetricus*, occurring along the southwest Australian coast from Shark Bay to near Cape Le Grand (Amor and Hart, 2021; Moltschaniwskyj and Hall, in press a). The ensemble model was able to accurately predict its present-day distribution but encountered issues of overprediction – mainly in the southern Australian shores, in disjunct areas along the Great Australian Bight. This species is considered not to occur in this region, which has been associated with sharp drops in sea surface temperature that prevent paralarvae dispersal and settlement, maintaining the allopatric distributions between the now *O. djinda* and *O. tetricus* to the east (Amor et al., 2014). Regarding the areas of present-day distribution, the future projections exhibited the same trend of decreasing mean habitat suitability in lower latitudes, with a (limited) increase in southern areas. In this context, the species is projected to lose some of its northern distribution, potentially expanding into the Great Australian Bight if conditions become more suitable over time. This pattern was consistent and exacerbated with increasing RCP severity. However, from RCP6.0 to RCP8.5, the areas of projected loss outpace those of potential expansion. Overall, the present results suggest a potential for southward expansion of *O. djinda* towards the shallow areas of the Great Australian Bay, since this species typically occurs in temperatures between 17-25°C and depths of up to 80 m (Amor and Hart, 2021). This could potentially lead to increased chances of overlap with other octopus species (*e.g.*, *Octopus kaurna*,

Stranks 1990) and top-down pressure in these habitats. At the same time, since *O. djnda* supports a highly productive fishery (Moltschaniwskyj and Hall, in press a), local fishermen communities may also be affected by these shifts.

O. sinensis was accurately predicted to occur over most of its present-day distribution. Indeed, this species is known to occur in the South China Sea (Gleadall, 2016), from the region of Taiwan (Lü et al., 2013; Reid and Wilson, 2015), northward into Japan in southern Hokkaido and the East China Sea (Sauer et al., 2021). Our projections translated into two positive (RCP-2.6 and 8.5) and two negative scenarios (RCP-4.5 and 6.0), with the least and most extreme emission scenarios both resulting in a considerable expansion of *O. sinensis* towards the most northern regions of its distribution (on the Sea of Japan and the Yellow Sea). This poleward expansion to newly suitable habitats would be beneficial to the species. On the other hand, the two middle-emission scenarios exhibited a considerable loss of habitat suitability and distribution, over most of its present latitudinal extent. Given that this species is of major economic importance in the southern waters of China and Japan (Sauer et al., 2021), this may lead to a loss of fishery grounds of this species. Indeed, in the 20th century, *O. sinensis* was already over-exploited in certain regions of Japan (Hamabe et al., 1976) and, despite current regulations, these synergistic pressures may undermine the sustainable use of this species in the region.

Lastly, *O. tetricus* also exhibited considerable overprediction of its area of present-day distribution. Indeed, this species was predicted to occur along most of the southeastern, southern, and southwestern shores of Australia, as well as most of eastern and northwestern New Zealand. However, its present distribution only encompasses the east coast of the Australian mainland, in shallow waters across the Tasmanian Sea, and in northern New Zealand (Amor et al., 2014; Amor and Hart, 2021; Moltschaniwskyj et al., in press b). Overall, until the end of the century, the ensemble models projected localized decreases in mean habitat suitability in northern latitudes, much as *O. djnda*, with consequent losses in distribution in these regions. For RCP-2.6, the species is inclusively projected to expand into the Great Australian Bight, together with increases into further depths in New Zealand and Tasmania. If this emissions scenario occurs, it is possible that this species could clash with other competitor species and further increase top-down pressure in these novel habitats. However, for the most extreme scenarios, together with shifts towards greater depths, the species is projected to be particularly pressured in its northern distribution latitudes in mainland Australia, with the Australian Bight becoming unsuitable for colonization (with the models projecting severe loss by 2100 for this area), and small expansions in the area west of the Bass Strait. Warming is known to significantly affect *O. tetricus* egg development speed at 25°C - the upper limit of their temperature range (Spreitzenbarth and Andrew, 2021). As such, with increasing temperatures over time, this species is particularly susceptible to negative effects imposed by warming, which would lead to a contraction of the species range in mainland Australia, pushing the species into being further pressured by other anthropogenic stressors, despite its recent poleward expansion into Tasmanian waters (Ramos et al., 2014). Indeed, in New Zealand, egg development for this species has been shown to cease above 21°C (Anderson, 1994), which further emphasizes the threat of warming in terms of reproductive fitness.

This study's results highlight the looming threat of marine climate change to ectotherm species, and namely in octopods. Indeed, temperature was one of the main contributing variables (either surface, benthic, or both) to determining habitat suitability in the ensemble models. The four RCP scenarios employed in this analysis encompass the wide gradient of possible climatic futures, mainly in terms of global temperature rise. The first scenario, RCP-2.6, projects the global temperature rise to stay below 2°C by the end of the century, requiring a global CO_2 emission decline until zero by the year 2100. The intermediate scenario RCP-4.5 projects peaking CO_2 emissions by the middle of the century, resulting in a 2-3°C increase in global temperatures. RCP-6.0 is a high emissions greenhouse gas scenario, resulting in a potential temperature increase of between 3 to 4°C. Lastly, the "worst case scenario" RCP-8.5 projects temperatures to increase by over 4°C due to continued increasing emissions during the 21st century (IPCC, 2014; Schwalm et al., 2020). In the present analysis, temperature was one of the most contributing variables for the majority of the OVSC species, emphasizing its potential future impacts to these species' worldwide distribution. Indeed, temperature (and thermal stress) has been shown to promote a decrease in egg incubation time, hatchling size, weight at first sexual maturity, and average generation time (Andre et al., 2010). In this sense, it is likely that habitats featuring the greatest thermal pressure over time will significantly decrease in terms of habitat suitability, leading to potentially deleterious effects on octopus populations and promoting poleward shifts whenever possible. In this context, decreasing habitat suitability and potential distribution contraction in tropical and subtropical areas were to be expected, since the thermal tolerance of marine animals in these regions is closer to the environmental temperature limits in their habitats (Tewksbury et al., 2008; Nguyen et al., 2011; Rosa et al., 2014). Salinity was also particularly relevant, being the most contributing variable for *O. vulgaris* and the second most to *O. americanus*. Indeed, salinity gradients are of a high importance for the *O. vulgaris* complex (Hermosilla et al., 2011; Moreno et al., 2014; Iglesias et al., 2016), for instance in determining the preferred areas of recruitment. Changes to the salinity profile could, then, further condition the habitats that are suitable for these species. Likewise, with temperature increase, temperate and subtemperate areas become more suitable for these species, opening more habitats at increasing latitudes for these species to move into [*i.e.*, poleward shifts (Burrows et al., 2011;

Poloczanska et al., 2013). The present study revealed a potential poleward shift in *O. americanus* and poleward expansion in *O. sinensis*, while also projecting severe range contractions in lower latitudes for *O. tetricus* and *O.* aff. *vulgaris*, and to a lesser extent for *O. djinda*. This is in line with previous research (Xavier et al., 2016; Schickele et al., 2021; Boavida-Portugal et al., 2022), and contributes to the notion of cephalopod borealization (Xavier et al., 2016). This type of change in the distribution of species which are both important predators and preys can lead to significant ecosystem-level impacts which must be addressed. First, areas which are projected to undergo octopus range contraction could very well suffer a decrease in top-down control of benthic communities, while projected poleward shifts could increase this top-down control on lower trophic levels, in higher latitude areas (Allen, 1971; Colléter et al., 2012; Schickele et al., 2021). However, the present models do not account for adaptation, and recent abundance increases in commercially important cephalopods at a global scale suggest the existence of potential benefits under an already changing environment (Doubleday et al., 2016). It is still uncertain if this pattern of abundance increase will be maintained under further, more prolonged, and severe environmental changes, with distinct species bound to have different responses. Notwithstanding, suitable habitats are projected to be shifting for most of the OVSC species, as have been for coastal cephalopods (Boavida-Portugal et al., 2022), which emphasizes the need for further research, namely featuring field validation of said projections to clarify the potential road ahead for this marine group.

As with all modelling projections, there is the need to address potential caveats of the SDM analysis. First, all models were subject to some degree of over or under prediction in all species. The first issue is common, since SDMs assume that a species will completely occupy areas predicted to be climatically suitable, ignoring potential real-world limitations such as unaccounted environmental predictors, the existence of geographical barriers preventing dispersion, etc. (Araújo et al., 2005). Underprediction, however, is linked to the nature of the occurrence point data, in terms of sample size and spatial biases. Occurrence data retrieved from online databases, such as OBIS and GBIF, are unlikely to represent a complete sample of a species known distribution, since data in these databases is highly dependent on sampling effort, which is frequently geographically skewed (Beck et al., 2013). Also, regarding occurrence data from online databases, species misidentification may introduce some bias in the data retrieved. Notwithstanding, this issue is not likely to be prevalent in most species, as there is no co-occurrence with other highly similar species. This issue may be more pressing in the case of *O. americanus* however, the overall trends projected for the future are not invalidated. Finally, the relatively low sample size for *O. djinda* and *O. sinensis* (post-environmental filtering sample sizes of 19 and 28 respectively; Table 1), could also be introducing uncertainty in the predictions, since sample sizes under 50 to 100 occurrence points suffer from relative decreases in predictive accuracy, depending on the algorithm and modelling conditions used (Stockwell and Peterson, 2002). However, all model runs for each species were maintained by the 'megaSDM' package during evaluation, suggesting reliable performance. Another issue refers to the assumption of climatic niche conservation between native and non-native ranges, which could potentially introduce biases and lead to inaccurate predictions (Pearman et al., 2008). As was already mentioned, the high phenotypic flexibility and strong adaptation potential of cephalopods (Boyle et al., 1996; Liscovitch-Brauer et al., 2017) suggest that *Octopus* species could adapt to future climate change-related conditions. Notwithstanding, this issue is minimized by the reduced geographical extent of the present study's projections and the fact that predictions were primarily made within each species present-day distribution. In this context, future research should aim to increase sample size whenever possible, as well as increasing the ecological relevance of environmental predictors used. In this sense, the inclusion of dissolved oxygen, pH, and the distribution and ecological relationships between the OVSC species and potential predators and competitors would contribute towards more ecologically relevant models (Warton et al., 2015; Abdulwahab et al., 2022). Despite these limitations, the overall tendencies of the present study's projections reflect major climatic drivers of change, and thus are bound to be ecologically meaningful (Garcia et al., 2016).

Final remarks

The present study contributes to the body of research on the biogeographical impacts of climate change on cephalopod species, particularly regarding the impacts on species complexes which are often treated as single species. In this regard, the results of this study highlight the potentially differential responses exhibited by each of the OVSC species. While results suggest increased pressure and potential extirpation at lower latitudes for some species (*e.g.*, *O. americanus* and *O.* aff *vulgaris*), widespread distribution contraction were also identified (*e.g.*, *O. vulgaris* and *O. tetricus*), and the potential for poleward distribution shifts as well (*e.g.*, *O. americanus* and *O. sinensis*). These different responses require that research conducted on the *O. vulgaris* complex be adapted to include the different environmental requirements of each species, as well as the different abiotic pressures they will be subjected to in the future. This is of paramount importance for the correct evaluation and management of the fisheries stocks of the OVSC species, and the sustainability of regional octopus' fisheries. The present study aimed to contributed towards fisheries management and conservation, by providing a set of future projections where the identification of potential areas of increase/decrease in habitat

suitability and the consequent potential changes in each species distribution towards the end of the century are possible. By identifying such areas, it is possible to complement potential stock evaluation and stock management to predict increased environmental stress which could further increase anthropogenic pressure already present through fisheries exploitation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

RR and FB conceptualized the study; FB was responsible for data gathering and curation; FB conducted the SDM analysis and post-analysis processing; FB wrote the original draft of the manuscript; FB, MG, CS, JP, and RR reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1018766/full#supplementary-material

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Bathyal octopus, *Muusoctopus leioderma*, living in a world of acid: First recordings of routine metabolic rate and critical oxygen partial pressures of a deep water species under elevated pCO_2

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Elevated atmospheric CO_2 as a result of human activity is dissolving into the world's oceans, driving a drop in pH, and making them more acidic. Here we present the first data on the impacts of ocean acidification on a bathyal species of octopus *Muusoctopus leioderma*. A recent discovery of a shallow living population in the Salish Sea, Washington United States allowed collection *via* SCUBA and maintenance in the lab. We exposed individual *Muusoctopus leioderma* to elevated CO_2 pressure (pCO_2) for 1 day and 7 days, measuring their routine metabolic rate (RMR), critical partial pressure (P_{crit}), and oxygen supply capacity (α). At the time of this writing, we believe this is the first aerobic metabolic data recorded for a member of *Muusoctopus.* Our results showed that there was no change in either RMR, P_{crit} or α at 1800 µatm compared to the 1,000 µatm of the habitat where this population was collected. The ability to maintain aerobic physiology at these relatively high levels is discussed and considered against phylogeny and life history.

KEYWORDS

routine metabolic rate, aerobic metabolism, ocean acidfication, muusoctopus, critical oxygen partial pressure, bathyal, oxygen supply capacity

Introduction

Presently atmospheric CO_2 levels are over 400 ppm (Dunn et al., 2020) well above pre industrial levels of approximately 275 ppm (Macfarling et al., 2006). The world's oceans absorb as much as one-third of annual anthropogenic CO_2 (Doney et al., 2009), causing an increase in oceanic partial pressure of CO_2 (pCO_2). The increased pCO_2 drives a decrease in pH, causing oceanic pH to decline from its pre-industrial revolution level of 8.2 to a current average below 8.1, in a process termed ocean acidification (OA) (Caldeira and Wickett, 2003).

Initial studies of the impact of OA on marine organisms focused largely on challenges faced by calcifying organisms (Fabry et al., 2008). However more recently there has been a number of studies that examine how OA impacts the physiology of an array of organisms. Changes in pH have been shown to impact respiratory physiology (Miller, 1985; Bridges, 1995; Widdicombe and Spicer, 2008; Seibel, 2016). Negative impacts to respiratory physiology make it more difficult to obtain oxygen from the environment and may limit aerobic energy production. This has been shown in crab, squid, fish, and sipunculids (Portner and Zielinski, 1998; Langenbuch and Portner, 2002; Metzger et al., 2007; Rosa and Seibel, 2008; Munday et al., 2009; Walther et al., 2009).

Studies within cephalopods have shown various, and sometimes conflicting, responses to environmental hypercapnia and the resulting low pH. At environmentally relevant ranges of 700–1700 µatm adult Cuttlefish *Sepia officinalis* show no change in aerobic metabolic rate (Gutowska et al., 2008) whereas embryonic *S. officinalis* showed an increase in routine metabolism (Rosa et al., 2013). In squid, the vertically migrating epipelagic squid *Dosidicus gigas* showed conflicting responses with some showing metabolic depression (Rosa and Seibel, 2008) and others showing no effect (Birk et al., 2018). *Sepioteuthis lessoniana* had no aerobic metabolic response below 2000 µatm pCO_2 (Hu et al., 2014). Alternatively the benthic, inter- and sub-tidal octopus *Octopus rubescens* had a short term (1 day) increase in routine metabolic rate (RMR) which then returned to pre-exposure levels within 1 week. Additionally, critical oxygen partial pressure (P_{crit}) was significantly higher after long term exposure to elevated pCO_2 (Onthank et al., 2021).

In studies where pCO^2 is pushed above 2,000 µatm there is a continued variation in response. Cuttlefish increase calcification of their cuttlebone (Gutowska et al., 2008; Gutowska et al., 2010), whereas squid statoliths are malformed and more porous (Kaplan et al., 2013). Above 4,000 µatm squid continue to show a decrease in aerobic metabolic rate (Hu et al., 2014).

The majority of studies which examine the impact of OA on cephalopods have focused primarily on cuttlefish and squid. As of this writing there has only been one other study we are aware of that explored how OA affects octopus and no studies on bathyal occurring species of octopus. Here, we examine the impact of OA on the Smoothskin octopus *Muusoctopus leioderma* (Berry, 1911). Like all other members of Family Enteroctopodidae, *M. leioderma* is a deep living species. It can be found in the Northern Pacific from the Sea of Okhotsk off Siberia to California and are reported to live on muddy or silty bottoms at meso and bathyal depths ranging from 250 to 1400 m

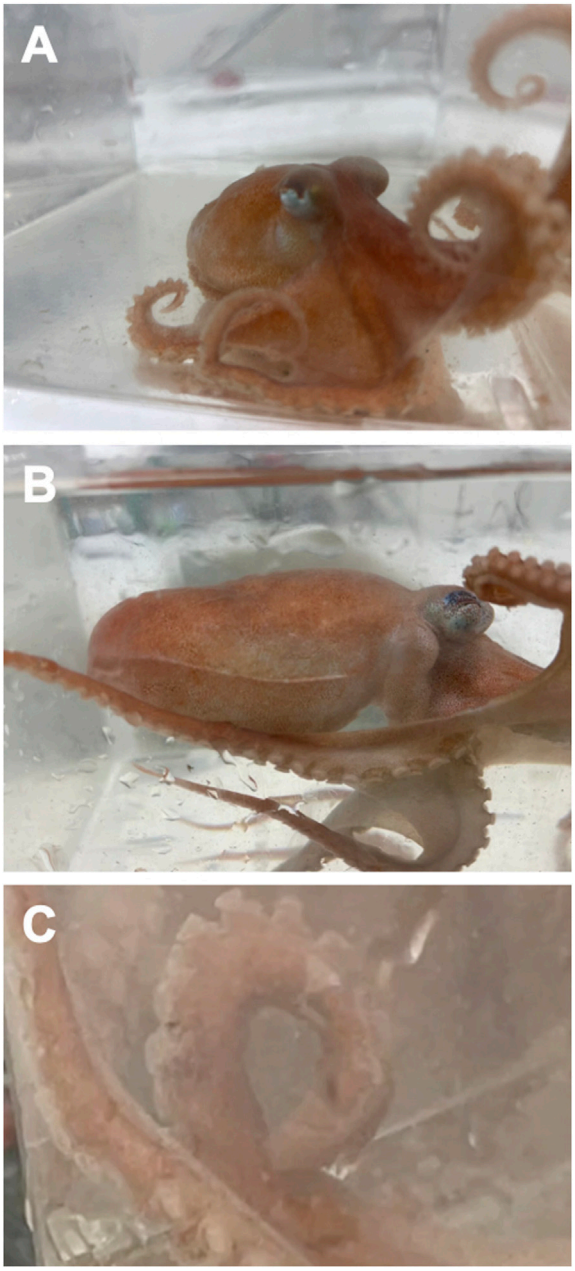

FIGURE 1
Muusoctopus leioderma collected at Burrows Bay, Anacortes Washington, United States **(A)**. Note the well-developed ridge of skin on the periphery mantle **(B)** and hectocotylized arm on third armright **(C)**.

(Conners et al., 2016). This range overlaps with an oxygen minimum zone that has both low oxygen levels as well as pCO_2 levels in excess of 2000 µatm (Kamykowski and Zentara, 1990; Paulmier et al., 2011). The greatest frequency of occurrence for *M. leioderma* has been reported between 450–650 m (Conners et al., 2016). However, it has previously been reported as shallow as 70 m (Hochberg, 1998). Recently a population has been found at depths reachable by SCUBA (10–15 m) in Burrows Bay, Skagit County, Washington, United States, the shallowest record for any individuals in the genus *Muusoctopus*, a major deep water octopus genus with 28 recognized species. Morphological and genetic data were used to confirm the species identity of this population as *Muusoctopus leioderma* (Onthank unpublished data) (Figure 1).

As part of a marine environmental physiology course at the Rosario Beach Marine Lab (RBML) *Muusoctopus leioderma* were collected and held at 1,000 or 1800 µatm for 1 day and 7 days. Routine metabolic rate, critical partial pressure (P_{crit}) and oxygen supply capacity (α) were recorded for each treatment. The Salish Sea, where the RBML is located, is a unique location for OA studies as CO_2-rich water from the California Undercurrent wells up into this shallow basin producing persistent hypercapnic conditions (Murray et al., 2015). The pCO_2 regularly reaches 1,000 µatm (Onthank et al., 2021). Habitats such as the Salish Sea will experience accentuated acidification due to local hypoxia and eutrophication (Cai et al., 2011; Melzner et al., 2013). The goal of this study was to examine the impacts of short-term and prolonged exposure to hypercapnia on aerobic metabolism of a bathyal associated species of benthic octopus. The population of *M. leioderma* we used for this study was found at depths, and locations near populations of *O. rubescens*, and likely experiences similar environmental conditions. Because of this we hypothesized that *M. leioderma* would show a similar response to elevated pCO_2 as *O. rubescens* having a short term increase in RMR after 24h exposure, and a return to pre-exposure RMR and elevated P_{crit} with a prolonged 7-day exposure to hypercapnic conditions. This is the first publication of aerobic metabolic rate for any species in the genus *Muusoctopus*. This is also the first study of effects of ocean acidification in any deep water living species of octopus.

Methods

Field seawater pH measurement

This research was carried out at the Rosario Beach Marine Laboratory in Anacortes, Washington United States. Water samples were taken at the octopus collection site in Burrows Bay. Samples were taken at depth where the octopus were collected *via* SCUBA. A 50 ml high-density polyethylene sample container filled with air was opened at depth and filled with a water sample, excluding all air bubbles. A screw top lid was used to cap the sample. All water samples were immediately transported to the RBML where pH on the total scale (pH_T) was measured using the *m*-cresol purple spectrophotometric method (Dickson et al., 2007) within 3 h. Alkalinity was determined by open-cell titration (Dickson et al., 2007), and alkalinity values were calculated from titration data using the at() function in the "seacarb" package version 3.2.14 in R (Gattuso et al., 2015). The resulting measured alkalinity and pH were used to calculate the pCO_2 using the carb() function in the "seacarb" package in R. Samples from the collection site had pCO_2 ~1,000 µatm.

Octopus collection

Seventeen *Muusoctopus leioderma* (mass = 2.5–70.0 g) were collected in June through August of 2021 from Burrows Bay, Skagit County, Washington State, United States by SCUBA at depths of 10–20 m (Figure 2). Octopuses were found on the sediment bottom during night dives. Individuals were placed in plastic resealable bags for transport to RMBL. At RMBL octopuses were placed in holding aquaria with sediment that had previously been collected from Burrows Bay. The holding aquaria were supplied with unmodified seawater directly from the lab seawater system which uses seawater pumped from Rosario Bay.

pCO_2 level selection

The Salish Sea has persistent hypercapnic conditions that reach 1,000 µatm CO_2 (Murray et al., 2015) and in the future may increase by an additional 800 µatm (Barry et al., 2010; Cai et al., 2011; Melzner et al., 2013; Bianucci et al., 2018). At the time of octopus collection, water samples from Burrows Bay were approximately 1,000 µatm, which is consistent with values recorded previously by Onthank et al. (2021) at surface and 15 m depth. The selected pCO_2 range allowed us to examine whether the Burrows Bay population that regularly experiences 1,000 µatm CO_2 would be impacted by the predicted increased CO_2 levels resulting in 1800 µatm CO_2 locally.

Hypercapnia exposure

After being held in aquaria for at least 1 day, octopuses were transferred to treatment tanks as previously described (Culler-Juarez and Onthank, 2021). Each tank (113.5 L) was made using an insulated cooler with an overhead window in the lid to allow observation of octopus. The tank system included an Active Aqua AACH10HP chiller to maintain temperature, and a venturi injector to keep tank water oxygenated. Each tank had a slow constant water exchange from the lab sea water system which flowed fresh seawater into the tank at ~100 ml min^{-1} and drained

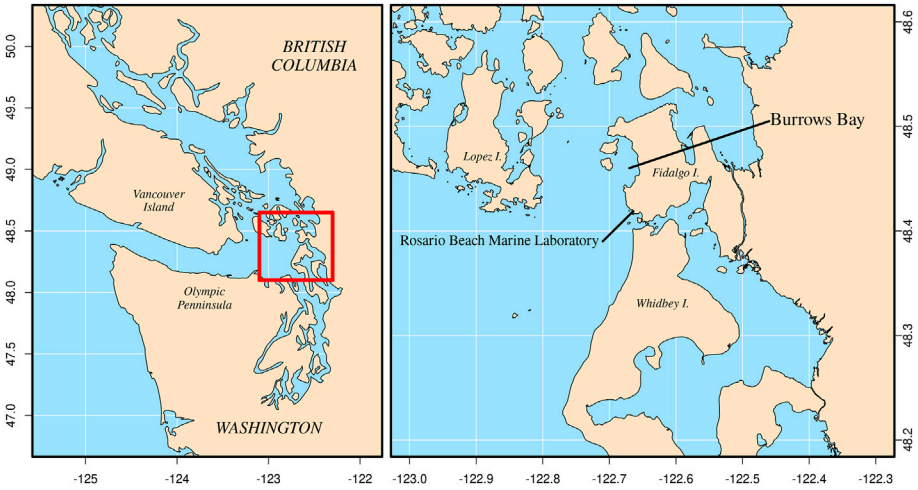

FIGURE 2
Location of Burrows Bay, Anacortes, Washington, United States where *Muusoctopus leioderma* were collected for this study.

from an overflow port. This prevented building up waste in each tank without having to perform large, full-tank water changes. Temperature and pCO_2 of each tank was controlled with a pH-stat system (https://open-acidification.github.io/) which received temperature input from a PT-100 temperature probe and pH input from a single junction glass pH electrode. The pH of aquaria was measured daily using the *m*-cresol purple spectrographic method (Dickson et al., 2007), and that measured aquarium pH used to perform a one-point calibration of the pH electrode. Temperature probes were calibrated using a 0.1°C resolution alcohol thermometer. To modify pCO_2 pure gaseous CO_2 was slowly bubbled into the tank by a solenoid controlled *via* the Open Acidification Tank Controller. Temperature was controlled by powering on/off a chiller controlled by the same Tank Controller.

In addition to pH measurements, the total alkalinity (A_T) was measured weekly using a modified open cell titration based on Dickson et al. (2007). Off gassing time was increased from 6 to 10 min with vigorous stirring *via* a stir bar and motor. Titrations were verified against certified reference material (CRM, supplied by Andrew Dickson, Scripps Institution of Oceanography, San Diego, CA, United States). The alkalinity values were calculated from titration data using the "seacarb" package in R (Gattuso et al., 2015). The measured alkalinity and desired pCO_2 of each tank were used to calculate pH setpoints and tank pH set points were updated weekly. The pH was calculated from raw spectrographic data using the specpH(), function in the OTools package in R (https://github.com/KirtOnthank/OTools), alkalinity and PCO_2 were determined using the at() function and carb function respectively in the seacarb package in R (https://CRAN.R-project.org/package=seacarb).

After at least 1 day acclimating in holding aquaria, octopus were transferred to treatment tanks. One octopus was placed in a treatment tank and held at either 1,000 µatm or 1800 µatm for 1 day and then its RMR and P_{crit} were measured (methods described below) and it was then returned to the same treatment tank and held for additional 6 days, for a total of 7 days exposure to its treatment pCO_2. Routine metabolic rate and P_{crit} were then measured again after the 7th day of exposure. During treatment feeding was done by placing purple shore crabs in the treatment tank after the first 1 day RMR data collection and removed 1 day prior to RMR and P_{crit} measurements on day 7.

Routine metabolic rate measurement

Routine metabolic rate were measured after fasting for 24 h. Octopus were placed in 1 L or 120 ml as body size required, flow through water-jacketed respirometers. Experimental temperatures were between 13°C and 14°C. The same type of pH-stat system that was used in the treatment tanks was used to adjust the pH of the seawater for the flow through respirometry. PyroScience Firesting or Presense O_2 flow-through optode cells and robust temperature probes were placed on the incurrent and excurrent channel of each respirometer. A peristaltic pump was used to cycle water through the system. Flow rates for each respirometer were measured at the start and end of each respirometry run by measuring output of water mL for 1 minute. Flow rates for 1 L respirometers had a mean rate of 30 ml min^{-1} and 120 ml respirometers had a mean flow rate of 7 ml min^{-1}. Octopuses were placed in the respirometers for 24 h and aerobic metabolic rates were measured throughout; during analysis, the first 3 hours of each RMR run was trimmed out to account for any

handling stress on metabolism. Because the octopus were able to spontaneously move in their respirometer, though they typically did not, we termed our metabolic rates routine metabolic rate instead of standard metabolic rates. After RMR and P_{crit} experiments were completed, the octopuses were removed and oxygen consumption was measured in the empty respirometer to determine back-ground respiration. After the first eight runs, background respiration was consistently 5% or less of octopus respiration, and was therefore not recorded for the remainder of the study. After background respiration was measured, inflow and outflow optodes were connected immediately in series to evaluate drift. Throughout all experiments no drift was detectable. RMR was calculated from raw oxygen data using the resp.open() function in the OTools package in R (https://github.com/KirtOnthank/OTools). In brief, this function calculates RMR by subtracting the oxygen concentration of the outflow water from the oxygen concentration of the inflow water, multiplying this by the flow rate, and dividing by the mass of the octopus.

Oxygen supply capacity and critical oxygen pressure measurement

Following 24 h RMR measurements, the respirometer was closed by connecting the inflow of the respirometer to the outflow. Oxygen concentration in the respirometer was allowed to fall to at least 50 μmol O_2 l^{-1}. Oxygen supply capacity (α) and P_{crit} was determined from aerobic metabolic rate (R) as function of oxygen partial pressure (P_{O2}). We used the calc_alpha function to determine α and determined the P_{crit} *using the* α-method described in Seibel et al. (2021) using the calc_pcrit() function in the "respirometry" package in R (https://CRAN.R-project.org/package=respirometry).

Statistical analysis

The effects of pCO_2 exposure on the log of RMR, α, and P_{crit} were examined using repeated-measures linear mixed effect models with log of mass, pCO_2 and duration in treatment included as fixed factors and octopus ID as a random factor using the lme() function in the "nlme" package in R (Pinheiro et al., 2022). Estimated marginal means (covariate-corrected means, in this case, mass) were determined for each pCO_2 category using the emmeans package in R (Lenth, 2018).

Data availability

All of the raw datasets, data ID files, and R scripts underlying all statistical analysis and figures presented in this study can be found at the Zenodo online repository: https://doi.org/10.5281/zenodo.7058934.

Results

Control treatments had a measured pCO_2 of 1,083 ± 48 μatm; high CO_2 treatments had a measured pCO_2 of 1767 ± 94. (Table 1).

There was a significant effect of mass (linear mixed-effects model, x^2 = 5.84, df = 1, p = 0.01565), but not of pCO_2 (linear mixed-effects model, x^2 = 0.19, df = 1, p = 0.6621) nor day of measurement (linear mixed-effects model, x^2 = 2.18, df = 1, p = 0.13939) on the RMR of *Muusoctopus leioderma*. Routine metabolic rate for 1 day exposure showed an estimated marginal mean (EMM) at 1,000 μatm pCO_2 = 2.60 μmol O_2 g^{-1} hr^{-1} and at 1800 μatm pCO_2 = 2.79 μmol O_2 g^{-1} hr^{-1} and for 7 days exposure RMR 1000 μatm pCO_2 = 2.64 μmol O_2 g^{-1} hr^{-1} and at 1800 μatm pCO_2 = 2.84 μmol O_2 g^{-1} hr^{-1} (Table 2; Figure 3).

Routine metabolic rate was significantly affected by body mass (M), decreasing mass specific RMR with increased body mass in all four treatments as follows: 1,000 μatm 1 day RMR = 8.06 $M^{-0,39}$, 1,000 μatm 7 day RMR = 19.17 $M^{-0.68}$, 1800 μatm 1 day RMR = 10.61 $M^{-0.46}$, and 1800 7 day RMR = 9.36 $M^{-0.41}$ (Figure 3; linear mixed-effects model, x^2 = 5.84, p = 0.0157).

Critical oxygen partial pressure was not significantly affected by elevated pCO_2, in 1 day treatments (1,000 μatm P_{crit} = 3.64 kPa, 1800 μatm P_{crit} = 3.14 kPa) or 7 day (1,000 μatm P_{crit} = 5.00, 1800 μatm P_{crit} = 4.50) (Figure 4; linear mixed-effects model, x^2 = 0.4646, p = 0.4954). However, it did show a significant increase from day 1 and day 7 within both 1,000 μatm and 1800 μatm pCO_2 treatments (Figure 4; linear mixed-effects model, x^2 = 10.53, p = 0.001).

Oxygen supply capacity was not significantly affected by elevated pCO_2, in 1 day treatments (1,000 μatm α= 0.80 μmol O_2 g^{-1} hr^{-1} kPa^{-1}, 1800 μatm α = 0.89 μmol O_2 g^{-1} hr^{-1} kPa^{-1}) or 7 day (1,000 μatm α = 0.53 μmol O_2 g^{-1} hr^{-1} kPa^{-1}, 1800 μatm α = 0.46 μmol O_2 g^{-1} hr^{-1} kPa^{-1}) (Figure 5; linear mixed-effects model, x2 = 0.06, p = 0.8080). However, it did show a decrease from day 1 and day 7 within both 1,000 μatm and 1800 μatm pCO_2 treatments (Figure 5; linear mixed-effects model, x^2 = 17.35, p = 0.000031). There was an effect of mass on oxygen supply capacity in all four treatments as follows: 1,000 μatm 1 day α = 1.47 + (−0.025M), 1,000 μatm 7 day α = 1.09 + (−0.021M), 1800 μatm 1 day α = 1.29 + (−0.015M), and 1800 7 day α = 0.39 + 0.003M (Figure 5; linear mixed-effects model, x2 = 9.84, p = 0.00171).

Discussion

This study is the first to investigate the effects of near-future ocean acidification on the physiology of a deep-water octopus. The Salish Sea is an excellent location for studying the effects of ocean acidification. It has historically had a persistent elevated pCO_2, attributed in part to upwelling from the California Undercurrent (Murray et al., 2015). It is common to see

TABLE 1 Carbonate system parameters of control and experimental tanks. The pH was measured daily for each of the four control and four experimental tanks, (n = 29 per tank), Alkalinity and salinity were measured weekly including day one and the last day of the experiments (n = 6 per tank). Means are presented with ± as standard error.

Treatment	pCO_2 (µatm)	pH	Alkalinity (µmol kg^{-1})	Salinity (PSU)
Control	1,083 ± 48	7.621 ± 0.019	2068 ± 7	30 ± 0.4
Elevated CO_2	1767 ± 94	7.422 ± 0.022	2066 ± 4	30.1 ± 0.4

TABLE 2 Estimated marginal means for routine metabolic rate of *Muusoctopus leioderma* held at either 1,000 or 1800 µatm pCO_2 for 1 day and 7 days. These values are corrected for mass of 18.3 g, the value that corresponds to the mean of the logged masses from the linear mixed effects analysis. For all treatments n = 8.

pCO_2 (µatm)	day	RMR (µmol O_2 g^{-1} hr^{-1})	RMR 95% CI
1,000	1	2.60	1.75–3.87
1800	1	2.79	1.88–4.16
1,000	7	2.64	1.77–3.93
1800	7	2.84	1.91–4.23

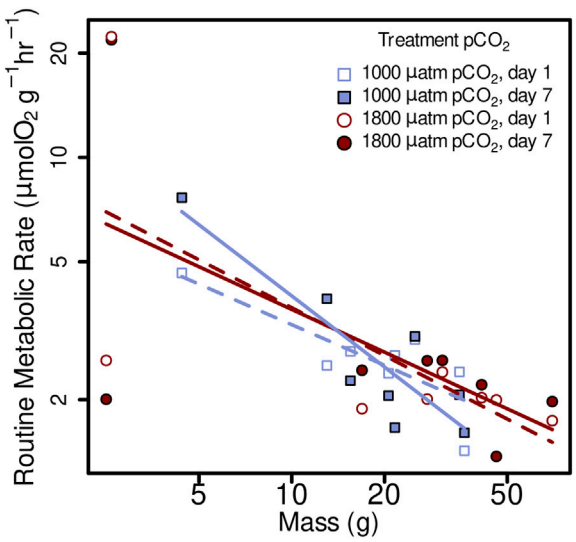

FIGURE 3
Routine metabolic rate (RMR) from *Muusoctopus leioderma* in Burrows Bay, Anacortes Washington held for 1 day (open symbols) and 7 days (closed symbols) at 1,000 µatm (purple squares) or 1800 µatm (maroon circles) pCO_2 expressed as a function of body mass (M). Routine metabolic rate was significantly affected by body mass (M), decreasing mass specific RMR with increased body mass in all four treatments as follows: 1,000 µatm 1 day RMR = 8.06 $M^{-0.39}$, 1,000 µatm 7 day RMR = 19.17 $M^{-0.68}$, 1800 µatm 1 day RMR = 10.61 $M^{-0.46}$, and 1800 7 day RMR = 9.36 $M^{-0.41}$. Note that axes are plotted on a log scale.

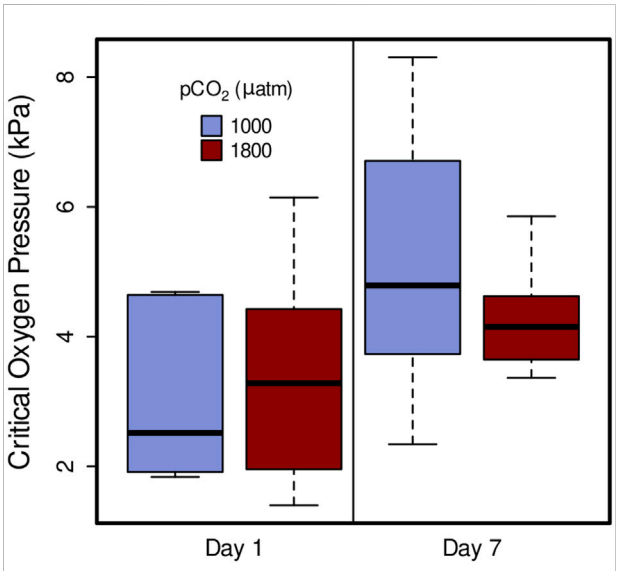

FIGURE 4
Critical oxygen pressure (P_{crit}) of *Muusoctopus leioderma* after 1 day and 7 day exposure to carbon dioxide partial pressure (pCO_2) of 1,000 µatm (purple, Day 1 n = 7, Day 7 n = 7) and 1800 µatm (maroon, Day 1 n = 8, Day 7 n = 7). Critical oxygen pressures are not significantly different between pCO_2 treatments after long-term exposure, however, they did show an increase from day 1 and day 7 within both 1,000 µatm and 1800 µatm pCO_2 treatments (linear mixed-effects model, x2 = 10.53, p = 0.001).

pCO_2 above 1,200 µatm which exceeds all but the most extreme atmospheric pCO_2 projections for the end of this century (Onthank et al., 2021). Organisms that live in the Salish sea are persistently exposed to elevated pCO_2 throughout their life history, making them excellent study subjects to explore the impact OA may have on such organisms after many generations.

Previous work on cephalopods has shown conflicting outcomes, with squid having a decrease in RMR and increase in P_{crit} when exposed to pCO_2 above 1,000 µatm (Rosa and Seibel, 2008; Rosa and Seibel, 2010; Hu et al., 2014; Seibel, 2016), as well as no effect (Birk et al., 2018). The observed negative effect on aerobic metabolism has been postulated to be the result of challenges to respiratory physiology. Cephalopods rely on hemocyanin as a respiratory pigment which has a pronounced Bohr effect, resulting in a decrease in oxygen affinity in areas of low pH. Because of this, it has previously been predicted that cephalopods would not tolerate large changes in environmental

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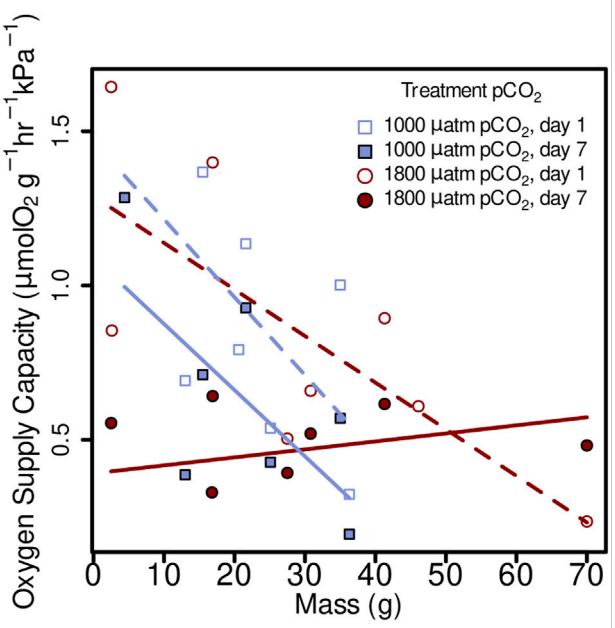

FIGURE 5
Oxygen supply capacity (α) from *M. leioderma* in Burrows Bay, Anacortes Washington held for 1 day (open symbols) and 7 days (closed symbols) at 1,000 µatm (purple squares) or 1,800 µatm (maroon circles) pCO_2 expressed as a function of body mass (M). Oxygen supply capacity was significantly affected by body mass (M), decreasing α with increased body mass in three of four treatments as follows: 1,000 µatm 1 day α = 1.47 + (−0.025)M, 1,000 µatm 7 day α = 1.09 + (−0.021)M, 1800 µatm 1 day α = 1.29 + (−0.015)M, and 1800 7 day α = 0.39 + 0.003M.

pH (Portner and Zielinski, 1998; Rosa and Seibel, 2008; Seibel, 2016). However recent work by Birk et al. (2018) suggests that epipelagic squid blood oxygen carrying capacity is minimally impacted by changes in environmental pH. Yet recent work in octopus suggests this may not be valid for all cephalopods. *Octopus rubescens* exposed to elevated pCO_2 shows a short-term elevation in RMR, however there was no change in RMR after 5 weeks and a significant increase in P_{crit} at the same time point, indicating a reduction in oxygen supply capacity resulting in decreased hypoxia tolerance (Onthank et al., 2021). This deviation from the prediction of Birk and Seibel's model may be linked to the difference in Bohr coefficient found in some species of octopus as compared to those found in squid. In their proposed model squid were assigned a Bohr coefficient of −1.5 as being a worst-case scenario for considering impacts of OA on cephalopods. However octopus hemocyanin has an even larger Bohr effect with Bohr coefficients of −1.7 in *Enteroctopus dofleini* (Miller, 1985) and −1.99 in *Octopus macropus* (Lykkeboe and Johansen, 1982). This suggests that elevated pCO_2 may have a greater effect on octopus blood oxygen binding and aerobic metabolism.

Here we found, unlike the decreased RMR reported in squid (Rosa and Seibel, 2008) or the short term elevated RMR in response to hypercapnia observed in *Octopus rubescens* (Onthank et al., 2021), *Muusoctopus leioderma* RMR was not significantly impacted when exposed to 1800 µatm. Additionally there was no significant change in α or P_{crit} as a result of exposure to hypercapnia. This does not follow most of the trends observed in other octopus or squid species previously studied. Our results do however show a change in both α and P_{crit} in both CO_2 treatments with day 7 P_{crit} values being higher than those recorded for 1 day (Figure 4). It could be that these changes are the result of repeated exposure to low oxygen in subsequent P_{crit} measurements, however, this would be contrary to previous work showing a decrease in P_{crit} with repeated hypoxia exposure in octopus (Onthank, 2008). The combination of no change in RMR, indicating the animals are not significantly stressed, with changes to α and P_{crit} are particularly puzzling. This octopus species spends a considerable proportion of time in subsurface sediment burrows, which, as discussed below, would likely expose them to regular bouts of hypoxia. This would likely drive increases in supply capacity, such as elevating the amount of respiratory pigments to meet metabolic demands. This hypothesis is supported by our finding the oxygen supply capacity in *M. leioderma* (α = 0.80 µmol O2 g-1 hr-1 kPa-1 under control conditions) is substantially higher than other shallow living octopus species for which α is known (*Octopus vulgaris*: 0.30 µmol O2 g-1 h^{-1} kPa^{-1}, *Octopus bimaculoides*: 0.34 µmol O_2 g^{-1} hr^{-1} kPa^{-1}, *Octopus rubescens*: 0.32 µmol O_2 g^{-1} hr^{-1} kPa^{-1}, as well as the deep sea octopus *Octopus californicus*: 0.23 µmol O_2 g^{-1} hr^{-1} kPa^{-1} (Table 3) (Seibel and Childress, 2000; Valverde and García, 2005; Seibel and Deutsch, 2020; Onthank et al., 2021). However, similar to how terrestrial organisms decrease hematocrit as they acclimate to elevated oxygen pressure at lower elevations (Keys et al., 1986; Zubieta-Calleja et al., 2007; Borras et al., 2010), it is possible that the introduction of these octopuses to the well-oxygenated tanks in the lab initiated acclimation processes which lower oxygen supply capacity, such as a reduction of blood hemocyanin concentration or a reduction in hemocyanin oxygen affinity, while not impacting RMR. Such a decrease in oxygen affinity of hemocyanin when exposed to elevated oxygen concentrations has been demonstrated in the shore crab *Carcinus maenas* (Lallier et al., 1987). Unlike those in other treatments, octopuses in 1800 µuatm pCO_2 at 7 days showed a positive relationship between oxygen supply capacity and mass. The reasons for this are unclear, but the unusual responses at the extremes of octopus body masses may be driving this. The largest individual (70 g) in 1800 µatm pCO_2 is the only one that showed an increase in oxygen supply capacity, while the smallest individual (2.5 g) in 1800 µatm pCO_2 showed the largest decrease in oxygen supply capacity in the study. Combined, this suggests a mass specific and pCO_2 specific response in alpha to time in treatment, but more data would be required to make a firm conclusion.

TABLE 3 Comparative routine metabolic rate (RMR), critical partial pressure (P_{crit}) and oxygen supply capacity (α) for species of cephalopods from the literature compared to those recorded for *Muusoctopus leioderma* in the present study.

Species Name	RMR (μmol O_2 g^{-1} hr^{-1})	P_{crit}	α	Temperature (°C)	References
Octopuses					
Muusoctopus leioderma	2.6	3.64	0.80[a]	13–14	Present study
Octopus californicus	0.6		0.23[b]	6	Seibel and Childress, (2000)
Octopus bimaculoides	0.73	2.13	0.34[c]	10	Seibel and Childress (2000)
Octopus rubescens	1.49	4.71	0.32[c]	11	Onthank et al. (2021)
Octopus vulgaris	2.36	8	0.30[c]	25	Wells et al. (1983a), Wells et al. (1983b), Valverde and García (2005)
Other Cephalopods					
Vampyroteuthis infernalis	0.07	0.96	0.07[c]	5	Seibel et al. (1997)
Dosidicus gigas	5.91	1.6	3.69[c]	10	Trueblood and Seibel, (2013)
Nautilus pompilius	1.09	6.47	0.17[c]	21	O'dor et al. (1990), Staples et al. (2000)

Methods of calculating alpha.
[a]Estimated marginal mean (co-variate corrected mean) from linear mixed effects model.
[b]Calculated directly from data extracted from figure.
[c]Estimated by dividing mean RMR, by mean P_{crit}.

Muusoctopus leioderma from this study lives in close geographic proximity to *Octopus rubescens*, and as such they encounter similar abiotic environmental factors. It would be reasonable to assume then these two species should have a similar response to elevated pCO_2, yet they do not. The observed variation in responses to elevated pCO_2 may be linked to differences in phylogenies and behavior between the two species.

Phylogenetic influence

Muusoctopus leioderma has historically been considered a meso to bathyal benthic species, residing between 200–1,500 m depth, with a greatest occurrence between 400–650 m (Conners et al., 2016). This species is part of the Family Enteroctopodidae which includes three genera and a total of ~33 species (Ibáñez et al., 2016; Jereb et al., 2016; Sanchez et al., 2018). Of these, all are deep living species with very few exceptions such as most of the species of genus *Enteroctopus* which are found as deep as 1,500 m, but which can also be found in shallow water. There is one instance of *Muusoctopus eureka* found at 20 m (Laptikhovsky et al., 2011) and *Muusoctopus leioderma* at 70 m (Hochberg, 1998) and at 10 m in the present study. Otherwise, all other species occupy depth ranges between 200 and 1,500 m. Along the west coast of North America there is a pronounced oxygen minimum zone, marked by low oxygen and high levels of CO_2 above 2000 μatm (Paulmier et al., 2011) that occurs between approximately 300–800 m (Kamykowski and Zentara, 1990). These depths overlap with the depth of greatest abundance of *M. leioderma* (Conners et al., 2016). The population of *M. leioderma* used in this study is the shallowest occurrence recorded for any species in the genus *Muusoctopus* that we are aware of. Yet this population of *M. leioderma* presents classic deep sea features, having no ink sac, minimal chromatic change, large eyes, and can only be found out of its burrows in the dark. As a member of Family Enteroctopodidae, there is a long evolutionary history of deep occurrence. It is logical that as *M. leioderma* seemingly has adapted anatomy for the deep sea it's physiology would have as well.

Conversely, *Octopus rubescens,* a member of family Octopodidae, is the most abundant shallow water octopus along the west coast of North America. Its distribution ranges from the mouth of the Gulf of California, Mexico to the Gulf of Alaska. It is common in the intertidal and has been found as deep as 300 m (Hochberg, 1998). These depths are shallower than the depths of OMZs along the west coast of North America typically occur (Kamykowski and Zentara, 1990). Like other shallow water species *O. rubescens* has an ink sac, performs dynamic skin color and texture change, (Packard and Hochberg, 1977), and is most active during the day (Humbert et al., 2022). This species and its shallow water congeners have not experienced the same environmental hypoxia and hypercapnia of species who live at depths corresponding to OMZs and thus may not have the same physiological adaptations.

The Salish Sea receives water input from the upwelling of the California Undercurrent (Murray et al., 2015). As the CU upwells there is additional consumption of oxygen and production of CO_2 from respiration in shelf sediments (Connolly et al., 2010; Bianucci et al., 2018) resulting in pCO_2 levels in excess of 2,100 μatm and oxygen levels below 30% air saturation (Murray et al., 2015). It is probable that the population from this study was originated by individuals brought into the Salish

Sea from CU upwelling. The P_{crit} we recorded at 1,000 and 1800, (Figure 4), are close to P_{crit} values recorded for other species of cephalopods who regularly experience enter OMZs, such as *Dosidicus gigas* (Trueblood and Seibel, 2013). While oxygen supply capacity is substantially lower than *D. gigas*, a transient of the OMZ, it is higher than that of *Vampyroteuthis infernalis*, a resident of the OMZ, and more than double that of shallow living octopods as discussed above (Table 3). This supports that *M. leioderma* may have adapted its oxygen supply capacity for the hypoxic waters like those of the CU. Similarly, the lack of significant change in RMR, P_{crit} and α when exposed to 1800 µatm supports this population is adapted to life in environmental hypercapnia, similar to what is found in the CU. The response observed in *O. rubescens* by Onthank et al. (2021) at 1,500 µatm may reflect that this level of pCO_2 is above historical levels this shallow living species has encountered, but is within the range it is able to acclimate to.

Denning and burrowing behavior

Octopus rubescens, like other species of octopus, spends the majority of each 1 day period in a den (Kayes, 1973; Mather, 1988; Humbert et al., 2022). While their natural dens in the wild have not been studied extensively, they are regularly observed using rocky outcroppings, large empty barnacle shells, beer bottles, and other hard containers of sufficient size (Anderson, 1997; Anderson et al., 1999). These containers allow for reasonable water exchange, and minimal background respiration besides that of the occupying octopus. As such, it is unlikely that *O. rubescens* would typically experience extreme hypoxia or hypercapnia while occupying its den.

Muusoctopus leioderma spend an appreciable amount of time in burrows as well. During this study we only observed them on silt bottoms at night, never during the day. Observations in the lab show their burrows to be small, not much larger than the volume of the individual's body, and having a small narrow opening at least one mantle length below the surface of the substrate. The interior of silty coastal bottoms such as those found in Burrows Bay are often areas of low oxygen and elevated CO_2 (Middelburg et al., 2005). By creating and occupying a small burrow and spending large portions of each day there, it is likely that *M. leioderma* regularly experiences hypoxia and hypercapnia and as a result has physiological adaptations for both.

Invasion potential of deep-water fauna

In retrospect, it is unsurprising that our treatments resulted in no significant change in RMR or P_{crit} given the typical high pCO_2 of most of *M. leioderma*'s depth range. Despite being first described in 1911 (Berry, 1911), and collected frequently as bycatch in various trawl fisheries (Conners et al., 2016), this species has only recently been found as shallow at 10 m depth.

Depth of occurrence, and the resulting zonation of species has been attributed to environmental factors such as temperature, oxygen, and/or food availability, which impact the physiology of organisms, and competition (Rex, 1976; Collins et al., 1999; Carney, 2005; Yeh and Drazen, 2009). Deep-water animals living in higher pCO_2 environments will likely be more robust to higher pCO_2 than their shallow water counterparts. As pCO_2 continues to rise in shallow waters, this could potentially shift balances that may have historically excluded deep-water animals from shallow-water environments. If this is true, the first locations you would expect to see such shallow-water invasions would be in areas that already experience relatively high pCO_2, and recent anthropogenic inputs pushing CO_2 levels even higher, such as the Salish Sea (Murray et al., 2015; Onthank et al., 2021). In addition, the first animals you would likely see invading into shallow waters would be, like octopuses, highly mobile and highly adaptable. Together, the finding that an octopus most commonly found in 450–650 m at the unprecedented depth of 10 m of water is relatively robust to elevated pCO_2 could both partially explain its recent discovery in shallow water and also be a harbinger of future deep-to-shallow water invasions.

Conclusion

Our data is the first of its kind, examining the effects of OA on the aerobic metabolism of a bathyl species of octopus. We show that *Muusoctopus leioderma* maintained its RMR and P_{crit} at elevated levels of 1800 µatm pCO_2, even after 7 days of exposure. The ability to maintain aerobic physiology at these relatively high levels shows this species has physiological adaptations that are likely linked to its phylogeny and life history. It is unique to find this species in the shallow depths recorded in this study. However, it may be that their robust tolerance of elevated pCO_2 has allowed them to survive in the hypercapnic shallow waters of the Salish Sea. Further research is needed to clarify the mechanism that drives this species tolerance to high environmental pCO_2. Because of their resilience to acidification, and relative ease of collection and maintenance in the lab, *Muusoctopus leioderma* make an excellent model system to further study how some organisms may compensate for future levels of environmental pCO_2.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.5281/zenodo.7058934 Zenodo.org.

Ethics statement

Ethical review and approval was not required for the animal study because Cephalopods do not require an ethical review committee in the United States of America. Because of this our institutions do not require, nor conduct review of invertebrate subjects.

Author contributions

LT and KO contributed to conception and design of the study. All authors contributed to data collection. KO organized the data and performed the statistical analysis. LT wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1039401/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Flow chart of experimental procedures for examining the effect of elevated pCO_2 on Muusoctopus leioderma from Burrows Bay, Washington United States.

SUPPLEMENTARY FIGURE 2
Diagram of flow through respirometry system used for measuring routine metabolic rate (RMR) and critical partial pressure (P_{crit}). A firesting optode system (Pyroscience GmbH Aachen Germany) was used to measure oxygen content of water at the inlet and outlet of the respirometer. A peristaltic pump was used to pull water through the system, blue arrows denote direction of water flow. Flow rates for each respirometer were measured at the start and end of each respirometry run by measuring output of water in mL for 1 min. The respirometer system was submerged in a water bath to maintain temperature. To measure Pcrit the system was closed by connecting the inlet and outlet ports.

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Lifecycle, culture, and maintenance of the emerging cephalopod models *Euprymna berryi* and *Euprymna morsei*

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Cephalopod research remains limited by the inability to culture species under laboratory conditions for multiple generations to provide continuous access to animals at all stages of the life cycle. Here, we describe a multi-generational laboratory culture system for two emerging cephalopod models: the hummingbird or Berry's bobtail squid, *Euprymna berryi* Sasaki, 1929, and Morse's bobtail squid, *Euprymna morsei* Verrill, 1881, which are primarily found off mainland Japan. *E. berryi* wild adults were spawned and raised to the third filial generation, and *E. morsei* wild adults were spawned and raised to the second filial generation in a closed system at 20°C. We report growth and survivorship data for a cohort of 30 individuals across the first generation raised in captivity. *E. berryi* and *E. morsei* grew exponentially during the first 90 and 60 days post-hatching, respectively. Survivorship at the first spawning event for *E. berryi* and *E. morsei* was 90% and 77%. *E. berryi* and *E. morsei* females spawned after days 112 and 71 days post-hatching, respectively. We describe the life history of each species and how to distinguish sexes. We discuss the challenges of cephalopod culture and how culturing these species address those problems.

KEYWORDS

***Euprymna*, *Euprymna berryi*, *Euprymna morsei*, cephalopod, bobtail squid, model organism, aquaculture, developmental biology**

Introduction

Cephalopods are widely recognized as the most behaviorally complex invertebrates, attracting attention in the fields of neuroscience, development, and evolution (O'Brien et al., 2018). They have unique characteristics, including adaptive camouflage (Chiao et al., 2015), efficient motor control relative to other mollusks (Levy et al., 2017), and high levels of RNA editing (Liscovitch-Brauer et al., 2017) and transposon activity (Albertin et al., 2015). Furthermore, their capacity to perform complex tasks resembling that of some vertebrate species promoted their inclusion in European Union legislation for animal experimentation and welfare under Directive 2010/63/EU at the same level as vertebrate organisms (European Parliament and Council of the European Union, 2010; Sykes et al., 2012; Smith et al., 2013; Fiorito et al., 2015).

The study of cephalopod development and evolution is a growing area of research that has led to increasing demand for embryos and animals at all stages of their life cycle (Lee et al., 2003; Peyer et al., 2014; Koenig et al., 2016; Navet et al., 2017; Tarazona et al., 2019). While for many purposes wild-caught animals can be studied, and hatchings raised to juvenile or later stages in the laboratory, multigenerational cultures have only been initiated for some cephalopods including octopus (Iglesias et al., 2004; Rosas et al., 2014; Vidal et al., 2014; Maldonado et al., 2019; Grearson et al., 2021), sepioids (Minton et al., 2001; Walsh et al., 2002; Nabhitabhata, 2014), sepiolids (Boletzky et al., 1971; Nabhitabhata et al., 2005; Jones and Richardson, 2010; Nabhitabhata and Nishiguchi, 2014; Sanchez et al., 2019), and the myopsid squid *Sepioteuthis lessoniana* (Forsythe et al., 1994). Large-scale multigenerational cephalopod culture systems are not only a necessity for forward genetics but is also desirable for targeted approaches like CRISPR-Cas genome editing (Jinek et al., 2012; Doudna and Charpentier, 2014). In cephalopods, gene knockouts by genome editing have been accomplished in the progeny of wild-caught *Doryteuthis pealeii* (Crawford et al., 2020). Multigenerational cultures will thus help to move forward the field of development and evolution on cephalopods.

In general, each cephalopod species has unique biological characteristics, morphology, and lifestyle that determine which phenomena can be readily studied, as well as disadvantages in terms of difficulty of culture conditions and difficulty of maintenance (Figure 1). For example, cephalopods generally have a high metabolism and food conversion rate but limited fat

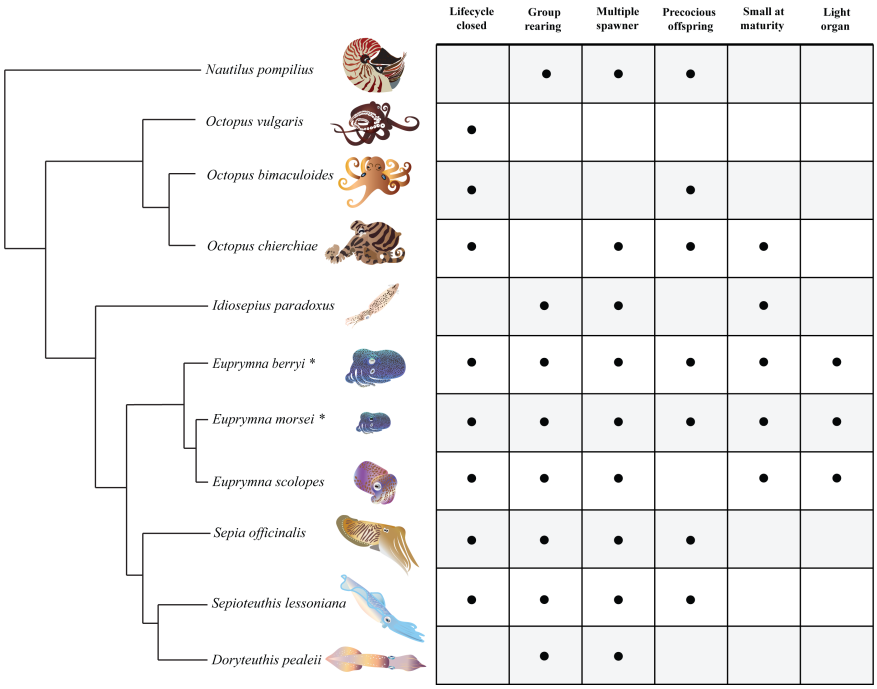


FIGURE 1
Advantageous Culture Traits of Several Cephalopod Models. Comparison of cephalopod species previously used in laboratory experiments. "Lifecycle closed" refers to a species being cultured across at least one generation. An animal is considered capable of group rearing if minimal aggression and cannibalism is observed, and the stress of group rearing prevents successful culturing efforts. "Multiple spawner" indicates normal multiple spawning events completed by one female. "Precocious offspring" refers to hatchling behaviors similar to adults (including predation). "Small at maturity" refers to an animal with a dorsal mantle length less than 6 cm. Some cephalopod species have evolved a light organ that is bioluminescent. The tree is based on results published by Anderson and Lindgren (2020).

reserves, requiring frequent feeding (Iglesias et al., 2014; Vidal et al., 2014). Physically larger species such as *Sepia officinalis*, *Sepioteuthis lessoniana*, and *Octopus vulgaris* therefore require correspondingly large aquaria and amounts of food which rapidly become impractical for many laboratory budgets without dedicated marine facilities. Furthermore, most cephalopods are active visual hunters that prefer live prey, which can be costly and labor intensive to provide (Villanueva et al., 2017). Moreover, many cephalopods have evolved different ranges of sociality, with most of the octopus species being solitary and many squids performing group-like behaviors (Sugimoto et al., 2013; Iglesias et al., 2014). Some species may even practice cannibalism (Ibáñez and Keyl, 2010), preventing the culture of more than single animals per tank. Cephalopods that are relatively small at maturity, avoid cannibalism, and are not entirely solitary therefore have advantages for small-scale laboratory culture.

Reproductive (Rocha et al., 2001) and life history traits, including early mode of life (Boyle, 2005; Villanueva et al., 2016), vary among cephalopod species. For most cephalopods, the diet during their early life in their natural habitats is still unknown, limiting the selection of suitable prey to raise them. Some cephalopods lay only very few eggs, e.g., *Eumandya parva* (Sanchez et al., 2019), and others can produce hundreds or thousands of immature planktonic paralarvae with high mortality, e.g., *Octopus vulgaris* (Villanueva, 1995), and whose size is too tiny to feed with standard prey in laboratory settings.

Bobtail squid from the subfamily Sepiolinae, i.e., sepiolida clade (Anderson and Lindgren, 2020), are a group of nocturnal cephalopods with relatively small size, correspondingly limited nutritional requirements, short life span, benthopelagic early mode of life, and ability to live at high densities without cannibalism. Female bobtail squid can also mate several times with different males and store spermatangia for around two months for future spawning (Squires et al., 2013; Drerup et al., 2020). These characteristics make them suitable for laboratory culture and a potential model organism for developmental, physiological, behavioral, and genetic assays. Their small size is also ideal for advanced imaging (Kerbl et al., 2013).

Thanks to the pioneering efforts of McFall-Ngai and Ruby (Boettcher and Ruby, 1990; Montgomery and McFall-Ngai, 1994; Boettcher et al., 1996; Ruby, 1996; Ruby and Lee, 1998; McFall-Ngai, 1999), the Hawaiian bobtail squid *Euprymna scolopes* Berry 1913 has been widely adopted as a model for bacterial-metazoan symbiosis in which luminescent *Allivibrio fischeri* colonize the light organ of these and related species (Boettcher et al., 1996; McFall-Ngai, 1999; McFall-Ngai, 2014). These efforts fostered studies of bobtail squid diversity (Jones et al., 2006) and development (Lee et al., 2003) both morphologically (Lee et al., 2009b) and at the molecular level (Callaerts et al., 2002; Hartmann et al., 2003; Lee et al., 2003; Sanchez et al., 2021). The genome of *E. scolopes* (Belcaid et al., 2019) has become a reference to study other species in the *Euprymna* clade (Heath-Heckman and Nishiguchi, 2021; Schmidbaur et al., 2022). Many bobtail squid have also been investigated in studies of associative learning, behavior, and the heritability of personality and fitness traits (Steer et al., 2004; Sinn and Moltschaniwskyj, 2005; Sinn et al., 2006; Sinn et al., 2008; Zepeda et al., 2017). Despite these advantages, a disadvantage of *E. scolopes* for multigenerational culture is the high mortality of its larval stage (Lee et al., 2009b).

Two species of bobtail squid that are abundant in mainland Japanese waters have the potential to become laboratory models: the hummingbird or Berry's bobtail *Euprymna berryi* Sasaki, 1929, and Morse's bobtail *Euprymna morsei* Verrill, 1881 (Figures 2A–G). The distribution of these sympatric species extends from Japanese waters southward along the coast of China and westward into the Indian Ocean (Raj and Kalyani, 1971; Okutani and Horita, 1987; Reid and Jereb, 2005; Sundaram and Sreeram, 2008). The spawning season for *E. berryi* is late April to July in Aichi, Japan (Choe, 1966a), and March and December in Taiwan (Huang, 2006). Adults of *E. berryi* have been found from April to June in the southern and the Pacific Ocean side of mainland Japan swimming near the water surface at night, while adults have been found as deep as 60 m on the Pacific side of mainland Japan. In a trawl survey off Nobeoka Bay in Miyazaki prefecture, Toriyama et al. found a relatively similar amount of *E. morsei* across the year with the highest catch from April to June and the lowest from January to March (Toriyama et al., 1970). However, some trawl surveys could have confused both species due to their very similar morphology. Although most fishermen do not discriminate between the two species, they are distinguished by several morphological differences as well as molecular markers (Sanchez et al., 2019). *E. morsei* (mantle length ≤ 4 cm) is considerably smaller than *E. berryi* (mantle length ≤ 5cm) (Reid and Jereb, 2005). *E. morsei* males have enlarged suckers on the ventral sucker rows of arms II, III, and IV, whereas *E. berryi* have enlarged suckers on both dorsal and ventral sucker rows of arms II and IV (Okutani and Horita, 1987; Norman and Lu, 1997). *E. morsei* have chromatophores on the dorsal surface of the fins, while *E. berryi* have chromatophores on both dorsal and ventral surfaces (Okutani and Horita, 1987). Finally, the tentacular suckers in *E. morsei* have a cylindrical shape but in *E. berryi* resemble a smoking pipe (Okutani and Horita, 1987; Reid and Jereb, 2005; Huang, 2006).

Several previous studies described culturing attempts of *E. berryi*, *E. morsei*, and other members of the genus *Euprymna* (Supplementary Table 1). *E. berryi* was reared for two months (Choe, 1966a), and *E. morsei* was raised to reproductive maturity (Ikeda et al., 2003). *Euprymna scolopes* was successfully raised to the second generation (Hanlon et al., 1997). Several species of *Sepiola* and *Euprymna* have been cultured to the second generation (Boletzky et al., 1971; Jones and Richardson, 2010; Sanchez et al., 2019). Further, *Euprymna tasmanica* and

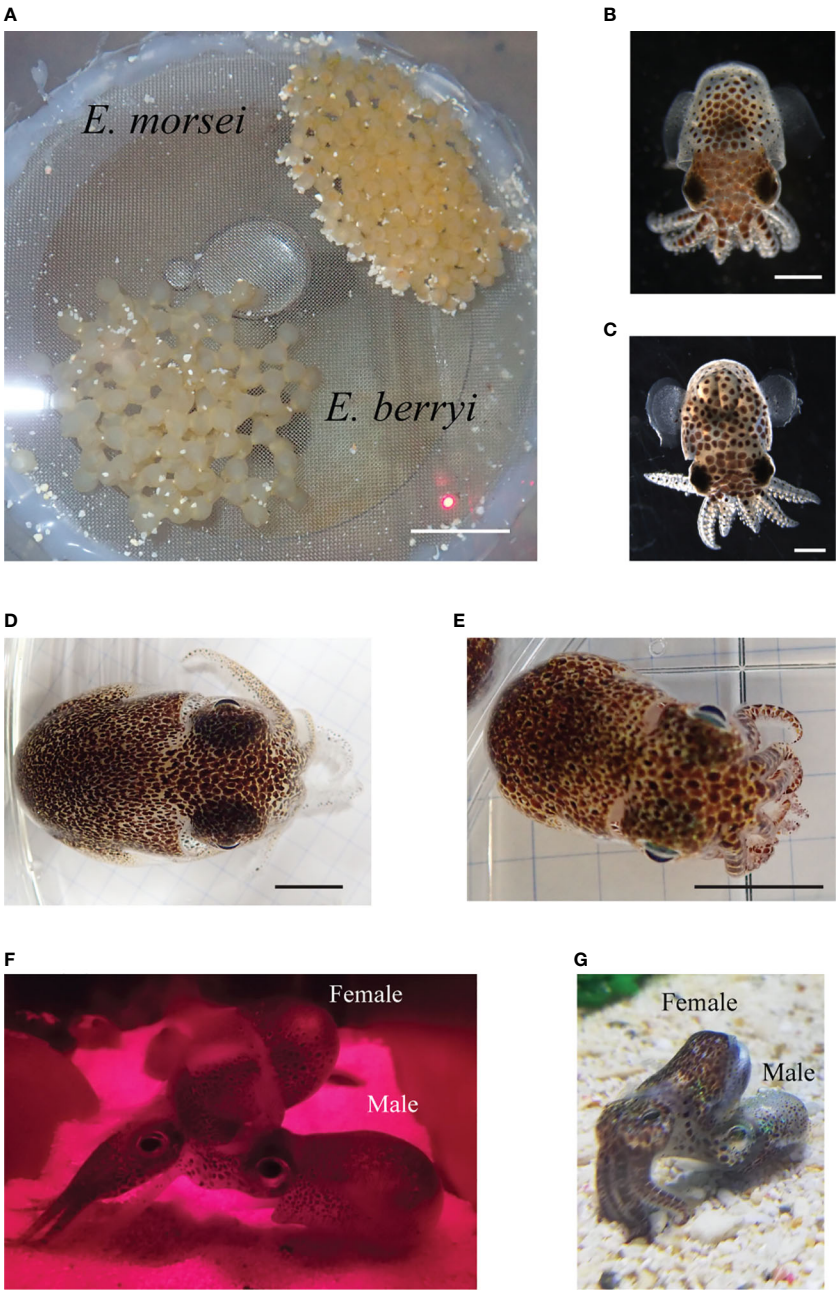


FIGURE 2
E berryi and *E morsei* at different life stages. **(A)** Egg clutches of *E berryi* (left) and *E morsei* (right). Scale bar is 5 cm. **(B)** *E berryi* at one day post-hatching (dph). Scale bar is 1 mm. **(C)** *E morsei* at one dph. Scale bar is 1mm. **(D)** Mature *E berryi* at 130 dph. Scale bar is 1 cm. **(E)** Mature *E morsei* at 70 dph. Scale bar is 1 cm. **(F)** *E berryi* mating. The male (on the right) grasps the female from the ventral side to engage mating. **(G)** *E morsei* mating. The smaller male on the right grasps the female from the ventral side to engage mating.

Euprymna hyllebergi have been cultured to the third generation (Nabhitabhata et al., 2005; Nabhitabhata and Nishiguchi, 2014).

Here we report our efforts to develop multigenerational cultures of *E. berryi* and *E. morsei*. We closed the lifecycle of both species in a recirculating aquaculture system and measured growth and survivorship for thirty individuals of each species under the same aquarium conditions. This work, along with the development of genomic resources for *E. berryi* (Gavriouchkina et al., 2022), provides a foundation for the future development of *E. berryi* and *E. morsei* as laboratory model organisms.

Materials and methods

Broodstock collection

Both *E. berryi* and *E. morsei* were obtained from vendors or from wild collections in southern mainland Japan. Adults are available from vendors seasonally. Adults survived long-distance shipping with commercial couriers using oxygen-saturated seawater and styrofoam-insulated packaging. Adult females of *E. berryi* and *E. morsei* were collected from Mie prefecture, Japan from February to June with a set net, and transported using overnight shipping services. Animals were individually packed in 15 L round-bottom transparent 3 mm plastic bags containing 5-7 L of oxygen saturated filtered seawater with excess volume filled with pure oxygen and shipped in expanded polystyrene foam boxes similar to *E. scolopes* (Hanlon et al., 1997; Cecere and Miyashiro, 2022). Transit time until arrival in the lab was less than 48 h. Upon arriving in Okinawa, animals were acclimated to the temperature (20°C or 23°C) and salinity (~35 gL^{-1} i.e., parts per thousand) of our culture system. Adults from each species were housed separately. Upon spawning, eggs were removed to two-liter tanks like previous methods (Sanchez et al., 2019). Recently spawned eggs – within the first ten days post spawning - were shipped internationally within 72 h using similar methods to shipping adults apart from using 5 L bags containing 1.5-2 L oxygen saturated filtered seawater. To prevent widespread fouling, eggs were monitored on a daily basis and nonviable eggs removed. Hatchlings were housed in two liter tanks for approximately the first month after hatching.

Culture system

The tank system assembled for culturing bobtail squids is a closed tank system that consists of a 200 L filter tank, five 70 L tanks (60 cm x 35 cm x 35 cm), five 2 L tanks (20 cm x 13 cm x 13 cm), two protein skimmers, an ultraviolet sterilizer, and contains filtered natural seawater from the OIST Seragaki Marine Science Station (Supplementary Figure 1). Flow rate for larger tanks is 4.5 to 5 L min^{-1}, and 160 to 180 ml min^{-1} for smaller tanks. Cleaning and partial exchange of 10% seawater are performed daily, and larger 50% water changes are performed biweekly. Water temperature from 2017 to February 2018 was maintained at 23°C with a chiller and heater and thereafter maintained at 20°C. All measurements were taken from animals kept at 20°C. The following water parameters were maintained between the ranges and monitored daily: salinity - 33 to 37 gL^{-1}, pH - 8.2 to 8.4. The following parameters were measured at least weekly nitrate - 0 to 20 mg L^{-1}, i.e., parts per million (ppm), nitrite - 0 to 0.5 mg L^{-1} (ppm), ammonia (NH_3/NH_4) - 0 to 0.25 mg L^{-1} (ppm).

Artificial plants, coral rubble, and PVC pieces were added to tanks to provide egg laying substrate and refuge. Beach sand collected from nearby beaches was added to aquarias. Sand was autoclaved and rinsed in reverse osmosis-treated water before introducing into aquaria. Enough substrate was given to allow the animals to bury completely. Without any burying substrate, skin lesions can form on the ventral side of the animal from friction with the aquarium floor. Aquaria were spot cleaned daily with siphons. The use of coarse sand collected from beaches or from vendors is adequate to allow the animal refuge while remaining easy to siphon.

Blue light-emitting diodes (450 nm wavelength) were used to create twelve hour light-twelve hour dark diurnal cycles in the laboratory. The photoperiod was shifted similarly to previous methods (Franklin et al., 2014) so "night" begins at noon local time to facilitate feeding and experimentation. We used red light-emitting diodes (665 nm) to observe and feed animals during "night" when they were most active.

Feeding and maintenance conditions

Animals were fed *ad libitum*, with new shrimp added once daily. From hatching to 40 dph (days post-hatching), both species were fed mysids (*Neomysis* spp.). Mysids were maintained in a separate tank and fed *Artemia* sp. nauplii once a day prior to being fed to hatchlings. After 40 dph, both species were fed glass shrimp (*Palamonetes* spp.), and the freshwater marsh shrimp-*Caridina* spp.

Both species were also trained to consume cut frozen shrimp (e.g., black tiger prawn) upon reaching maturity.

Frozen shrimp were thawed and cut before presenting to a squid with forceps and moved to simulate living prey. Afterward, contact was made between frozen food and the inner portion of the squid's arms until the squid either showed signs of stress or voluntarily grasped the frozen food (Supplementary Video 1). After approximately one week of continuous training, squids could attack falling frozen food spontaneously.

In ongoing culture we provide adequate space, refugia, and burying substrate to minimize stress. Adults are kept in a ratio at or greater than 1:1 males to females. Our heuristic for determining appropriate space is to ensure each animal has at least two mantle lengths distance between animals. For our tank dimensions (approximately 70 L, 60 cm x 35 cm x 35 cm and water height of 31 cm), we do not exceed eight fully mature *E. berryi* individuals per tank (eight squids per 2,100 cm^2 floor area, 65,100 cm^3 water volume). Assuming a maximum potential mantle length of 6 cm at maturity (Okutani and Horita, 1987), each animal is therefore given a cube of water that is 12 cm on each side, equivalent to 1700 cm^3. *E. morsei* reaches a smaller size at maturity, with a dorsal mantle length (DML) less than 4 cm (Okutani and Horita, 1987; Reid and Jereb, 2005), and therefore can be kept at higher densities than *E. berryi*. To avoid reproductive attempts and aggression from males, females can be separated from males after mating. We have not observed

any overt changes in behavior when adults of either species are isolated.

Survivorship and growth rate

Thirty F1 hatchlings of *E. berryi* and *E. morsei* were isolated on the first day after hatching and reared to monitor their survivorship and growth rate. To measure growth rate, wet weight (WW, g) and DML (mm) were measured in five randomly selected individuals approximately every 10 d to maturity to prevent additional stress due to handling. We measured only five individuals each to minimize handling stress on the cohorts. Squid were placed in a transparent reservoir containing aquarium water atop graph paper and imaged using an Olympus TG-5 camera. The FIJI variant of ImageJ (Schindelin et al., 2012) was used for image calibration and DML measurement. All data is expressed as Mean ± SD. Survivorship was calculated (Leverich and Levin, 1979) as the percentage of surviving individuals, I(t) by:

$$I(t) = 100\left(\frac{Ns(t)}{No}\right)$$

Where $N_s(t)$ is the number of survivors at time t and N_o is the initial cohort size.

At 115 dph, eleven *E. berryi* were removed from the study due to lack of space and prey items, and $N_o$was then adjusted from 30 to 19 for *E. berryi*. All 27 surviving *E. berryi* were first removed from the aquaria, then eight of each males and females were selected and placed back in the aquaria. The animals selected were the first animals to be collected. The other 11 *E. berryi* were removed and euthanized *via* overdose to the anesthetic ethanol (Abbo et al., 2021). Animals were immersed in a bath of 1% ethanol in filtered seawater. Over a period of thirty minutes, ethanol was gradually introduced until reaching a final concentration of 5% followed by mechanical destruction of the brain (Fiorito et al., 2015; Abbo et al., 2021).

Observations

Behavioral observations of *E. berryi* were made visually from March 2017 to May 2020. *E. morsei* observations were from March 2018 to May 2020. Both species were cultured during that time for other experiments.

Results

Culture

We cultured *E. berryi* and *E. morsei* under conditions similar to those used for other bobtail squid (Hanlon et al., 1997; Nabhitabhata et al., 2005; Jones and Richardson, 2010; Sanchez et al., 2019) (**Methods**). We spawned wild-caught adults of both species and cultured *E. berryi* to the third filial generation and *E. morsei* to the second filial generation. Growth rate and survivorship were tracked for the first filial generation (Figure 3).

Egg masses

Both *E. berryi* and *E. morsei* were collected during the Japanese spring season and we observed mating and spawning of wild-caught adults in the laboratory. We shipped recently spawned eggs internationally and they hatched and were raised without overt abnormalities. Both species laid a large number of eggs per clutch, usually exceeding 200 eggs for *E. berryi* and 100 eggs for *E. morsei* (Figure 2A). Wild-caught *E. berryi* laid an average of 235 ± 75.8 eggs per clutch (n=7), while wild-caught *E. morsei* laid 153 ± 26.5 eggs per clutch (n=4). Eggs are encapsulated within a jelly coat and laid individually in a clutch. The jelly coat of eggs of both species have an orange tint due to a dye secreted by the maternal accessory nidamental gland. Non-viable eggs have an opaque white appearance.

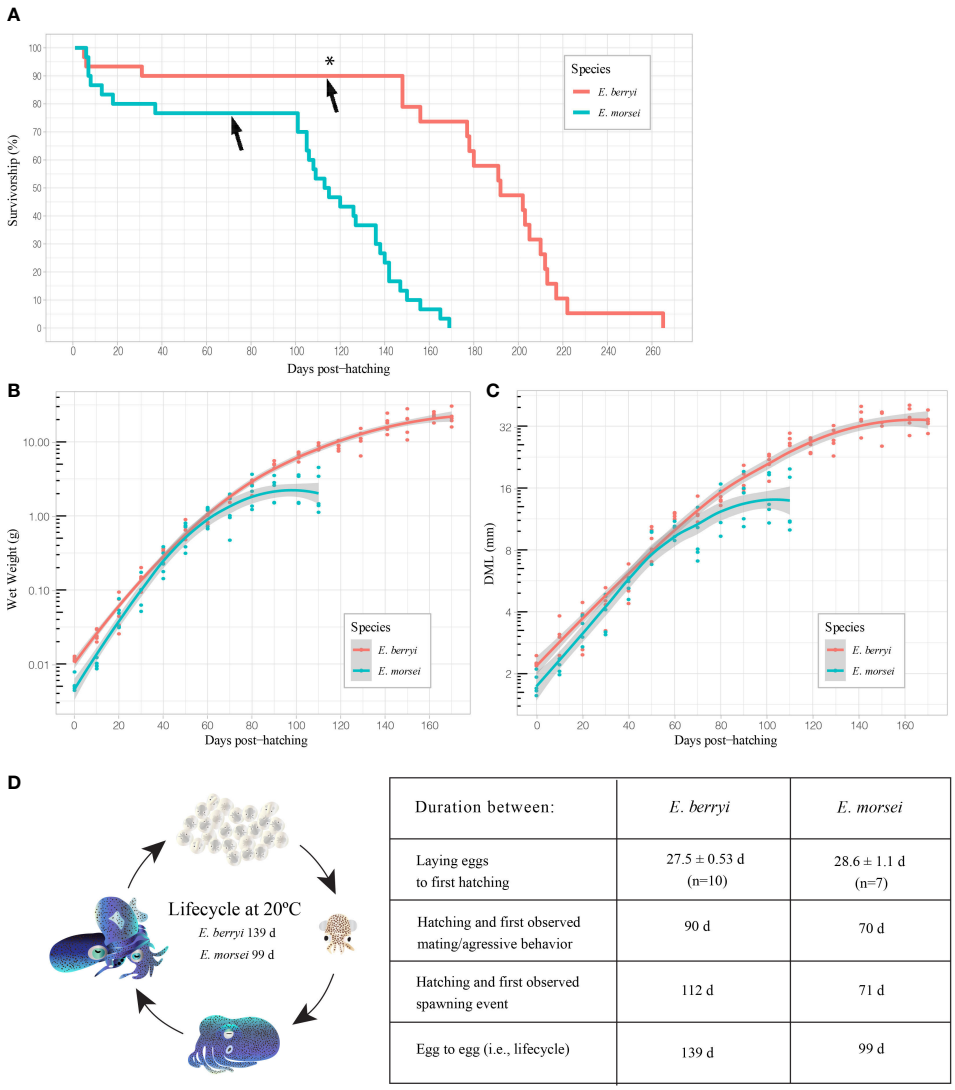
The period between spawning events for wild *E. berryi* at 20°C was 6.7 ± 2.7 d (n=18) and was not recorded for *E. morsei*. Both species demonstrated intermittent terminal spawning and spawned separate clutches continually once reaching sexual maturity (Figure 3A). On one occasion one isolated wild-caught female *E berryi* laid 9 fertilized clutches over a period of 59 d without any additional mating in the laboratory (presumably using stored spermatangia). *E. berryi* was observed to live longer in captivity after capture than *E. morsei* and benefited from more spawning events. *E. berryi* has higher survivorship and fecundity than *E. morsei* (Figure 3).

We observed no physical or behavioral abnormalities in sequential generations; however, survivorship of later generations of *E. berryi* and *E. morsei* immediately after hatching was noticeably reduced for some clutches. On some occasions, we observed eggs laid outside of the jelly coat and some clutches with many unfertilized eggs.

Growth

Growth of *E. berryi* and *E. morsei*, measured by WW or DML, was approximately exponential for the first 90 and 60 dph (Table 1) before reaching species-specific plateaus by 80 and 140 dph, respectively (Figures 3B, C).

We observed a large range of both DML and WW at later stages in both *E. berryi* and *E. morsei* (Figures 3B, C). Average *E. berryi* WW and DML were 17.32 ± 3.82 g and 32.14 ± 4.90 mm for males (n=16), and 23.06 ± 4.90 g and 36.26 ± 3.67 mm for females (n=14). Average *E. morsei* WW and DML were 1.41 ± 0.15 g and 11.45 ± 1.24 mm for



Duration between:	*E. berryi*	*E. morsei*
Laying eggs to first hatching	27.5 ± 0.53 d (n=10)	28.6 ± 1.1 d (n=7)
Hatching and first observed mating/agressive behavior	90 d	70 d
Hatching and first observed spawning event	112 d	71 d
Egg to egg (i.e., lifecycle)	139 d	99 d

FIGURE 3
Survivorship, growth rate, and developmental timelines for *E berryi* and *E morsei*. **(A)** Survivorship for each species. For each species, initial population size was 30 individuals. The asterisk (*) represents an artificial reduction in total population size for *E berryi* from 30 to 19 individuals due to limited tank space. Arrows indicate the first spawning event for each species. **(B)** Growth rate comparing wet weight (g) to dph on semi-log scale. **(C)** Growth rate comparing dorsal mantle length (mm) to age on semi-log scale. **(D)** Comparison of the lifecycle and time between developmental landmarks of both *E berryi* and *E morsei*.

males (n=6), and 3.70 ± 0.42 g and 18.95 ± 2.20 mm for females (n=6). The average female *E. berryi* weighed 1.33 times larger than males and were 1.13 times longer. The average female *E. morsei* weighed 2.63 times larger than males and were 1.65 times longer.

Survivorship

Survivorship was 93% for *E. berryi* and 80% for *E. morsei* for the first 30 days after hatching (Figure 3A). Survivorship was stable until shortly after spawning began. Thereafter, survivorship declined steadily from ~101 dph in *E. morsei* and ~148 dph in *E. berryi*. The oldest *E. berryi* and *E. morsei* in our laboratory culture were males and lived 265 dph and 169 dph, respectively. *E. berryi* outlived *E. morsei* and took longer to reach spawning age by 42 dph.

Mating and spawning

Sexual maturity was noted when males became aggressive towards conspecifics. No courtship behavior was observed in

TABLE 1 Exponential growth curve equations for *E. berryi* 0-90 days post hatching (dph) and *E. morsei* 0-60 dph.

Exponential growth curve from 0 to 90 dph	**a**	**T**	**R^2**
E. berryi WW (g) = a $e^{d/T}$	0.015 g	14.8 d	0.984
E. berryi DML (mm) = a $e^{d/T}$	2.31 mm	42.6 d	0.977
Exponential growth curve from 0 to 60 dph			
E. morsei WW (g) = a $e^{d/T}$	0.0054 g	10.85 d	0.985
E. morsei DML (mm) = a $e^{d/T}$	1.70 mm	33.2 d	0.991

a is the growth parameter either wet weight (WW, g) or dorsal mantle length (DML, mm), T is time (d), R^2 is the coeffient of determination.

either species. Aggression appeared similar to mating, i.e., a male would assault, grapple, and possibly bite a conspecific. At 20°C this was first observed at 90 dph in *E. berryi* and 70 dph in *E. morsei*. During mating the female is first attacked by the male and the male attempts to grab the ventral head of the female, i.e., the male-to-female neck position. The male maintains control of the female and inserts the hectocotylus holding spermatophores into the mantle of the female (Figures 2F, G).

Spawning events began shortly after the night cycle began. Females spawned on the substrate provided including the tank walls, PVC pipes, rocks, and on imitation plants. As described above, for both species, the female laid each egg individually as part of a large clutch (Supplementary Video 2). Spawning began at night and continued into the day. Females have been observed laying eggs cooperatively on the same substrate simultaneously. Females typically consumed less prey one day before spawning. Females repeatedly laid egg clutches every few days until reaching a late senescent life stage (Figure 3A). Some individuals spawned within three days of shipment. Mature *E. berryi* females were observed spawning fertilized clutches repeatedly over a period of 100 days at 20°C. No parental care was observed in either species. Egg clutch morphology was different for both species. *E. morsei* eggs were more densely packed in a clutch, whereas *E. berryi* eggs were more spaced out (Figure 2A).

Sexual dimorphism

Sexual dimorphism was visually evident at 100 dph for *E. berryi* and 70 dph for *E. morsei*. The sex of the animal can be determined by its side profile, size, suckers, and the morphology of the first left-arm (Supplementary Figure 2). Males are smaller than females for both species. The size difference is more pronounced in *E. morsei* than *E. berryi*. The side mantle profile in males is sharper in males than in females (Supplementary Figures 2A, B). Fully mature females generally have a bulbous mantle, because of the presence of oocytes in their ovaries, and can be distinguished from males visually in a minimally invasive manner. Males of both species can further be distinguished from females by observing the first arm pair (Supplementary Figure 2C). Males have a modified first left arm known as the hectocotylus which is shorter than the opposing arm and curls slightly outward. Males of both species have large suckers on some rows of certain arms and modified suckers on the hectocotylus (Supplementary Figure 2D), whose patterns can be used to discriminate species of *Euprymna* (Norman and Lu, 1997). Females have uniform sucker sizes. Female first arms and suckers (Supplementary Figure 2E) are indistinguishable from one another (Norman and Lu, 1997).

Senescence

Males of both species generally outlived females and displayed similar signs of senescence. Characteristics of early senescence include nonfunctional and faded chromatophores, greater susceptibility to infections, and loss of appetite. Signs of later stages of senescence include complete cessation of eating and burying, loss of equilibrium, and continuous labored ventilation.

Discussion

Two promising cephalopod model organisms

The utility and prominence of *E. scolopes* as a model cephalopod species was discussed by Lee et al. (2009c) who also suggested that, as genomic information becomes available for different cephalopod species, the availability of broodstock and embryos becomes a primary factor in choosing a model system. Here we have explored the culturing of two related Japanese bobtail squid species, *E. berryi* Sasaki, 1929, and *E. morsei* Verrill, 1881. We find that *E. berryi* and *E. morsei* have comparable life cycles in captivity to *E. scolopes* (Hanlon et al., 1997), *E. hyllebergi* (Nabhitabhata et al., 2005), *E. tasmanica* (Nabhitabhata and Nishiguchi, 2014), *E. parva*, and *E. brenneri* (Sanchez et al., 2019) (Table 2). Both *E. berryi* and *E. morsei* can be raised in laboratory settings and are intermittent terminal spawners, spawning repeatedly once reaching sexual maturity (Figure 3A). They are therefore well-suited for evo-devo studies,

physiological assays, behavioral assays, laboratory culture, and have the potential to be used for gene editing (Crawford et al., 2020). *E. berryi* has higher survivorship and fecundity than what is reported for other sepiolids including *E. morsei*, *E. scolopes* (Hanlon et al., 1997), and *S. atlantica* (Jones and Richardson, 2010). These characteristics are crucial for establishing genetic lines with mutations that potentially decrease fitness.

Broodstock

Adult wild *E. berryi* and *E. morsei* can both be shipped using commercial couriers from their native range in southern mainland, with oxygen-saturated seawater and styrofoam-insulated packaging as described for *E. scolopes* (Cecere and Miyashiro, 2022), and acclimate well to aquarium conditions. *E. berryi* and *E. morsei* are usually caught using a set net round 30m deep, although both species have been collected with dip nets at night near the surface. *E. berryi* is also caught from the shore by recreational fishermen, and by commercial fishermen by trawling for sale to fish markets. An existing commercial fishery is potentially useful to obtain large numbers of specimens either living, for seeding propagation in captivity or studying behavior, or dead animals for morphological comparisons, isotope analysis, and population genetics. Because *E. morsei* is a relatively smaller cephalopod, this species is less familiar to the fishing community, which hinders the collection of wild specimens. *E. morsei* is also similar in size to the adult forms of the sympatric species *Lusepiola birostrata* and *Eumandya parva* making identification challenging for nonexperts (Takayama and Okutani, 1992; Bello, 2020). Differences in egg clutch morphology have been used to distinguish sympatric species (Sanchez et al., 2019), and can aid in identifying and collecting eggs in the field.

Proper care of sepiolid eggs is necessary to prevent fouling and maintain high hatching rates (Lee et al., 2009a). Females of *E. scolopes* host bacterial consortium in their accessory nidamental gland that is secreted to eggs during spawning to protect them from predation (Kerwin et al., 2019). We indirectly observed the same feature of *E. berryi* and *E. morsei*; specifically, we noted orange-dyed accessory nidamental glands, whose pigments are generated from carotenoids produced by symbiotic bacterial communities (Pichon et al., 2005). Eggs can be kept in incubating tanks with constant water flow in dark conditions to further inhibit microbial growth (Choe, 1966a). Our eggs were maintained at constant conditions with minimal disturbance as failure to do so can cause premature hatching and decreased survivorship (Hanlon et al., 1997).

The stocking density and male:female ratio are important to consider in cephalopod culture. Crowding has been shown to induce stress and decrease fecundity in *Sepia officinalis* (Forsythe et al., 2002), *Sepioteuthis lessoniana* (LaRoe, 1971; Boal and Gonzalez, 1998) and *Euprymna scolopes* (Hanlon et al., 1997). As most bobtail squid adopt benthic lifestyles quickly (Table 2), the ratio of animals to floor area is also relevant. A low male:female ratio can reduce stress from mating events and prevent forced copulation. We achieved mating and spawning with a low (1:1-1:2) male to female ratio though it is preferable to separate males from females as males become aggressive, similar to *E. tasmanica* (Nabhitabhata and Nishiguchi, 2014).

Few studies exist on the effects of inbreeding depression on cephalopod culture. *Sepia officinalis* grown for seven consecutive generations developed decreased fertility in later generations, and the seventh generation failed to produce viable offspring (Forsythe et al., 1994). There are also accounts of a decreased size

TABLE 2 Comparison of life cycle and culture traits of cultured *Euprymna* spp.

Species	*E. berryi*[a]	*E. morsei*[a]	*E. scolopes*[b]	*E. tasmanica*[c]	*E. hyllebergii*[d]	*E. brenneri*[e]	*E. parva*[e]
Known distribution	West Pacific, East Indian Oceans	West Pacific, East Indian Oceans	Central Pacific/Hawaii	South Indopacific/ Australia	East Indian Ocean/Thailand	West Pacific Ocean/Okinawa	West Pacific Ocean/East Asia
Temperature (°C)	20	20	23	20	28	24	24
Clutch size (eggs)	137-362	121-175	50 - 250	25-500	108-464	–	47
Embryonic Phase (d)	28	29	20	29	14	–	22
Survivorship - First 30 days	93%	80%	73%	–	–	–	–
Hatchling behavior	Benthic	Benthic	Planktonic	Benthic	Planktonic	Planktonic	Benthic
Exponential Growth Phase (d)	90	60	83	44	30	–	–
First Mating Behavior (d)	90	70	61	60	66	83	–
Lifecycle (d)	139	99	80	–	80	–	90
Max Lifespan (d)	265	169	139	–	125	99	–

(a - this study; b - Hanlon et al., 1997; c - Nabhitabhata and Nishiguchi, 2014; d - Nabhitabhata et al., 2005; e - Sanchez et al., 2019).

at maturity for cephalopods cultured to multiple generations (Iglesias et al., 2014). Thus, maintaining the genetic diversity of a colony, by careful interbreeding of separate subpopulations, or the introduction of new alleles by the steady addition of wild animals to the culture, may be necessary to support healthy laboratory colonies. *Euprymna hyllebergi* and *Euprymna tasmanica* were cultured for three generations without the introduction of wild-caught specimens. Growth rates were similar across generations and no obvious abnormalities relating to inbreeding were observed (Nabhitabhata and Nishiguchi, 2014). No physical or behavioral abnormalities were observed in both species in sequentially cultured generations; however, sometimes survivorship immediately after hatching was noticeably reduced for some clutches due to some unknown phenomenon similarly reported in *E. scolopes* (Hanlon et al., 1997). Additionally, rare clutches contained many unfertilized eggs and aberrant jelly coats, and more work should be done to understand and improve these traits. Based on our findings, it should be feasible to maintain a culture of both *E. berryi* and *E. morsei* for several generations. Genetic diversity can be maintained by introducing wild caught individuals seasonally (February to June) when vendors in Japan are able to supply additional animals.

Prey and hunting

Activity patterns were similar to what is described for other *Euprymna* species (Hanlon et al., 1997; Nabhitabhata et al., 2005; Nabhitabhata and Nishiguchi, 2014; Sanchez et al., 2019; Drerup et al., 2020) and animals became active at "night" - emerging from the substrate and discarding the sand coat for hunting and mating. During the "day", animals bury themselves under the sandy substrate for shelter and to avoid predators (Hanlon et al., 1997; Rodrigues et al., 2010; Drerup et al., 2020). An alternating 12 hour light-dark cycle is sufficient to mimic natural diurnal cycles (Franklin et al., 2014). As for other *Euprymna*, *E. berryi* and *E. morsei* cover their body, head, and arms with sand but leave their eyes exposed (Nabhitabhata et al., 2005; Hanlon and Messenger, 2018; Sanchez et al., 2019; Drerup et al., 2020).

While adults can be fed frozen shrimp, hatchlings and juveniles require live food, similar to other *Euprymna* spp. (Choe, 1966b; Hanlon et al., 1997; Ikeda et al., 2003; Nabhitabhata and Nishiguchi, 2014; Sanchez et al., 2019). To feed hatchlings of either species, mysids can be collected from inshore locations and reared on a diet of *Artemia* spp. (Lussier et al., 1988). Hatchlings of both species attacked adult mysids often larger than themselves similar to other species of *Euprymna* (Okutani and Horita, 1987; Hanlon et al., 1997; Nabhitabhata et al., 2005; Sanchez et al., 2019). While adults were taught to spontaneously grasp frozen prey, they would sometimes ignore non-moving prey. Fully mature adult females consumed more food relative to adult males possibly due to ongoing egg production.

Hatchling behavior

For both *E. berryi* and *E.morsei*, hatching from an egg clutch occurs over a period of several days. *E. berryi* settled and established a benthic lifestyle shortly after hatching in agreement with (Choe, 1966a) and similar to *Euprymna tasmanica* (Nabhitabhata and Nishiguchi, 2014) and *Eumandya parva* (Sanchez et al., 2019). *E. berryi* and *E. morsei* exhibited a brief nektobenthic paralarval stage similar to what is described for *Euprymna hyllebergi* (Nabhitabhata et al., 2005) and unlike hatchling behavior of *Euprymna scolopes*, *Eumandya pardalota*, and *Euprymna brenneri* (Hanlon et al., 1997; Sanchez et al., 2019) which displayed surface swimming phototaxic paralarval stages the first month after hatching (Table 2). In our culture, *E. berryi* and *E. morsei* could consume prey within 24 hours after hatching, two days earlier than previously reported for *E. berryi* (Choe, 1966a).

Growth and sexual dimorphism

E. berryi and *E. morsei* followed similar growth patterns to other *Euprymna* spp., including early growth stages of *E. scolopes* (Hanlon et al., 1997) and *E. hyllebergi* (Nabhitabhata et al., 2005) (Table 2). For comparison, *E. scolopes* raised at 23°C experienced exponential growth from hatchling to 83 dph (Hanlon et al., 1997). *E. hyllebergi* demonstrated an exponential growth phase the first 30 dph when raised at 28°C (Nabhitabhata et al., 2005). *E. tasmanica* raised at 20°C experienced exponential growth from 7 to 44 dph was followed by approximately linear growth from 58 to 140 dph (Moltschaniwskyj and Carter, 2010). As with other bobtail squid, adult males of *E. berryi* and *E. morsei* can be definitively distinguished from females by their characteristically modified first left arm, the hectocotylus, which is shorter than the opposing arm and curls outward; female left and right first arms are indistinguishable (Okutani and Horita, 1987; Norman and Lu, 1997). Sexual dimorphism becomes visually evident ~90-100 dph for *E. berryi* and ~70 days for *E. morsei*, concurrent with aggressive behavior in males.

Survivorship

Both *E. berryi* and *E. morsei* recorded higher survivorship in the first 30 dph (93% and 80%) compared to the 73% survivorship reported for *E. scolopes* (Hanlon et al., 1997). Neither species exhibited long-lived pelagic paralarval stages after hatching, which could contribute to higher survivorship in captivity relative to *E. scolopes*. *E. morsei* was previously reared at

22.5°C and survived for 97 to 128 dph (Ikeda et al., 2003). Our *E. morsei* grew more slowly and lived longer, possibly due to being cultured at a lower temperature (20°C) and thus having a lower metabolic rate (Iglesias et al., 2014). *E. berryi* recorded the longest lifespan of any cultured *Euprymna* spp. (Table 2).

Reproductive maturity and mating behavior

Mating behavior is similar to what was observed in other *Euprymna* spp. without any obvious courtship behavior (Moynihan, 1983; Hanlon et al., 1997; Nabhitabhata et al., 2005; Squires et al., 2013; Sanchez et al., 2019; Drerup et al., 2020). Females stored spermatangia deposited during matings similarly to other *Euprymna* spp. (Hanlon et al., 1997; Squires et al., 2013). As females were observed laying eggs cooperatively on the same substrate simultaneously; it is necessary to separate females to track parental lineage without genotyping. Male squids were sometimes aggressive towards conspecifics; therefore, crowding should be avoided especially as squids reach sexual maturity.

Spawning

Both *E. berryi* and *E. morsei* are multiple spawners similar to other members of the genera *Euprymna* and *Sepiola* (Huang, 2006; Rodrigues et al., 2011; Squires et al., 2013). Adult females of both species laid egg clutches every few days until reaching a late senescent life stage similar to other *Euprymna* species (Hanlon et al., 1997; Nabhitabhata et al., 2005; Squires et al., 2013). Spawning events were observed within three days of shipment of wild animals, possibly stimulated by the stress of transport (Cecere and Miyashiro, 2022). Mature *E. berryi* were observed spawning fertilized clutches repeatedly over a period of 100 days at 20°C. Egg clutch morphology differs across sepiolids, and may be used to differentiate sympatric species (Sanchez et al., 2019). Similarly, we found eggs more densely packed in *E. morsei* clutches than in *E. berryi* (Figure 2A).

Concluding remarks

Protocols established for *E. scolopes* are readily adapted for *E. berry* and *E. morsei*. Some existing protocols, including *in situ* hybridization (Lee et al., 2009e), micro-CT (Kerbl et al., 2013), immunohistochemistry (Lee et al., 2009d), and hemocyte collection (Collins and Nyholm, 2010) have already been described in *E. berryi* (Gavriouchkina et al., 2022), and protocols for infection with symbiotic bacteria (Naughton and Mandel, 2012), behavioral assays, injury treatment, and electrophysiology (Howard et al., 2019) are expected to be transferable from *E. scolopes* other bobtail species.

Established cultures of *E. berryi* and *E. morsei* will allow for comparative studies among bobtail squids in the genus *Euprymna*. Genomic and transcriptomic data are publicly available for both *E. berryi* and *E. morsei* (Sanchez et al., 2019) and other related species (Sanchez et al., 2021), and a genome sequence of *E. berryi* has recently been reported (Gavriouchkina et al., 2022). The widespread distribution of *E. morsei* and *E. berryi* in conjunction with the ability to ship adults and recently spawned eggs should allow more researchers access to these model bobtail squids, and also offers opportunities to find adaptations acquired by different populations.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://figshare.com/search?q=10.6084%2Fm9.figshare.21063211.

Ethics statement

Japan has no specific regulations regarding cephalopods used for research purposes and cephalopods do not fall under the Japanese legislation 'Act on Humane Treament and Management of Animals (Ogden et al., 2016). All procedures were approved by the OIST Animal Care and Use Committee (approval ID: 2018-204). Procedures and animal cultural protocols followed the guidelines set by Directive 2010/63/EU for cephalopods (Fiorito et al., 2015) and animal welfare guidelines set by OIST Animal Care and Use Committee. Efforts were made to provide the highest quality care and reduce the suffering of animals.

Author contributions

JJ led the project and all aspects of husbandry. JJ, YH, LZ, RK, GS, and CS were involved in husbandry efforts. YH and CS were involved in obtaining wild specimens. JJ, GS, and YH identified species. JJ and CS took growth measurements. DG, FM, GS, and DR provided guidance. JJ, GS, and DR wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1039775/full#supplementary-material

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The geographic problem in cephalopod genomics

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Publications describing genomes of various cephalopod species have recently proliferated. Some papers have involved large geographic distances between the collection locality of sequenced specimens and the type locality of the presumed species. However, cryptic species have been demonstrated in many cephalopods. Therefore, even if the sequenced specimen is very similar morphologically to the species in question, the likelihood that it is a member of the species in question decreases with increasing distance from the type locality. An associated problem is that many publications do not provide information adequate to determine the source locality for the genomic sequence. We reviewed a decade of literature on mitochondrial genomes of cephalopods and found a total of 43 publications containing 48 species within 23 genera. Of the 48 species, only 17 could be evaluated for our geographic question. Distances between sampling locality and type locality of the named species ranged from 0 nautical miles (sampled at type locality) to half-way around the world. Where data were present for distance calculation, the average for the 17 species was 3785 km (2044 nmi).

KEYWORDS

biogeography, genomics, species complex, type locality, sampling

Introduction

Determination of genetic sequences has revolutionized understanding of evolutionary relationships. Increasingly sophisticated methods have allowed this revolution to progress greatly throughout the last few decades to include inferences about entire genomes. Accordingly, the literature describing cephalopod genomes, especially those of the mitochondria, has increased greatly over the past 10 years (O'Brien, 2018). The primary goal of most of these publications has been to resolve phylogenetic relationships within extant Cephalopoda.

Another result of widespread use of genetic sequencing, including "barcodes" and other sequences shorter than entire genomes, has been an increasing recognition that

species with distributions once considered to be very broad or even global were actually complexes of morphologically similar species with geographic ranges resembling a patchwork within the broad range of the species complex. Some examples include taxa within the families Sepiolidae (Fernandez-Alvarez et al., 2021), Loliginidae (Sales et al., 2017), Chtenopterygidae (Escanez et al. (2018), Ommastrephidae (Fernandez-Alvarez et al., 2020; Xu et al., 2020a), Spirulidae (Hoffmann et al., 2021), and Octopodidae (Avendano et al., 2020; Amor and Hart, 2021). Because of these species complexes, both currently recognized and possibly to be discovered in the future, a substantial potential exists for misidentification of specimens collected for genomic sequencing (e.g., Lima et al., 2017; Salvi et al., 2021). This misidentification potential is especially true if the genomic specimen is not collected within the normal range of nominal sequenced species (i.e., named based on morphological identification). We are concerned that authors, using specimens from the nearest convenient area to sample a presumed species or from sources where the actual collection locality cannot be verified (e.g., fish markets, aquarium dealers), could be using a different species than what they report and, as a result, sequences in genomic databases may be misidentified.

Materials and methods

We surveyed the past decade of genomic literature on Cephalopoda for comparison of collection locality with designated type localities of the nominal species to determine the extent of this potential problem. Only publications describing complete mitochondrial genomes were analyzed. Each publication was examined to determine the collection locality of the specimen used for genomic analysis. Type localities for the nominal species are available online. We converted both sample locality and type locality to latitude/longitude and then calculated distance between them using NOAA Latitude/Longitude Distance Calculator (https://www.nhc.noaa.gov/gccalc.shtml). The repository and accession numbers for published genome sequences were also recorded (Table 1).

TABLE 1 Mitochondrial genome sequences for cephalopods in recent literature.

Species	Reference	Genome repository	Locality of specimen	Type locality of species	Separation*
Nautilus pompilius	Wang et al., 2018	GenBank KY794928	Not given	Ambon Island, Indonesia	n/a
Sepia officinalis	Akasaki et al., 2006	GenBank AB240155	Tsukiji Fishery Market, Japan	"Oceano" [NE Atl. O.]	n/a
Sepia aculeata	Guo et al., 2016	GenBank KF690633	Not given	Java, Indonesia	n/a
Sepia apama	Kawashima et al., 2013	GenBank AP013073	Not given	Port Adelaide, Australia	n/a
Sepia esculenta	Yokobori et al., 2007	DDBJ genbank AB266516	Tsukiji Fishery Market, Japan	Yokohama Fishery Market, Japan	n/a
Sepia latimanus	Kawashima et al., 2013	GenBank AP013074	Not given	Port Dorey, New Guinea	n/a
Sepia latimanus	Lu et al., 2019	GenBank MK347498	Naozhou, China	Port Dorey, New Guinea	**1890**
Sepia lycidas	Kawashima et al., 2013	GenBank AP013075	Not given	Canton Fishery Market, China	n/a
Sepia lycidas	Guo et al., 2018	GenBank KJ162574	Zhanjiang fishing grounds, SE China	Canton Fishery Market, China	n/a
Sepia pharaonis	Kawashima et al., 2013	GenBank AP013076	Not given	Gulf of Suez, Red Sea	n/a
Sepia pharaonis	Song et al., 2021	ERZ1300763	Ningbo City fishfarm, China	Gulf of Suez, Red Sea	n/a
Sepia pharaonis	Wang et al., 2014	GenBank KC632521	Not given	Gulf of Suez, Red Sea	n/a
Metasepia tullbergi	Lee et al., 2021	GenBank MT974497	NE Taiwan	Nagasaki, Japan	**632**
Sepiella inermis	Wang et al., 2015	GenBank KF040369	Not given	Bombay [Mumbai], India	n/a
Sepiella maindroni	Zheng et al., 2016	GenBank KR912215.1	Not given	Pondichery, India	n/a
Semirossia patagonica	Kawashima et al., 2013	GenBank AP012226	Not given	Portland Bay, Patagonia	n/a
Spirula spirula	Strugnell et al., 2017	GenBank KU893141	Queensland, Australia	America	n/a
Loligo beka	Jiang et al., 2016e & 2018	GenBank KT254309	30.1oN 122.4oE, E. China Sea	Kojima Bay, Japan	**585**
Loligo chinensis	Jiang et al., 2017b & 2018	GenBank KT362380	30.1oN 122.4oE, E. China Sea	Canton Fishery Market, China	n/a
Loligo duvauceli	Jiang et al., 2016a & 2018	GenBank KR051264	30.1oN 122.4oE, E. China Sea	Syntypes India & Sumatra	n/a
Loligo edulis f. budo	Takemoto & Yamashita, 2012	GenBank AB675081	Multiple locations	Multiple locations, Japan	n/a
Loligo edulis f. kensaki	Takemoto & Yamashita, 2012	GenBank AB675080	Multiple locations	Multiple locations, Japan	n/a
Loligo japonica	Jiang et al., 2017b & 2018	GenBank KU568467	Hakodate, Japan	Yokohama Fishery Market, Japan	n/a

(Continued)

TABLE 1 Continued

Species	Reference	Genome repository	Locality of specimen	Type locality of species	Separation*
Loligo opalescens	Jiang et al., 2016d & 2018	GenBank KP336703	30.1oN 122.4oE, E. China Sea	Puget Sound, Washington, USA	**4975**
Uroteuthis chinensis	Xu et al., 2020b	GenBank MN687903	Minnan–Taiwan Bank	Canton Fishery Market, China	n/a
Loliolus (N.) uyii	Jiang et al., 2016b & 2018	GenBank KP265013	30.1oN 122.4oE, E. China Sea	Kagoshima Bay, Japan	**432**
Sepioteuthis lessoniana	Akasaki et al., 2006	GenBank AB240154	Tsukiji Fishery Market, Japan	Not designated	n/a
Watasenia scintillans	Akasaki et al., 2006	GenBank AB240152	Tsukiji Fishery Market, Japan	Misaki[?], Japan	n/a
Watasenia scintillans	Hayashi et al., 2016	GenBank KJ845633	Toyama Bay, Japan	Misaki[?], Japan	n/a
Chiroteuthis picteti	H. Kim et al., 2018	GenBank MG833837	east sea of Korea	Ambon Island, Indonesia	**2450**
Bathyteuthis abyssicola	Kawashima et al., 2013	GenBank AP012225	Not given	46o16'S 48o27'E, Southern Ocean	n/a
Thysanoteuthis rhombus	Tang et al., 2021	GenBank MT733875	South China Sea	Strait of Messina, Sicily	**5070**
Illex argentinus	Jiang et al., 2016c	GenBank KP336702	Not given	Patagonia, 39oS 55oW	n/a
Todarodes pacificus	Akasaki et al., 2006	GenBank AB240153	Tsukiji Fishery Market, Japan	Hokodate, Japan	n/a
Sthenoteuthis oualaniensis	Xu et al., 2020c	GenBank MT661575	17o59'N 111o59'E, China Sea	Oualan Island, Caroline Islands	**3080**
Vampyroteuthis infernalis	Yokobori et al., 2007	DDBJ genbank AB266515	Ogasawara Island Chain, Japan	1o56.7'S 7o40.6'E, Atlantic Ocean	**7715**
Amphioctopus aegina	Zhang et al., 2017	GenBank KT428877	Haikou Fishery Market, China	Not designated	n/a
Amphioctopus fangsiao	Lashari et al., 2020	GenBank MF029678-029691	9 separate localities in China	Japan	n/a
Amphioctopus marginatus	Tang et al., 2018	GenBank KY646153	Haikou Fishery Market, China	Kamae, Oita Prefecture, Japan	n/a
Amphioctopus neglectus	Tang et al., 2019	GenBank MF447873	Nanning Fishery Market, China	Ko Phuket, Thailand	n/a
Amphioctopus rex	Tang et al., 2019	GenBank MF447874	Wenzhon Fishery Market, China	Ko Food, Trat Province, Thailand	n/a
Octopus bimaculatus	Dominguez et al., 2016	GenBank KT581981	N. Gulf of California, Mexico	Syntypes; 3 localities	n/a
Octopus conispadiceus	Ma et al., 2016	GenBank KJ789854	Haishenwai, Amur Bay, Russia	Sapparo Fishery Market, Japan	n/a
Octopus dollfusi	Yan et al., 2018	GenBank KX108697	Zhanjiang, Guangdong, China	"Indochina"	n/a
Octopus fitchi	Magallon-Gayon et al., 2020	GenBank MK450541	Bahia Magdalena, Mexico	N. Gulf of California, Mexico	**990**
Octopus minor	B. Kim et al., 2018	SRA database SRX3462978	Not given	Suruga Bay, Japan	n/a
Octopus minor	Cheng et al., 2012	GenBank HQ638215	Weihai, Shandong Province, China	Suruga Bay, Japan	**785**
Octopus mimus	Magallon-Gayon et al., 2020	GenBank MN078094	Zihuatanejo, Guerrero, Mexico	Iquique, Chile	**2925**
Octopus ocellatus	Akasaki et al., 2006	GenBank AB240156	Tsukiji Fishery Market, Japan	"China Sea"	n/a
Octopus sinensis	Li et al., 2021	GenBank MT712046	Zhoushan, China	Oyano Island, Ariake Sea, Japan	**446**
Octopus vulgaris	Zarrella et al., 2019	Not listed	Bay of Naples, Italy	"Mediterranean Sea"	n/a
Cistopus chinensis	Cheng et al., 2013	GenBank KF017606	coastal Xiamen, China	Xiamen, China	**0!!**
Cistopus taiwanicus	Cheng et al., 2013	GenBank KF017605	"coastal Taiwan"	Miaoli, Taiwan	**close+/-**
Hapalochlaena fasciata	Kim et al., 2020	GenBank MT497543	Southern coastal Korea	Port Jackson, Australia	**1165**
Hapalochlaena maculosa	Morse et al., 2018	Not listed	8 South Australia localities	"Australia"	n/a
Argonauta argo	Hirota et al., 2021	DDBJ genbank LC596061	Oki Island, Sea of Japan	Syntypes; Red Sea + Mediterranean	n/a
Argonauta hians	Chiu et al., 2018a	GenBank KY649285	Kenting, Taiwan	Ambon Island, Indonesia	**1600**
Tremoctopus violaceus	Chiu et a.l, 2018b	GenBank KY649286	Taiwan	Not designated	n/a

Approx. distance between specimen location and type locality calculated using https://www.nhc.noaa.gov/gccalc.shtml Separation* is calculated as nmi. (1nmi = 1.852 km). Shaded boxes: data absent or too general to be analyzed.
Bold numbers in the last column highlight the publications for which included information was adequate for distance calculation.

Results

An online search of the previous ten years of Cephalopoda genomic literature found a total of 58 genomic descriptions within 43 publications containing 48 different species in 23 genera (Table 1). For many species sequenced (70%), either collection locality or type locality (from the original description) was missing or was too general (e.g., Australia). In addition, if either locality was indeterminate (e.g., Tsukiji fishery market); or there were multiple type localities (ex. syntypes); or the genome was derived from combined specimens from multiple localities, the sequence was not included in our distance analysis.

Of the 48 species sequenced, only 17 could be evaluated for our geographic question (Table 1). Distances calculated ranged from 0 km (sampled at type locality) to half-way around the world in a different ocean basin. The average distance between sampling locality and type locality for the 17 species for which data were adequate for distance calculation, was 3785 km (2044 nm).

Incidentally, as we reviewed this literature for geographic information, we also noticed that very few of the publications included any indication that voucher specimens or unprocessed tissue were preserved in established archival collections for future research. For example, of the 17 species mentioned above, only 5 (29.4%) had vouchered specimens. Thus, 10.4% of species accounts included both adequate geographic information and archived specimens.

Discussion

Our point here is not that any of these publications is wrong. Rather, we want to highlight the potential for taxonomic errors in publications where the sampling area is very distant from the species' type locality. As pointed out by one of the reviewers, for coastal cephalopod species in complex habitats, such errors are possible even at very small distances. Any taxonomic error introduced by this geographic mismatch may be compounded when the sequence is archived in a genomic database and the database is used for other investigations.

We therefore recommend selection of specimens for genomic sequencing collected from as close to the type locality of the species as possible. Although we recognize that it may not always be possible to sample the type locality, we recommend that the genomic sample be from the same biogeographic province (e.g., GOODS, 2009 or subsequent modifications by various authors) or "Large Marine Ecosystem" (LME – Sherman and Duda, 2011) as the type locality. The collecting locality should always be included in any publication resulting from DNA sequencing. Furthermore, specimens should not be selected for sequencing from a source where the actual collecting locality cannot be determined confidently (e.g., not from fishery landings, etc.). Also, although our primary purpose here is to highlight the need for sequenced specimens to come from as close to the type locality as possible, we also recommend that specimens sequenced and any unprocessed tissue be vouchered in an established archival collection. Relevant information about archived material (e.g., museum catalogue number) should be included in resulting publications.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the animal study because this is a literature review. No animals (live or preserved) were used.

Author contributions

MS conceived the idea. MV and MS analyzed the data. PR accumulated the references. MV wrote the first draft. All authors contributed to the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Vessel sound causes hearing loss for hummingbird bobtail squid (*Euprymna berryi*)

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Anthropogenic activity and its associated sounds have been shown to incur adverse effects on the behaviour and physiology of a wide range of aquatic taxa, from marine mammals to fishes. Yet, little is known about how invertebrates detect and respond to anthropogenic sound. The hummingbird bobtail squid (*Euprymna berryi*) has a short lifespan (< 6 months), grows to sexual maturity around 90 days post hatching and its small size (< 5 cm mantle length) makes the species an ideal candidate to examine potential effects of sound exposure under laboratory conditions. Hearing and behavioural observations were made before, during and after 15 minutes of vessel sound playback, and aural sensitivity curves were determined using auditory evoked potentials. A significant decrease in relative ventilation rate was observed during and post sound exposure. Auditory sensitivity before and after vessel sound exposure was also examined for three different ages: juveniles, mid- and late adults. Baseline audiograms indicated that there was a decrease in aural sensitivity with age. All three age groups showed similar, significantly decreased hearing sensitivity following sound exposure, however auditory sensitivity recovered within two hours. Globally, anthropogenic sounds have become louder and more persistent, therefore there may be limited time for these animals to recover from sound exposure. Given their ecological and economic importance, cephalopods should be considered in management and policy on underwater noise owing to potential adverse effects of anthropogenic sound on behaviour and physiology.

KEYWORDS

cephalopods, hearing, underwater sound, noise pollution, threshold shift, *Euprymna berryi*

1 Introduction

Underwater sound is used by aquatic life to navigate their environment, to find suitable habitats, locate food and avoid predators, as well as communicate with conspecifics. In recent years, animals have been exposed to increasing amounts of anthropogenic sound which may negatively affect their behaviour (e.g., foraging, movements, predator/prey

interactions and mating (Myrberg, 1990; Shannon et al., 2016; Erbe et al., 2018) and physiology (e.g., heart rate, oxygen consumption and hearing)(Williams et al., 2015; de Soto and Kight, 2016) . However, most bioacoustics research has focussed on marine mammals and fishes, while the extent to which sound produced by human activity might be affecting invertebrates has been largely overlooked (Hawkins et al., 2015; Popper and Hawkins, 2018).

Ecologically, cephalopods use sound as they occupy many of the same niches as other acoustically sensitive fishes. Shipping, geophysical activity and construction all produce low frequency (< 1000 Hz) sound, which may overlap the hearing sensitivity of cephalopods, contributing to masking of biologically relevant stimuli or inducing acoustic trauma (Mooney et al., 2010) . Behavioural reactions to a sound stimulus can range from exhibiting a momentary awareness of the sound, to small movements, or escape responses (Hawkins et al., 2015). Cephalopods occupy a key position in the food web (Boyle and Rodhouse, 2008: and if their population declines or migrates, for example to avoid a noisier environment, disruptive effects on the trophic structure potentially could occur (e.g., Tyack et al., 2011). Sound exposures may also induce physiological damage and more severe impairments to function and fitness such as permanent or temporary hearing loss (Hawkins et al., 2015).

Cephalopods possess hair filled statocysts that regulate equilibrium and balance, and these organs have been associated with sound detection (Offutt, 1970; Budelmann, 1990; Williamson, 1995). Each statocyst contains three lobes positioned in the x, y and z planes, which contain heavily innervated hair cells coupled to the statolith, a dense particle within the statocyst (Budelmann, 1990; André et al., 2011). During underwater sound wave propagation, regions of compression and rarefaction generated by local particle motion are produced in conjunction with pressure fluctuations. The statocyst detects sound through the differential displacement of the heavier statolith in contrast to the surrounding tissues and endolymph (Budelmann, 1992). The statocyst is further divided into a macula that aids orientation, and a crista-cupula that acts as an angular accelerometer (Budelmann, 1990; Mooney et al., 2010).

Auditory evoked potentials (AEPs) have been employed to investigate the auditory responses of animals that are challenging to study *via* conventional psychophysical experiments, such as fishes and invertebrates (Higgs and Radford, 2016; Sisneros et al., 2016). AEPs reflect synchronous neural activity as afferent responses are conducted from the auditory end organ to higher centres (Burkhard et al., 2007). It was recently determined that adult cephalopods can detect low frequency vibrations using the AEP technique. *Sepiotethis lessoniana* (bigfin reef squid) and *Octopus* sp. (common octopus) were sensitive to frequencies between 400 and 1600 Hz (Hu et al., 2009), while *Dorytheuthis pealeii* (longfin squid) showed auditory sensitivity to frequencies between 30 and 500 Hz, with the lowest threshold between 100 and 200 Hz (Mooney et al., 2010). A behavioural study on *D. pealeii* also showed sensitivity to 200 - 400 Hz sound (Mooney et al., 2016). The modality and frequency range of hearing suggests that squid probably detect acoustic particle motion stimuli from both predators and prey, as well as low frequency environmental sound signatures that may aid navigation (Mooney et al., 2010). However, these studies focussed solely on adults, and it is unknown to what extent newly hatched and juvenile cephalopods use sound. In other marine invertebrates, juveniles are often hypersensitive to external stimuli or stressors. For larval crustaceans, sound plays an important role for juveniles to orientate and settle on suitable reef habitat (Montgomery et al., 2006; Stanley et al., 2012). Squid statocysts are fully formed in hatchlings (Hanlon et al., 1983) indicating that cephalopods may have the capacity for sound sensitivity from an early age.

The hummingbird bobtail squid (*Euprymna berryi*) has a short lifespan (< 6 months), grows to sexual maturity around 90 days post hatching and its small size (< 5 cm mantle length) makes the species an ideal candidate for examining the ontogeny of cephalopod hearing. Additionally, the benthic *E. berryi* inhabit shallow waters (< 20 m) throughout the Indo-Pacific, preferring sandy or fine sediment substrate where they bury themselves during the day to avoid predation. The benthic lifestyle may make *E. berryi* more susceptible to the negative effects of sound exposure than mobile species that can swim away from the source, especially as anthropogenic sounds often peak during daylight hours.

The aims of this study were to determine, under laboratory conditions, the auditory sensitivity of the hummingbird bobtail squid (*E. berryi*) and to investigate the potential impacts of noise on the behaviour and auditory sensitivity of different life stages.

2 Methodology

2.1 Animal husbandry

Hummingbird bobtail squid (*E. berryi*) were cultured at the Marine Biological Laboratory's Cephalopod Breeding Centre in Woods Hole, MA to three different ages [juvenile 45 – 60 days (n = 16); mid adult 90 – 100 days (n = 15); late adult 125 – 140 days (n = 14)] for the experiments (Table 1). These age groups were

TABLE 1 Number of animals used for the behavioural and hearing experiments (baseline; noise exposure; recovery).

Age group	Number of experimental animals				Age range (days)
	Baseline	Noise exposure	Recovery*	Total	
Juvenile	8	8	3	16	45 – 60
Mid adult	6	9	2	15	90 – 100
Late adult	8	6	–	14	125 – 140

*Recovery animals (tested 2 hours post exposure) were a subset of the individuals used for noise exposure.

chosen to reflect pre and post sexual maturity (juvenile and mid adult) as well as potential senescence (late adult).

All squid were housed in tanks on a semi-open system where natural seawater was conditioned (mechanically, chemically, and biologically filtered) and maintained at 24°C, 8.5 pH, 32 ppt salinity, 0 ppm ammonia/nitrite and< 10 ppm nitrate. Each age group was housed separately in holding tanks that measured 21.5 cm x 11.0 cm for juveniles, 45.5 cm x 23.5 cm for mid-adults and 44.0 cm x 76.0 cm for late adults. The sound pressure level in the holding tanks was recorded using a hydrophone (Soundtrap300; Ocean Instruments, NZ, sampling rate 24 kHz), and the median root mean squared sound pressure level was 80 dB re. 1µPa (10 Hz – 12 kHz).

All experiments were conducted between June 1st and September 2nd, 2022, in a rectangular fibreglass experimental tank (56 x 40 x 30 cm). The experimental tank was enclosed within an acoustic isolation box (76 x 68 x 66 cm) covered on all sides with 2.2 cm thick insulating foam panels to reduce background sound and placed on a vibration isolation table (TMS Vibration Control 63-543 = 122 x 76 cm) to minimise vibrations. All trials were conducted during the daytime, and squid were tested individually for each trial.

This animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the Marine Biological Laboratory, Woods Hole, MA, USA protocol number 22-13F, in compliance with the EU Directive 2010/63/EU on cephalopod use and AAALAC guidelines on the care and welfare of cephalopods (Fiorito et al., 2014, 2015; Lopes et al., 2017).

2.2 Behavioural observations

To assess changes in behaviour in relation to sound, video recordings were taken of unanaesthetised baseline and sound exposed individuals (Table 1). On the day of the experiment, individual squid were hand-transferred from their holding tanks into the experimental tank and placed inside a soft mesh arena (10 x 10 x 4 cm) where individuals remained in the centre of the tank. Squid were allowed to acclimate to the experimental tank for five minutes (preliminary trials showed this time was sufficient as squid remained quiescent). A high-resolution camera (PixeLINK PL-E533CU, Pixelink, Ontario, Canada) was positioned 28 cm above the experimental tank to observe behaviour under an infrared light (850 nm, S75, SmartVision) with viewing field encompassing the entire 10 x 10 cm experimental arena (Figure 1). PixelLinkCapture software (PixeLINK, Ontario, Canada) was used to capture video recordings at 19 frames per second at a resolution of 1980 x 1020 pixels.

Baseline individuals were observed *via* video recordings for 15 minutes with no sound exposure. Sound exposed animals were recorded for three different 15-minute sessions before, during and after presentation of vessel sound. Vessel sound [sound pressure (re 1 µPa) = 150 dB; particle acceleration (re. 1 ms^{-2}) = -8.6 dB (both measured between 100 and 1000 Hz)] was emitted from the underwater speaker (UW30; Lubell Labs Inc, USA) submerged in the centre of the experimental tank, 16 cm below the squid. Sound exposure level was limited by the maximum capacity of the underwater speaker however, the exposure level was deemed suitable given that vessel source levels have been documented up to 195 dB in the field (McKenna et al., 2013). The exposure duration was also designed to replicate exposure to anthropogenic sound in the natural environment. Audio files of the vessel [an idling 15 m research vessel (Detroit Diesel 12 V-71 engine; power output: 7 – 1193 kW; single screw)] sound used for exposure experiments were recorded on a hydrophone (Soundtrap 300; Ocean Instruments, NZ). The hydrophone was positioned midwater (water depth 2 m) ca. 1 m away from the propellor of the research vessel while it was idling dockside (Mackiewicz et al., 2021).

Behaviours of the mid- and late-adult animals (baseline and sound exposure individuals) were observed by the author *via* the video recordings *post hoc*. No jetting, inking or colour change was observed from any of the animals. The number of breaths per minute was recorded using a manual hand-held counter (respiration rates provided in Supplementary Tables 1, 2). Individual's ventilation rate (breaths per minute) was divided by the average value of the 15 minutes of its pre baseline counts to provide a relative value for comparing between the different age groups and different exposure conditions (before, during and after sound exposure). Juveniles underwent the same experimental sound exposure treatments. However, due to equipment availability, video was not recorded for juvenile behavioural observations.

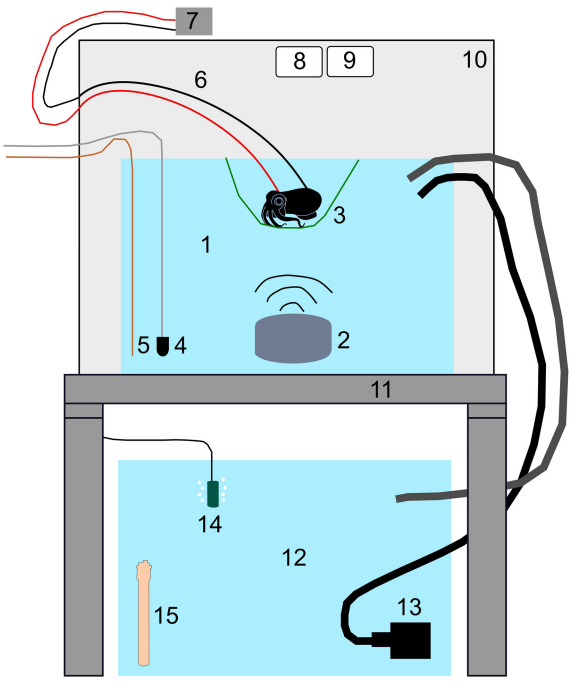

FIGURE 1
AEP experimental tank setup (not to scale). 1: Experimental tank; 2: Underwater speaker (Lubell Labs UW-30); 3. Mesh sling with animal suspended 4cm below water surface; 4: Hydrophone (B&K 8103); 5. Ground for experimental tank; 6. Recording and reference electrodes; 7. Headstage (Dagan); 8. Camera (Thorlabs); 9. IR light; 10. Acoustic isolation chamber; 11. Vibration isolation table (TMS Vibration Control); 12. Sump tank; 13. Pump and associated tubing; 14. Bubbler; 15. Heater.

2.3 Auditory evoked potential recordings

Immediately after the initial behavioural observations, auditory evoked potential (AEP) recordings were used to measure the hearing sensitivity of the baseline and sound exposed individuals across the same three different age groups (juvenile, mid-adult, and late-adult; Table 1). To examine potential recovery of auditory sensitivity, a subset of the sound exposed individuals were re-tested 2 hours post sound exposure. All AEP recordings were performed in the same rectangular fiberglass experimental tank used for behavioural observations (Figure 1).

Following behavioural observations, squid were anesthetised by immersion in a bath of $MgCl_2$ solution [7.5% (75 g dissolved in 1 L distilled water) mixed with home tank sea water in a ratio of 1:3 $MgCl_2$: sea water]. $MgCl_2$ has been identified to have no effect on cephalopod evoked responses and allows the animals to continue breathing (Messenger et al., 1985; Preuss and Budelmann, 1995; Mooney et al., 2010). State of anesthesia was confirmed once the individual stopped responding to a physical stimulus (a gentle pinch with a pair of blunt forceps on the mantle). Additional signs of anesthesia included slowed respiratory rate, pale colour, and loss of sucker attachment.

Once sedated, animals were moved from the anaesthetic bath back into the experimental tank. Inside the experimental tank, the individual was supported by a nylon mesh sling 16 cm directly above the underwater speaker at a fixed position of 4 cm water depth. Briefly, the AEP procedure entailed placing two subdermal electrodes (Rochester Electro-Medical Inc., USA) just under the skin of the animal following the methodology of Mooney et al., 2010. The recording electrode was placed at the posterior margin of the head, medial to the statocyst, using a micromanipulator (World Precision Instruments; M3301-M3). The reference electrode was inserted in the muscle of the cephalopod's body, at least 1 cm away from the head. Only the tips of the electrodes were uncoated to allow for evoked potential recordings from the desired location. The rest of the electrode and wire leads were waterproofed to allow for recording underwater.

Pure tone stimuli (50 ms duration; 500 repetitions; 3 ms silence between repetitions) between 100 and 1000 Hz were presented to the animals *via* an underwater speaker in the experimental tank; the sound output was monitored in real time using an oscilloscope (Tetronix, USA). Individuals were tested in 100 Hz increments to produce an audiogram. For the sound stimulus, a programmable attenuator (CED 3505; Cambridge Electronics Design, UK) and biological amplifier (AS-35; Accusonic Corp, Canada) controlled sound amplitude in 3 dB re. 1 µPa steps up to the maximum sound level of the underwater speaker (150 dB re. 1 µPa sound pressure level, -8.6 dB re. 1 ms^{-2} particle acceleration).

It is important to note that the difference between sound pressure and particle motion cannot be easily predicted in small tanks (Nedelec et al., 2016; Sisneros et al., 2016; Jézéquel et al., 2022). Therefore, prior to the start of each AEP trial, particle motion and sound pressure were calibrated at the position normally occupied by the experimental animal to allow audiograms to be produced for the two measures. Particle motion was measured using a tri-axial accelerometer (PCB Piezotronics Inc., USA; sensitivity: X = 10.47 mV ms^{-1}; Y = 10.35 mV ms^{-1}; Z = 10.29 mV ms^{-1}, frequency response 0.5 – 2000 Hz), modified to be neutrally buoyant using a polystyrene float to counterbalance the weight of the device, connected to a signal conditioner (482C; PCB Piezotronics Inc., USA). The accelerometer was placed such that its x-axis corresponded to the anterior-posterior, the y-axis to the left-right, and the z-axis to the dorsal-ventral positions. For a given frequency, particle acceleration measurements were made across the corresponding sound intensity range throughout the attenuation range. Sound pressure was measured using a hydrophone (8103; Bruel and Kjaer, Denmark) connected to an amplifier (Nexus Conditioning Amplifier 2692-01s; Bruel and Kjaer, Denmark). All data were recorded using Power Lab data acquisition system and analysed offline as the voltage root mean square (V_{rms}) using LabChart software (Version 8, AD Instruments, USA).

V_{rms} values measured with the hydrophone were converted into dB_{rms} (Equation 1).

$$dB_{rms}\ re\ 1.\mu Pa = 20\log_{10}(V_{rms}) \qquad \text{Equation 1}$$

V_{rms} values for each axis (X, Y and Z) of the particle accelerometer were calibrated to the sensitivity of the accelerometer and used to calculate the magnitude of particle acceleration in dB scale (equation 2) (Vetter et al., 2015, 2018; Nissen et al., 2019).

$$dB_{rms}\ re.\ 1ms^{-2} = 20\log_{10}(\sqrt{X^2 + Y^2 + Z^2}) \qquad \text{Equation 2}$$

During the AEP recordings, signals from the implanted electrodes were amplified with a headstage (gain = 10 x) connected to an extracellular amplifier (gain = 100 x; EX1; Dagan Corporation, Minneapolis, MN, USA), with a 0.05 to 10.0 kHz band pass filter. A data acquisition system (micro3, 1401; Cambridge Electronic Design Ltd., UK) administered sound stimuli and a custom Spike 2 script (Cambridge Electronic Design; UK) collected and averaged responses.

The presence of an AEP was initially assessed visually by observation of the characteristic double frequency AEP wave (Maruska et al., 2007). Secondly, the presence of an AEP was verified quantitatively by fast Fourier transform (FFT) power spectrum analysis (1024 pt, Hanning window) of the averaged waveform response. The auditory threshold was defined as the lowest sound intensity that elicited an observable and repeatable AEP, with the presence of a significant peak (FFT level ≥ 0.001 µV) at the second harmonic of the stimulation frequency (Egner and Mann, 2005; Higgs and Radford, 2016; Bhandiwad et al., 2017) because of the opposed orientation of the hair cells (Budelmann, 1990; Ladich and Fay, 2013; Sisneros et al., 2016) (Figure 2).

At the conclusion of each AEP experiment, individuals were euthanised with an overdose of $MgCl_2$ solution (1:1 dilution of 7.5% $MgCl_2$ in home tank seawater). Individuals remained in the solution for a minimum of 10 minutes following the cessation of respiration. A recently euthanised squid was used as a control animal to ensure all detected waveforms from the anesthetised squid were biological in origin.

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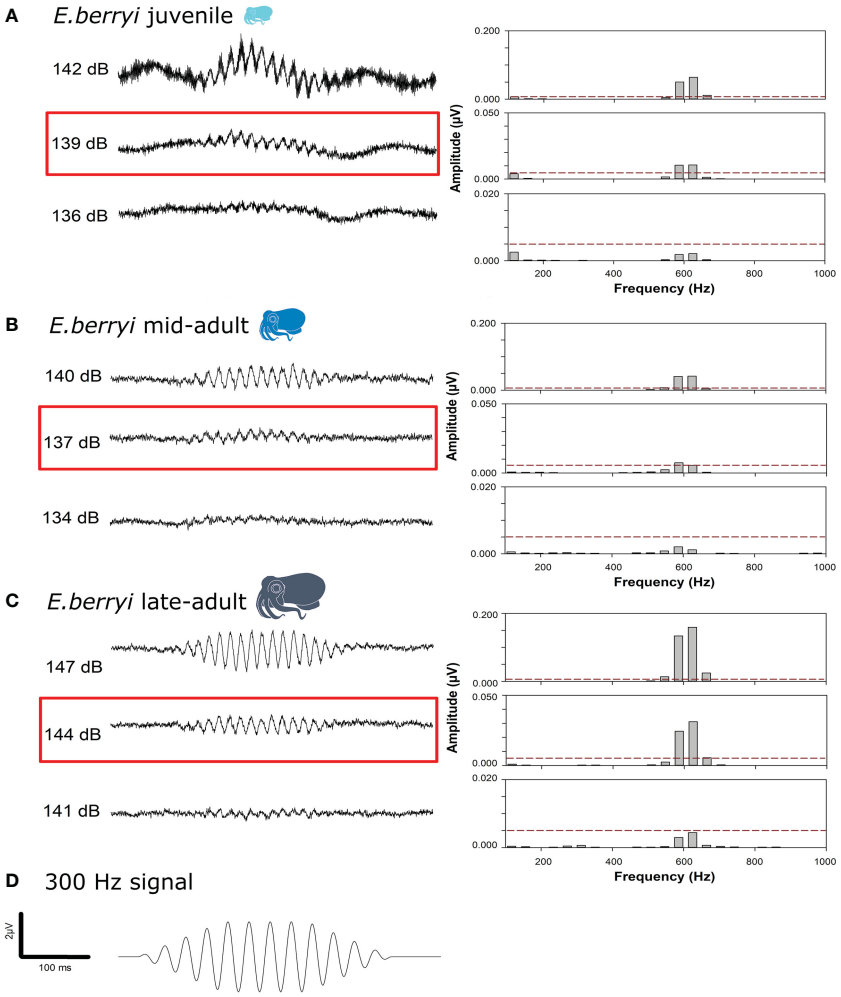

FIGURE 2
Auditory evoked potential (AEP) response for **(A)** juvenile; **(B)** mid-adult and **(C)** late-adult hummingbird bobtail squid (*E. berryi*) to **(D)** the 300 Hz signal presented. Each panel **(A–C)** displays the average AEP trace (500 repetitions) for the indicated sound pressure level (dB re. 1 µPa) on the left and the fast Fourier transformation (FFT) on the right. Please note the y axis for the FFT data varies. FFT peaks are twice the stimulus frequency (600 Hz) owing to the opposed orientation of the hair cells. AEP thresholds (0.005 µV) for each age group are highlighted in red.

2.4 Statistical analysis

All statistical analyses were performed using Sigmaplot software (Version 14). Ventilation rate data was calculated as a percentage, to determine the effects of sound exposure on the observed ventilation rates of squid; the data was arcsine transformed and analysed using a Kruskal-Wallis one-way ANOVA on ranks. A Tukey *post hoc* test determined significant differences between time periods for each age group ($\alpha = 0.05$).

Auditory evoked potential data passed normality (Shapiro-Wilk $P > 0.05$) and equal variance (Brown-Forsythe) testing therefore all sensitivity data are reported as mean ± 1 SD. To determine whether there was a difference in baseline auditory sensitivity between juvenile, mid-adult and late-adult squid, a two-way analysis of variance (ANOVA) with frequency (Hz) and age as factors, and sensitivity measurements [either sound pressure level (SPL) or particle acceleration level (PAL)] as the dependent variable was performed. A Holm-Sidak *post hoc* test determined significant differences between ages for each frequency ($\alpha = 0.05$).

Additionally, to determine the effects of sound exposure and recovery on the auditory sensitivity of squid, a two-way repeated measure ANOVA with frequency (Hz) and time (baseline, sound exposure or recovery) as factors, and sensitivity measurements (either sound pressure level or particle acceleration level) as the dependent variable was performed. A Holm-Sidak *post hoc* test determined significant sensitivity shifts from baseline for each frequency ($\alpha = 0.05$).

3 Results

3.1 Behavioural observations

Background sound levels were significantly lower, across all frequencies, than the sound exposure playback (Figures 3A, B). For example, at 300 Hz, the background sound power spectral density was 68 dB re 1 $\mu Pa^2\ Hz^{-1}$ compared to 118 dB re 1 μPa^2 for sound exposure.

The ventilation rates of mid- and late-adult squid were observed before, during and after sound exposure. There was a significant

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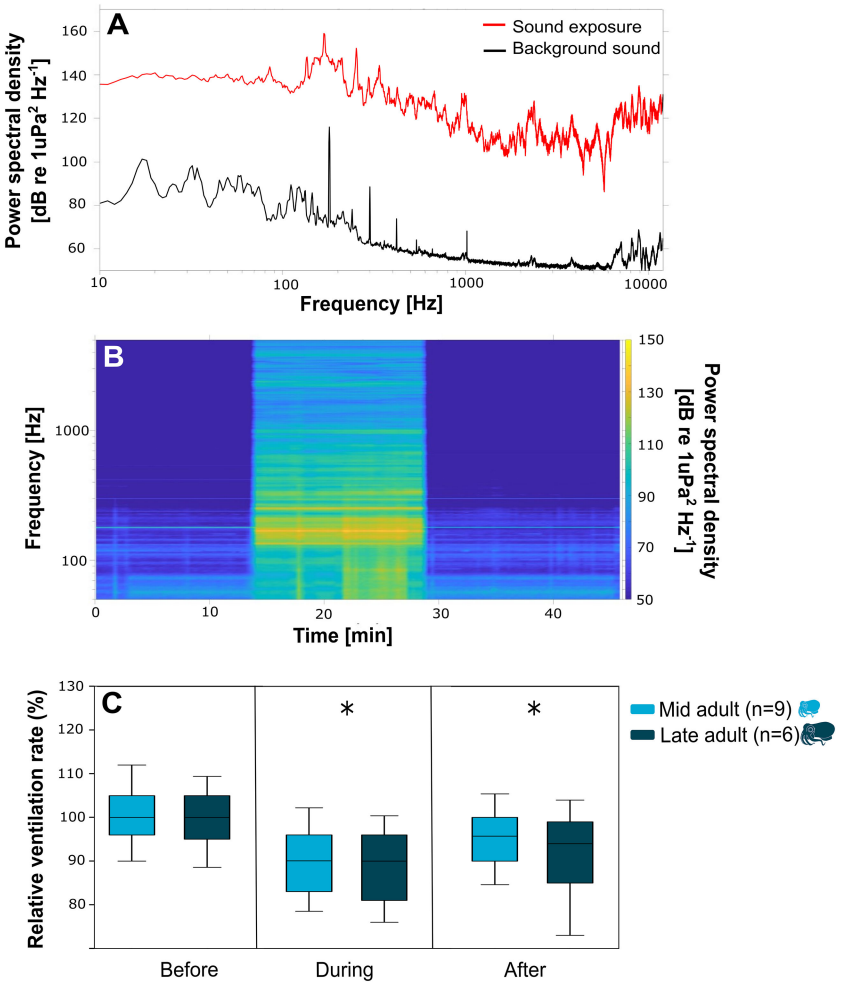

FIGURE 3
(A) Power spectral density (dB re. 1 μPa^2 Hz^{-1}) curves versus frequency for the background sound of the experimental tank (black) and sound exposure (red). **(B)** Spectrogram for the sound exposure trial versus time. **(C)** Box plots (maximum, 75%, median, 25%, minimum) for relative ventilation rates (breaths min^{-1}/average pre) of the mid adult (light blue) and late adult (dark blue) versus sound treatment (before; sound exposure, after), asterisk indicates significant difference to before.

decrease in the relative ventilation rate both during and after sound exposure compared to before levels for the two age groups tested (mid adult: one-way ANOVA, H = 80.681, d.f. = 2, P< 0.001; late adult: one-way ANOVA, H = 30.735, d.f. = 2, P< 0.001) (Figure 3C). No significant difference was found between the two ages groups observed (Figure 3C). Additionally, there was no significant difference in the relative ventilation rates between individuals within each age group (Supplementary Figure 1).

3.2 Baseline audiogram

Bobtail squid responded to all tested frequencies between 100 and 1000 Hz, and all age groups showed greatest auditory sensitivity between 300 and 500 Hz. Sensitivity decreased up to 1000 Hz and AEPs above 1000 Hz were not detectable at the maximum sound pressure levels (150 dB re. 1 μPa). There was a significant difference in both PAL (two-way ANOVA, d.f. = 2, F = 9.593, P = 0.002) and SPL (two-way ANOVA, d.f. = 2, F = 5.011, P = 0.020) auditory sensitivity curves when the different age groups were compared (Figure 4). *Post-hoc* tests (Holm-Sidak, p< 0.05) showed significant differences in PAL were observed at 200, 300, 400, 900 and 1000 Hz (Figure 4A). For example, at 300 Hz, juvenile squid were most sensitive (-32.5 ± 1.0 dB re. 1ms^{-2}), followed by mid-adult (-21.2 ± 1.9 dB re. 1ms^{-2}) and late adult (-14.5 ± 4.6 dB re. 1ms^{-2}). Significant differences were also observed between the different ages at 200 and 300 Hz when thresholds were measured in SPL (Figure 4B). For example, at 300 Hz, mid-adults were most sensitive (SPL: 135.3 ± 2.9 dB re. 1 μPa) followed by juveniles (SPL: 140.3 ± 1.2 dB re. 1 μPa) and late-adults (141.0 ± 2.9 dB re. 1 μPa). Individual SPL and PAL threshold values for all frequencies and ages tested are provided in Supplementary Tables 3, 4.

3.3 Sound exposure

Following 15 minutes of vessel sound exposure, tone pips of higher PALs were needed to evoke a response in juvenile and late

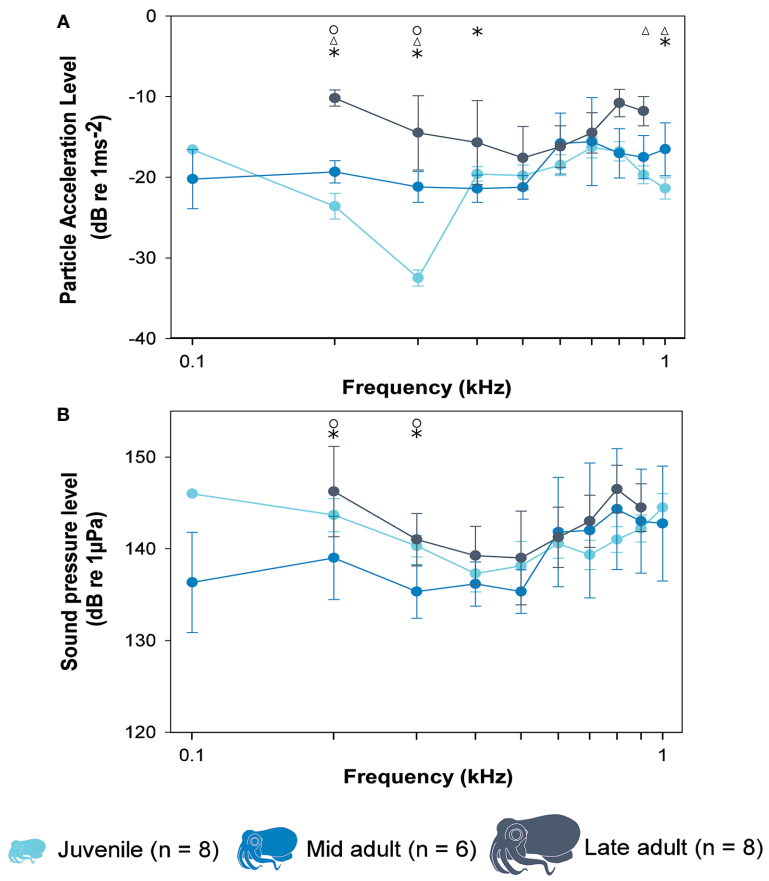

FIGURE 4
The thresholds of **(A)** particle acceleration level (dB re. 1 ms^{-2}) and **(B)** sound pressure level (dB re. 1 µPa) needed to evoke an AEP response plotted against frequency (Hz) for three different aged hummingbird bobtail squid (*E. berryi*). Data are plotted as mean ± SD. Statistical difference in sensitivity at that frequency indicated by circles (O) between juveniles and mid adults, triangles (Δ) between juvenile and late adult, and asterisks (*) between mid adult and late adults.

adults (Figure 5). This also corresponded to similar, higher SPLs for all age groups post sound-exposure. Juveniles exhibited a significant decrease in auditory sensitivity following sound exposure at 400, 500, 600 and 800 Hz in both SPL and PAL sensitivity audiograms (Figures 5A, B); whereas mid-adults had a significant decrease in auditory sensitivity for SPL audiograms at 200, 300, 400 and 500 Hz but no significant difference in PAL audiograms (Figures 5C, D). Late-adults had significant differences in auditory sensitivity for PAL at 200 Hz and for SPL at 400, 500 and 600 Hz (Figures 5E, F).

Juvenile and mid-adult bobtail squid were also tested following a two-hour recovery period after sound exposure. There was no significant difference between the baseline and recovery auditory sensitivity curves (for both SPL and PAL) across all frequencies tested (Figures 5A–D).

4 Discussion

Human activity, such as recreational and commercial shipping, often heavily utilizes the areas where many species of cephalopod, including the hummingbird bobtail squid (*E. berryi*), inhabit. In this study, auditory evoked potentials showed that *E. berryi* were sensitive between 100 – 1000 Hz to both particle motion and sound pressure stimuli, with greatest sensitivity observed in response to 300 – 500 Hz. This low frequency sensitivity matches the frequency range of both abiotic and biotic sound sources of shallow water marine environments (McKenna et al., 2021), as well as the low frequency range of vessel noise (Duarte et al., 2021). In this study exposure to short duration (fifteen minutes) and high intensity (150 dB re. 1 µPa sound pressure level, -8.6 dB re. 1 ms^{-2} particle acceleration) vessel sound was found to have significant effects on both the behaviour and auditory physiology of hummingbird bobtail squid.

For all age groups tested, auditory sensitivity was impaired following exposure to vessel sound, with a higher sound level needed to evoke a response. A change in auditory sensitivity has potential implications for cephalopods because it may reduce their ability to assess their environment, find suitable settlement habitat, avoid predators, or detect prey. Importantly, we noted that exposure to short term vessel sound caused temporary hearing loss (or temporary threshold shift – TTS) because, auditory sensitivity returned to pre-exposure baseline levels following a two-hour recovery period. The number of individuals retested following the recovery period was limited owing to the number of animals

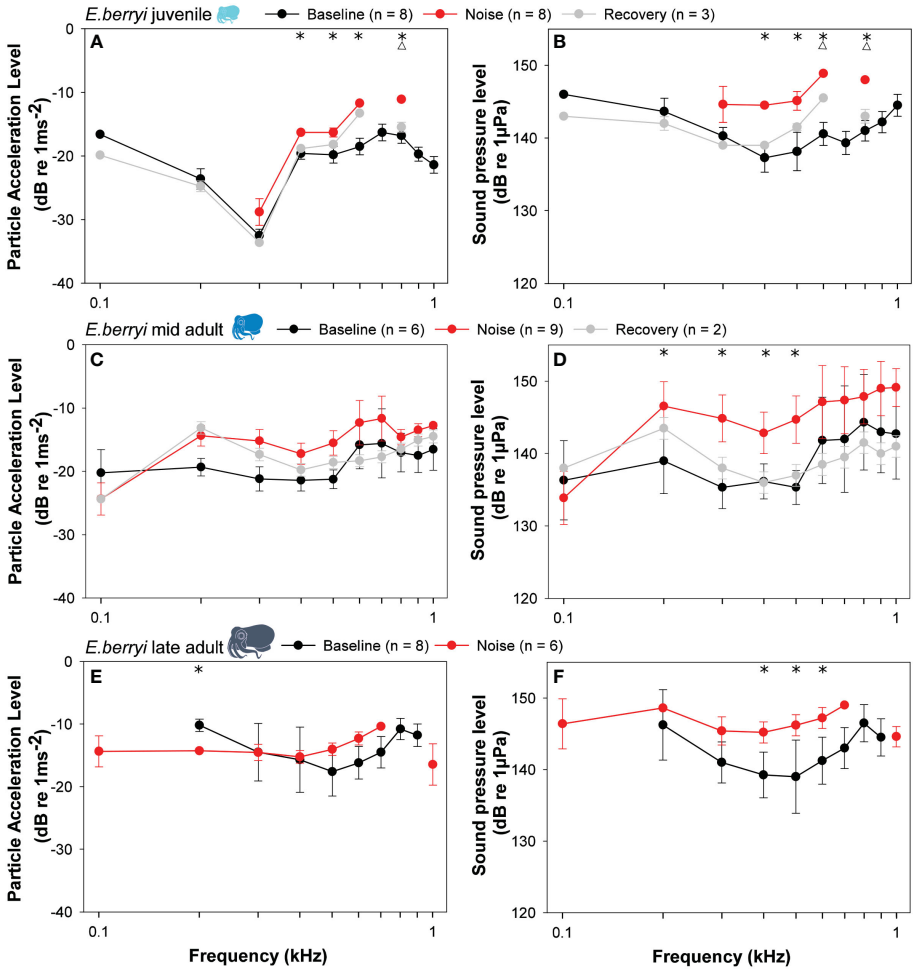

FIGURE 5
The thresholds of **(A, C, E)** particle acceleration level (dB re. 1 ms^{-2}) and **(B, D, F)** sound pressure level (dB re. 1 µPa) needed to evoke an AEP response plotted against frequency (Hz) for three different aged hummingbird bobtail squid (*E. berryi*) groups **(A, B)** juveniles; **(C, D)** mid-adult and **(E, F)** late adult. Data are plotted as mean ± SD. Statistical difference (Holm-Sidak< 0.05) in sensitivity at that frequency indicated by asterisks (*) between sound exposure and baseline, and triangles (Δ) between sound exposure and recovery.

available. Yet, the data collected provides preliminary evidence that vessel sound exposure did not cause a permanent threshold shift (PTS) where sensory hair cells were damaged beyond repair. Acoustic trauma, including lesions, hair cell loss and neuron swelling, have been reported in other cephalopod species (*Loligo vulgaris, Sepia officinalis, Octopus vulgaris* and *Illex coindetti*) following two hours of sound exposure (50 – 400 Hz, 157 ± 5 dB re 1µPa, although the particle acceleration levels were not noted) (André et al., 2011). A range of exposure durations and sound exposure levels should be tested to investigate when PTS occurs in cephalopods. Fishes can repair and replace damaged hair cells following exposure to intense sounds or ototoxic drugs (Lombarte et al., 1993; Lombarte and Popper, 1994) therefore, potential regenerative mechanisms in cephalopod models also needs to be explored.

Sound exposure had a significant effect on behaviour because the relative ventilation rate of mid and late adults significantly decreased both during and post sound exposure compared to baseline rates. Such a response could be a potential defence mechanism to avoid detection by predators (Mader et al., 2010). Anti-predator defence has been identified in many animals (Sih, 1987; Kavaliers and Choleris, 2001; Alcock, 2005), including the webfoot octopus (*Octopus ocellatus*) that suppressed its respiration when exposed to 50, 100 and 150 Hz tones (Kaifu et al., 2007). Alternatively, startle responses have been observed in cephalopods exposed to rapid-onset air gun sounds (147 - 151 dB re 1 µPa sound exposure level) (Fewtrell and McCauley, 2012). Body pattern changes, inking, jetting and startle responses (Jones et al., 2020) and fewer prey captures (Jones et al., 2021) were also observed in longfin squid exposed to impulsive pile driving sound (190 – 194 dB re 1 µPa). Sustained reductions in feeding behaviour due to anthropogenic stressors could lead to reduced survival, especially in regions with patchy prey distributions or limited prey abundance (Jones et al., 2021). However, behavioural impacts, including alarm jetting, may be short lived and have minimal impacts on squid energetics (Cones et al., 2022). It is also important to consider the effects of noise exposure on cephalopods in the context of their natural environment. The soundscape of the Indo-Pacific, where

E.berryi lives, includes sounds from fishes, marine mammals (such as the Indo-Pacific dolphin, *Sousa chinensis*) and anthropogenic activity. For example, a recent study found that vessel sound was present in up to 30% of recordings taken at ten shallow water locations (Xu et al., 2020). *E.berryi* buries in the sand during the day and emerges at night to feed. This lifestyle means that they may be exposed to anthropogenic sound regularly and repeatably throughout the daytime, compared to open water species that could leave the area during exposure. More soundscape studies are needed to confirm the sound levels that different species are exposed to as well as spatial and temporal trends in anthropogenic noise exposure. While there is some evidence of habituation to acoustic stimuli in cephalopods (Samson et al., 2016; Cones et al., 2022), most behavioural experiments on the effect of noise on aquatic life have been conducted in the laboratory where conditions can be controlled, and individuals observed. Yet, the caveats of working in a laboratory environment must be noted, as small experimental tanks have complex acoustic fields with overlapping reflection and refraction (Jézéquel et al., 2022). The sound exposure methodology used in this study was chosen to alleviate some of the complications of small tanks by placing the squid in a small arena within the experimental tank and calibrating at the location of the animal. In other studies, sound exposure chambers have been used to control the relative magnitudes of particle motion and sound pressure by placing sound projectors at either end of a steel tube (Martin and Rogers, 2008; Halvorsen et al., 2012) . It is recommended that future research efforts strive to conduct experiments in the wild because individuals may behave differently depending upon location, water temperature, physiological state, motivation, age, body size and previous exposure (Popper et al., 2022). Furthermore, cephalopods inhabit shallow waters to deep oceans (Boyle and Rodhouse, 2008), and will be subject to different sound exposure levels from anthropogenic activity depending on sound propagation properties, bolstering the need to investigate behavioural and physiological effects using field experiments at a range of locations and water depths.

To the authors' knowledge, this is the first study that compared auditory thresholds between different ages of a cephalopod species. The different age groups behaved similarly, remaining quiescent in the daytime during the experiments and increasing activity at night or when feeding in their holding tanks. Both mid and late adults were observed to have a significant decrease in ventilation rate with anthropogenic sound exposure. In terms of AEPs, for particle acceleration there was a loss of sensitivity with age and for sound pressure juveniles and mid adults were more sensitive than late adults. To extend this study, it would be interesting to test the auditory sensitivity of recently hatched individuals (< 30 days old) and determine if they are more sensitive than juveniles. Additionally, cephalopods undergo senescence and can exhibit physiological and behavioural changes, including a loss of coordination and a reduction in feeding (Roumbedakis and Guerra, 2019) . The late-adults used in this study were 125 – 140 days old, and older individuals (up to 180 days) could be tested to examine whether further loss of auditory sensitivity occurs with age. Furthermore, microscopy techniques may be able to establish if there is evidence of degradation in the sensory hair cells of the statoliths during senescence.

The auditory sensitivity data collected on *E.berryi* in this study aligns with previous behavioural and physiological results that the cephalopod statocyst detects low frequency sound (Mooney et al., 2010; Samson et al., 2016. Continued research focussed on understanding how animals detect and use particle motion is critical to the future evaluation and regulation of sound in the natural environment. It is likely that substrate born vibrations, are also important to these cephalopods and certainly to other invertebrates. Addressing how these squid perceive and utilize this cue could greatly expand our understanding of their sensory ecology, and how they may be impacted by noise. Substrate-borne vibrations can travel great distances with little attenuation, allowing for potentially improved sensitivity and a wider range of noise-based impairments. In this study, frequencies< 100 Hz were not tested owing to the limitations of the use of an underwater speaker as the sound source. Yet cephalopods likely detect infrasound (< 20 Hz) (Packard et al., 1990) and given that many anthropogenic noise sources persist into these lower frequencies, squid sound sensitivity to infrasound should be examined, as particle motion at these frequencies may propagate through both the water and sediment (Popper and Hawkins, 2018).

5 Conclusion

This study expands knowledge on cephalopod hearing highlighting that exposure to short duration, high intensity anthropogenic sound can cause significant shifts in the behaviour and physiology of *E.berryi*. There was some evidence that the reduction in auditory sensitivity was a temporary threshold shift however, human produced sounds have become louder and more persistent and there may be limited time for these animals to recover from noise exposure. Understanding baseline sensitivity and potential physiological effects of noise on cephalopods is an important step towards establishing guidelines for management and policy to protect cephalopods from noise pollution. Auditory thresholds for marine mammals and fishes have been established and are regularly used in environmental impact assessments globally (Popper et al., 2014; National Marine Fisheries Service, 2018) . Now, guidelines based on both sound pressure and particle motion are needed to support the inclusion of cephalopods within management and policy because anthropogenic activities and associated sound levels in the ocean are increasing, while the role sound plays in cephalopod life history is only just beginning to be understood.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the Marine Biological Laboratory, Woods Hole, MA, USA.

Author contributions

RP: Conceptualization, Methodology, Formal Analysis, Visualization, Writing - original draft. TM: Conceptualization, Methodology, Writing - review and editing. AM: Conceptualization, Resources, Writing - review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1151605/full#supplementary-material

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Cuttlefish color change as an emerging proxy for ecotoxicology

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Lately, behavioral ecotoxicology has flourished because of increasing standardization of analyses of endpoints like movement. However, research tends to focus on a few model species, which limits possibilities of extrapolating and predicting toxicological effects and adverse outcomes at the population and ecosystem level. In this regard, it is recommended to assess critical species-specific behavioral responses in taxa playing key roles in trophic food webs, such as cephalopods. These latter, known as masters of camouflage, display rapid physiological color changes to conceal themselves and adapt to their surrounding environments. The efficiency of this process depends on visual abilities and acuity, information processing, and control of chromatophores dynamics through nervous and hormonal regulation with which many contaminants can interfere. Therefore, the quantitative measurement of color change in cephalopod species could be developed as a powerful endpoint for toxicological risk assessment. Based on a wide body of research having assessed the effect of various environmental stressors (pharmaceutical residues, metals, carbon dioxide, anti-fouling agents) on the camouflage abilities of juvenile common cuttlefish, we discuss the relevance of this species as a toxicological model and address the challenge of color change quantification and standardization through a comparative review of the available measurement techniques.

KEYWORDS

behavior, body pattern, camouflage, cephalopod, chromatophore, crypsis, mollusk, neurotoxicity

1 Introduction

For ethical reasons and given the growing need for risk assessment of environmental pollutants, the field of ecotoxicology is undergoing substantial changes (Campbell et al., 2022). In line with the 3Rs principle (Replace, Reduce, Refine), alternatives are being sought to replace conventional toxicity tests, which frequently measure mortality as an endpoint. Among them, the development of sublethal behavioral endpoints is not new (Dell'Omo, 2002), but attracts increasing attention. Indeed, it has been shown that a variety of behavioral responses to contaminants, both in vertebrates and invertebrates, appear to reflect alterations in sensory, hormonal, neurological, and/or metabolic systems (Saaristo et al., 2018; El-Gendy et al., 2021). Several authors have suggested that behavior could be among the most sensitive, flexible and conspicuous expressions of an animal's integrated physiological response, making it a suitable endpoint for early

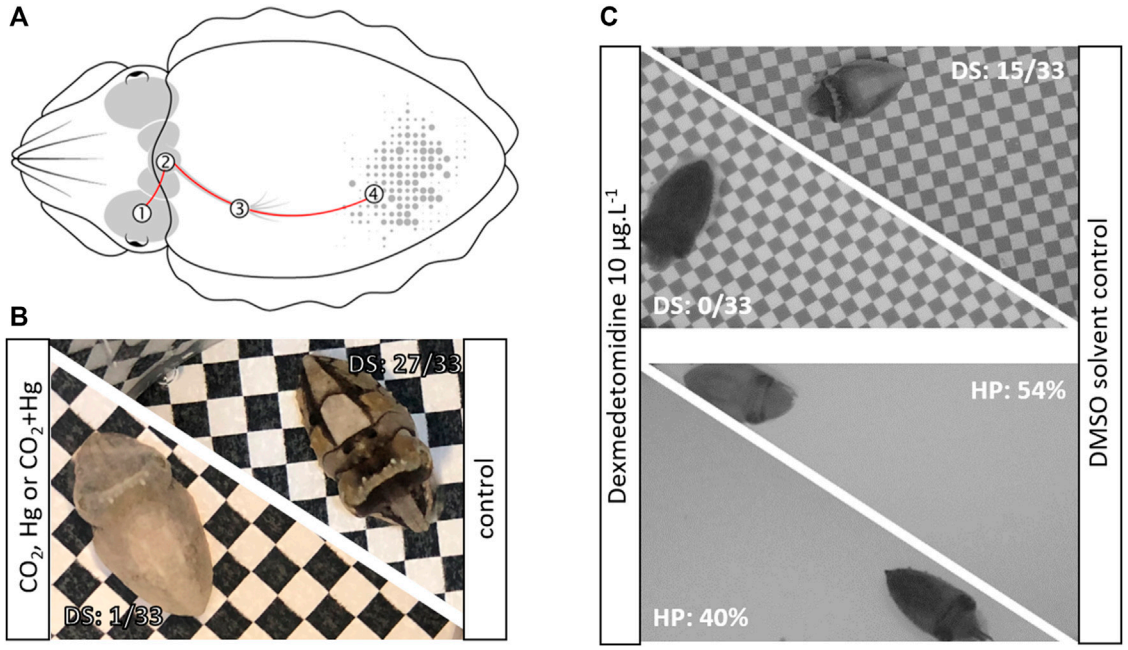

FIGURE 1
(A). Diagram of the neuronal control of chromatophores in the European cuttlefish: The visual information is transmitted from the eye (1) to the central nervous system (through the optic lobes, the lateral basal lobes, then the chromatophore lobes) that controls the motor activity of chromatophores (4) through the stellate ganglion (3), **(B)**. Example of cuttlefish hatchlings exposed for 1 month to CO_2 (i.e., pH 7.54), Hg (i.e., 3 µgg^{-1} dry weight in muscle) or CO_2+Hg, with a low disruptive score (left) compared to a cuttlefish from control conditions (i.e., pH 8.02; right) with a high disruptive score, **(C)**. Example of cuttlefish hatchlings exposed for 3 days to dexmedetomidine during 3 days displaying a dark uniform pattern on both checkerboard and uniform backgrounds compared to cuttlefish from solvent control conditions (DMSO: dimethyl sulfoxide; DS: disruptive score; HP: homochromy percentage).

toxicity testing (e.g., Clotfelter et al., 2004; Hellou, 2011; Melvin and Wilson, 2013; Peterson et al., 2017).

A large proportion of the research conducted so far in aquatic species has targeted behaviors such as predator avoidance, locomotor activity, exploration and anxiety (Melvin and Wilson, 2013). They are doubtless highly relevant to assess ecological risks as they reflect a wide range of biological functions, including predation, feeding, migration and mating (El-Gendy et al., 2021). Moreover, some behavioral tests using these endpoints allow high-throughput screening assays in laboratories and can be adapted to various species. However, diversifying the approaches, toxicological endpoints and model organisms is necessary (1) to account for inter-specific differences in sensitivity and toxicodynamics and (2) to make extrapolations and predictions of toxicological and ecological effects possible (Segner and Baumann, 2016). In this regard, some critical species-specific behavioral responses must be assessed in species playing key roles in trophic food webs, such as cephalopods. As mollusks—an important phylum among marine invertebrates—they certainly offer a particularly large and rich behavioral repertoire, including remarkable color changes that may serve as a proxy, notably for neurotoxic effects of environmental chemicals (Hanlon and Messenger, 2018).

Color change is a widespread ability among animals, known to fulfill various biological functions such as thermoregulation, UV protection, crypsis or communication (Figon and Casas, 2018). It is generally achieved either by the production, degradation or chemical modification of pigmented structures (morphological color change) or by changes in intracellular pigment distribution within specialized skin organs called chromatophores (physiological color change). In cephalopods, the chromatophores consist of pigment-containing elastic sacculi attached to a set of neuromuscular fibers, whose mechanical action controls the dispersion or the concentration of pigments (Messenger, 2001; Figure 1A). These basic structural *elements* are organized in *units*, themselves organized in chromatic *components*, which, combined with textural (i.e., expression of skin papillae), postural (e.g., arm posture) and locomotor components, form a palette of species-specific *body patterns* (Hanlon and Messenger, 2018).

Multiple experiments have shown that the choice of body pattern relied on a fine visual analysis of the animal's immediate surroundings, considering, not only the nature of the substrate, but also the presence of objects, conspecifics, prey or predators (Allen et al., 2009; Barbosa et al., 2012). This information is processed and integrated by the brain which generates a motor program that selectively activates the expansion/retraction of chromatophores (Figure 1A; Supplementary Figure S1).

A range of environmental stressors might interfere with these neurally controlled mechanisms and result in the alteration of chromatophores' activity—and, subsequently, of color change. Therefore, the quantitative measurement of physiological color change in cephalopod species could provide new opportunities for toxicological risk assessment. Following a body of research that has evaluated the effect of various environmental stressors

on camouflage in juvenile European common cuttlefish, we here discuss the relevance of this species as a toxicological model and address the challenge of color change quantification and standardization through a comparative review of the available measurement techniques.

1.1 *Sepia officinalis* as an emerging model in behavioral ecotoxicology

The European common cuttlefish (*Sepia officinalis*) is one of the most studied cephalopod species, especially for the exploration of color change and advanced cognitive functions (Darmaillacq et al., 2014). This necto-benthic species heavily relies on rapid adaptive camouflage to avoid being detected by predators. Cuttlefish are known to exploit a combination of background color matching, disruptive coloration, masquerade and distractive markings, involving more than 30 different chromatic components (Hanlon and Messenger, 1988). Although *S. officinalis* could theoretically produce billions of body patterns, it appears that some combinations of components are preferentially used (Osorio et al., 2022), resulting in the expression of three main camouflage patterns called *uniform*, *mottle* and *disruptive* (Hanlon and Messenger, 2018). Uniform patterns are usually displayed on uniform backgrounds, whereas cuttlefish exhibit more complex patterns such as mottle pattern on sandy bottoms or disruptive pattern on contrasted substrates containing gravel, pebbles or shells. These body patterns are said to be chronic (i.e., maintained over long time periods) as opposed to acute body patterns (i.e., displayed during seconds to minutes for communication, predation or defensive purposes). Their efficiency can be improved by behavioral stillness and sand-digging (Mather, 1986).

The chromatophores of *S. officinalis* are known to be under the control of serotonin, which induces a retraction of the pigment-containing sacculi, and glutamate, which induces their expansion. This antagonistic system would control rapid color changes for the production of acute body patterns, whereas FMRFamide-related peptides and nitric oxide would be involved in the maintenance of chronic patterns by modulating the action of glutamate (Loi and Tublitz, 2000; Mattiello et al., 2010). A proven malfunction of these control systems could therefore reveal a neurotoxic effect or neuroendocrine disruption.

At the time of hatching, the chromatophores are functional and the juveniles are immediately capable of color changes—although their camouflage abilities significantly improve with age (Dickel et al., 2006). This gives the opportunity to implement behavioral tests on the earliest-life stages, with several practical advantages: (1) hatchlings can be available in large numbers from egg clusters collected in the environment, (2) the hatching success is close to 100% and (3) juveniles are relatively easy to maintain in laboratory conditions. Their small size (about 10 mm in dorsal mantle length at hatching against 45 cm for the adults) requires little space for rearing facilities (0.005 m^2 and 2 L per animal according to Fiorito et al., 2015) and experimental devices. Most importantly, the use of early-life stages is particularly relevant from an ecotoxicological and ecological point of view. Indeed, hatchlings and juveniles are more vulnerable to environmental contaminants than adults, since they are at key stages of their cerebral, cognitive and behavioral development (Dickel et al., 2006). They are also more exposed to contaminants as they spend all embryonic stages and the first post-hatching months in coastal waters subjected to anthropogenic pollutants, before migrating offshore to their wintering grounds (Miramand et al., 2006). In addition, the digestive gland, a key organ for detoxification, has not yet reached its full physiological development in the first month of juvenile life, raising the question of its efficiency to cope with the toxic effects of contaminants (Lacoue-Labarthe et al., 2016).

More generally, the recent interest in using cephalopods in ecotoxicological studies lies in their potential for bioaccumulation (Bustamante et al., 1998b), which should be favored by their short life span and rapid growth (i.e., the accumulation of contaminants followed by reproduction is less energetically expensive than their elimination for survival purpose). In addition, cephalopods are recognized vectors for chemicals transfer along the trophic web as they are predators and prey of numerous marine organisms (Bustamante et al., 1998a). In cuttlefish, bioaccumulation occurs from different uptake pathways, including seawater, sediment (for sand-digging species) and trophic routes (see Bustamante et al., 2002, Bustamante et al., 2004). Consequently, the concentrations of contaminents vary along the environmental conditions affecting bioavailability and physiology (e.g., temperature, pCO_2) and many life traits, such as age, size or diet and trophic levels (for reviews: Lacoue-Labarthe et al., 2016; Penicaud et al., 2017).

To date, neither cuttlefish nor any cephalopod species has been recognized as a model species *per se*. However, *S. officinalis* was acknowledged as an emerging model for research in the fields of cognitive neuro-ethology, sensory ecology and behavioral ecotoxicology (Bassaglia et al., 2013). With an effort toward the harmonization of existing analytical tools and resources, studies devoted to cephalopods could benefit a wider scientific community.

1.2 The challenge of color change quantification

The experimental analysis of cuttlefish color change requires (1) properly designing the visual stimuli meant to elicit specific behavioral responses (color change or body pattern) and (2) collecting images of sufficient quality, allowing (3) relevant quantitative image analysis.

To meet the first requirement, fundamental research conducted on cuttlefish camouflage is a valuable resource. While cuttlefish are known to integrate multiple visual features from their surroundings to elaborate a camouflage response, it has been demonstrated that the use of simple artificial substrata can reproducibly elicit typical body patterns. For example, the disruptive body pattern is routinely induced in response to black and white checkerboards displayed on the bottom and walls of the arena. A top requirement is that the checkerboard squares are between 40 and 120% of the size of the animals' white square component area (see Barbosa et al., 2007). The use of smaller or larger checkerboard squares is likely to induce mottle or uniform body patterns (Chiao and Hanlon, 2001).

The photographs used for color change analyses must also meet certain criteria. The topic was covered by Stevens and collaborators (2007), who emphasize the utmost importance of manually controlling the white balance and light exposure. The lighting

should also be tuned so as not to stress tested individuals (<350 lux at water surface according to Fiorito et al., 2015) while being as close as possible to the spectrum of sunlight to offer ecologically relevant testing conditions.

Finally, the camouflage patterns can be characterized using a range of descriptors (see Table 8 three from Josef and Shashar, 2014 for a review). For ecotoxicological purposes, the simple discrimination of uniform, mottle and disruptive patterns can already provide ample information. This can be done manually (Dickel et al., 2006) but also automatically, based on their distinct spatial frequency spectra (Barbosa et al., 2008), or with the help of learning algorithms (Orenstein et al., 2016).

The analysis can be extended to an evaluation of the efficiency of each camouflage. The uniform camouflage pattern can be assessed by a measurement of homochromy, i.e., the matching of the animal mantle compared to the background luminance according to the mean grey value (see Poirier et al., 2005; Di Poï et al., 2014; Supplementary Figure S2). In this case, the standard deviation of the grey values of the animal's body can also be considered as a descriptor of the heterogeneity of the uniform pattern. Finally, the disruptiveness can be assessed by assigning a score—from 0 (not expressed), 1 (weakly expressed), 2 (moderately expressed) to 3 (strongly expressed)—to each of the eleven chromatic components commonly forming disruptive patterning (Barbosa et al., 2008). Individual cuttlefish can then be assigned a total grade, called disruptive score, ranging from 0, for maximal homochromy, to 33, for highly disruptive patterning (Supplementary Figure S2).

It is also possible to add a temporal dimension to the analyses. It may consist in assessing the latency of a chronic pattern (Court et al., 2022) or its stability over short time periods (Di Poï et al., 2014; Chabenat et al., 2021; Gouveneaux et al., in prep.). Otherwise, the comparison of camouflage quality at different ages during the first months is a simple way to assess whether the typically observed post-embryonic maturation of camouflage abilities is altered (Dickel et al., 2006).

These tests are relatively quick and inexpensive to implement, not to mention that photographic imaging is non-invasive. The reproducibility of image analysis is also facilitated by the fact that cuttlefish usually stay still on the substrate and, therefore, exhibit a portion of its mantle relatively unchanged in size and extension (as opposed to octopodiforms which take on shapes and postures that usually conceal part of their skin). This feature could facilitate the adaptation of more complex quantification methods (e.g., the analysis of grey values profiles along some body axes; Court et al., 2022 adapted from Chiao and Hanion, 2001) to reveal more subtle color change alterations. Indeed, image analysis can be technically challenging and time-consuming, especially if the method is manual and several annotators have to analyze in parallel to avoid bias on the results.

1.3 Recent applications in ecotoxicology

A number of environmental stressors have been shown to alter *Sepia officinalis*' color change abilities. Several studies have focused on the effect of waterborne fluoxetine and venlafaxine, two antidepressants of the class of selective serotonin reuptake inhibitors and serotonin-norepinephrine reuptake inhibitors, respectively (Table 1). Changes were mainly observed in the efficiency of the uniform pattern, which appeared more heterogeneous in animals exposed to one or the other of these compounds (Bidel et al., 2016a, 2016b). However, a mixture of both resulted in a more uniform pattern (i.e., lower disruptive score) when calculated in animals tested on uniform backgrounds (Chabenat et al., 2021). Although venlafaxine did not appear to modulate the serotonin levels in the central nervous system of cuttlefish (Bidel et al., 2016a), it was observed to cause the dose-dependent relaxation of chromatophore-associated muscles in isolated skin patches, as observed in response to serotonin (Gouveneaux et al., in prep).

More recently, these tests have been adapted to monitor the effects associated with trophic mercury, a potent neurotoxin under its methylated form (MeHg) (Minet, 2022; Figure 1B), as well as the effects of suspected neuroendocrine disruptors (i.e., dexmedetomidine) (Gouveneaux et al., in prep). In contrast to previously cited studies, juvenile cuttlefish were shown to struggle mostly with the disruptive pattern: the chronic exposure to MeHg resulted in the display of a light uniform pattern on black and white checkerboards. In contrast, acute exposure to the antifouling agent dexmedetomidine resulted in a dark uniform pattern regardless of the type of background presented (light or dark grey uniform backgrounds, black and white checkerboards; Figure 1C). Finally, seawater carbon dioxide (pCO_2) is of growing interest due to its effects on nervous system and behaviors in cephalopods (e.g., Spady et al., 2018).

It must be noted that, while one-month-old juveniles exposed to elevated pCO_2 (~1,600 µatm) failed to produce a disruptive pattern on the black and white checkerboards (Minet, 2022), exposure of hatchlings to ~1,000 µatm did not affect the camouflage display as assessed with a different quantification method regardless of the substrate nature (Court et al., 2022). This raises the question of how the animals' response depends on its age, the contaminants' pressure levels and the descriptors used for color patterns. In sum, this emphasizes the need to harmonize the camouflage assays.

2 Discussion

Cephalopods combine the advantages of being keystone species in marine food webs, important fishing and aquaculture resources, and valuable experimental animals. As such, they stand at the crossroads of several of the environmental problems of the Anthropocene. As a matter of fact, human activities have substantially changed the world's marine environments in recent decades. While some populations and species are paying a high price for these changes, up to the point of collapse or extinction. Cephalopods, however, seem to take advantage of these major changes (Doubleday et al., 2016). This success is partly attributed to the "live fast, die young" life history strategies of cephalopods, which confer them great plasticity and adaptability to environmental changes (O'Brien et al., 2018). However, these overall trends of populations can mask a continuous decrease in the European cuttlefish stocks in the last decade (FAO, 2019), suggesting that this species may be vulnerable to multiple, co-acting stressors (Clarke, 1996; Bustamante et al., 1998b). Therefore, their study might shed light on our understanding and assessment of the risks

TABLE 1 Summary of uses of body patterns in ecotoxicology (dph: days post-hatching; NE: no effect).

Stressors	Concentrations	Exposure duration	Body patterns	Endpoints	Results	References
Fluoxetine (antidepressant)	1, 10 and 100 $ng.L^{-1}$	15 days pre-hatching to 32 dph	Uniform	*Homochromy percentage Heterogeneity index*	decrease NE	Di Poï et al. (2014); Bidel et al. (2016b)
			Disruptive	*Disruptive score*	NE	
Venlafaxine (antidepressant)	5 and 100 $ng.L^{-1}$	20 dph	Uniform	*Heterogeneity index*	increase	Bidel et al. (2016a)
			Disruptive	*Disruptive score*	NE	
Fluoxetine + venlafaxine	5 $ng.L^{-1}$ fluoxetine 2.5 + 2.5 or 5 + 5 $ng.L^{-1}$ of the mixture	29 dph	Uniform	*Disruptive score*	decrease	Chabenat et al. (2021)
			Disruptive	*Disruptive score*	NE	
Mercury + pCO_2	0,3 and 3 $\mu g.g^{-1}$dry weight in muscle ~1,600 µatm	30 dph	Uniform	*Homochromy percentage Heterogeneity index*	NE	Minet (2022)
			Disruptive	*Disruptive score*	light uniform pattern	
Dexmedetomidine (anesthetic, antifouling agent)	10 $\mu g.L^{-1}$	3 dph	Uniform	*Homochromy percentage Heterogeneity index*	dark uniform pattern	Gouveneaux et al., in prep
			Disruptive	*Disruptive score*	dark uniform pattern	

associated with pollutants and related stresses in marine environments.

In this perspective, the quantification of physiological color change in cuttlefish is emerging as an easily accessible, non-invasive, sensitive and holistic ecotoxicological endpoint. Indeed, color change determines camouflage, whose efficiency depends on (1) visual abilities and acuity (from eye functioning to pigments structure), (2) information processing (nervous system) and (3) control of chromatophore dynamics (nervous and hormonal control pathways) (see Reiter and Laurent, 2020) with which many contaminants can interfere. Previous studies, notably on fish, crustaceans and cephalopods (e.g., Lennquist et al., 2010; Ford and Feuerhelm, 2020; Chabenat et al., 2021), have highlighted various effects of contaminants on color change, ranging from impaired acclimatization abilities to changing visual environments up to persistent darkness or paleness. The underlying causes of these alterations remain poorly understood, although in most cases they are likely explained by the disruption of color change control mechanisms. It appears so for venlafaxine, whose topical effects on *S. officinalis* (i.e., the concentration of pigments and skin paling), are consistent with its mode of action, namely, an increase in serotonin levels (Gouveneaux et al., in prep).

Yet, unusual mantle color changes or discoloration are commonly used in cephalopods—although quantitative assessment methods are rarely implemented—as indicators of anesthesia or general ill-being (Polese et al., 2014; Fiorito et al., 2015). As it generally goes with behavior, this emphasizes that color change is the expression of an integrated physiological state and carries the potential to reveal a wide spectrum of disruptions beyond those affecting the chromatophore control mechanisms themselves. This points to the possibility of confounding factors, which exist for many toxicological endpoints. Thus, the assessment of color change must be completed by sensory tests (such as visual acuity assessment; see Cartron et al., 2013) but also neurotransmitters quantification or genomic approaches, to highlight neurotoxic effects.

The use of *S. officinalis* as a model proved to be convenient, with fundamental studies about its color change offering a solid knowledge base and a wide range of analytical tools. However, scoring methods, such as the disruptive score, are still time-consuming and experimenter-dependent with a qualitative interpretation of the expression of chromatic components. Thus, one of the main challenges in the development of cuttlefish color change as a proxy for ecotoxicology will be the development of automatized and, above all, standardized methods to ensure the reproducibility and comparability of tests. This applies to the methods of image acquisition and analysis, but also to the conditions of exposure to contaminants, as well as the age of the animals tested. Finally, it is essential to develop positive controls, i.e., to use chemical compounds with known modes of action, such as neuromodulators, to characterize and mark out the nature and magnitude of effects that can be expected on cuttlefish color changes.

Most studies conducted so far have focused on the description of chronic body patterns and their components. However, a variety of subtle color changes and body patterns, including the repertoire of acute body patterns displayed by *S. officinalis*, remain to be assessed under stressful conditions for possible derivation as toxicological endpoints. Besides, finer scale studies could lead to a better understanding of the hierarchical organization of chromatophores and thus of the impact of contaminants on neuronal activity. This could rely on existing methods for automatic analysis of chromatophores activity, which have significantly improved (Goodwin and Tublitz, 2013; Hadjisolomou and El-Haddad, 2017). In fact, it is currently

possible, using deep learning algorithms, to perform automatic delineation and tracking of individual chromatophores, to classify them as light or dark and to discriminate overlapping structures (https://git.unicaen.fr/nicolas.elie/redpol-open). Such a tool was recently developed from video recordings of *S. officinalis*' skin explants, following the bioassay protocol implemented by Loi and collaborators (1996) for highlighting the pharmacological basis of chromatophore control. It will likely be able to process high-resolution images acquired from motile cuttlefish, as recent works have shown (Reiter et al., 2018; Hadjisolomou et al., 2021). Yet, the number of animals needed for *ex vivo/in vitro* tests is reduced by the potential for high-throughput using of several skin patches from one animal only.

In conclusion, color change appears to be an integrated endpoint of the subtle effects of contaminants that can interact at different processing levels (visual perception, neuro-hormonal control, motor dynamic of chromatophores, or even decision-making) and thus provide early warning information of the cuttlefish vulnerability to environmental stressors. In turn, improving the knowledge on the toxicodynamics of contaminants targeting specific pathways would help understand the complex biological processes governing camouflage. Finally, the ecological consequences of affected camouflage abilities on cuttlefish fitness remain to be explored.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Comité d'éthique Normandie en matière d'expérimentation animale (CENOMEXA).

Author contributions

AG developed the initial idea for the article. AG and AM organized manuscript sections, directed editing, made the figures and finalized the manuscript. All authors have made a substantial and direct intellectual contribution to this work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1162709/full#supplementary-material

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Understanding species responses in a changing world by examining the predatory behaviour of southern calamari to changes in temperature

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Predator–prey interactions are key drivers in structuring communities, with the potential to substantially impact the whole ecosystem when important predators and prey are involved. Squid are voracious predators and also important prey for other top predators. To date, the available data suggests that under current and projected ocean warming, the behaviour of ectotherms could be modified (for example, through individual movement, predator avoidance and escape speed), yet little is known of the influence of temperature on the predatory behaviour of cephalopods. Here, the predatory behaviour of adult southern calamari (*Sepioteuthis australis*) under different thermal scenarios was examined demonstrating that squid exhibited different behaviour and performance capabilities across temperature treatments. Overall, attempts of squid to capture prey were faster and more persistent at higher temperature treatments (25°C), suggesting that individuals need to increase their food consumption rate, presumably associated with the higher energetic costs of living at elevated temperatures. However, we also observed a possible decrease in capture efficiency and increased prey handling time at higher temperatures suggesting that implications for energetic balance are not straightforward and that trade-offs need to be carefully explored. As cephalopods are ecologically important species acting as key links in food webs around the world, the results here could have important implications for the dynamics of many marine ecosystems in future.

KEYWORDS

acclimation, cephalopods, ocean warming, predation, species interaction, squid

1 Introduction

Marine ecosystems across the world are being affected by climate change, with impacts ranging from changes in species' life histories (e.g. growth and development), distribution and interactions, through to shifts in ecosystem composition, stability and function (Hoegh-Guldberg and Bruno, 2010; Doney et al., 2012; Vergés et al., 2014; Payne et al.,

2016; Pecl et al., 2017; Vergés et al., 2019). Ocean warming, induced by anthropogenic CO_2 emissions, is expected to increase by between 2 and 4°C by the end of the century (IPCC, 2022), yet the velocity and magnitude of change differs regionally around the world.

In south-eastern Australia, ocean warming is occurring almost four times faster than the global average due to the intensification and poleward extension of the East Australian Current (Ridgway, 2007; Oliver et al., 2015), creating a regional 'hotspot'. Such fast-warming regions can act as natural laboratories for climate change as climate-driven biological and ecological changes may be accelerated, occurring ahead of such changes in other regions (Hobday and Pecl, 2014). Across those waters, particularly Tasmania, extensive alteration of marine ecosystems has been linked with both heatwaves and trends of temperature increases, for example in the loss of habitat-forming species like kelp and the range extensions of many 'new' species (Johnson et al., 2011; Last et al., 2011; Pecl et al., 2019). However, most of the research effort has focused on understanding species current and projected distributional responses to warming, whereas very little work has been undertaken on how species might perform within their existing ranges and interact with existing or with novel species in response to warming waters. In this part of the world, squid have been identified as having a strong effect on ecosystems, acting as a link between food webs in different habitats (de la Chesnais et al., 2019). Consequently, squid performance under different thermal regimes could alter the stability and strength of the biotic interactions, leading into modification of whole marine community dynamics under warming conditions across waters.

Worldwide, cephalopods are considered marine keystone species, serving as important predators and prey (Villanueva et al., 2017; Chen et al., 2022). They are voracious carnivores and opportunistic animals, feeding on a wide variety of live prey including fish, cephalopods, gastropods, bivalves or crustaceans (Hanlon and Messenger, 2018). Furthermore, as fast-growing animals with the potential to respond quickly to environmental changes (Steer et al., 2003; Pecl et al., 2004), they are likely to play a significant role in the response of marine ecosystems to climate change (Pecl and Jackson, 2008; de la Chesnais et al., 2019). Therefore, the study of how temperature affects behavioural performance (e.g. predation and locomotion) (Gilman et al., 2010), in ecologically important species such as squid, could improve our capacity to more accurately forecast the dynamics of their associated ecosystems.

Ectotherms are particularly vulnerable to ocean warming, as temperature plays a vital role in physiological regulation and behavioural performance (Amarasekare, 2015) with the potential to impact ecological communities by altering the strength and stability of trophic interactions (Rall et al., 2010; Grigaltchik et al., 2012; Gilbert et al., 2014). Modification of these interactions could have significant consequences for whole marine communities when important predator or prey species are affected. As interactions of key species can determine the flux of nutrients among individuals through communities and ecosystems (Dell et al., 2014; Horwitz et al., 2020).

Predation is a key process in structuring communities with cascading effects across trophic levels (Steffan and Snyder, 2010; Warren et al., 2017). Furthermore, predation pressure can alter prey morphology, and regulate population size, as well as prey species composition by trophic cascades (Warren et al., 2017). Any encounter between predator and prey depends on a complex interaction of physiological and behavioral capacities such as locomotion, escape speed and foraging patterns (Lienart et al., 2014; Öhlund et al., 2015). Different factors may affect the motivation to make an attack, including predation risk, hunger or prey availability (Sentis et al., 2012). Yet, the behavioural functional response still mainly depends on two parameters – search rate and handling time (the time that a predator takes to kill and consume the prey), and both parameters are directly related to the water temperature (Sentis et al., 2012). Handling time is the time taken to capture and kill the prey, and the rate of gut clearance (Jeschke et al., 2002), whereas searching is an active predator's behaviour, directly associated with locomotor performance. For example, in *Stylocheilus striatus* (sea hare), an increase in water temperature (from 28 to 31°C) leads to a reduction in locomotion speed (Horwitz et al., 2020), and in the predator *Macquaria novemaculeata* (Australian bass), the number of attacks increases under warm conditions (25°C) in comparison with cold acclimation (15°C) (Grigaltchik et al., 2012).

In previous studies of fish and marine invertebrates, predator behaviour has been shown to be affected by temperature (Kidawa et al., 2010; Grigaltchik et al., 2012; Sentis et al., 2012; Horwitz et al., 2020), and some species of squid have responded to other environmental stressors like acidification (Spady et al., 2018), yet the behavioural responses of cephalopods to warming waters have been under-represented in the literature (Higgins et al., 2012). To date, studies have examined how changes in temperature affect the anti-predatory behaviour or escape speeds of cephalopods (Neumeister et al., 2000). However, to the best of our knowledge, no studies have examined the effect of acclimation temperature on their predatory behaviour. This is an important gap in our understanding of how future warming conditions could affect ecologically important species, with potential flow-on effects for community stability.

The main objective of this study was to examine the effect of acclimation temperature on the predatory behaviour of southern calamari (*Sepioteuthis australis)*. Specifically, the predatory interaction with a common prey, Australian salmon (*Arripis trutta*), under different thermal scenarios, simulating current (13°C, 16°C, 19°C) and possible future (22°C, and 25°C) environmental conditions was examined. Southern calamari are a large loliginid species, endemic to southern Australia and northern New Zealand. They are commonly found in shallow waters (< 20m deep) over seagrass meadows or sandy habitats with a lifespan of an approximately one year (Moltschaniwskyj and Steer, 2004; Pecl, 2004). Australian salmon can be found sharing a similar geographical distribution to southern calamari across south-eastern Australia, from western Victoria and Tasmania to north New South Wales, although Australian salmon distribution extends around southern Queensland (Paulin, 1993). Within their geographical Tasmania range, both species experiences

average summer temperatures of 19°C with a maximum of 21°C, while in winter the average temperature is 12.5°C with a minimum of 10°C. Still, Australian salmon could encounter water temperatures as high as 24.6°C on their northern distribution. The study of how environmental temperature affects a species' behavioural performance in key predator–prey interactions is crucial to potentially identify any climate-driven major changes in species interactions, which may in turn influence future population trends and ecosystem dynamics in response to climate change.

2 Material & methods

2.1 Squid collection and holding conditions

A total of 100 adult squid were caught (mantle length 110–247 mm with a mean of 244 ± 91.67 g in weight) by hand-jigging from the south-east coast of Tasmania (43°00'27.0"S 147°19'32.5"E) from December 2018 to May 2019, with groups of 10 individuals captured within 24-hour periods. Collection depths varied from 5 to 15 m, as southern calamari are a largely inshore species. For acclimation to temperature and the experimental procedures, squid were transported to the IMAS Taroona research facilities at the University of Tasmania. During the animals' transport, as well as their initial time in the holding tanks, water temperature was maintained at the same temperature as that of collection (± 0.5° C), which ranged between 14°C and 19°C over the capture months. In total, five temperature treatments were conducted (13°C, 16°C, 19°C, 22°C, and 25°C), representing current and possible future conditions, with 20 squid acclimated per treatment.

Once at the research facility, 10 individuals were placed in a holding tank (2.2 m diameter x 0.8 m high, two per temperature treatment) connected to a recirculating system supplied with ocean water subjected to multiple stages of filtration (drum filter, foam fractionator, biofilter, and UV treatment) and a heater/chiller unit (Aquahort heat pump LWH030SC). After the first 12 hours of the squid being in the holding tank, temperatures were increased/decreased progressively by 1°C every 12 hours until the treatment temperature was reached to avoid any possible thermal shock to the animals. Squid were acclimated to the treatment conditions for a full week prior to any procedures being undertaken. Water quality parameters in the holding tanks (checked 3 times per day) were kept constant and at a suitable level for the squid; salinity 34–36‰; NO_3 <10 mg L^{-1}; NO_2 <0.1 mg L^{-1}; NH_4< 0.25mg L^{-1}; and oxygen saturation < 110%, and the water delivery was maintained at a constant 30 ± 2 L/h. Additionally, a photoperiod cycle of 13 hr/11 hr light–dark was established, with 30 minutes each of programmed sunset and sunrise. Squid were fed daily (approximately 1:30 pm) with small live fish (body size >150 mm), including locally caught Australian salmon (*Arripis trutta*) or garfish (*Hyporhamphus melanochir*).

2.2 Experimental set-up and trial

The experimental behavioural arena (Figure 1) consisted of a circular tank (1.0 m diameter and 0.5m high) receiving water supply at ~ 20 l/h. Temperature in the arena was controlled by a heater/chiller unit (Aquahort heat pump VL130R), and an air stone kept the dissolved oxygen at around 100%. The arena was divided by a transparent acrylic window, with a GoPro HERO 4 camera mounted behind the window to record a lateral view of the squid during trials. A GoPro HERO 5 was mounted on top of the tank at a height of 0.5 m and two lamps (10 W RGB LED) were mounted parallel to but 5 cm higher than the GoPro HERO 5 to eliminate the shadow of the squid. The arena was completely covered with a black curtain, to isolate it from any external stimuli.

Prior to behavioural trials, squid were exposed to metabolic activity trials for 22 hours refer to Peinado (2021) for more information on the methodology. During these trials, squid were exhausted in order to obtain the maximum metabolic rate (MMR), with squid swimming until they could no longer maintain equilibrium. As a result, mortality increased at higher temperature treatments, reducing the sample size to 38 squid across all the treatments, resulting in an unbalanced design (n = 6, 10, 10, 5 and 7 individuals at 13°C, 16°C 19°C, 22°C and 25°C, respectively). Additionally, individuals that showed unusual behaviour in the arena, such as curled arms or resting on the bottom for periods longer than two minutes were discarded from the trials.

To begin each trial, a single squid was moved from the respirometry chamber, placed in the behavioural arena and allowed to acclimate for 1 hour. Predatory trials started within 13-14 hours after exhaustive exercise was conducted. Furthermore, to increase hunger levels they were fasted for 47 hours (24 h starvation + 22 h of metabolic experiments + 1 h acclimation) prior the predator–prey trial commencing. Cameras began recording two minutes before the prey was introduced to record squid activity. A single live prey (Australian salmon) was then placed into the arena, and the resulting interaction was recorded for a further 15 minutes. Individual fish were always introduced into the tank in the same position with less than a second of air-exposure before the individual was placed in the behavioural arena. Once the fish was introduced into the tank, squid could immediately attack

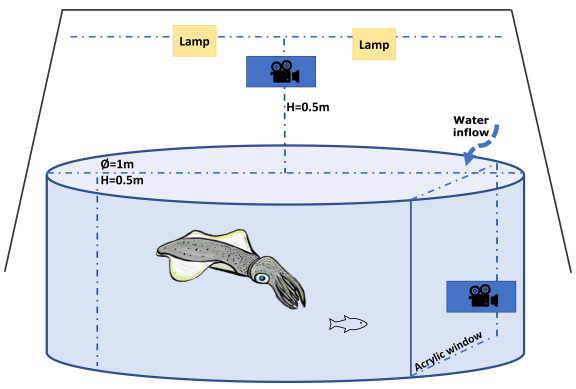

FIGURE 1
Diagram of the experimental arena used to measure predatory behaviour in southern calamari (examples of the video recording of interaction predatory-prey trial can be found in the Supplementary Materials).

the prey. In the event that a squid did not eat the prey during this period, it was removed immediately and placed back into the holding tank. This period was chosen due to our previous observations as well as other studies (Sugimoto and Ikeda, 2013; Spady et al., 2018), where squid are generally able to capture their prey within 10 minutes. All the Australian salmon (103 ± 27.6 mm in length) used in the experiments were acclimated for one week to the same water temperature and conditions as the particular squid treatment in question.

2.3 Behavioural analysis

The behavioural parameters chosen for this study were established in previous studies as good proxies of squid predation behaviour (Jantzen and Havenhand, 2003; Sugimoto and Ikeda, 2013; Spady et al., 2018).

Individual behavioural parameters observed included:

- Number of attacks – number of attempts the squid made to capture the prey.
- Latency time to attack (min) – time between the introduction of the prey in the arena and the first attack.
- Attack distance (mm) – the distance between the end of the arms (immediately before the tentacle's extension) and the prey (Figure 2).
- Tentacle elongation (mm) – the length of the tentacles when fully extended to capture the prey. To measure tentacle elongation and attack distances ImageJ software was use it. For that, images were extracted for the videos and three measures of the distance were taken. The data present here is the mean of the three replication measures.
- Attack direction/position – the orientation of the squid's body in relation to the prey when the attack occurred. Attack position was classified as 'horizontal arms', 'downward pointing' and 'upward pointing'. The 'Horizontal arms' position is when the arms and tentacles are held together in a horizontal body plan. 'Downward pointing' and 'upward pointing' postures are defined as the orientation when the whole body exceeded 45° from the horizontal plane in the corresponding direction (Jantzen and Havenhand, 2003) (Figure 2).
- Body pattern – display of body pigmentation once in the attack position, categorised as 'clear (transparent)', where chromatophores are reduced in size, and 'dark', where most of the chromatophores are expanded (Jantzen and Havenhand, 2003; York and Bartol (2016)).
- Handling time – the time that squid took to kill the prey.

Additionally, the proportion of squid that attacked the prey, captured the prey, and captured the prey on the first attempt were recorded.

FIGURE 2
Southern calamari (*Sepioteuthis australis*) parameters observed in the experimental arena; **(A)** Attack distance and body pattern 'dark', **(B)** 'downward pointing' attack position, **(C)** 'upward pointing' attack position, **(D)** 'horizontal arms' attack position.

2.4 Data analysis

R software (www.r-project.org) was used to perform all the statistical analysis in this study. Generalized linear models (GLMs) with a binomial distribution and a logic link function were used to investigate the relationship between acclimation temperature (13°C, 16°C, 19°C, 22°C and 25°C) and the proportion of squid that attacked the prey and the capture success. GLMs with a negative binomial distribution were used to compare the effect of acclimation temperature on the number of strike attempts. Preliminary analyses were performed for those variables using generalized linear mixed models (GLMM), including capture group (date and location caught) and acclimation days as random effects. The variance and standard deviation were zero and consequently, they were dropped from the model, fitting simple GLM models.

For the remainder of the behavioural performance measures (attack position, distance, body pattern, latency and handling time) results were only examined for the individuals that successfully captured the prey. As a result of the small numbers of squid attacking the prey (n=1) at lower temperature treatments (13°C and 16°C), both these treatments were necessarily eliminated from further statistical analysis. GLMs with a binomial logistic regression were used to examine the relationship between acclimation temperature, the ability to capture the prey at the first strike, and body pattern, where the attack position was examined using a multinomial logistic regression. To investigate the relationship between attack distance, and tentacle elongation, GLMs with a gaussian distribution and a log link were used, and GLMs with a gamma distribution and a log link were used to model latency and handling time.

Additionally, squid mantle length and total weight were included together with the acclimation temperature as a factor as well as the relation between predator and prey size in all models. Those factors show no statistical differences across variables. Akaike information criterion (AIC) values were used to establish the best fit of the model for each measure of performance (data not shown), and model assumptions were verified by examining residuals compared to the fitted values by inspection of the residual-fit plots.

3 Results

Acclimation temperature had a significant effect on the probability of a squid making an attack ($X^2 = 12.4$; df=1, $P < 0.0001$), as well as the capture success ($X^2 = 7.1$; df= 1; $P < 0.001$). The proportion of squid that attacked the prey increased substantially between treatments. Around 30% of the squid acclimated to lower temperatures (13°C and 16°C) made an attack strike, increasing to 80% at 19°C and 22°C, and reaching 100% at 25°C (Figure 3). Similar percentages were observed in the proportion of squid that successfully captured the prey, with 16.6% and 10% of the squid capturing the prey at 13°C and 16°C respectively, compared to 50% at 19°C, 80% at 22°C and 100% at 25°C. The number of strikes also increased significantly with acclimation temperature ($X^2 = 4.8$; df=1, $P < 0.05$) (Figure 3), where 71.4% of the individuals at 25°C strike the prey more than twice in compared with 16% at 13°C, 20% at 16°C and 22°C and 40% at 19°C.

For individuals that caught the prey at 19°C, 22°C and 25°C, there were no statistical differences in the proportion of squid that successfully captured the fish at the first strike attempt ($X^2 = 1.4$; df=1, $P > 0.05$). Although an overall decrease occurred with temperature from 60% (3 out 5 individual) at 19°C, and 75% (3 out 4 individuals) at 22°C, to 28.5% (2 out 7 squid) at 25°C. The lack of statistical significance could be related to the low sample size. Latency and handling time were also different among treatments ($X^2 = 57.3$; df=1, $P < 0.001$; $X^2 = 24.3$; df=1, $P < 0.001$). At higher temperatures (22°C and 25°C), the time that squid took to initiate the attack was reduced compared to individuals acclimated at 19°C, in contrast to the increase in handling time at higher temperatures (Figure 4). At 19°C, squid took between 1 and 11 min to attack, whereas squid at 22°C and 25°C started the attack only seconds after the prey had been placed in the arena (0.15 min ± 0.08; 0.07 min ± 0.01, respectively). Furthermore, individuals at 19°C only handled the prey for a mean of 0.5 min (± 0.3 SD), rising to 1.5 min (± 0.6 SD) and 2.2 min (± 0.7 SD) in squid acclimated to 22°C and 25°C, respectively. The distance from which squid started the attack, and the elongation of tentacles, also significantly differed across temperature treatments ($X^2 = 10.3$; df=1, $P < 0.01$; $X^2 = 8.7$; df=1, $P < 0.01$), increasing with acclimation temperature (Figure 5). Individuals at 19°C chose to be closer to the prey when the attack occurred (66.1 ± 30 mm), compared with individuals at 22°C (96.5 ± 30.9 mm) and 25°C (106.1 ± 24.3 mm). Moreover, the elongation of the tentacles was also shorter at 19°C (78.9 ± 36.1 mm), while it was 102.1 mm (± 24.6) at 22°C and 108.5 mm (± 14.6) at 25°C.

Neither attack position nor body color choice differed between treatments ($X^2 = 2.0$; df=2 P=0.7; $X^2 = 0.11$; df=1 P=0.73). However, the preferred body pattern of southern calamari during the attack, across the three treatments, was the dark color display, with 80%, 75% and 71% of the squid choosing it at 19°C, 22°C, and 25°C respectively (Figure 5). Additionally, there was no effect of mantle length or squid body weight for any of the behavioural parameters (data not shown).

4 Discussion

Southern calamari predatory behaviour differed depending on acclimation temperature, suggesting a potentially major influence of temperature on predator-prey dynamics under current and future climate change. Two of the most notable differences between temperature treatments was the proportion of squid attacking the prey and the capture success, with both measures increasing with the temperature of acclimation. All individuals acclimated to 25°C successfully attacked and captured 100% of prey provided, whereas at low temperatures (13°C and 16°C) only 30% of squid attacked the prey and with less than 16% success. These results suggest that environmental temperature has a profound effect on the predatory behaviour of southern calamari, with greater consumption rate at higher acclimation temperatures. This could be a function of physiological changes that affect the squid's ability to recognize

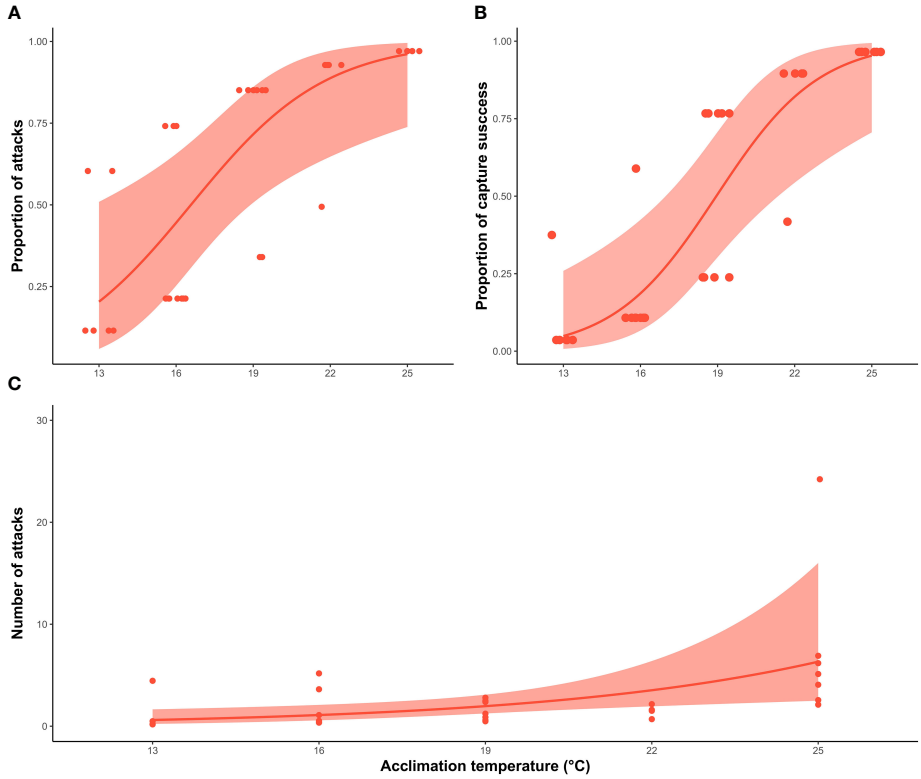

FIGURE 3
Relationship between behavioural responses of southern calamari (*Sepioteuthis australis*) and the five different acclimation temperatures (13, 16, 19, 22 and 25°C). **(A)** GLM with binomial distribution of the probability that individual squid will make an attack. **(B)** GLM with binomial distribution, of capture success of squid per treatment **(C)** GLM with a negative binomial distribution modelling the total number of strike attempts, including when squid were not successful. The thick line represents the expected value of the model for the variable measured. The shadow band corresponds to the 95% confidence interval, and the individual squid values are represented by the red points.

and strike prey, however, it is likely linked to increased metabolic demand at higher temperatures. Cephalopods are well known for having a high metabolic rate (O'Dor and Webber, 1986; O'Dor et al., 2002), and in a previous study, metabolic activity (routine metabolic rates) of southern calamari increased with temperature by 30% at 25°C (471.1 ± 91.7 mg O2 h-1 kg-, mean ± SD) compared to 13 and 16°C (312.2 ± 112.8 and 324.3 ± 37.1 mg O2 h-1 kg-1, respectively) (Peinado, 2021). Squid will thus be expected to have a higher energetic requirement to support basal and maintenance processes under future warming conditions (Peinado, 2021) and the predatory behaviour results observed here in the current study suggest that squid will need to increase food consumption rates to compensate for the energetic costs associated with living at elevated temperatures. Similar results have been found in other species of marine fish (Bethea et al., 2007; Grigaltchik et al., 2012) and invertebrates (Morón Lugo et al., 2020), where individuals at elevated acclimation temperatures were more motivated to attack than those at lower temperatures.

The decrease in number of individuals that attack at lower temperature (13°C and 16°C) may also be a consequence of a reduction decreased (~ 30%) in southern calamari metabolism (Peinado, 2021), having reduced energy/feeding requirements and possibly saving energy by not trying to capture the prey. It has also been suggested that similar reductions in other species of squid could be linked to the depression of metabolic rates due to environmental factors (Spady et al., 2018). Additionally, even if southern calamari across Tasmania experience minimum temperatures of 10°C, 13°C could be at the lower end of their thermal window which might limit their fitness and other physiological performances (e.g., olfactory clues or vision), triggering the no or slow response of squid here. Yet, due to the limited understanding of the lower thermal limits and their associated performance in cephalopods, it is difficult establish the main cause of the attack decrease. Future research should consider the role of cold adaptation in squid to identify if cephalopods respond with the same magnitude to cold or warm acclimation temperatures.

Australian salmon were also acclimated to the same temperature as the squid, which might influence the prey performance during the experiments, as temperature could influence locomotion in ectotherms (Angilletta et al., 2002) as well as escape responsiveness (Preuss and Faber, 2003). Here, we attempted to measure prey response to the squid attack, however, in many of the cases, the squid strike was successful at the very first attempt eliminating the opportunity for the prey to react and consequently the potential to record meaningful prey response data.

Even with the limitations of our experiment, behavioural differences were found in the squid which attacked the prey at the different temperature treatments (19°C, 22°C and 25°C). An increase in acclimation temperature greatly reduced the latency

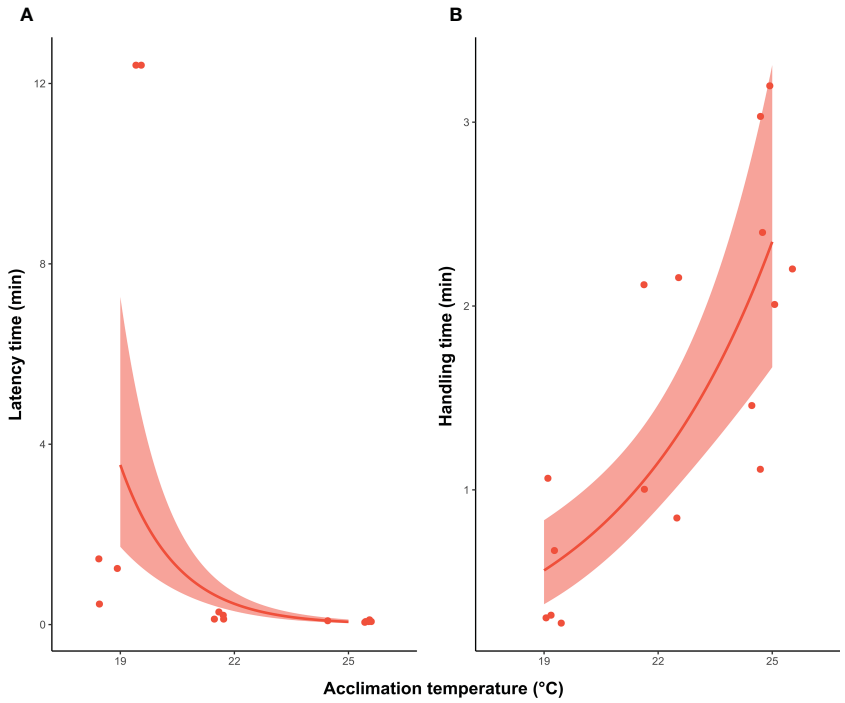

FIGURE 4
Southern calamari (*Sepioteuthis australis*) which successfully attack prey at different temperatures of acclimation (19, 22 and 25°C). **(A)** GLM model with a gamma distribution of latency time (min) that squid took to make the first attack **(B)** GLM with a gamma distribution of the handling time (min) to capture and kill the prey. Thick line represents the expected value from the model for the variable measure. The shadow band corresponds to the 95% confidence interval, and the individual values are represented by the red points.

time of squid to attack the prey. At 22°C and 25°C, individuals strike the prey in seconds, whereas at 19°C squid took between 1 and 11 min to attack. Time until first attack could be interpretated as measure of predation motivation (Grigaltchik et al., 2012), consequently results here suggest the motivation of squid to make an attack is directly influenced by temperature. In contrast, the handling time that squid needed to kill the prey increased with temperature, rising to over a min at 22°C and above 2 min at 25°C compared with a 0.5 min at 19°C. Furthermore, the capacity of squid to successfully strike the prey on the first attempt was also reduced at 25°C, with only 28% (2 out of 7) of squid capturing the prey in the first attempt compared with 60% and 75% at 19°C and 22°C, respectively. These results indicate that the physical capabilities of squid might be impacted at elevated temperatures, reducing their ability to handle (capture and kill) the prey effectively, although at this time, any potential prey co-response cannot be assessed. In other marine invertebrates, for example the Antarctic sea star (*Odontasted validus*), some physical abilities such as motor coordination or speed were also reduced at increased temperatures (Kidawa et al., 2010). Findings of a possible decrease in capture efficiency and increased prey handling time suggest that further research should consider the energetic balances and trade-offs involved in the need for increased predation among squid at elevated temperatures. Nevertheless, the effect of acclimation temperature on the prey could potentially be linked with the increase in handling time and the success rate of strikes on the first attempt, as higher temperatures may increase prey mobility. Future studies exploring these effects could examine how changes in water conditions affect the prey, to determine if those effects could be influencing squid ability to capture their prey.

Attack distance and tentacle elongation of squid increased at higher temperatures; this could also be related to the impatience of squid to capture the prey instead of a predatory strategy per se. Yet, greater attack distance could be beneficial for the squid if prey mobility is increased due to warmer waters by better disguising the incoming attack and reducing the chances of escape. In some cephalopod species, environmental stresses like acidification also lead to an increase in striking distances (Spady et al., 2018). Regarding their other predatory tactics, neither the attack direction nor body pattern showed differences between temperature treatments. However, across treatments 19°C, 22°C and 25°C, the most preferable body pattern was a dark color. This pattern (banded or totally dark) possibly acts as a disrupting coloration, distracting the attention from the extending tentacles (York and Bartol, 2016; Hanlon and Messenger, 2018) which might confuse the prey, and confer an advantage to the squid.

As the metabolic demands of squid are likely to increase due to ocean warming, *in situ* individuals might have different options for maintaining their energetic balance and overall fitness including, for example, consuming prey with higher energetic/nutritional content or increasing their feeding rates (Horwitz et al., 2020). If the individuals choose the latter option, squid may be more willing to actively search for prey, perhaps resulting in bolder behaviour and exposing them to a higher predation risk (Biro and Stamps, 2010; Killen et al., 2011; Careau and Garland, 2012; Cornwell et al., 2020). If individuals are more active, more energy would be allocated to

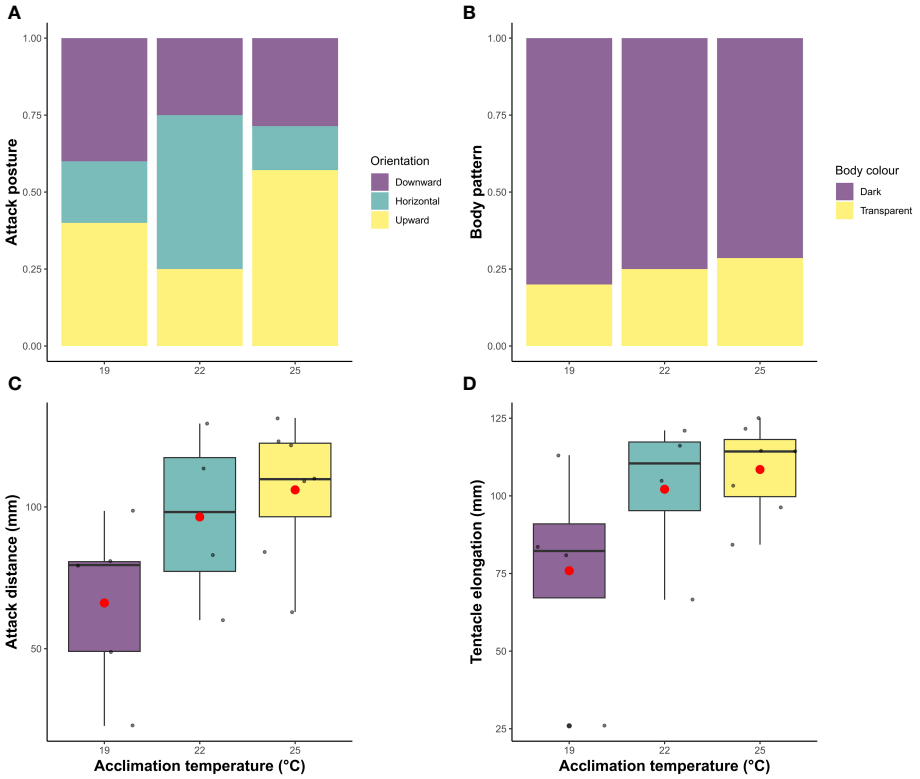

FIGURE 5
Behavioural measures of southern calamari (*Sepioteuthis australis*) that successfully attacked prey at different temperatures of acclimation (19, 22 and 25°C). **(A)** the proportion of squid exhibiting the different positions of attack (downward, horizontal arms, and upward). **(B)** the proportion of squid exhibiting different body display patterns (dark and transparent). **(C)** boxplots of the attack distance (mm) and **(D)** tentacle elongation (mm). In the boxplot, points indicate individual values and boxes represent the first and third quartiles. Within each box, the median is represented by the solid line and the mean by the red point.

locomotion, limiting energy available for other important traits and processes such as reproduction or growth. Furthermore, the metabolic thermal optimum (T*opt*) indicated by the aerobic scope for this species is between 19°C and 22°C (Peinado, 2021), beyond that point individual performance maybe be limited (Peck et al., 2009; Pörtner and Peck, 2010).

Modifications to predatory performance could alter the outcome of predator–prey interactions, potentially resulting in a cascade effect throughout food webs (Warren et al., 2017). Cephalopods are important and opportunistic predators, where changes in their feeding rates could have significant consequences for the structure of ecosystems (Spady et al., 2018; de la Chesnais et al., 2019). Southern calamari are likely to increase feeding intake rates as ocean warming continues in Tasmanian waters, putting greater pressure on their prey and potentially triggering a cascade effect on the food web, altering community and ecosystem stability.

To date, in an ocean warming context, information on feeding rates (especially in the wild), energy budgets and prey preferences in cephalopods is limited and future research could further examine these key concepts to better understand species performance under the various climate change scenarios. In the experiments reported here, trends across treatments were clear and consistent. However, further work could expand our results by exploring differences with sex, life stages and growth rate. This would be influential in more accurately predicting the responses of this critical animal group to future environmental challenges. Moreover, marine ecosystems are not only being altered by ocean warming, including changes in size structures (Audzijonyte et al., 2020), but also acidification, pollution, changes in productivity, and oxygen content, as well as overfishing (Hoegh-Guldberg and Bruno, 2010; Payne et al., 2016). The physical capacities of other species of squid to capture their prey were reportedly not impacted by environmental stressors such as acidification, although their decision-making processes and strategies have been shown to be affected (Spady et al., 2014; Spady et al., 2018) However, we have an extremely limited understanding of how multiple stressors will combine to impact the predatory behaviour of this group – an important gap in our knowledge of how future squid populations will respond to climate change over coming decades(Pecl and Jackson, 2008).

5 Conclusion

Ocean warming will modify ecosystems by affecting the interactions of existing predator–prey combinations, as well as creating novel ones as new species enter regions and existing species depart (Pecl et al., 2017; Bonebrake et al., 2018), In this context, it is important to examine how species predatory

performance alters due to environmental changes (Twiname et al., 2020). Our study demonstrated that environmental temperature has a major impact on the behaviour of southern calamari. Overall, our findings indicate that elevated temperatures, in line with those predicted for the future, could alter the predatory behaviour of southern calamari by increasing consumption rates, as well as potentially increasing the number of attempts they need to capture their prey, and their prey handling times. Individuals would then need to be more active to capture prey to maintain energetic balance and as a result they will be more exposed to predation risk, as well as experiencing greater trade-offs.

Data availability statement

The original contributions presented in the study are includedin the article/Supplementary Material. Further inquiries can bedirected to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by University of Tasmania Animal Ethics Committee (A0017463).

Author contributions

PP, QF, JS, ST, and GP designed the study. PP conducted the experiments, analyzed the data and drafted the first manuscript. QF, JS, ST, and GP reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1113984/full#supplementary-material

TABLE S.1
Data form this study.

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Genetic confirmation of *Octopus insularis* (Leite and Haimovici, 2008) in South Florida, United States using physical features and *de novo* genome assembly

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The distribution of octopuses within the *Octopus vulgaris* species complex remains inadequately understood. Species determination can be complex and involves characterizing a specimen's physical features and comparing its genetic makeup to other populations. In this study, we present the first genetic confirmation of *Octopus insularis* (Leite and Haimovici, 2008) inhabiting the coastal waters of the Florida Keys, United States. We employed visual observations to identify species-specific body patterns of three wild-caught octopuses and used *de novo* genome assembly to confirm their species. All three specimens exhibited a red/white reticulated pattern on their ventral arm surface. Two specimens displayed body pattern components of deimatic display (white eye encircled by a light ring, with darkening around the eye). All visual observations were consistent with distinguishing features of *O. insularis*. We then compared mitochondrial subunits COI, COIII, and 16S in these specimens across all available annotated octopod sequences, including *Sepia apama* (Hotaling et al., 2021) as a control outgroup taxon. For species exhibiting intraspecific genomic variation, we included multiple sequences from geographically distinct populations. Laboratory specimens consistently clustered into a single taxonomic node with *O. insularis*. These findings confirm *O. insularis* presence in South Florida and suggest a more extensive northern distribution than previously assumed. Whole genome Illumina sequencing of multiple specimens enabled taxonomic identification with well-established DNA barcodes while also generating the first *de novo* full assembly of *O. insularis*. Furthermore, constructing and comparing phylogenetic trees for multiple conserved genes is essential for confirming the presence and delineation of cryptic species in the Caribbean.

KEYWORDS

Brazilian reef octopus, *Octopus vulgaris*, Octopus americanus, genomic assembly, cryptic species, cephalopod genetics, *de novo* assembly, body patterns

1 Introduction

The taxonomy of benthic octopuses in the shallow waters of the tropical western Atlantic Ocean, Gulf of Mexico, and Caribbean Sea is complex. Numerous octopus studies focus on characterizing a single species' life history traits, trophic interactions, or ecological role (Ambrose, 1988; Forsythe and Hanlon, 1988; Aronson, 1989; Anderson et al., 2008; Leite et al., 2009; de Beer and Potts, 2013). However, multiple species coexist in shallow areas and can exhibit similarities in morphology and behavioral phenotypes (Hanlon et al., 2008; O'Brien et al., 2021). Consequently, they can be easily mistaken for the same species or misidentified, leading to inaccurate reports of biodiversity, population size, and food web dynamics. These cryptic octopus species coexist in regions where molecular research has been limited until recently (Norman, 2003).

Octopuses have experienced significant taxonomic revision and expansion, particularly in the western Atlantic Ocean, Gulf of Mexico, and Caribbean regions (Norman, 2003; O'Brien et al., 2021). The genus *Octopus*, once viewed as a "catch-all" genus, has been discovered to be polyphyletic (Guzik et al., 2005), and now encompasses numerous cryptic species—morphologically similar yet genetically distinct octopus species (Knowlton, 1993; Amor et al., 2016). *Octopus vulgaris* (Cuvier, 1797), originally described by Cuvier (1797) from the Mediterranean, was once considered a cosmopolitan species distributed across tropical, subtropical, and temperate waters. Due to geographic and temperature boundaries (while still exhibiting morphologically similar traits), type species names based on location were assigned to *O. vulgaris*: *sensu stricto* (Mediterranean, northeast Atlantic), Type I (western Atlantic), Type II (southwest Atlantic: Brazil), Type III (South Africa and Indian Ocean), and Type IV (east Asia). However, the taxonomic resolution remained elusive. Mitochondrial genes have been used to distinguish *O. vulgaris* species on both coasts of the Americas (COIII and 16s, Söller et al., 2000; Warnke et al., 2002; 2002), in the Atlantic Ocean, South Africa, Japan, and Taiwan Province of China (16S and COIII, Warnke et al., 2000), from Brazil (16S, Leite et al., 2008), and from Amsterdam and Saint Paul islands (COI and COIII, Guerra et al., 2010). By integrating molecular and morphological data, studies have been able to identify multiple distinct species Xu et al., 2022; Santana-Cisneros et al., 2021. However, results still indicate the existence of a single, widely distributed *O. vulgaris*.

Advancements in technology, including underwater photography, videography, and morphological and molecular tools, has led to further recent taxonomic revisions, and resulted in the naming, renaming, and redescribing of numerous octopus species throughout western Atlantic and Caribbean (Amor et al., 2016; Guerrero-Kommritz and Camelo-Guarin, 2016; Guerrero-Kommritz and Rodriguez Bermudez, 2019; Avendaño et al., 2020a; O'Brien et al., 2021). As a result, *O. vulgaris* and close relatives have formed the *Octopus vulgaris* species complex including at least six species: *O. vulgaris sensu stricto*, *O. vulgaris* Type III, *Octopus sinensis*, *Octopus tetricus, O. cf. tetricus*, and *O. americanus* (Amor et al., 2016; 2017; 2019; Gleadall, 2016; Avendaño et al., 2020a). Both once described as *O. vulgaris*, *Octopus insularis* (Leite and Mather, 2008) is shown to be morphologically distinct and genetically distant from the complex (although still mentioned as a member of the *O. vulgaris* group) and *O. americanus* (Froriep, 1806) is proposed to be the reinstated name for conspecifics *O. vulgaris* Type I and Type II due to genetically distant results from *O. vulgaris sensu stricto* (Ritschard et al., 2019; Avendaño et al., 2020a; Lima et al., 2020b). These molecular, morphological, and behavioral observations and revisions spanning over two decades highlight the challenges of cryptic species identification and emphasize the need to explore innovative methodologies while optimizing existing phylogenetic techniques for octopuses. As arguably the most intelligent invertebrates, octopuses possess a genome size several times larger than other sequenced molluscan and lophotrochozoan genomes (Albertin et al., 2015; Amor et al., 2019).

Octopus insularis was historically believed to inhabit only the tropical waters of reef and rocky substrates off the coast of Brazil. However, recent findings have identified this species in various locations, including the Gulf of Mexico, Turks and Caicos, the Bahamas, Bermuda, and southeastern Florida (Leite et al., 2008; Leite et al., 2009; Sales et al., 2013; Lima et al., 2017; González-Gómez et al., 2018; Rosas-Luis et al., 2019; Lima et al., 2020a; O'Brien et al., 2021; Lima et al., 2023). The presence of *O. insularis* in these newly discovered ranges was determined through underwater photography and videography, identifying species-specific body pattern components and habitat features (Lima et al., 2017; Gonzalez-Gomez et al., 2018; O'Brien et al., 2021). While these methods are useful for investigating species presence in new areas, genetic analysis is crucial for studying cryptic species found in the western Atlantic. Furthermore, understanding both the known and proposed expanded distribution in the region and connecting the identified populations requires genetic analyses of sampled animals (e.g., Ritschard et al., 2019). For example, genetic samples were recently used to confirm *O. insularis* occurrence off the coast of West Africa (Lima et al., 2023).

Genetic tools such as DNA barcodes have become increasingly important for confirming species identity in octopuses. However, to optimize the detection of interspecific differences, previous studies have employed multiple established barcodes to distinguish between species (Hollenbeck et al., 2017; Avendaño et al., 2020a). Sequences used as barcodes typically meet the following criteria: 1) they exhibit significant variation between species; 2) they are flanked by highly conserved regions, allowing the use of universal PCR primers to excise these regions; and 3) they are short regions (less than 1 k bp) that can be easily amplified (Kress and Erickson, 2008). With the advancement of next-generation sequencing technologies, the cost of generating long reads has decreased dramatically, enabling researchers to characterize biodiversity across the animal kingdom in an unprecedented way (Hotaling et al., 2021). Whole genome sequencing is especially important in octopod studies, as they possess expansions of certain subsets of gene families exceeding that of other invertebrates (Albertin et al., 2015).

De novo genome assembly, a technique for constructing an entire genome from raw sequencing data of a specimen without referencing a known species' complete genome, has been employed to reconstruct the genomes of various octopus species Zarrella et al., 2019. This approach offers insights into the genetic differences and evolutionary trajectories of different taxa (Kim et al., 2018; Li et al., 2020). It is particularly valuable for accurately characterizing cryptic

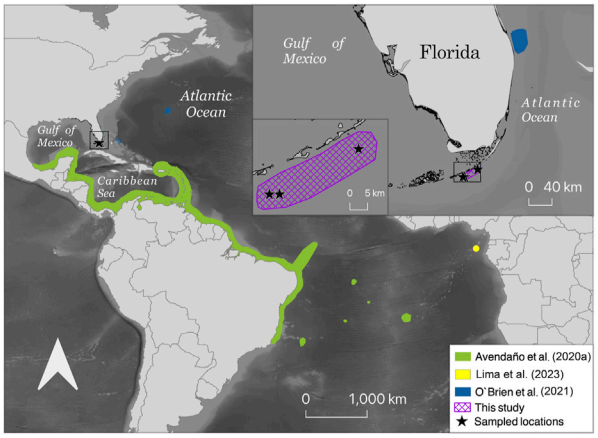

FIGURE 1
Map of collected *Octopus insularis* in the Florida Keys, Florida, United States, and the proposed expansion of the known range of *O. insularis*. All three octopuses sampled in this study were collected in shallow waters off the Florida Keys where the Gulf of Mexico meets the Atlantic Ocean. The main map shows the distribution range of *O. insularis* from O'Brien et al. (2021); Avendaño et al. (2020a), and Lima et al. (2023) with new locations from this study added. The inset map at the top right shows the distribution range of *O. insularis* from O'Brien et al. (2021) and this study.

complexes, as it minimizes the chance of bias introduced by aligning reads to the reference genome of a closely related taxon. The objective of *de novo* genome assembly is to obtain the most contiguous and accurate representation of the original genome by sequencing millions of DNA fragments (reads) assembling overlapping reads into longer contiguous sequences (contigs). Scaffolds, larger genomic sequences with gaps between contigs, are then constructed by identifying where reads present in the contigs are arranged larger fragments with known length, but undetermined sequence (Waterston et al., 2002; Baker, 2012). Assembly is further refined through error correction and consensus building. By sequencing and assembling the genomes of individuals from different populations, researchers can identify unique genetic signatures specific to each species. These genetic signatures can then be employed to differentiate cryptic species and provide evidence of their distinctiveness. Examining single nucleotide changes across conserved regions allows for an understanding of how evolutionary selection rates vary across the genomes of related species (Wu et al., 2018). In addition to characterizing trends across large genomic regions, shorter barcode regions within these sequences can be compared across species for identification. This approach echoes previous methods by allowing for comparison with all species for which barcodes have been generated.

In this study, we report the presence of *O. insularis* in the Florida Keys, United States, firstly by visual identification (body patterns and components) and secondly by confirmation through genetic analysis. Our research includes a description of the body patterns and components of the sampled animals, and the first use of *de novo* genome assembly for species confirmation of *O. insularis*. We discuss the significance of *O. insularis*' presence in areas bordering the Gulf of Mexico and the western Atlantic Ocean as well as this molecular tool in identifying cryptic cephalopod species.

2 Methods

2.1 Animal origins and housing

All animals were collected off the coast of the Florida Keys, Florida between January 8 and 15, 2021 (Figure 1) by Dynasty Marine. Octopuses were caught in shallow waters (6–7.5 m depth) on light-colored bottom substrates of patch reefs, locally referred to as "whip patches," i.e., thin strips of limestone and coral reef with small aggregations of vertebrates and invertebrates. Octopus dens were in areas with rocks of ~30 cm heights and gorgonians on the bottom. These individuals were acquired and maintained for behavioral studies. Species identification using genomic and genetic information were needed to confirm the study species before reporting on behavioral findings in the laboratory experiments (Ramos et al., 2023).

The three octopuses (specimens A, B, and C) were shipped from the Florida Keys to the Laboratory of Integrative Neuroscience and the Comparative Biosciences Center at The Rockefeller University in Manhattan, New York. Octopuses were housed individually in 120-gallon glass aquariums (Aqueon, 121.9 cm length × 45.7 cm width × 71.1 cm height) with various shelters and sources of enrichment. Each tank was connected to a closed circulation system for filtration centered in a 36 gallon three-partition sump (91.4 cm length × 34.6 cm width × 38.1 cm height) (Trigger 36 Crystal Sump) housed underneath the aquarium. Water temperature was regulated with a heating element (Finnex Titanium) and a digital temperature controller (Aqua Logic). Water quality was rigorously monitored and maintained. Waste was extracted from filter socks and cleaned daily. The system also regulated the automated 12 h/12 h light/dark cycle of the overhead light fixtures to simulate natural light conditions. Lighting consisted of LED light bulbs in conical aluminum light fixtures, two 250-W bulbs provided white light in the daytime (07:00–19:30 h) and two 36-W bulbs provided deep red light (660 nm) at night (19:30–07:30 h). Water quality parameters were manually tested multiple times daily for pH, salinity, water temperature, and the concentration of ammonia, nitrates, nitrates using API testing kits and handheld electronic sensors (Hanna Instruments, Woonsocket, RI) to maintain optimal levels for the animal (pH: 8.2–8.5; salinity: 32–34 ppt; water temperature: 23–25 C; ammonia: 0 ppm; nitrates: <20 ppm; nitrites: 0 ppm). The sand was cleaned daily with a siphoning gravel washer to remove organic material including sucker cuticles shed by the octopus, animal excrement, and other small organic material left from food remains and uneaten food. Each octopus was fed 50–150 g pieces of thawed frozen shrimp or whole shrimp once to twice a day (11:00 and 16:00 h). Animals were housed for up to 4 months and died of natural death without any indication of distress or illness. After postmortem inspections of the bodies, they were stored at −20°C. A 0.5-g tissue punch was taken from the arm of specimen A and B, while 0.5 g of both kidney and gill tissue

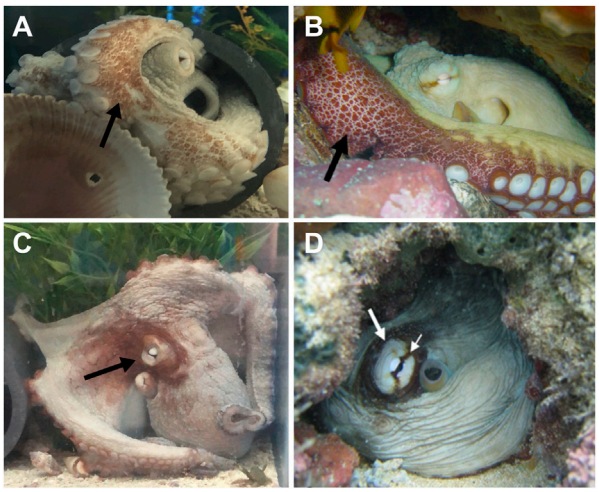

FIGURE 2
Images of different behaviors and body patterns observed in captive *Octopus insularis* species studied here **(A,C)** and images of the species identified through imagery from O'Brien et al. (2021), images **(B,D)**. All three individuals displayed species-specific patterning for *O. insularis* on the ventral surface of their arms. Deimatic display was confirmed in two animals. **(A)** The red-white reticulated pattern on the ventral surfaces of the arms appeared as patches on a light background and were primarily visible when the animal was within its den. **(C)** Octopus in deimatic display with a white eye, encircled by a light ring, and darkening of the head around the eye. This individual did not regularly present a distinct dark eye bar unlike the wild *O. insularis* **(B,D)**.

were extracted from specimen C. Samples were kept frozen at −80°C until DNA extraction.

2.2 Visual characteristics of *Octopus insularis*

Digital video and imagery were used to characterize the physical appearance of the three *O. insularis*. We intermittently filmed the live octopuses with a Canon EOS 80D digital SLR camera fitted with a 55–150 mm lens (1,080 p, 30 fps) or a Sony FDR-AX53 4K Ultra HD Handycam Camcorder (2,160 p, 60 fps). Cameras were mounted on a tripod and positioned <1 m from the tank at a height of 1.5 m, oriented directly at the stationary animal. Imagery was reviewed to identify species-specific body patterns and components enabling confirmation of *O. insularis* (O'Brien et al., 2021). Two distinguishing features that were used to identify *O. insularis* from *O. americanus* (Figures 2A–D): 1) the coloration and patterning of the ventral arm surface described as dark red to purple to brown patches or "red/white reticulate" (Leite and Mather, 2008; Figures 2A, C) and 2) the deimatic display in each species. The deimatic display is found in different octopus species with species-specific characteristics (Packard and Sanders, 1971; Leite and Mather, 2008). In *O. insularis*, the deimatic display consists of a white eye, a pale ring encircling the eye, a dark eye bar, and a broader darkened area around the eye (Leite and Mather, 2008).

2.3 *De novo* genome assemblies for genetic species confirmation

Postmortem, all specimens were stored intact at −20°C for use in future studies. For DNA extraction, a 0.5 g tissue punch was taken from the arm of specimen A and B respectively, while 0.5 g of both kidney and gill tissue were extracted from specimen C. Tissue samples were kept frozen at −80°C until further processing. High molecular weight DNA was extracted from each tissue sample using the MagAttract system from Qiagen, which uses a silicon based magnetic bead technology to minimize shearing. Extracted DNA from each animal was then sequenced using the Illumina TruSeq DNA PCR Free kit on a NovaSeq6000. For each specimen, reads were assembled on the Galaxy Project Server, a community-driven web-based analysis platform (Afgan et al., 2018). Reads were trimmed and cleaned prior to assembly using trimmomatic to remove Illumina adapter sequences prior to assembly (Bolger et al., 2014). Raw reads were then checked prior to assembly using the NCBI Foreign Contamination Screen (FCS, 2023) tool (NCBI, 2022). Paired reads from each specimen were then assembled using the St Petersburg genome Assembler (SPAdes) algorithm version 3.9. SPAdes utilizes a de Bruijn graph methodology to optimize the assembly of Illumina short reads (Bankevich et al., 2012; Prjibelski et al., 2020).

2.4 Genetic barcode comparison

Expanding on previous work in octopus genetics, we employed a DNA barcoding protocol to compare the sequences of Cytochrome c oxidase subunit I (COI), Cytochrome c oxidase subunit III (COIII), and 16s ribosomal RNA gene (16s). These standard mitochondrial barcode regions have been widely used for species differentiation in octopods (Söller et al., 2000; Warnke et al., 2002; Warnke et al., 2004; Warnke et al., 2004; Leite et al., 2008; Guerra et al., 2010; Allcock et al., 2011; Amor et al., 2016; Avendaño et al., 2020b). To identify these regions in each whole-genome assembly, we locally aligned the sequence of *Sepia apama* (Hotaling et al., 2021), an outgroup species to the order Octopoda, to each assembly using the NCBI Blast + algorithm 2.13 (Altschul et al., 1990; Madden and Comacho, 2008). These sequences were then aligned to annotated sequences within Octopoda in the NCBI nucleotide database. For species with annotated sequences from multiple distinct geographic locations, we included at least two distinct sequences for each gene. To determine percent sequence identity, we aligned all available sequences across octopods to that of *S. apama*, as well as our three experimental specimens, using the Multiple Alignment Fast Fourier Transformation (MAFFT) v7.490 (Katoh et al., 2002; Katoh and Standley, 2013) to calculate phylogenetic relation ad pairwise sequence identity. From this alignment, we generated a phylogenetic tree using a Tamura-Nei neighbor-joining model to calculate the distance between sequences based on their percent shared identity (Tamura et al., 2004). We implemented this model through the PhyML 3.3.20180621 phylogenetic tree-generating program (Guindon et al., 2010).

TABLE 1 Assembly statistics of lab specimen *de novo* genome assemblies and the current *Octopus vulgaris* reference genome (*Octopus vulgaris* (ID 12157)—Genome—NCBI, n.d.).

	Specimen A	Specimen B	Specimen C	*O. vulgaris*
Total length	1,637,756,730	1,622,104,405	1,617,965,393	1,772,957,336
# Contigs	777,909	755,111	845,134	786,906
N50	2,537	2,624	2,178	3,040
L50	181,381	168,259	207,630	137,635
GC%	36.18	36.07	36.22	36.79
Reference Free?	Yes	Yes	Yes	No

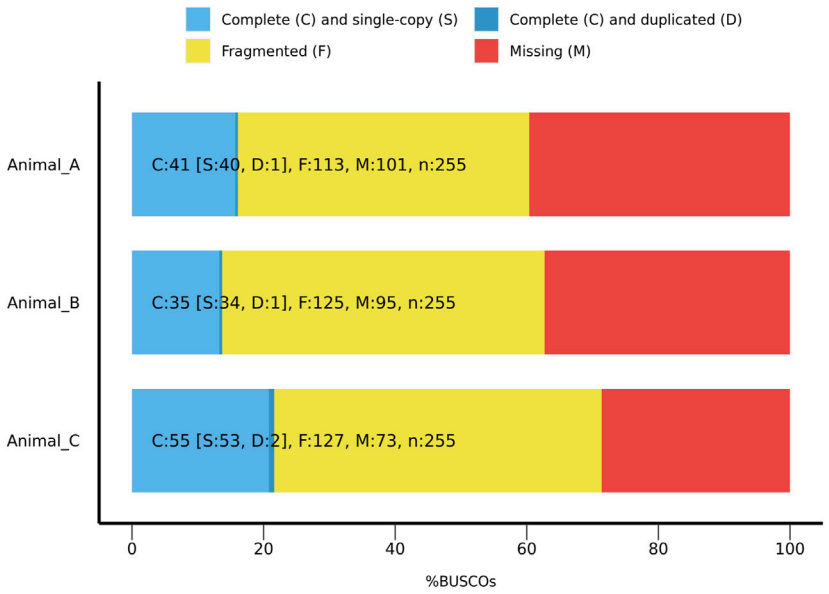

FIGURE 3
BUSCO ratios for complete, fragmented, and missing orthologs from the eukaryota database 10 across all animals sequenced for 255 orthologous genes, for all three specimens.

3 Results

3.1 Visual observations

Similar to previous descriptions of *O. insularis* (Leite et al., 2008; Leite and Mather, 2008; O'Brien et al., 2021), all three octopuses in this study exhibited distinct configurations of patches ranging from dark red to purple to brown on their arms. This feature is described as a "red/white reticulate on ventral arms" (Leite and Mather, 2008; Figures 2A, C). The red/white reticulated pattern on the ventral arm surface was easily identifiable when the animal was resting or sitting in its den with its anterior arm pairs exposed. Two animals were observed displaying a deimatic behavior, presenting a white eye encircled by a light ring within a darkened area on the octopus's head (Figure 2B). In contrast to previous reports in wild *O. insularis*, individuals in this study did not consistently display a dark eye bar (Figure 2D; O'Brien et al., 2021).

3.2 Assembly quality assessment

Across the independent *de novo* assemblies of each specimen, there was a consensus in general statistics, which were generated using gfaststats version 1.3.6 (Formenti et al., 2022). The specimen assemblies were of comparable quality to the currently available reference genome for *O. vulgaris* (Table 1). Among the *de novo* assemblies presented in this study, specimen B generally exhibited the highest quality. It had the fewest contigs in the assembly of the three specimens, despite having the greatest total length, indicating that it is the most intact of the three. Furthermore, the N50, a weighted mean statistic such that 50% of the assembly is composed of reads this length or greater, is highest in specimen B, while the L50, the minimum number of contigs whose length sum 50% of the total assembly size, is lowest in this sample. Across all specimens, the GC% content is approximately 36%, indicating agreement in sequence composition between the distinct assemblies.

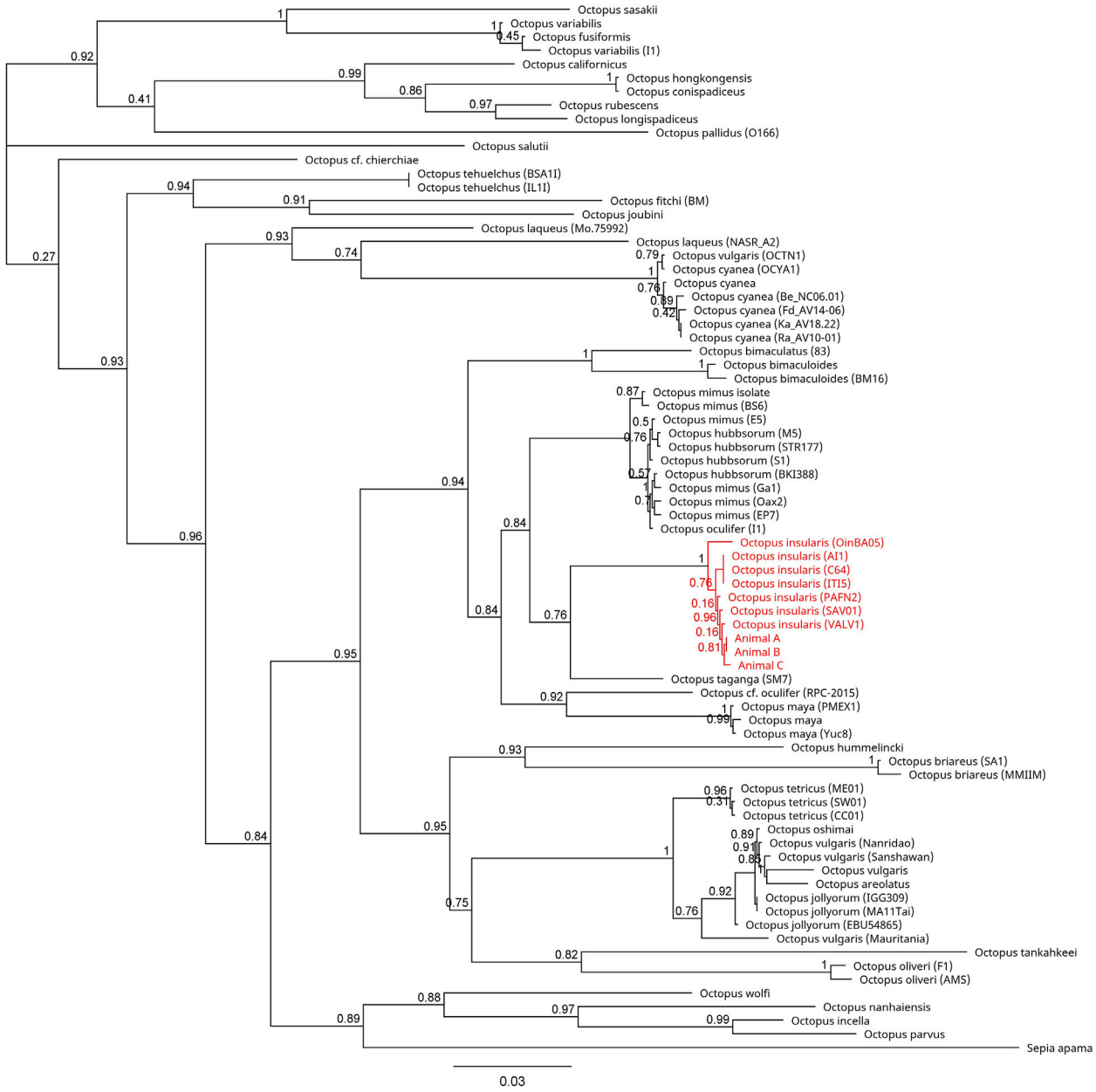

FIGURE 4
Phylogenetic tree of 77 available COI sequences in octopods and the outgroup *Sepia apama*. Branch length is scaled to indicate the average number of nucleotide substitutions per site. The node capturing the laboratory specimens and *Octopus insularis* is highlighted in red text.

To assess accuracy and intactness, these assemblies were screened using the Benchmarking Universal Single-Copy Orthologue (BUSCO) tool version 5.4.6. (Simão et al., 2015). Using the eukaryota ortholog database version 10, each assembly was screened for a set of orthologous genes shared across eukaryotes. Of the 255 orthologs screened, between 13%–22% were found to be intact in each assembly, with 44%–50% of orthologs present in a fragmented form (Figure 3). Raw reads for each specimen had 21-mer histograms generated with Meryl Version 1.3, a genomic kmer counter which assesses the frequency for all reads of a given length *k* (Rhie et al., 2020). To estimate size of the final assembly from the raw data, 21-mer frequency histograms were generated using Genomescope 2.0 (Vurture et al., 2017). From these histograms, Phred score, a measure of consistency of raw read kmer frequency in final assembly, was generated using Merqury version 1.1 (Rhie et al., 2020). All assemblies had a Phred score (QV) greater than 20, indicating a greater than 99% accuracy in assembly of raw reads (Supplementary Table S4; Supplementary Figure S5).

3.3 Data availability

All assemblies have been developed under the NCBI BioProject ID PRJNA938087. Currently, assemblies for Specimen A and B are

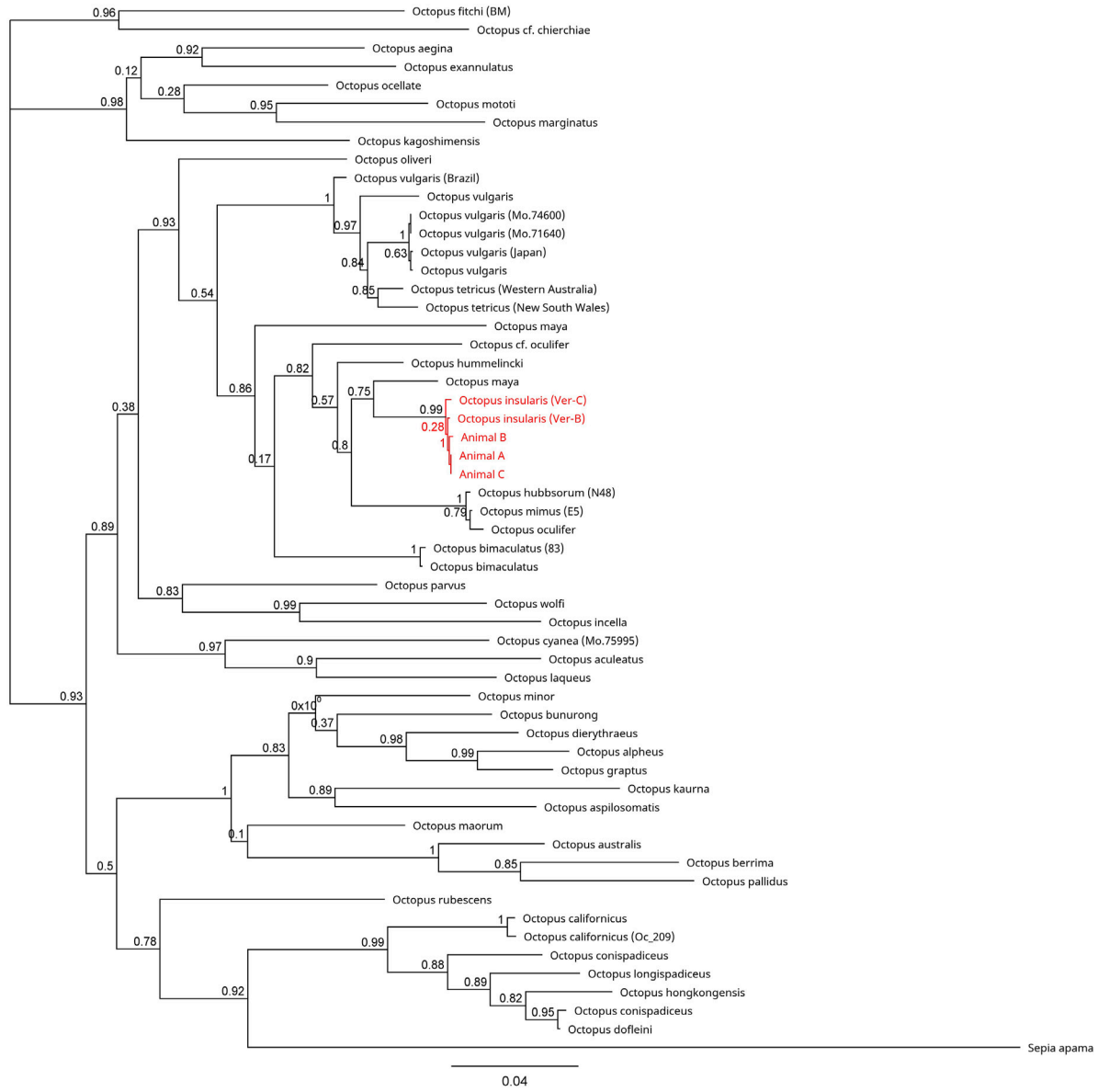

FIGURE 5
Phylogenetic tree of 57 available COIII sequences in octopods and the outgroup *Sepia apama*. Branch length is scaled to indicate the average number of nucleotide substitutions per site. The node capturing the laboratory specimens and *Octopus insularis* is highlighted in red text.

under review. Specimen C, the first to be sequenced, has passed review and been released into GenBank and is publicly available under accession number JARUKP000000000 (https://www.ncbi.nlm.nih.gov/nuccore/JARUKP000000000).

3.4 Barcode region comparisons

Across all mitochondrial regions compared, COI sequences exhibited the highest degree of genetic conservation, sharing 84.8% pairwise identity and 32.0% identical sites across 75 specimens representing 49 unique species (Figure 4; Supplementary Table S1). In contrast, available COIII sequences displayed 66.1% pairwise identity and only 1.5% identical sites across 57 sequences from 40 distinct species (Figure 5; Supplementary Table S2). The 16S region showed 78.8% pairwise identity with 10.0% identical sites across 65 specimens, consisting of individuals from 42 distinct species (Figure 6; Supplementary Table S3). In all three mitochondrial barcode regions, laboratory specimens A, B, and C clustered with each other. All three specimens shared highly similar sequences, with only a single nucleotide variation present between specimen C as compared to specimens A and B, which were identical to each other. For all mitochondrial barcodes assessed, the laboratory specimens formed a single clade that exclusively shared a node with the available partial CDS of *O. insularis*.

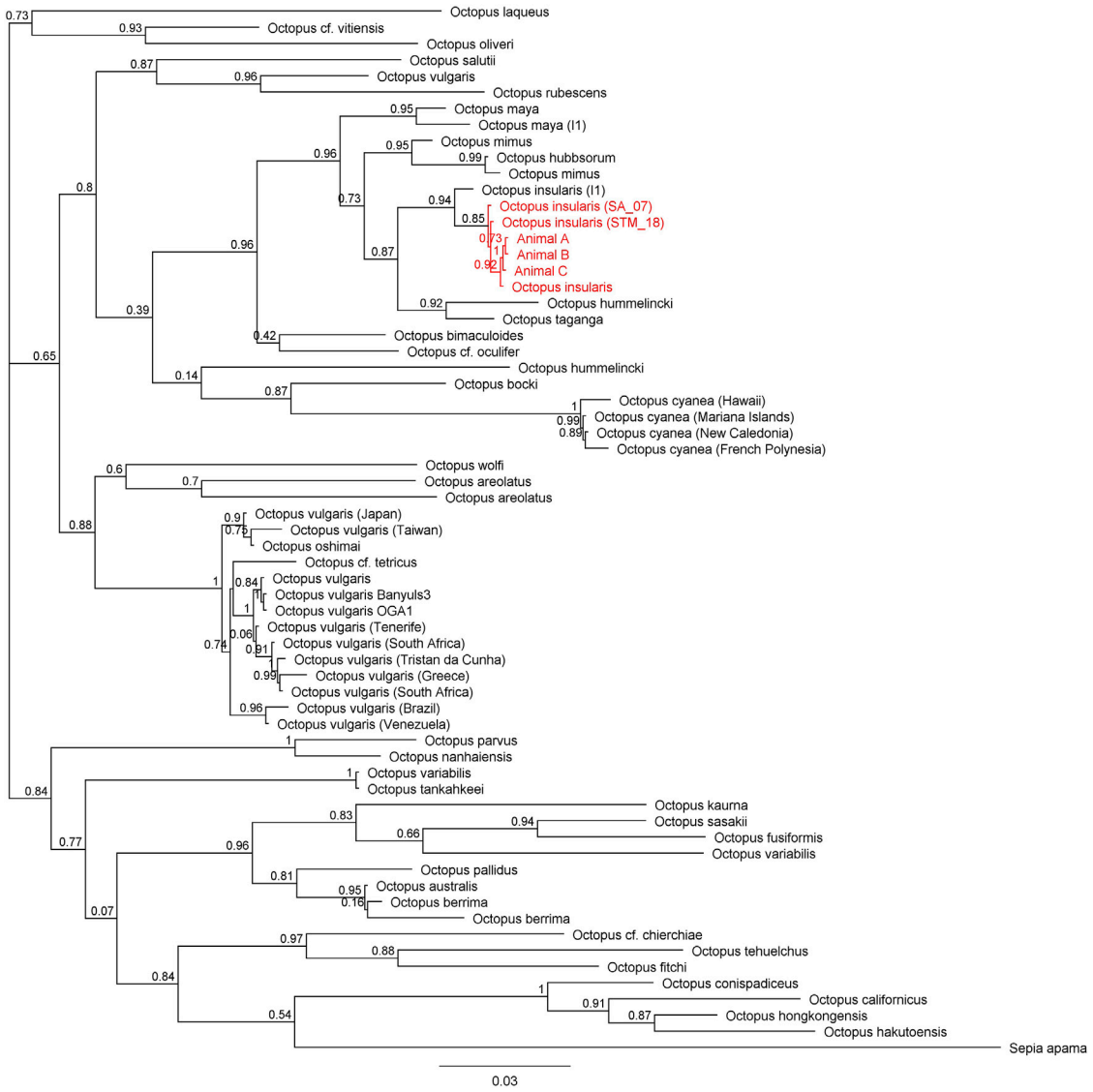

FIGURE 6
Phylogenetic tree of 65 available 16S sequences in octopods and the outgroup *Sepia apama*. Branch length is scaled to indicate the average number of nucleotide substitutions per site. The node capturing the laboratory specimens and *Octopus insularis* is highlighted in red text.

4 Discussion

Here we report the first record of full genomic sequencing for *Octopus insularis* and confirmation of its presence in South Florida. Three specimens were collected from the shallow tropical waters of the Florida Keys and visually identified as *O. insularis* based on distinguishing characteristics of this species (Leite et al., 2008; Leite and Mather, 2008; O'Brien et al., 2021). All mitochondrial barcodes (COI, COIII, 16S) clustered to a monophyletic group with *O. insularis* specimens, supporting evidence of this species in Florida.

O. insularis has been described as a generalist, living at a range of depths, temperatures, salinities, habitat types, and now newly proposed geographic areas. O'Brien et al. (2021) first described the sightings of O. *insularis* in its newly proposed western Atlantic northern range that was recently used as occurrence data for ecological niche models and dispersal simulations in conjunction with molecular methods (*Octopus* spp. collected from Africa) to determine possible transport and dispersal routes for *O. insularis*. Models and molecular results suggest that the distribution of *O. insularis* in America occurs from Florida to Brazil with potential suitable regions throughout the Gulf of Mexico and the Caribbean and trans-Atlantic distribution to Africa (Lima et al., 2023). Our study genetically confirms that the occurrence data for building such models is appropriate for the dispersion of *O. insularis* to Florida. Since these reports, additional sightings of this species have been documented in South Florida (C. O. Bennice, pers. observ).

Although now proposed as a widely dispersed cryptic species, morphological traits and body pattern features have only been described in detail for *O. insularis* in Brazil (Leite et al., 2008; Leite and Mather, 2008). Despite estimates of divergence between *O. insularis* and members of the *O. vulgaris* complex (19-41 million years) morphology of these taxa is relatively conservative except

where closely related species occur in sympatry and ecological character displacement may be used as a strategy to reduce resource overlap and facilitate species coexistence (Brown and Wilson, 1956; Amor et al., 2016). However, these sympatric cryptic species in Brazil (*O. vulgaris* and *O. insularis*) have maintained very similar morphology and it is now reported that they coexist in Florida where previous reports of high-density species coexistence occurs (Bennice et al., 2019; Bennice et al., 2021). Morphological traits have been used to successfully distinguish greater species-level diversity within the *O. vulgaris* species complex; however, broad sampling across known distributions to ensure robust morphological analyses has been suggested (Amor et al., 2016). Since both species are generalists, they may compromise and partition resources with little character displacement; however, given *O. insularis* recent geographic expansion and sympatry with many closely related species still warrants investigation of morphological and behavioral traits to ensure species are not misidentified when genetic tools may not be possible or in addition to genetic analysis.

For decades, *O. vulgaris*-like species have been and continue to be inaccurately classified under the species name *O. vulgaris*. Cryptic species are common among octopuses, which may possess subtle or indistinguishable morphological, behavioral, and color pattern traits. Moreover, these distinguishing features may not be adequately captured in video or photo or could be distorted during specimen preservation. We advocate for the inclusion of multiple species recognition mechanisms, encompassing genetic barcoding methods and full genomic sequencing. Numerous mitochondrial barcodes have been established for identifying octopus species, but discrepancies between mitochondrial barcodes still exist (Avendaño et al., 2020a). Additional genes, particularly nuclear genes, have received limited attention and warrant further investigation to determine their efficacy as molecular tools for identification, diversity, and divergence assessment (Amor et al., 2019; Avendaño et al., 2020a). While barcodes enable the comparison of a single region across species of interest, full genome assembly offers the same capability to compare barcodes across species, maximizing the genetic information that can be extracted from such valuable samples. As sequencing technologies become more accessible and cost-effective, whole genome approaches will enable researchers to better characterize variation within cryptic species and their closest relatives.

There is a crucial need for accurate baseline studies to comprehend the distribution, resource utilization, and population biology of cryptic species (Lima et al., 2017; Ángeles-González et al., 2021). The Food and Agriculture Organization (FAO) reports that approximately 100 octopus species are harvested; however, global statistics only identify four (*O. vulgaris*, *Octopus maya*, *Eledone cirrhosa*, and *E. moschata*), with the remaining likely categorized as unidentified octopuses. This study focuses on regions where artisanal fisheries play a significant role, but its findings also have wider implications for the importance of precise species identification in global octopus fisheries. These fisheries are valued at an estimated US$1.07 billion for exports and US$1.33 billion for imports, surpassing many finfish fisheries (Norman and Finn, 2016). Understanding how species baselines change due to environmental pressures, such as climate change, is vital for determining distribution and population connectivity. Genetic analyses are essential for gaining deeper insights into the ecosystem roles and effective fisheries management for these species (Bickford et al., 2007; Lima et al., 2017; Sauer et al., 2020).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA938087.

Ethics statement

The animal study was reviewed and approved by the IACUC at the Rockefeller University.

Author contributions

FY collected the specimens and supplied location and habitat descriptions. CB provided initial species identification and insight to perform genetic confirmation for this species in the proposed new northern geographic range. ER, BM, and MM conceptualized the study, collected initial data, and drafted the manuscript. ER and CB analyzed imagery for visual confirmation of species identity. BM conducted genomic analyses. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author FY was employed by Dynasty Marine.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1162807/full#supplementary-material

SUPPLEMENTARY FIGURE S5
Merqury table of kmer multiplicity in raw reads and across the assemblies of our samples. Merqury copy number spectrum plots for assemblies of laboratory specimens. Gray regions represent all 21-mers present only in the raw reads and red regions represent all kmers detected in the assembly. **(A)** Spectra ASM ST for Specimen A, **(B)** Spectra ASM ST for Specimen B, and **(C)** Spectra ASM ST for Specimen C.

SUPPLEMENTARY TABLE S1
Shared sequence identity across all COI sequences compared in this study.

SUPPLEMENTARY TABLE S2
Shared sequence identity across all COIII sequences compared in this study.

SUPPLEMENTARY TABLE S3
Shared sequence identity across all 16S sequences compared in this study.

SUPPLEMENTARY TABLE S4
Kmer based statistics of 21 base pair sequences in final assemblies.

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Bioluminescence in cephalopods: biodiversity, biogeography and research trends

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Numerous terrestrial and marine organisms, including cephalopods, are capable of light emission. In addition to communication, bioluminescence is used for attraction and defense mechanisms. The present review aims to: (i) present updated information on the taxonomic diversity of luminous cephalopods and morphological features, (ii) describe large-scale biogeographic patterns, and (iii) show the research trends over the last 50 years on cephalopod bioluminescence. According to our database (834 species), 32% of all known cephalopod species can emit light, including oegopsid and myopsid squids, sepiolids, octopuses, and representatives of several other smaller orders (bathyteuthids, and the monotypic vampire "squid", *Vampyroteuthis infernalis* and ram's horn "squid", *Spirula spirula*). Most species have a combination of photophores present in different locations, of which light organs on the head region are dominant, followed by photophores associated with the arms and tentacles and internal photophores. Regarding the biogeographic patterns of cephalopod species with light organs, the most diverse ocean is the Pacific Ocean, followed by the Atlantic and Indian Oceans. The least diverse are the Southern and the Arctic Oceans. Regarding publication trends, our systematic review revealed that, between 1971 and 2020, 277 peer-reviewed studies were published on bioluminescent cephalopods. Most research has been done on a single species, the Hawaiian bobtail squid *Euprymna scolopes*. The interest in this species is mostly due to its species-specific symbiotic relationship with the bacterium *Vibrio fischeri*, which is used as a model for the study of Eukaryote–Prokaryote symbiosis. Because there are many knowledge gaps about the biology and biogeography of light-producing cephalopods, new state-of-the-art techniques (e.g., eDNA for diversity research and monitoring) can help achieve a finer resolution on species' distributions. Moreover, knowledge on the effects of climate change

stressors on the bioluminescent processes is nonexistent. Future studies are needed to assess such impacts at different levels of biological organization, to describe the potential broad-scale biogeographic changes, and understand the implications for food web dynamics.

KEYWORDS

bioluminescence, mollusks, Cephalopoda, diversity, distribution, systematic review

1 Introduction

Bioluminescence is the production of visible light by a living organism (Wilson & Hastings, 1998; Haddock et al., 2010). This phenomenon results from a chemical reaction that involves the release of energy—in this case, light (Hastings, 1995; Wilson & Hastings, 1998). It usually involves a light-producing molecule called luciferin and an enzyme to control the reaction, such as a luciferase or a photoprotein (Shimomura, 2006; Haddock et al., 2010). Light production generally occurs in an organ usually referred to as a "photophore" or "light organ" (Haddock et al., 2010); such organs can be autogenic, where the organism itself produces light, or bacteriogenic, where an organism is in symbiosis with bioluminescent bacteria and benefits from their light production.

Bioluminescence has evolved independently in several lineages including bacteria and fungi, and has arisen multiple times in animals (particularly within marine groups), such as arthropods, cnidarians, fishes, and cephalopods. Its functions are diverse since it can be used for attraction purposes (predation or reproduction), as a defense mechanism, or for communication (Widder, 1999; Haddock et al., 2010). Very few freshwater organisms are capable of light emission, and only a few representatives live on land, making the marine realm the most diverse environment in terms of bioluminescent organisms. Given that the oceans are vast and encompass a wide variety of environments—from the cold waters of the poles to the warm waters of the tropics and from the warm well-lit surface waters to the cold and dark deep sea—the diversity of bioluminescent biota in the ocean realm is probably underestimated. In fact, for most marine organisms, bioluminescence is the sole source of light in their environment, with a tendency to increase in occurrence below the euphotic zone (Widder, 1999; Haddock et al., 2010).

Cephalopods are one of the marine invertebrate groups that contain light-producing species. They include both autogenic (Takahashi & Isobe, 1994; Tsuji, 2002) and bacteriogenic organisms (Ruby & McFall-Ngai, 1992; Guerrero-Ferreira & Nishiguchi, 2009; Anderson et al., 2014) and the bioluminescence can be expressed in different locations throughout their body (Herring et al., 1981; Robison & Young, 1981; Herring et al., 2002; Robison et al., 2003; Kubodera et al., 2007). For example, the photophores can be internal and associated with the ink sac (e.g., *Euprymna scolopes*, Figure 1A-A'), around the mouth of the animal (e.g., *Japetella diaphana*, Figure 1B-B'), on the integument (e.g., *Histioteuthis heteropsis*, Figure 1C-C') or even ocular (e.g., *Abralia veranyi*, Figure 1D-D'). In some cephalopods, photophores have developed from modified suckers (e.g., *Stauroteuthis syrtensis*, Figure 1E-E'); in others, they may be embedded within the tissue of the arms and/or tentacles (e.g., *Chiroteuthis calyx*, Figure 1F-F') or at the tip of their arms (e.g., *Taningia danae*, Figure 1G-G'). Cephalopods use bioluminescence as a camouflage strategy or as an anti-predation method (Dilly & Herring, 1974; Herring et al., 2002; Robison et al., 2003; Jones & Nishiguchi, 2004; Bush et al., 2009). Some also use light as a communication or attraction mechanism (Robison & Young, 1981; Kubodera et al., 2007; Bush et al., 2009; Burford & Robison, 2020).

In squids, autogenic photophores probably arose in a pelagic common ancestor and eventually diversified considerably in both morphology and position. In octopods, on the other hand, it appears that luminescence evolved separately in three different lines (Lindgren et al., 2012). The bacteriogenic photophores present in Loliginidae and Sepiolidae also appear to have evolved separately (Lindgren et al., 2012).

While many studies have been made on bioluminescence in cephalopods, a hundred years have passed since knowledge on this subject was first reviewed. Berry (1920a; 1920b) gathered information from 32 families to provide a guide on light production in this group. Out of these 32 families, he described 14 light-producing families (43.7%), including 128 bioluminescent species, out of a total of the 595 cephalopod species known at the time (21.3%) (Berry, 1920a; Berry, 1920b). Furthermore, Berry surveyed the positions and structures of photophores, showing that photophores could be found at several places in cephalopods, with the surface of the eyeball being the most common (Berry, 1920a; Berry, 1920b). Afterward, Herring published two reviews focused on cephalopods and other light-producing marine taxa (Herring, 1977b; Herring, 1988). More specifically, in 1977, Herring described luminescence found in several cephalopods (e.g. Teuthoidea comprising oegopsid and myopsid squids, Sepioidea and Vampyromorpha), with comparisons to luminescent fishes. He described: i) whether the luminescence was autogenic or bacteriogenic, ii) the position of the light organs as well as their structure, iii) how the light is produced and its functions, and iv) some information on the vertical distribution of some species (Herring, 1977b). Later, Herring combined knowledge about the luminous organs in other mollusks as well, reviewing bioluminescence in gastropods, bivalves, and cephalopods. He also gave more insights into the spectral emission found in luminous cephalopods (Herring, 1988).

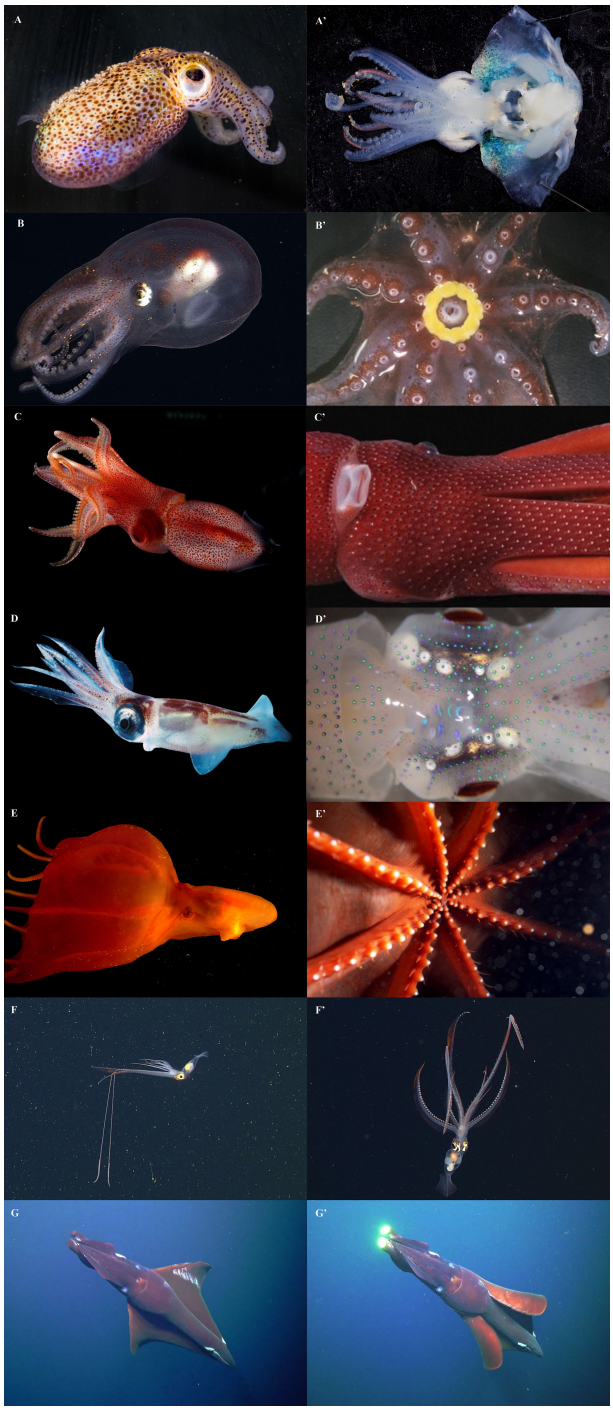

FIGURE 1
Bioluminescent cephalopods and the position of their respective light organs. **(A-A')** The Hawaiian bobtail squid, *Euprymna scolopes*, with an internal light organ. **(B-B')** The diaphanous pelagic octopod, *Japetella diaphana*, with a circumoral photophore. **(C-C')** The strawberry squid, *Histioteuthis heteropsis*, with integumental photophores. **(D-D')** the eye-flash squid, *Abralia veranyi*, with ocular photophores. **(E-E')** the glowing sucker octopus, *Stauroteuthis syrtensis*, with modified light-emitting suckers. **(F-F')** the swordtail squid, *Chiroteuthis calyx*, with photophores on the arms and tentacles. **(G-G')** the Dana octopus squid, *Taningia danae*, with photophores on the arm tips. Photo credits: Margaret McFall-Ngai and Edward Ruby **(A, A')**, MBARI **(B, F, F')**, Michael Vecchione **(B', D, D')**, Katie Thomas **(C)**, Henk-Jan Hoving **(C')**, Edith Widder **(E, E')**, and the Schmidt Ocean Institute **(G-G')**.

Since then, bioluminescence has been described in many more cephalopod species, including new species (Collins & Henriques, 2000; Norman & Lu, 2000; Lipinski, 2001; Jereb & Roper, 2005a; Jereb & Roper, 2005b; Jereb et al., 2005; Lu, 2005; Young & Vecchione, 2005; Bolstad, 2007; Kubodera et al., 2007; Bolstad, 2010; Lindgren et al., 2012; Braid & Bolstad, 2015; Braid, 2016; Bolstad et al., 2018; Judkins

et al., 2020; Reid, 2021; Lu & Okutani, 2022). Within this context, here we aim to: (i) present updated information on the taxonomic diversity of luminous cephalopods and morphological features; (ii) describe the global distribution of the bioluminescent cephalopods across different ocean basins (including coastal, epipelagic and mesopelagic realms), and last (iii) show the research trends (from 1971 to 2020) on bioluminescence in cephalopods (using a systematic PRISMA approach).

2 Diversity and morphology of luminous cephalopods

2.1 Diversity

Currently, the phylogeny of the Class Cephalopoda is still debated (Lindgren et al., 2004; Lindgren et al., 2012; Allcock et al., 2015; Allcock, 2017; Uribe & Zardoya, 2017; Sanchez et al., 2018; Anderson & Lindgren, 2021; Lindgren et al., 2023). In this review, we examined 10 extant orders, containing 50 families, with approximately 834 known species. Among them, 32% are presently understood to be bioluminescent (265 species, Figure 2, Supplementary Table 1 and Supplementary Material 1). At the order level, two are represented by a single species that is bioluminescent: the two monotypic orders Vampyromorpha (vampire "squid", *Vampyroteuthis infernalis*) and Spirulida (ram's horn "squid", *Spirula spirula*). Idiosepida, Nautilida, and Sepiida appear to be the only orders that do not contain bioluminescent species. The most diverse group of cephalopods, in terms of families and genera, are the oegopsid squids (24 families, 82 genera). At the species level, there are 249 oegopsid species, of which 71% are bioluminescent. The order Bathyteuthida (with 2 families and 9 species) contains 67% light-producing species. The Myopsida (2 families, 48 species) and Sepiolida (2 families, 89 species) include 33% and 63% bioluminescent species, respectively. In contrast, only 3% of octopod species (15 families, 308 species) produce light (Figure 2).

Important differences arise at the family level (Figure 3). For example, amongst the Sepiolida, bioluminescence is only present in "bobtail squids" (Sepiolidae, 71%). Among the myopsid squids, 32% of Loliginidae and the sole species of Australiteuthidae (*Australiteuthis aldrichi*) are bioluminescent. In the Oegopsida, several families are 100% bioluminescent, such as Brachioteuthidae, Cranchiidae, Cycloteuthidae, Enoploteuthidae, Histioteuthidae, Lycoteuthidae, Octopoteuthidae, Pyroteuthidae, and the following species, each the sole representative of its family: *Ancistrocheirus lesueurii* (Ancistrocheiridae), *Batoteuthis skolops* (Batoteuthidae), *Psychroteuthis glacialis* (Psychroteuthidae) and *Thysanoteuthis rhombus* (Thysanoteuthidae). A few other oegopsid families are partially luminescent: Chiroteuthidae (65%), Mastigoteuthidae (81%), Ommastrephidae (44%), Onychoteuthidae (38%), and one species of the Gonatidae (*Gonatus pyros*, 5%). Finally, *Stauroteuthis syrtensis* (Stauroteuthidae), *Japetella* spp. (3 species) and *Bolitaena pygmaea* (Amphitretidae, 50%), as well as *Cirroteuthis muelleri* and *Cirrothauma* sp. (2 species, Cirroteuthidae, 75%) are the only known bioluminescent species of the order Octopoda.

2.2 Photophores and spectra

To compare the diversity of bioluminescence among cephalopods, we assign photogenic tissue and secretions into six simplified categories: i) release of bioluminescent material to the surrounding environment; ii) strictly internal photophores that are associated with the ink sac or viscera; iii) circumoral photophores (around the mouth); iv) photophores on the arms and tentacles (comprising photophores on the arms and tentacles themselves, on the arm tips and tentacular stalks, at the base of the suckers and photophores with a sucker-like structure); v) photophores on the head region (combining the photophores on the head integument up to the base of the arms and internal photophores associated with the eyes and eyelids) and vi) photophores on the mantle region, comprising photophores both on the surface and embedded in the mantle, between and on the fins, as a strip along the ventral midline and/or on the funnel (Figure 4). In this comparison, we do not distinguish between bacteriogenic and autogenic photophores. We note that our grouping of photophores on the head integument with internal photophores associated with the eyes differs from previous reviews (Berry, 1920b; Herring, 1988), because they are thought to have mostly a similar role in counterillumination (Herring, 1988).

Most bioluminescent species have photophores at several different locations, with the head region being the most common position, followed by photophores associated with the arms and tentacles and internal photophores (Figure 5A). In the case of bathyteuthids, all luminous species have photophores on the head, and two of them (*Chtenopteryx sepioloides* and *C. sicula*) have an additional internal light organ (Figure 5B). On the other hand, the myopsid squids only possess internal photophores (Figure 5C). Octopoda is the only group with circumoral photophores, and some also possess light organs on the arms (Figure 5D). Oegopsid squids mainly present photophores on the head region and the arms and tentacles. However, many of these squids have additional light organs on the mantle region and internally (Figure 5E). Like the myopsids, sepiolid species possess internal photophores except for *Nectoteuthis pourtalesi*, the only sepiolid species to have photophores on the head region, and *Stoloteuthis japonica*, on the mantle region. Species of the genera *Heteroteuthis* (n=7) and *Sepiolina nipponensis* can also release bioluminescent material to their surroundings (Figure 5F). In the monotypic orders, *Spirula spirula* only presents photophores on the mantle area (Figure 5G), and *Vampyroteuthis infernalis* possesses photophores on the mantle region and the arms. The vampire squid can also release bioluminescent particles (Figure 5H).

The bioluminescent material released by some of the above-mentioned species is ejected when the animal is disturbed.

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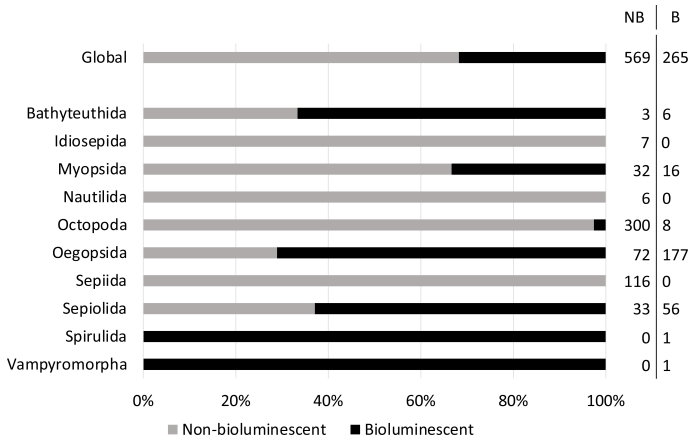

FIGURE 2
Percentage of known non-bioluminescent (grey) and bioluminescent (black) cephalopod species, globally and according to the cephalopod order. The number of non-bioluminescent (NB) and bioluminescent (B) species is detailed on the right of the figure.

Therefore, these luminous clouds are likely used as a defense mechanism to startle or distract predators and allow the animal to escape (Robison et al., 2003). In the case of the sepiolids (*Heteroteuthis* sp.), this fluid contains a high concentration of bioluminescent bacteria that are released to the animal's surroundings through the siphon, in combination with ink and mucus (Herring, 1977a; Herring, 1988; Herring, 2002). *Vampyroteuthis* releases bioluminescent material by a different process, ejecting glowing particles from the arm-tip photophores, in a stickier and more viscous matrix. This material can adhere to other surfaces, including potential predators, placing them at risk of secondary predation (Robison et al., 2003).

The function of the circumoral photophores remains unclear; the luminescence is only visible to the surrounding organisms and not to the emitting animal itself due to its position. Moreover, it is unlikely to be detectable by potential prey. Finally, as it is only present in female individuals, the most likely role of circumoral bioluminescence would be for sexual-recognition purposes (Herring et al., 1987). In terms of communication, the case of the oegopsid *Dosidicus gigas* ("Humboldt" or "jumbo" squid) is worth highlighting. In most cases, luminous cephalopods modify the light intensity for a single photophore, which usually glows in an outward direction. However, due to the tissue-embedded photophores of the Humboldt squid, in this case, light radiates through the tissues, causing the animal to luminesce entirely (Burford and Robison, 2020). This squid can reveal and conceal specific lighted body regions in any combination, using the overlying chromatophores. Such complex communication ability is probably crucial for group coordination and cohesion, which are usually only observed in well-lit environments (Burford and Robison, 2020).

Photophore position appears strongly correlated to cephalopods' habitats. Here, coastal species were considered to be associated with, but not necessarily restricted to, continental shelves (bottom depths <200 m) at late ontogenetic stages (as in Rosa et al., 2008a; Rosa et al., 2019). Pelagic species living outside the continental shelf were considered oceanic (as in Rosa et al., 2008b). In the present database, 97% of the coastal bioluminescent cephalopods possess internal photophores, and only a very few have external light organs. Only 9% of the coastal luminous species release bioluminescent material (Figure 6). In oceanic light-producing species, on the other hand, photophores are mostly present externally, on the head region (88%), on the arms and tentacles (62%), or on the mantle region (50%). Light organs associated with the ink sac and viscera are less common in this group (only 30% of the oceanic luminous species) compared to coastal bioluminescent organisms (Figure 6).

Interestingly, these patterns are maintained when we divide the groups according to light origin (autogenic vs. bacteriogenic photophores). Coastal light-producing cephalopods mainly comprise myopsid and bobtail squids, namely the Loliginidae and Sepiolidae (see also section 4), which have bacteriogenic photophores (Nishiguchi et al., 2004; Guerrero-Ferreira & Nishiguchi, 2009; Lindgren et al., 2012). By contrast, oceanic luminous species usually have autogenic light organs (Lindgren et al., 2012). Similar trends have been observed in fishes, where a higher proportion of bacteriogenic light organs is found in species that live closer to the ocean floor and the coastal areas, compared to oceanic fishes (Morin, 1983; McFall-Ngai and Toller, 1991; Haygood, 1993; Paitio et al., 2016).

Finally, the characteristics of the light produced should be considered. Cephalopods whose emission spectra have been measured produce light mostly within blue-green wavelengths (450-490mm; Herring, 1983; Haddock et al., 2010). However, more extreme values are possible in some species (Widder et al., 1983), ranging from 420 nm (*Chtenopteryx*; Herring, 1983; Herring, 1988) to 535 nm (*Abralia*; Herring, 1988). Some species appear to emit light outside even these values, either directly such as the bright yellow arm-tip photophores of *Taningia* (Figure 1G-G') or through colored filters (e.g., *Abralia*; Young and Tsuchiya, 2014).

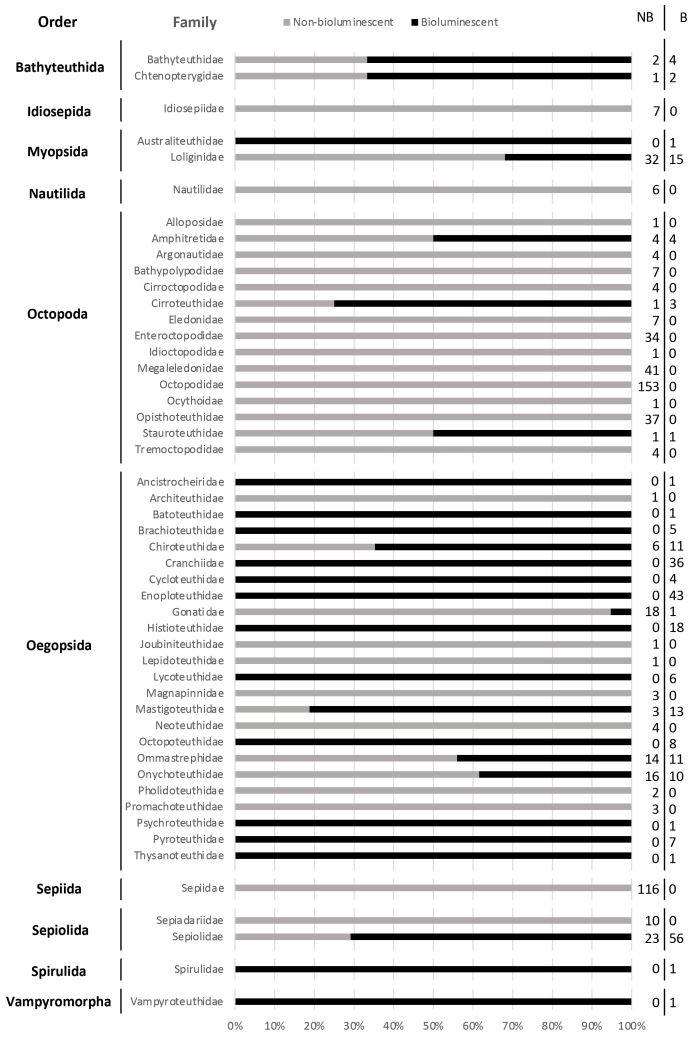

FIGURE 3
Percentage of known non-bioluminescent (grey) and bioluminescent (black) cephalopod species, according to each cephalopod family. The exact number of non-bioluminescent (NB) and bioluminescent (B) species is detailed on the right of the figure.

Some species of *Abraliopsis* and *Abralia* have also been observed to actively modify the emitted wavelength with temperature, which would correspond to temperature (and likely light intensity) variations encountered during vertical migration (Young and Mencher, 1980).

2.3 Bioluminescence disparities amongst cephalopod groups

In this section, we emphasize some ontogenetic, and inter- and intra-specific differences observed in cephalopod bioluminescence.

2.3.1 Bathyteuthida

Four species in the family Bathyteuthidae were considered bioluminescent, possessing photophores at the base of the arms. In 2020, the new species *Bathyteuthis inopinatum* and *B. devoleii* were described by Judkins et al. as lacking these basal arm photophores. In their third new species, *B. numerosus*, sexual dimorphism was observed, with photophores only present in males.

Within the Chtenopterygidae family, only *Chtenopteryx sepioloides* and *C. sicula* have been confirmed as bioluminescent, for which the large eyeball photophores can assist in identifying these species (Escánez et al., 2012; Escánez et al., 2018; Luna et al., 2021). The potential presence of photophores in *C. canariensis* remains debated (Escánez et al., 2012; Escánez et al., 2018; Luna et al., 2021).

2.3.2 Myopsida

Only 16 species (33%) of myopsid squids are bioluminescent; of these, all species except for one are from the genus *Uroteuthis* (Loliginidae). As in most neritic cephalopods, loliginids have bacteriogenic bioluminescence (Guerrero-Ferreira and Nishiguchi, 2009). In terms of phylogeny, *Uroteuthis* are considered as closely related to *Loliolus* (non-bioluminescent species). In fact, it is suggested that these loliginids had a common ancestor that

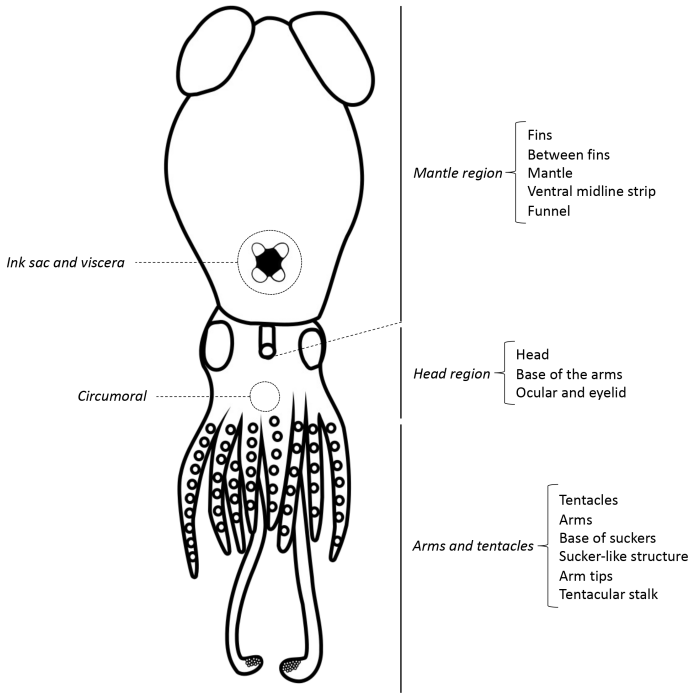

FIGURE 4
Schematic of all photophore positions. This schematic represents a fictitious cephalopod that combines all known photophore positions except for the release of bioluminescent material.

possessed bacteriogenic light organs, which were maintained in *Uroteuthis* but lost in *Loliolus* (Anderson et al., 2014).

Furthermore, it is also interesting to highlight that several different species of bioluminescent bacteria have been reported within a single squid species, *Sepioteuthis lessoniana*, which appears to be non-luminous (Zari et al., 2020). In this case, swabs were taken from the surface to the gut and ink sac of the non-bioluminescent "big-fin" reef squid (*S. lessoniana*). Although 5 luminous bacterial species were found, they do not appear to confer the capacity of light production to the squid (Zari et al., 2020).

2.3.3 Octopoda

Within the Octopoda, photophores are found in two locations, each unique to one evolutionary lineage and all potentially restricted to females. Circumoral photophores appear specific to the incirrate species (family Amphitretidae), while the cirrate octopods (Cirroteuthidae and Stauroteuthidae families) only have photophores on the arms (Jereb et al., 2005).

Stauroteuthis syrtensis is the only species of its genus confirmed to have bioluminescence, which is produced by modified suckers along the length of all arms. Only female *S. syrtensis* have been confirmed to luminesce (Collins and Henriques, 2000); if males prove non-luminescent, this species provides another example of sex-specific light production in cephalopods. Bioluminescence in *Stauroteuthis gilchristi* has not been reported to date, although its suckers appear similarly modified to those of *S. syrtensis* (Johnsen et al., 1999; Collins and Henriques, 2000).

The circumoral photophore found in *Japetella diaphana* and *Bolitaena pygmaea* (also previously known as *Eledonella pygmaea*), and potentially all derived species of the same genus, is also confirmed to be present only in mature females (Robison and Young, 1981; Herring et al., 1987). This strengthens the probability that sex-specific signaling is a primary function of octopod photophores.

2.3.4 Oegopsida

Within the cephalopods, oegopsid squids display the greatest variety of bioluminescent structures and positions, in some cases also varying with ontogeny. The diamondback squid, *Thysanoteuthis rhombus* (Thysanoteuthidae), is the only known bioluminescent cephalopod species where the photophore is present solely in immature squids and is lost by adulthood (Fernández-Álvarez et al., 2021). In this case, the light organ may play a role in pairing behavior, where immature male and female individuals pair up as young individuals and remain together through maturity (Guerra and Rocha, 1997; Jereb and Roper, 2005b).

For some oegopsids, bioluminescence is suspected but remains unconfirmed. For example, *Grimalditeuthis bonplandii* and *Psychroteuthis glacialis* were included as bioluminescent species in our analysis, due to the suspected photogenic nature of structures that develop at the arm tips of mature females, but further

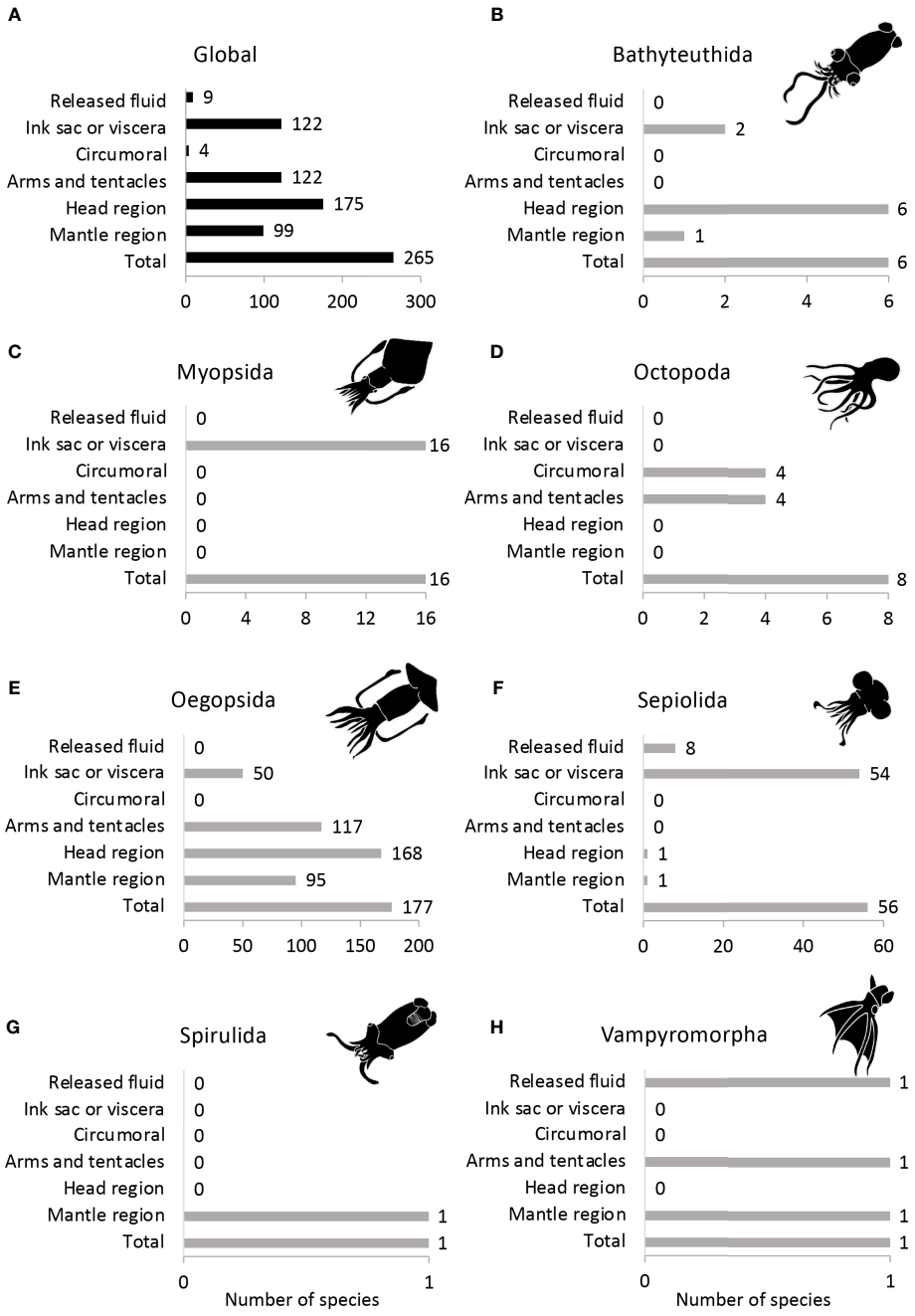

FIGURE 5
Morphological distribution of photophores in **(A)** all cephalopods, **(B)** Bathyteuthida, **(C)** Myopsida, **(D)** Octopoda, **(E)** Oegopsida, **(F)** Sepiolida, **(G)** Spirulida and **(H)** Vampyromorpha.

investigation into the nature of this tissue is needed (Jereb and Roper, 2005b; Hoving et al., 2013; Young and Roper, 2016). The onychoteuthid species *Ancistroteuthis lichtensteinii* has also been suggested (but not confirmed) to possess photogenic tissue on the ventral eye surface (Bolstad, 2010).

Another oegopsid worth mentioning is *Gonatus pyros*, the only luminescent member of the family Gonatidae (~20 species). This species possesses a large light organ on the ventral surface of each eye (Young, 1972; Bublitz, 1981). This raises questions about the evolution of its light organ, given that other members of Gonatidae (several sympatric with *G. pyros*) do not possess a photophore. Lindgren et al. (2005) reported *G. pyros* as being most closely related to several sequenced individuals attributed to *G. kamtschaticus* based on three mitochondrial loci, but the position of this clade within the family was not resolved and should be further investigated.

2.3.5 Sepiolida

Bioluminescence in Sepiolida is only present in the family Sepiolidae, which are hypothesized to have radiated from a

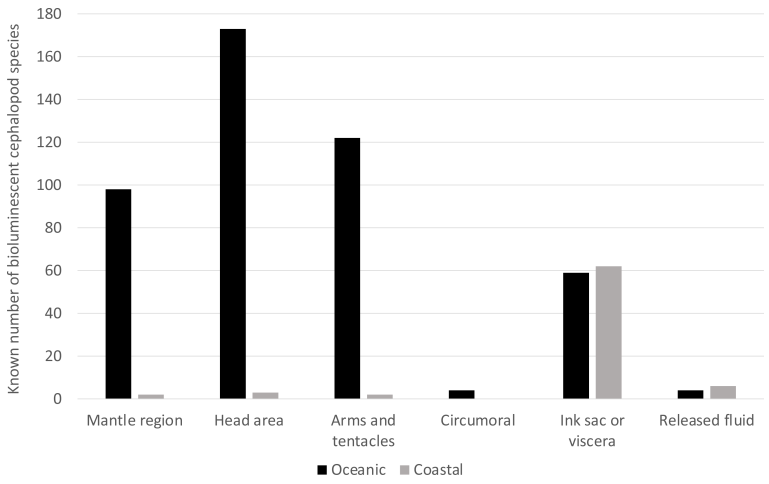

FIGURE 6
Diversity in photophore location according to the lifestyle of the cephalopods. Oceanic species are in black, and coastal ones are in grey.

common ancestor that possessed a bilobed light organ associated with bioluminescent bacteria. This structure was eventually lost for the genera *Inioteuthis* and *Sepietta* (Sanchez et al., 2021). In *Heteroteuthis*, however, where pelagic adults depart from the typical benthic sepiolid lifestyle, the autogenic origin of its bioluminescence suggests a convergent evolution with other pelagic bioluminescent cephalopods (Dilly and Herring, 1978; Lindgren et al., 2012; Sanchez et al., 2021). *Heteroteuthis* is also the only sepiolid known to secrete luminous fluid.

Interestingly, as in *Sepioteuthis lessoniana*, bioluminescent bacteria have been identified in a sepiolid species that is considered non-luminescent. *Neorossia caroli* lacks a light organ (Sanchez et al., 2021; Calogero et al., 2022); yet bioluminescent bacteria (*Photobacterium kishitanii* and *P. leiognathi*) were found in samples taken from the siphon and the mantle (Calogero et al., 2022). The relationships between the non-luminous cephalopods *S. lessoniana* and *N. caroli* and the respective bioluminescent bacteria found, in terms of light production, would then be facultative rather than obligate (Zari et al., 2020; Calogero et al., 2022). However, no studies have yet investigated such associations for purposes other than bioluminescence.

3 Biogeography

3.1 Database limitations and assumptions

The biogeographic patterns of bioluminescent cephalopod richness were derived using a conservative approach, using only species with well-known distributions (238 species, Supplementary Material 1) and excluding new or rare species [as described by Rosa et al. (2019)]. Moreover, it is important to note that many coastal and oceanic regions are still under-sampled, and the present patterns may also be driven by an underestimated cryptic diversity (Rosa et al., 2019). Here, we only included the coastal, epipelagic (<200m), and mesopelagic (between 200m and 1000m) realms, since they are the ones with a relatively solid global biogeographic classification (Spalding et al., 2007; Spalding et al., 2012 and Sutton et al., 2017, respectively). Yet, it is worth mentioning that at least half of the bioluminescent cephalopod species present in the mesopelagic realm can also be found in waters below 1000m depth.

3.2 Richness patterns per ocean and habitat

Our results show that the most diverse ocean, in terms of light-producing cephalopod species, is the Pacific Ocean (162 species), followed by the Atlantic (117 species) and Indian (112 species) Oceans. The least diverse oceans are the Southern and the Arctic Oceans (Figure 7A). These patterns are also consistent within the orders Bathyteuthida, Oegopsida, Spirulida, and Vampyromorpha (the two latter are not present in the Arctic or Southern Oceans (Figures 7B, E, G, H). However, in the case of myopsid squids, the highest number of luminescent species (14 species) are observed in the Indian Ocean, followed by the Pacific Ocean (10 species); they are absent from the three other Oceans (Figure 7C). On the other hand, bioluminescent octopods can be observed in all oceans, but mainly in the Atlantic and the Pacific Ocean (6 species, Figure 7D). Similarly, luminescent members of Sepiolida are mainly observed in the Atlantic Ocean (21 species), followed by the Pacific and the Indian Oceans (Figure 7F). Furthermore, one species (*Sepiola atlantica*) can be found as far north as Iceland, already part of the Arctic Ocean (Jereb & Roper, 2005a).

Using 231 luminous cephalopod species with a well-characterized habitat preference, we identified that most are found in the oceanic zone, while only 23% occur in coastal waters (Figure 8). Yet, it is important to note that most oceanic species undergo both diel and ontogenetic vertical migrations and are reported to be vertically distributed across pelagic zones (Judkins and Vecchione, 2020). In fact, 53% of the bioluminescent

cephalopod species are found both in the epipelagic and mesopelagic zones of the oceans, such as *Abralia* spp., *Histioteuthis* spp., and *Leachia* spp., amongst others.

Regarding coastal areas, and according to ecoregions (Spalding et al., 2007), the highest species richness of luminescent cephalopods is found in the Eastern Philippines ecoregion, with 13 species, followed by the Sulawesi Sea/Makassar Strait (11 species) and the ecoregion of Palawan/North Borneo (10 species, Figure 9A). These ecoregions are present in the delimitations of the Coral Triangle (Veron et al., 2009), a region of the ocean that is well known for being the most significant hotspot in terms of marine biodiversity (Bowen et al., 2013; Cowman et al., 2017), including cephalopods (Rosa et al., 2019). Following these three ecoregions, bioluminescent cephalopods are also highly present in the Aegean and Adriatic Sea, Ionian Sea, Alboran Sea, and Western Mediterranean ecoregions, with 8 or 9 species observed (Figure 9A). Indeed, bobtail squids (Sepiolidae) have the maximum species richness values in the Mediterranean Sea, constituting a biogeographic hotspot for this group (Bello, 2019; Rosa et al., 2019). Their high presence in the Mediterranean Sea can be explained by the separation 5.5 Mya of the Mediterranean Sea from the Atlantic as part of the "Messinian salinity crisis", which induced the extinction of stenohaline species and started an increase in endemism in the area (Rosa et al., 2008a; Rosa et al., 2019). Bioluminescent coastal species are represented mainly by the Sepiolidae and Loliginidae (*Uroteuthis* sp.), in addition to the oegopsid squids *Watasenia scintillans* and *Pyroteuthis margaritifera*. Overall, the general biogeographic trends described here for luminescent cephalopods follow those described by Rosa et al. (2019) for all coastal cephalopods worldwide. Therefore, the distribution of cephalopod luminescent fauna seems to be mainly driven by well-known historical (geological) processes and species-area–energy relationships (Rosa et al., 2008a), rather than adaptations to specific coastal habitats or unique geographic regions.

Regarding the epipelagic zone, and based on the biogeographic provinces of Spalding et al. (2012), most species are found in the Indo-Pacific Warmwater Realm (IPWR) and the Atlantic Warmwater Realm (AWR, Figure 9B). More specifically, the North Central Pacific (IPWR) and the North Central Atlantic (AWR) are the provinces with the highest concentration of bioluminescent cephalopods, with 58 species and 57 species, respectively. The Equatorial Atlantic and the Canary Current also present a high species richness, with 51 and 50 species, respectively, followed by the North Atlantic Current and the Southwest Pacific (49 species), the South-Central Pacific (48 species) and Northern Indian Ocean (46 species), the Equatorial Pacific and the Gulf Stream (45 species). The lowest species richness of bioluminescent cephalopods is found in the Arctic (Northern Coldwater Realm), with only two bioluminescent species observed (*Cirroteuthis muelleri* and *Brachioteuthis riisei*), and the Antarctic and Antarctic Polar Fronts (Southern Coldwater Realm), with 8 and 12 species observed, respectively. The epipelagic bioluminescent cephalopods are represented mainly by the oegopsid squids. The bioluminescent octopods *Japetella* spp. and *Bolitaena pygmaea*, in addition to the ram's horn squid *Spirula spirula*, are also present in the epipelagic zone.

Finally, within the mesopelagic realm, the main luminescent cephalopods are oegopsid squids and pelagic octopods, in addition to the vampire squid *Vampyroteuthis infernalis*. According to the provinces outlined by Sutton et al. (2017), the Northern Central Pacific ecoregion is the hotspot for mesopelagic light-producing cephalopod richness, with 84 species, followed by the Central North Atlantic, with 69 species (Figure 9C). Following these ecoregions, 50 to 62 bioluminescent species are present in the Coral Sea, the Northern Indian Ocean, the Mid-Indian Ocean, the Tropical and West Equatorial Atlantic, and the Southern Central Pacific ecoregions. The Arctic and the Antarctic/Southern Ocean have the lowest species richness of luminescent cephalopods, with 8 and 14 species observed, respectively.

Several large-scale biogeographic studies have been made on pelagic cephalopods, but following different approaches, namely by: i) displaying the exact location of verified species records (e.g., Nesis, 1982; Nesis, 1985; Voss et al., 1998; Nesis, 2003; Collins and Rodhouse, 2006); ii) using presence–absence matrices condensed into latitudinal bands (Ibáñez et al., 2019), or iii) choosing different regional spatial units (provinces) (Rosa et al., 2008b). Although none of these approaches is directly comparable with the present findings, Rosa et al. (2008b) pointed out, for the Atlantic Ocean, that the highest levels of cephalopod pelagic diversity were found in Benguela Current System and the Southern convergence, and described a diversity impoverishment in some western oceanic regions, namely the South Sargasso Sea and South Atlantic Subtropical. As a result, these authors found a significant positive relationship between ocean net primary productivity and oceanic richness at regional scales and maximum oceanic richness at intermediate sea surface temperatures. Here, by contrast, bioluminescent cephalopods show: i) a decreased longitudinal richness, at subtropical latitudes, from the western to the eastern margins of both Atlantic and Pacific Oceans, namely towards the most important eastern boundary upwelling systems (EBUS) and ii) a decreased richness at higher latitudes (Figure 9). These patterns seem to corroborate the findings of Ibáñez et al., (2019) in the Eastern Pacific, since pelagic richness was higher across the tropics and decreased steadily towards both poles, apparently not showing any correlation with ocean productivity. Angel (1993) also pointed out that high species richness in the pelagic ecosystems may be associated with zones of low productivity lacking marked seasonality. Oxygen availability may also be a key limiting factor for the distribution of bioluminescent cephalopods since oxygen is one of the primary components in the chemical reaction that induces light production (Tsuji, 2002; Haddock et al., 2010). Oceanic oxygen minimum zones (OMZs), where oxygen concentrations are limited, are usually formed by high productivity at the surface of the oceans due to upwelling, which degrades and falls to the bottom while depleting the oxygen present in the water column (Levin, 2003). These zones can create major boundaries for species since the lack of oxygen prevents mobile organisms from traversing or living within these ocean zones unless they are strongly adapted (Levin, 2003; Rosa and Seibel, 2008; Rosa & Seibel, 2010). In fact, the west coasts of Central America, Peru

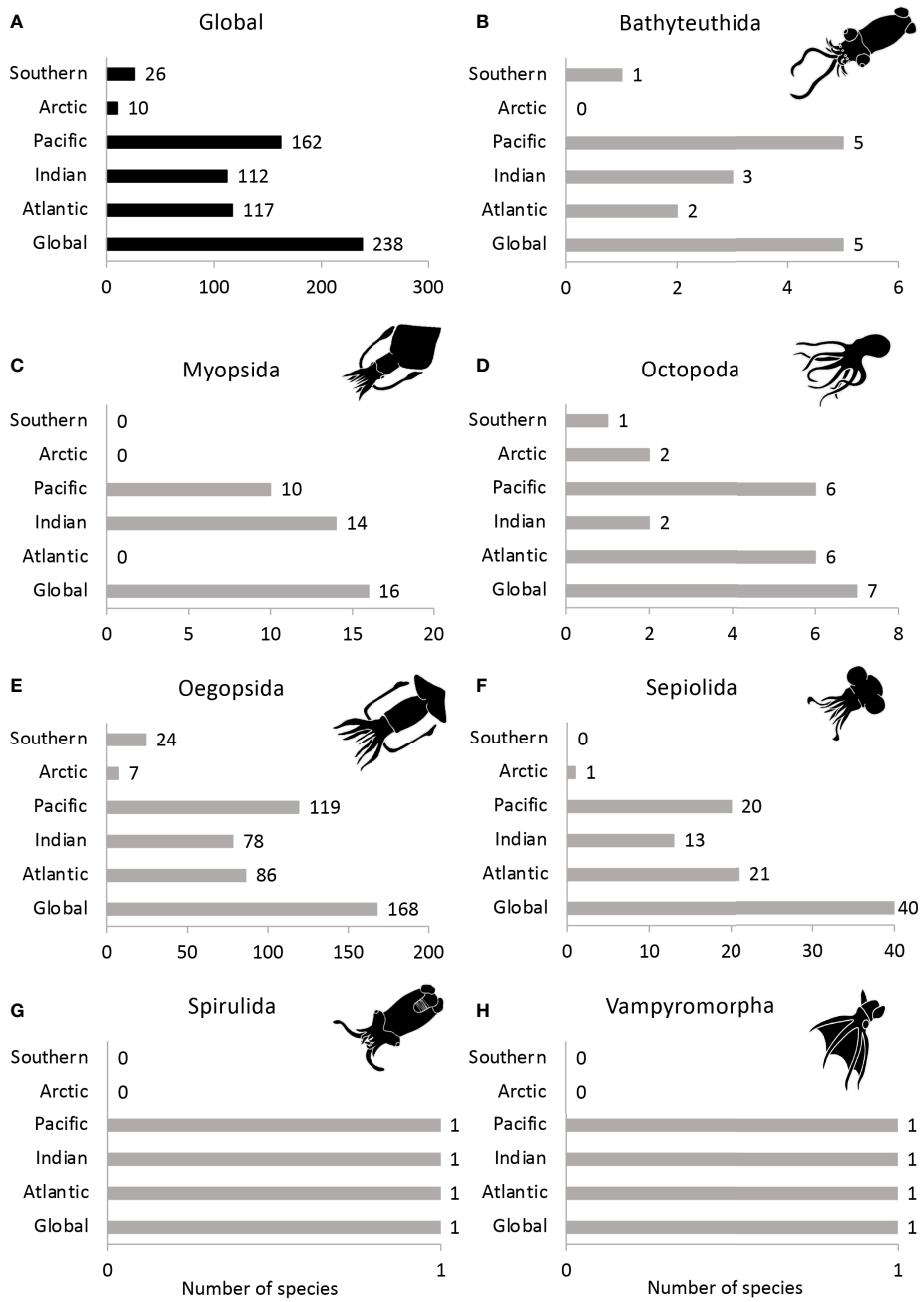

FIGURE 7
Total known number of bioluminescent cephalopod species for each ocean basin in **(A)** all cephalopods, **(B)** Bathyteuthida, **(C)** Myopsida, **(D)** Octopoda, **(E)** Oegopsida, **(F)** Sepiolida, **(G)** Spirulida and **(H)** Vampyromorpha.

(Humboldt Current), southwest Africa (Benguela Upwelling), the Arabian Sea and Bay of Bengal are well known for their seasonal or permanent OMZs (Levin, 2003; Stramma et al., 2008; Sutton et al., 2017). Although mid-water oxygen clines could explain the general longitudinal decrease in the diversity of light-producing cephalopods towards both EBUS (although not so clear along the northern epipelagic area of the California Current system) and the monsoon-influenced Arabian Sea (Figures 9B, C), it is worth noting that some bioluminescent cephalopods thrive within OMZs, including the vampire squid (*Vampyroteuthis infernalis*). This may indicate that productivity, and not oxygen *per se*, is the key environmental driver for the large-scale longitudinal trends in cephalopod oceanic diversity.

4 Research trends

Research on cephalopods and, more specifically, work focusing on light-producing species has increased substantially during the last 50 years (see below). Here, we aim to understand which research areas have dominated and identify the main studied species. To do so, we performed a systematic analysis to

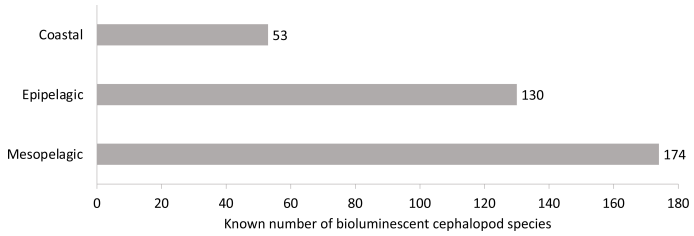

FIGURE 8
Known number of bioluminescent cephalopod species according to the ocean zone.

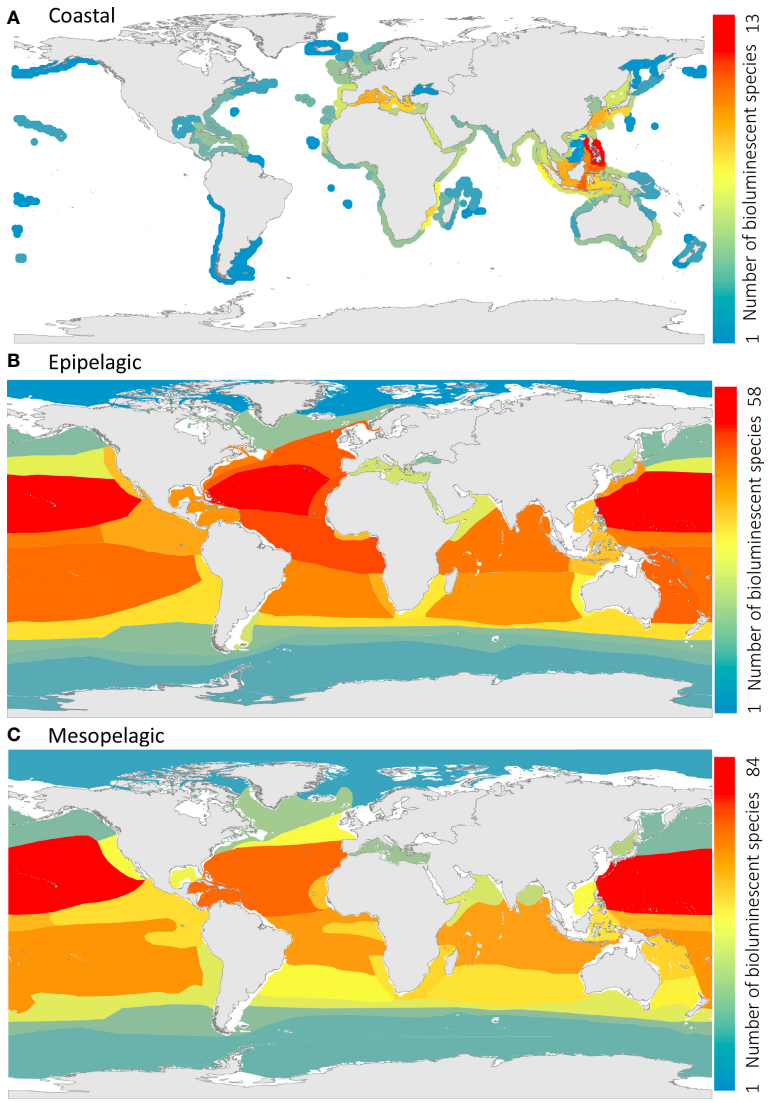

FIGURE 9
Species richness (known number of bioluminescent cephalopod species per ecoregion) of **(A)** coastal, **(B)** epipelagic, and **(C)** mesopelagic bioluminescent cephalopods. Ecoregions and provinces shown in the coastal panel, the epipelagic panel, and mesopelagic are based on Spalding et al. (2007); Spalding et al. (2012), and Sutton et al. (2017), respectively.

investigate the studies on bioluminescence in cephalopods, using the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) approach (Moher et al., 2010). The literature considered in the present analysis was restricted to the Scopus and Web of Science scientific databases (detailed methods are explained in Supplemental Material 2, 3 and Figure S1).

Between 1971 and 2020, 277 peer-reviewed studies were published on bioluminescent cephalopods. Since the 1980s, there

has been a steady increase in publications on this topic, with 126 studies (45% of the publications) published in the last decade (2011–2020; Figure 10A).

Studies on the fields of "Behavior" and "Developmental biology" within bioluminescent cephalopods have been decreasing over the years, being replaced by studies on genetics and -omics (e.g., genomic, transcriptomic, proteomic). In fact, the latter have been increasing since the 1990s (Figure 10B). The growing interest in this type of study comes along with the great improvements made in the field, e.g., the creation of GenBank® in 1982, and the notable reduction in cost for sequencing (Gužvić, 2013; Kulski, 2016). Together these factors have supported a vast increase in DNA and RNA sequences produced and uploaded to GenBank® since 1992 (Kulski, 2016).

Regarding the areas of "Microbiology" and "Biochemistry" of luminous cephalopods, the number of studies has largely held stable across the decades, except between 1971 and 1980, when the number of publications in "Microbiology" was lower compared to other areas. On the other hand, less than 10% of the studies included the fields "Aquaculture", "Taxonomy and Phylogeny", "Biogeography", and "Physiology" (Figure 10B). It is worth noting that one recent study opened the doors of light-producing cephalopods to the field of "Medicine", investigating the cytotoxicity effects of symbiotic bacteria against cancer cells (Luyon et al., 2017).

4.1 Focus species in studies on cephalopod bioluminescence

Overall, amongst the 277 studies listed in our analysis, the Hawaiian bobtail squid *Euprymna scolopes* is the most studied species by far, with 214 publications (77%). Other studied bioluminescent cephalopods include *E. tasmanica*, *Watasenia scintillans*, *Sthenoteuthis oualaniensis* and *Sepiola robusta*, but each only with 6 to 10 studies (Figure 11A). The category "others" comprises 49 species or groups of species.

Furthermore, it is interesting to highlight that nearly half of the studies about bacteriogenic bioluminescent species (221 studies) focused on the bacterial side of the symbiosis (49%, Figure 11B). In other words, these studies investigated the requirements of the bacterium, in order to successfully establish the symbiosis with the cephalopod. 24% of the studies focused on the cephalopod itself, and 28% focused on both symbiotic partners and their interaction with each other (Figure 11B).

4.2 *Euprymna scolopes*: the model organism

As most of the recent research on bioluminescent cephalopods focuses on *Euprymna scolopes*, we summarize in this section the state of knowledge for this species. *Euprymna scolopes* gains its bioluminescence through the single species-specific symbiosis with the bacterium *Vibrio fischeri* (McFall-Ngai and Ruby, 1998; Nyholm and McFall-Ngai, 2021). This sepiolid species is one of the best-studied cephalopods and both symbiotic partners have been the subject of research in the past decades (Nyholm and McFall-Ngai, 2021; Visick et al., 2021). In 2009, Lee et al. (2009) suggested considering this species a model organism as it offers many advantages for studies, including its overall small size, short lifespan, fast growth, and year-round availability. The interest in this species is mostly due to its species-specific symbiotic relationship with the bacterium *V. fischeri*, from which an easy comparison can be made for the study of eukaryote–prokaryote symbiosis (Nyholm and McFall-Ngai, 2004; Nyholm and McFall-Ngai, 2021). The symbiosis between these two partners is established at each generation, in the first hours after the bobtail squid hatches, and lasts for the entire life of the animal. When hatching, *E. scolopes* only possesses a rudimentary light organ associated with the ink sac, and the first encounter with the symbiont induces several steps, including organ morphogenesis, leading to a fully developed light organ and functional symbiosis (Nyholm & McFall-Ngai, 2004; Nyholm & McFall-Ngai, 2021). The first meeting between the two partners happens when the surrounding seawater, with free-living *V. fischeri*, flows through the mantle cavity of *E. scolopes* hatchlings (Visick et al., 2021). After aggregating at the surface of the light organ's pores, bacteria are brought into the light organ's crypts by the host's ciliary beats. This recruitment of bacteria subsequently induces a series of morphogenesis events of the light organ in the next four days post-hatching (Nyholm & McFall-Ngai, 2004; Nyholm & McFall-Ngai, 2021). Over the following four weeks, the host light organ becomes fully mature, and the bobtail squid's behavior changes from arrhythmic to nocturnally active (Nyholm & McFall-Ngai, 2004; Nyholm & McFall-Ngai, 2021). After hatching, the sepiolid quickly develops a diel behavioral pattern linked with the variation in bacterial density in the light organ, and the symbiont undergoes a metabolic change. The symbiont starts to shift its nutrient source to chitin at nighttime, causing the pH in the crypts of the light organ to decrease and, in turn, the luminescence to increase (Nyholm & McFall-Ngai, 2021). At dawn, bobtail squids expel between 90 and 95% of the symbiotic bacteria into the surrounding water, a process referred to as "venting". The animal then remains buried until dusk, by which time the 5–10% remaining bacteria have multiplied to fully re-occupy the light organ. Finally, bobtail squids rise out of the sand at night to hunt, with a fully restored bacterial population and using the bacterial bioluminescence as camouflage (Jones and Nishiguchi, 2004; Nyholm and McFall-Ngai, 2004; Nyholm and McFall-Ngai, 2021).

The regularity of this behavior makes *E. scolopes* a prime subject for understanding morphological, physiological, biochemical, molecular, and genetic pathways associated with such host–microorganism interactions. Moreover, the ease of rearing both partners, as well as the possibility of raising individuals in aposymbiotic conditions (without the bacterial symbiont), allow a deep understanding of such a relationship (Nyholm and McFall-Ngai, 2004; Lee et al., 2009; Nyholm and McFall-Ngai, 2021).

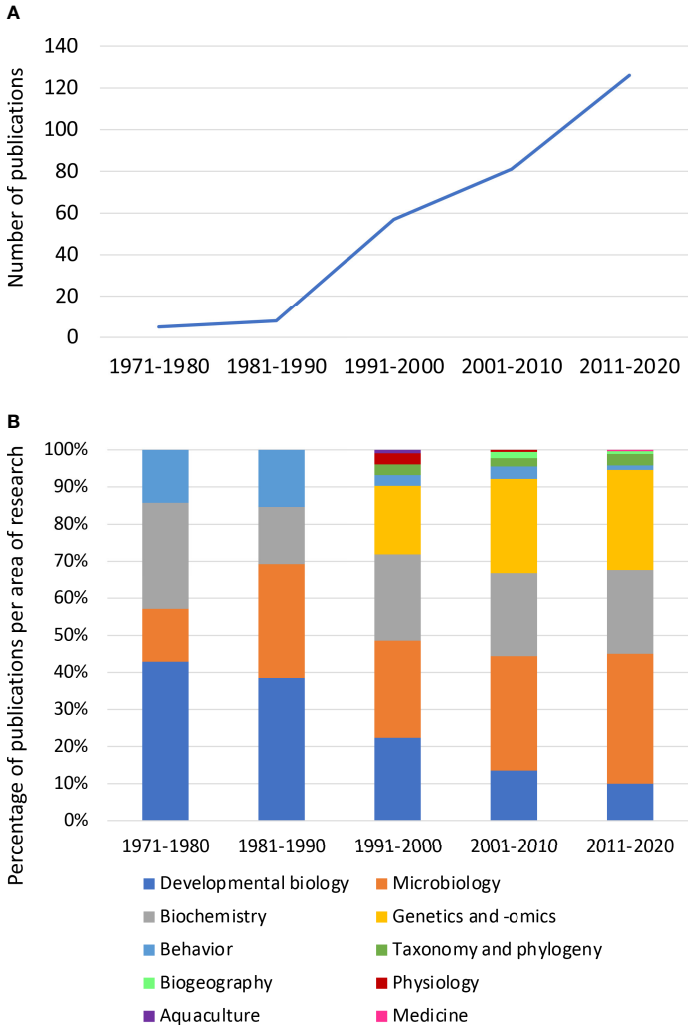

FIGURE 10
(A) Decadal publication trends (number of publications) on bioluminescence in cephalopods between 1971 and 2020. **(B)** Percentage of publications on bioluminescence in cephalopod research, between 1971 and 2020, according to the research area.

Euprymna scolopes' genome availability is a reference in the field and set the interest in other species of this genus (Belcaid et al., 2019; Jolly et al., 2022). In fact, thanks to *E. scolopes*, there are now additional emerging models such as *E. berryi* and *E. morsei* (Jolly et al., 2022).

Finally, *Vibrio fischeri* colonizes the epithelia of *Euprymna scolopes* extracellularly, along the apical surface of this epithelium. This process is similar to how the microbiome cells associate with gut epithelia, including in humans (Nyholm and McFall-Ngai, 2021). Therefore, the squid–*Vibrio* system can also be used to gain insight into the mechanisms underlying the recruitment of gram-negative bacteria by the epithelia and the mechanisms by which they persist over the life of the host.

5 Conclusions and future directions

This study reviews the biodiversity of light-producing cephalopods and provides insights into their geographic distribution, following earlier reviews by Berry (1920a; 1920b) and Herring (1977b; 1988). We show that out of the 834 currently known cephalopod species, 32% are luminous with light organs most often occurring on the head region. Both coastal and oceanic light-producing cephalopods show clear latitudinal gradients of species richness, with higher diversity at lower latitudes. To understand the future geographical distribution of luminous cephalopods, new state-of-the-art techniques such as environmental DNA (eDNA, DNA obtained from environmental samples rather than organisms directly and that is released and accumulated in the environment by these organisms; Thomsen and Willerslev, 2015) could help obtain more complete temporal and biogeographical data. This eDNA could also be used for biodiversity research and monitoring (Thomsen and Willerslev, 2015). Across the past few decades, research on bioluminescent cephalopods has mostly focused on three main research areas (genetics and -omics, biochemistry, and microbiology) and mainly on the bobtail squid *Euprymna scolopes*. We have little ability to predict how bioluminescent cephalopods may respond to future

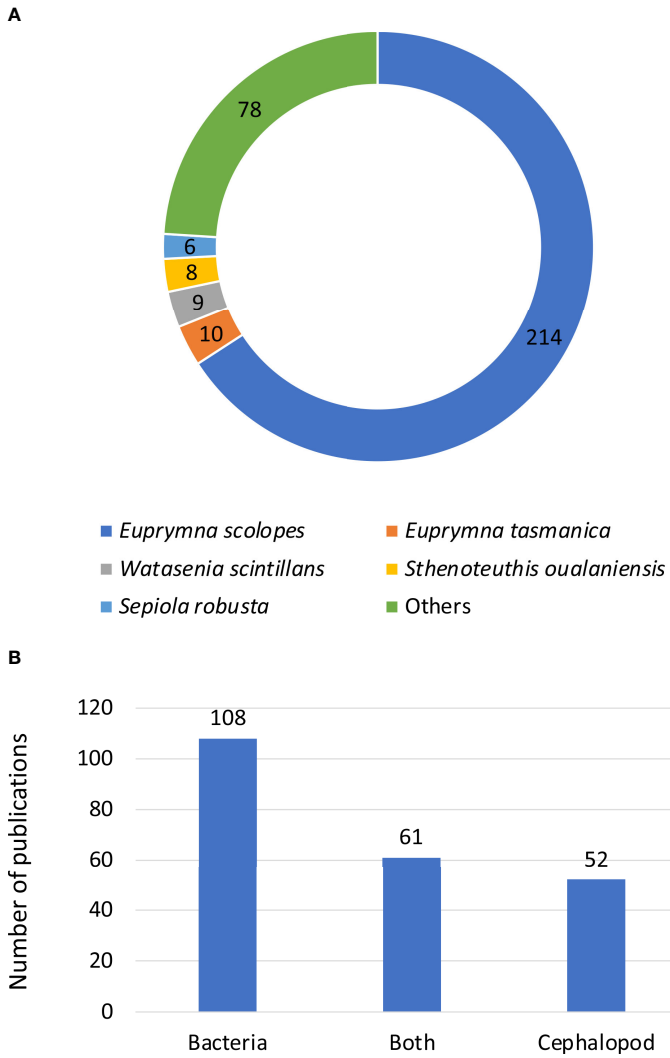

FIGURE 11
Number of publications on bioluminescent cephalopods: **(A)** according to the species of interest and **(B)** related to bacteriogenic bioluminescence, and according to the partner of interest, the symbiotic bacteria, the cephalopod or both partners.

anthropogenic pressures. At present, only two studies involving climate change-related stressors have been conducted to investigate the adaptation capabilities of *Vibrio fischeri* to temperature and pH stress, and how these affect the initiation of the symbiosis with *Euprymna scolopes* (Cohen et al., 2019; Cohen et al., 2020). Moreover, cephalopods, including bioluminescent species, are present in the diets of numerous top predators (Clarke, 1996; Klages, 1996; Smale, 1996). However, with climate change pressures, the geographical distribution of marine species such as cephalopods will change (Golikov et al., 2013), which would then affect food webs by impacting the top predators (Xavier et al., 2018). Thus, studying and understanding the marine food web dynamics in relation to future environmental climate is highly relevant. Many species-specific questions also need to be answered, such as: i) Which evolutionary processes led to a single species within a diverse family, such as *Gonatus pyros*, developing or retaining photophores? ii) which additional roles might luminous bacteria play within hosts that do not utilize their luminescence (e.g., *Sepioteuthis lessoniana* and *Neorossia caroli*)? iii) What additional bioluminescent cephalopods exist beyond those currently reported?

Finally, -omics research is becoming more and more dominant, bringing the possibility of a wide range of additional studies. The genome sequencing of cephalopods with bacteriogenic bioluminescence, such as *E. scolopes*, shows that these light organs can have a specific genomic signature, highlighting genes associated with host immunity and light mediation (Belcaid et al., 2019). However, as bacteriogenic species are limited to sepiolids and loliginids (Belcaid et al., 2019), more studies are required to analyze the genomic markers in cephalopods with autogenic bioluminescence. Moreover, such techniques have enabled, for example, a genus-level phylogenetic analysis of cephalopods, including bioluminescent species, using molecular markers (Sanchez et al., 2018). On the other hand, one striking feature of cephalopods is their ability to edit their messenger RNA, mostly converting adenosines to inosines (Alon et al., 2015; Albertin et al., 2022). While such research has mainly focused on non-luminous

species, it would be interesting to focus future studies on species that have the capacity for light production as a comparison.

Author contributions

EO and RR contributed to the design of the study. EO organized the databases. All authors contributed to populating the databases. EO performed the analysis of the database and produced the figures, and VP produced the biogeographical distribution maps. EO and RR wrote the original draft and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1161049/full#supplementary-material

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Cephalopod ontogeny and life cycle patterns

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Life cycle definitions provide the background for conceptualizing meaningful questions to address the mechanisms that generate different life cycle patterns. This review provides explicit definitions and explanations of the steps in a cephalopod life cycle, from fertilization to death. Each large step, or phase, is characterized by a particular developmental process and morphology. Each phase is composed of smaller developmentally distinct steps, or stages. The cephalopod life cycle is comprised of all or some of the following phases: Embryonic, Paralarval, Juvenile, Subadult, Adult and Senescent, and each life cycle is taxon-specific. All cephalopods have direct development and maintain a consistent body plan throughout ontogeny (i.e., no true larval phase and no metamorphosis). Most cephalopods have a life cycle marked by a long early life and a short adult life followed by senescence. Cephalopods have two developmental modes: they produce either small planktonic hatchlings as paralarvae, or large hatchlings as juveniles. All cephalopods go through a Hatchling stage soon after eclosion during which they rely on two modes of nutrition: endogenous (yolk) and exogenous (prey). Many cephalopods with planktonic paralarvae will become benthic early in their life cycle during their Settlement stage or remain pelagic during their Metapelagic stage. Juvenile growth is fast and ontogenetic changes (outside of gonadal maturation) generally cease at the end of the Juvenile phase. The Subadult phase begins when the definitive adult morphology (except for size and body proportions) is acquired (e.g., full complement of photophores). Sexual organs undergo most of their development during the Subadult phase. The Adult phase starts with spawning competency and concludes when gonads are spent. The Senescent phase begins with spent gonads and ends with death. Using this new terminology, we examine the patterns of cephalopod life cycles and find that there are four main patterns based on the presence of a Paralarval phase and the habitat occupied by each phase: Holopelagic (all phases are pelagic), Holobenthic (all phases are benthic), Merobenthic and Meropelagic (phases alternate between benthic and pelagic environments). In these two last patterns, the main difference is the presence of a Paralarval phase in Merobenthic species. The definitions and terminology proposed here provide a unifying framework for future ecological, evolutionary and life cycles research on cephalopods.

KEYWORDS

definitions, hatchling, meropelagic, metapelagic, morphology, Paralarva, senescent, terminology

1 Introduction

Metazoans have sequential developmental steps that are characterized by particular morphological, physiological, ecological and behavioral features (Roff, 1992; Stearns, 1992; Moran, 1994). The majority of marine invertebrates have complex life cycles with distinct steps or phases that differ radically from their adult forms (Levin and Bridges, 1995; Hejnol and Vellutini, 2017). Each phase can be subdivided into a series of stages that refers to any particular moment in development (Nesis, 1979; Eckman, 1996).

Molluscs possess a large diversity of body plans and developmental patterns. In non-cephalopod molluscs, development is indirect as it proceeds after hatching with a distinct larval phase (e.g., trochophore followed by veliger, pericalimma, or glochidia, Page, 2009; Ponder et al., 2019). These larvae undergo a radical metamorphosis where their entire body plan is reorganized to become a juvenile (Page, 2009; Ponder et al., 2021). Cephalopod life cycles and early development is dramatically different from other mollusks (Boletzky, 1987; Lee et al., 2003; Huan et al., 2020; Yang et al., 2020).

All cephalopods are direct developers. There is no larval phase or a true larva in the strict sense of the definition where larval parts are radically changed and new morphological features are formed from new anlagen (Geigy and Portmann, 1941; Nielsen, 2018) There is no metamorphosis. All structural features of late embryos are present in the post-embryonic and adult body plan with a few minor exceptions (Naef, 1928; Boletzky, 1974; Boletzky, 1987; Lee et al., 2003; Shigeno et al., 2010). All cephalopods hatch with a similar body plan: circumoral arm-crown, head with well-developed eyes and brain, beak and radula, ventral funnel, and a mantle covering the viscera (Figure 1) (Boletzky, 1974; Boletzky, 2003; Lee et al., 2003; Shigeno et al., 2010).

Cephalopods have two developmental modes. Some species hatch as planktonic paralarvae that are morphologically distinct from the adults and occupy a different habitat from their neritic, pelagic, or benthic adult conspecifics. Other species hatch as juveniles that are morphologically similar to the adults and found in the same habitat. Regardless of mode, development is direct. The body plan of the planktonic hatchlings is the same as all other phases; however, there are transient morphological structures that facilitate temporary planktonic and planktotrophic lifestyle. These species may have a prolonged period of development before the Juvenile phase is reached (Sweeney et al., 1992; McEdward, 2000).

Within the cephalopod literature, these two modes of development have caused considerable confusion and controversy and have contributed to a lack of consensus in terminology (e.g. Boletzky, 1974; Nesis, 1979; Young and Harman, 1988; Boletzky, 2003) (see Section 2). Often, the term "indirect development" is misused as a proxy for species with planktonic hatchlings, and "direct development" as a proxy for those that hatch as juveniles (e.g. Mangold-Wirz, 1963; Nesis, 1979; Fioroni, 1982; Arkhipkin, 1992; Nesis, 1995; Nesis, 1999; Nesis, 2002; Uriarte et al., 2011; Ibáñez et al., 2014; Robin et al., 2014; García-Flores et al., 2022).

Inconsistent terminology confounds interpretations of developmental processes and obscures the similarities and dissimilarities in morphogenesis among the cephalopods. Clarifying life cycle definitions and terminology based on established criteria can provide a common background for analyzing the developmental diversity and, the mechanisms that have generated different life cycles patterns in cephalopod (Boyle and Rodhouse, 2005; Rosa et al., 2013a, b; Vidal, 2014).

Here, we develop a consensus terminology for cephalopod life cycles that can be used regardless of taxon and across all research disciplines. Using these new, explicit terms we classify the main life cycle patterns of cephalopods, considering the transitions in habitat relationships among the phases of the life cycles.

2 Early life cycle terminology

Cephalopod development has been described most often as direct (Akimushkin, 1963; Boletzky, 1974; Chia, 1974; McEdward and Janies, 1993; Bonnaud-Ponticelli and Bassaglia, 2014; Nielsen, 2018), but also as indirect (Fioroni, 1982), and both (Mangold-Wirz, 1963; Nesis, 1979; Nesis, 1999; Nesis, 2002). Cephalopod early life phases have been called hatchlings, larvae, paralarvae, and juveniles, with varying degrees of attention to definitions (e.g., Robin et al., 2014; Fernández-Gago et al., 2019; García-Flores

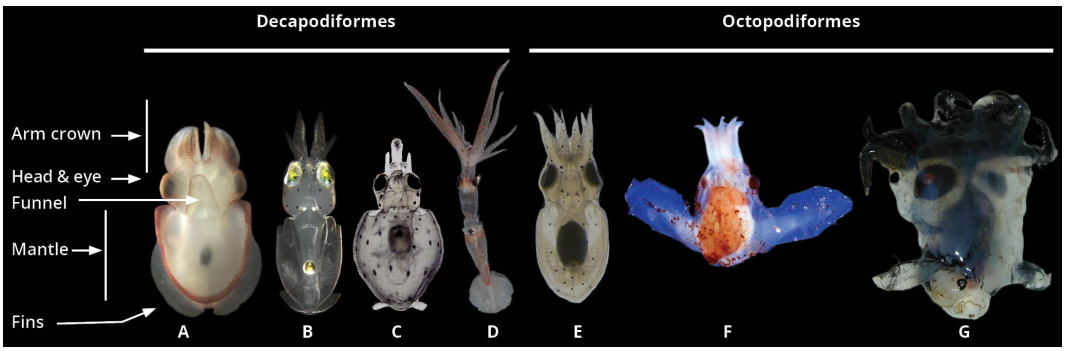

FIGURE 1
Body plan organization in coleoid cephalopod early life stages. Superorder Decapodiformes, **(A)** Sepiida, Sepiidae, **(B)** Myopsida, Loliginidae, **(C)** Oegopsida, Ommastrephidae, **(D)** Oegopsida, Chiroteuthidae and Octopodiformes, **(E)** Incirrata, Octopodidae, **(F)** Cirrata, Cirroteuthidae *Photo credit*: R.E. Young and **(G)** Vampyromorpha, Vampyroteuthidae.

et al., 2022; McCormick et al., 2022). Cephalopods have also been broken into functional groups separating adults (mature and maturing) from "juveniles" (paralarvae and immature) (Laptikhovsky et al., 2017b) to circumvent the difficulty of parsing out the different life cycle phases on a species-by-species basis. The few identification guides that exist for marine plankton occasionally include planktonic cephalopods as "larvae" (Todd et al., 1996; Boltovskoy, 1999), sometimes call them "paralarvae" (Johnson and Allen, 2012), and other times do not treat cephalopods at all (Newell and Newell, 1977).

In the mid-20th century, newly-hatched and small cephalopods were commonly called "larvae" (e.g., Allan, 1945; Rees, 1953; Akimushkin, 1963; Mangold-Wirz, 1963; Okutani and McGowan, 1969; Yamamoto and Okutani, 1975). Allometric analyses provided support for distinguishing between larvae and older conspecifics in oegopsid squids and suggesting important changes to the "mode of life", particularly behavior, habitat and predation (Clarke, 1966; Kubodera and Okutani, 1977).

Cephalopod researchers have long recognized a suite of morphological characters that are common to the early life stages including a sac-shaped body, paddle-shaped terminal fins, and large chromatophores (Sweeney et al., 1992; Nesis, 1999; Zaragoza et al., 2015). Other more distinctive features are family-specific, such as the fused tentacles in Ommastrephidae or the stalked eyes in Cranchiidae (Boletzky, 1974; Nesis, 1979; Fioroni, 1982). These distinctive features are often cited as major metamorphic differences but they are gradually modified and changed with ontogeny. Gradual modification of morphology is generally considered evidence of direct development (Page, 2009).

Categorizing and contextualizing the degree of morphological differences and whether these differences are significant enough to identify a distinctive larval phase of development has been the subject of much debate. The appropriateness of using larva in cephalopods was hotly debated during the 1985 Cephalopod International Advisory Council (CIAC) meeting in France.

Clearly cephalopods have no reorganization of the body comparable to that of other mollusks. Boletzky (1974) reasoned that many of the previously identified "larval" features (e.g., underdeveloped arm crown) are simply adult characters being expressed at an extremely small size. But Nesis (1979) argued that changes in growth coefficients could be construed as a type of metamorphosis, and continued to use "larva" to describe the early life stages.

Soon after the CIAC meetings, Young and Harman (1988) introduced the term "paralarva" to circumvent the developmental terms and to emphasize ecological concepts. The prefix "para" in Latin means "nearly" and paralarva was formally defined as "*a cephalopod of the first post-hatching growth stage that is pelagic in near-surface waters during the day and that has a distinctively different mode of life from that of older conspecific individuals*". The definition explicitly involved an ecological criterion - the daytime habitat differences of paralarvae and adults due to diel vertical migration (DVM) - which had not been previously considered (Boletzky, 2003).

Paralarva was not meant to replace developmental terms, but resulted in a confusing situation where an individual could be both a larva or a juvenile (developmental) and a paralarva (ecological). Using DVM as a marker of the end of the Paralarval phase was a way to distinguish between planktonic and nektonic animals, but requires fine-scale understanding of size-dependent depth distributions which are unknown for most open ocean species. Furthermore, it was later shown that coastal species (e.g., *Dorytheuthis opalescens* and *Octopus vulgaris)* perform DVM from the time of hatching (Zeidberg and Hamner, 2002; Roura et al., 2019), and can exert some influence over their fine-scale distribution (Vidal et al., 2018), suggesting that other species may be competent to undergo DVM even if available sampling equipment is not sufficient to document it.

Young and Harman (1988) also suggested morphometric data could mark the end point of the Paralarval phase, as could family-specific morphological features (e.g., fused tentacles in Ommastrephidae or the circular club in Chtenopterygidae). Ecological and morphological methods of identifying the endpoint of the Paralarval phase can be in conflict. Okutani (1989) argued that newly-hatched *Vampyroteuthis infernalis* could be considered a paralarva based on its morphological features, but hatchings are never found in near-surface waters. In addition, some holopelagic octopods have adults that are found in the surface waters during both the day and night (e.g., Argonautidae, Tremoctopodidae).

Strictly defining the Paralarval phase of a species using Young and Harman's DVM-based model also requires a compendium of species-specific data, including a well-resolved understanding of collecting depth, a large number of specimens of varying sizes to describe growth, a large set of measurement data, and specimens in good condition to identify a morphological marker (Shea and Vecchione, 2010). Because this is so difficult, "paralarva" has been generally adopted as a shorthand for "planktonic cephalopod" with varying degrees of attention to the original formal definition (see Boletzky, 2003 for multiple examples). Here, we adopt the community usage of paralarva as being roughly equivalent to "planktonic cephalopod", but we provide a more explicit and largely applied definition, and put this definition into context of the other phases of the cephalopod life cycle.

3 Cephalopod life phases and stages

Although both morphological and ecological factors are essential to understand life cycles (McEdward, 1995; Boyle and Rodhouse, 2005; Hanlon and Messenger, 2018), relying on morphology to define the life steps is consistent with past practice in embryology (Naef, 1928; Arnold, 1965) and maturity scales (Lipínski, 1979; Arkhipkin, 1992). Well-described morphological character states are easy to understand, are stable, and are thus broadly applicable and accessible. Using morphology is independent of any particular ecological framework (e.g., vertical migrations, feeding, survival), which is especially important because there are still fundamental knowledge gaps in many species. Finally, morphology may drive ecology (Vidal et al., 2018) or alternatively constrain behaviors (Kier, 1996; Boletzky, 1997; Hanlon and Messenger, 2018; Vidal and Salvador, 2019).

The terms life history and life cycle are often used interchangeably but are distinctly different. Life history describes how individuals, populations, or species survive, grow and reproduce in response to their physical environment. Important life history traits include longevity, age-at-maturity, and fecundity, all of which inform the ability of a species to survive from generation to generation. Alternatively, life cycle is used to describe the species-specific developmental sequence of morphological changes that begins with fertilization, includes all ontogenetic phases and stages, and ends with death. Life cycle traits may include the development or modification of a morphological feature and/or a change in habitat.

The cephalopod life cycle is composed of a series of major and minor steps that can be reliably delineated using morphology. Phases are major steps that are delimited by important milestone events in the life cycle (e.g. fertilization, eclosion, etc.) (Figure 2A). Each phase of life is marked by a particular developmental process and morphology. Each phase is composed of many smaller morphologically distinct steps called stages (Figure 2B) (Naef, 1928; Nesis, 1979; Eckman, 1996). Some stages are found in all taxa (e.g., Hatchling stage) but others may be species-specific (e.g., Settlement stage in small-egged octopods, see Section 3.7).

The timing of transitions between phases and stages is species-specific, with individual and sex-based variations (Nesis, 1979; Okutani, 1987; Vidal, 1994; Shea and Vecchione, 2002). Environmental conditions impact growth rates, but temperature and food availability are particularly important influences (Forsythe et al., 2001) over the size and age at which an individual will transition between phases and stages.

In the following sections, we propose standardized terminology and definitions that are applicable for all cephalopod life cycles.

3.1 Embryonic phase

Definition: The Embryonic phase of the cephalopod life cycle is enclosed in an egg. This phase begins with fertilization and ends with eclosion (=hatching) (Table 1).

The Embryonic phase begins after the egg is fertilized and cell differentiation starts. By the end of this phase, the definitive body plan of a cephalopod is attained.

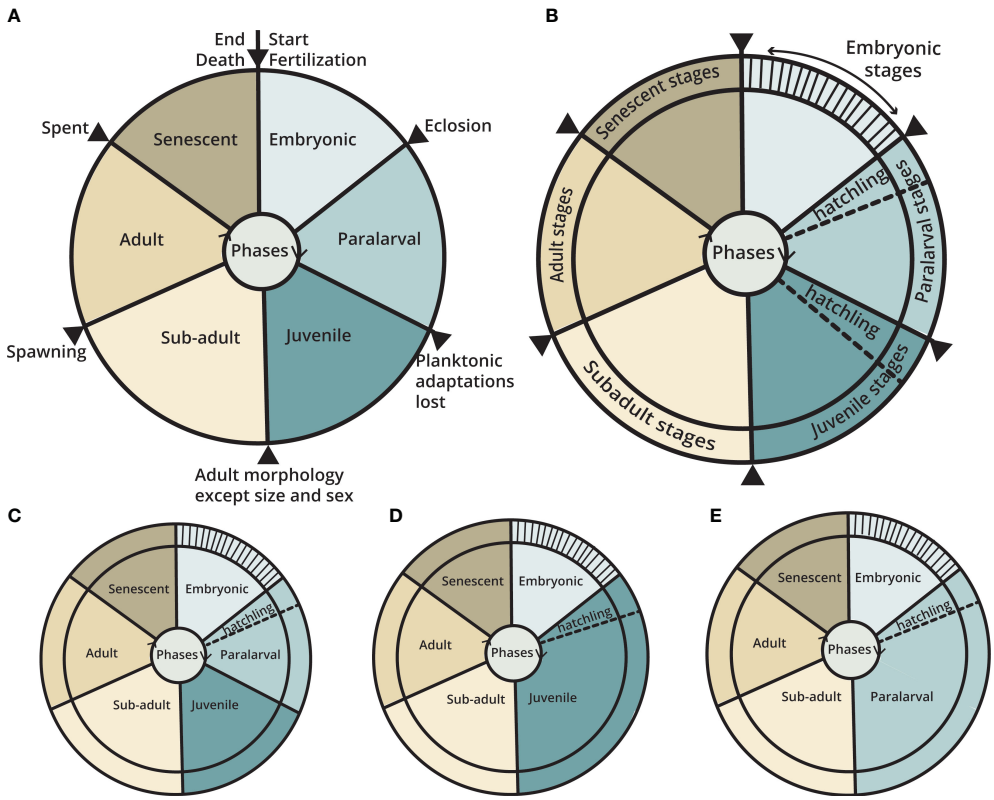

FIGURE 2
The phases and stages of a single cephalopod life cycle, from fertilization to death. **(A)** Phases are major divisions of the life cycle. Here, all phases are evenly distributed and color coded for easy comparison. The limit of each phase is marked by a black dividing line and arrowhead and is annotated with the important transitory events or milestones. **(B)** Stages are smaller, developmental steps within a phase. Here, they are indicated by subdivisions around the circle perimeter. The Hatchling stage is found either in the Paralarval or Juvenile Phase, whichever comes first. **(C)** Generalized life cycle of a species with a Paralarval phase **(D)** Generalized life cycle of a species that hatches as a Juvenile and, **(E)** Generalized life cycle of a species that hatches as a Paralarva and does not have a Juvenile phase. The duration of each phase in relation to the other phases or the whole life cycle are species-specific, and the length of an individual life cycle is influenced by abiotic factors such as temperature.

Cephalopods produce yolk-rich telolecital eggs with large meroblastic discoidal cleavage (Boletzky, 1987). The nutrition of the embryos is provided by yolk within the eggs and composed mainly of proteins, carbohydrates and lipids. Traditional stages of embryogenesis include cleavage, gastrulation, organogenesis and growth (Boletzky et al., 2016).

Descriptive embryology scales range from 18 - 30 morphologically distinguishable, developmental stages, depending on taxon and proposed scale (Naef, 1928; Arnold, 1965; Lemaire, 1970; Boletzky et al., 2016; Deryckere et al., 2020). The duration of development is species-specific, strongly dependent on temperature and egg size. Embryogenesis can take 10-15 days for sepiolid small eggs incubated at warmer temperatures (Nabhitabhata, 2014; Nabhitabhata and Nishiguchi, 2014) or many months to years in large-egged octopus incubated at colder temperatures (Uriarte et al., 2010; Robison et al., 2014; Khen et al., 2022). Within the optimal incubation temperature range for a given species, higher temperatures shorten the time between spawning and hatching and those outside the optimal range increase the incidence of abnormalities and death (Vidal and Boletzky, 2014). The interplay between egg size, incubation temperature and egg-laying timing will be the main determining factors of hatching time and will set the conditions for survival and growth of the hatchlings. There are, however, many other environmental factors that influence embryonic development and hatching success (Vidal et al., 2014).

3.2 Paralarval phase

Definition: The Paralarval phase begins at eclosion and ends when adaptations for life in the plankton have been lost and/or the individual is no longer planktonic. Individuals are morphologically distinct from older conspecifics (Table 1 and Figure 2C).

This phase is marked by unique transitory morphological specializations that are adaptations for dispersal, locomotion, feeding, defense and, camouflage in the plankton regardless of depth (Table 2; Figure 3). These specializations characterize and differentiate a paralarva from species that hatch from large eggs as juveniles and live in the same habitat as the adult conspecific (e.g., *Haliphron atlanticus*, O'Shea, 2004).

During the Paralarval phase the main developmental process is morphological development. Changes can vary from minor differences in proportions (e.g., Sepiolidae) to pronounced changes in structure and overall morphology (e.g., Chiroteuthidae).

After eclosion, paralarval body parts develop fast and allometry operates at high rates. The arm crown and fins are often rudimentary or underdeveloped at eclosion but develop quickly (e.g. Boletzky, 1974, 2003; Okutani, 1987; Vidal, 1994; Villanueva, 1995; Shea and Vecchione, 2002; Wakabayashi et al., 2005), particularly tentacular clubs and stalks (Sweeney et al., 1992; Vidal and Salvador, 2019). In some species development may lead to degeneration of tentacles (e.g. *Gonatopsis*, Okutani, 1987). Chromatophores increase in number and patterns become more complex (e.g. Young et al., 1985; Young

TABLE 1 Definitions, main developmental process and limits of each phase of the cephalopod life cycle.

Life phases	EMBRYONIC	PARALARVAL	JUVENILE	SUBADULT	ADULT	SENESCENT
Definition	Life cycle phase enclosed in an egg	First post-embryonic phase that is planktonic and morphologically different from older conspecifics	Either the first post-embryonic phase that is morphologically similar to the adults with the same mode of life as the adults or the phase of the life cycle after the Paralarval phase	Life cycle phase that have the definite adult morphology but are small and not competent for spawning	Life cycle phase with the attainment of full sexual maturity: mature eggs in oviduct (s) and mature spermatophores in Needham's sac or terminal organ	Life cycle phase with spent gametes
Developmental process	Differentiation and morphogenesis	Morphological development	Morphological development and somatic growth	Development of sexual organs and mass increase	Spawning	Organ systems deterioration
Starts	With fertilization	With eclosion (e.g., hatching)	With eclosion, or after the end of the Paralarval phase	With attainment of all diagnostic morphological features used to define the species other than those relating to sex and size	With competency for spawning	With spent gametes
Ends	With eclosion	When morphological adaptations for life in the plankton are lost	With attainment of all diagnostic morphological features used to define the species other than those relating to sex and size	With competency for spawning	With spent gametes	With death
Main features	Morphogenesis of the body plan of a cephalopod	Transitory morphological adaptations for life in the plankton	Acquisition of definite adult morphology, but different body proportions. Ontogenetic development is completed	Acquisition of adult body proportions and development of sexual organs	Spawning	Declining physiological condition

TABLE 2 Cephalopod paralarvae morphological adaptations for life in the plankton.

Feature	Function	Comments	Taxon	Reference
Kölliker organs	Hatching, locomotion, buoyancy, defense	No fins to aid in locomotion	Incirrata	Boletzky (1974); Villanueva et al. (2021)
Rudimentary, separated, paddle-shaped fins	Locomotion	Planktonic drifters	Idiosepiidae, Myopsida, Oegopsida, Spirulidae Vampyromorpha	Sweeney et al. (1992); Nesis (1999); Vidal et al. (2018)
Denticulated beak	Feeding	For grasping and tearing prey apart in low Reynolds number environments	Myopsida, Oegopsida, Octopodidae (small-egged species)	Boletzky, (1971); Boletzky, (1974); Franco-Santos and Vidal, (2014); Franco-Santos and Vidal, (2020)
Underdeveloped arm crown with few suckers	Feeding	Morphological constrains due to small hatching size	Idiosepiidae, Myopsida, Oegopsida (in general), Octopodidae (small-egged species), Spirulidae, Vampyromorpha	Boletzky, (1974); Boletzky, (2003); Sweeney et al (1992); Zaragoza et al. (2015)
Transparent body (chromatophores few and large)	Camouflage	Transparency is camouflage in euphotic zone	Most species with paralarva (exceptions Idiosepiidae, Thysanoteuthidae)	Nesis, (1979), Nesis, (1999); Sweeney et al. (1992)
Photophores, few or absent	Camouflage	Allows transparency	Most species with paralarva	Sweeney et al. (1992)
Unique transitory morphological features in particular families				
Feature	Function	Comments	Taxon	Reference
Mantle outer jelly coat with filaments/ mucus sheath	Unknown		Amphitretidae (*Japetella diaphana*)	Nesis (1979); Hochberg et al. (1992)
Circumbrachial cuff-shaped membrane (arm cuffs)	Unknown		Argonauthidae, Tremoctopodidae	Fioroni (1982); Hochberg et al. (1992)
Long neck	Buoyancy, feeding	Allow movement of arm crown towards prey	Brachioteuthidae	Young et al. (1985); Shea and Vecchione (2010)
Long neck and brachial pillar	Wider visual field, feeding buoyancy	Allow movement of head and arm crown independenty of the body, increasing visual field	Chiroteuthidae	Young (1991); Bitondo (2016)
Long tail with ornamentation	Buoyancy and body stabilization	Tail may contain structures with fluids lighter than seawater	Chiroteuthidae	Young et al. (2019)
Tentacular clubs	Feeding	Prey capture	Chiroteuthidae	Young (1991); Bitondo (2016)
Circular tentacular clubs	Feeding	Prey capture	Chtenopterygidae	Sweeney et al. (1992)
Eyes on stalks	Wider visual fied, buoyancy, feeding	Eyes move independently increasing visual field and perception distance	Cranchiidae	Young, (1975b)
Proboscis	Feeding	Feeding behavior with a proboscis is unknown	Ommastrephidae	Franco-Santos and Vidal (2020)

and Harman, 1985b; Sweeney et al., 1992; Vidal, 1994; Zaragoza et al., 2015), photophores might develop (Nesis, 1979; Wakabayashi et al., 2002). Although many paralarvae lack photophores as well as hooks, both may develop later in some species (Young, 1972; Boletzky, 2003).

Some paralarval specializations are unique to particular families or genera (Table 2; Figure 3). Chiroteuthide doratopsis paralarvae are marked by a branchial pillar, a long neck and a long tail (Figure 3D). In Ommastrephidae, the tentacles are fused into a proboscis (Figure 3B), which progressively split into the two tentacles. The circular club of Cthenopterygidae is unique (Figure 3G), as is the circumbrachial cuff-shaped membranes in Argonauthidae and Tremoctopodidae (Sweeney et al., 1992). Even more remarkable and morphologically distinct are the conspicuous stalked eyes of most Cranchiidae paralarvae (Figure 3A) and the two fins stage in Vampyroteuthidae (Young and Vecchione, 1999).

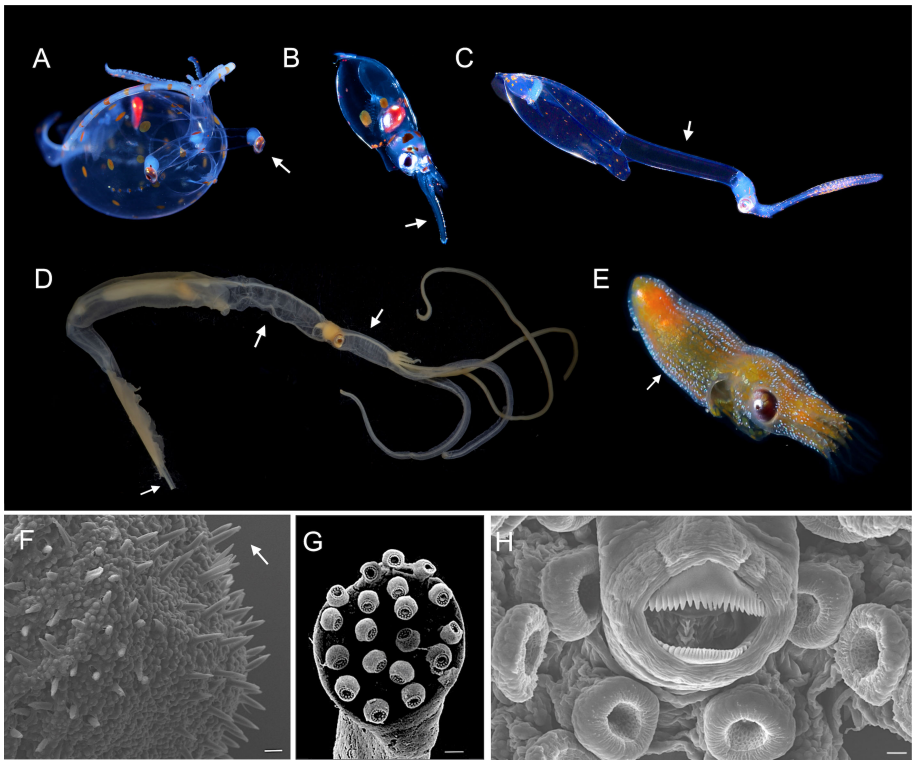

FIGURE 3
Paralarval transitory morphological adaptations. **(A)** Stalked-eyes in the cranchiid, *Leachia*, *Photo credit:* R. Minemizu. **(B)** Proboscis in ommastrephid rhynchoteuthion, *Photo credit:* L. Ianniello. **(C)** Neck in *Brachioteuthis*, *Photo credit:* R. Minemizu. **(D)** Neck, brachial pillar and long tail with most part broken off in *Chiroteuthis spoeli*, *Photo credit:* R.E. Young. **(E)** Kölliker organs in Octopodidae paralarvae (light blue points), *Photo credit:* R. Minemizu. **(F)** Kölliker organs on the posterior mantle of *Octopus americanus*, scale bar= 20 µm. **(G)** Circular club in *Chtenopteryx sicula*, scale bar= 15 µm. **(H)** Buccal mass showing denticulated upper and lower beaks in *Octopus americanus*, scale bar= 10 µm.

Other less obvious changes also occur during this time. In many families, the fragile, lightly pigmented and denticulated beaks of hatchling paralarvae change during this phase of life into more heavily pigmented, tooth-less beaks with pointed rostra. This change likely represents a change from external pre-digestion and suction of body fluids of crustacean prey to biting and tearing flesh (Franco-Santos and Vidal, 2014; Franco-Santos and Vidal, 2020). Thus, the set of characters that defines a paralarva include their relatively small hatching size and morphological adaptations to the planktonic habitat. These adaptations create a distinct morphology from older conspecifics, but are contained within the basic cephalopod body plan (Figure 1, 3).

Cephalopods with a Paralarval phase have larger dispersal potential and thus wider adult geographical distribution than species without a Paralarval phase (Villanueva et al., 2016). The length of time an individual spends as a paralarva is determined in part by the size at eclosion and environmental temperature, which in turn impacts the fitness, dispersal potential, trophic niche and mortality (Levin and Bridges, 1995; Morgan, 1995). The relative duration of the Paralarval phase has been directly observed for only a few coastal cephalopod species raised under laboratory conditions. Results show that hatchlings that are morphologically similar to the adults have a shorter Paralarval phase than species that are morphology dissimilar (Table 3).

The Paralarval pahse may be brief, lasting for hours to days with paralarvae found in roughly the same location as adults (e.g., Sepioloida, Idiosepiidae). At the other end of the spectrum, the Paralarval phase may last several months in colder waters species, which allows for considerable dispersal (e.g., *Enteroctopus dofleini*, Table 3). Although a longer planktonic period is often associated with increased mortality and advection (Morgan, 1995), it can also be advantageous for maximizing dispersal when benthic habitats are constantly changing or when habitat choice for settlement has important implications for survival. Some benthic octopods, such as *Octopus rubescens* (Hochberg et al., 1992), *Pteroctopus tetracirrhus* (Villanueva et al, 2020), and *Macrotritopus* spp. (Judkins and Vecchione, 2020) can attain large sizes in the water column in coastal and oceanic waters (Villanueva and Norman, 2008).

3.3 Juvenile phase

Definition: The Juvenile phase begins either after eclosion or after the Paralarval phase is complete and ends when all diagnostic morphological features used to define the species other than those relating to sex and size have been attained (Table 1).

In species that hatch as juveniles, the hatchlings are large, well developed and morphologically similar to the adults and often occupy the same habitat of the adults.

Complex behaviors are common in large juvenile hatchlings. For example, *Sepia officinalis* hatchlings are capable of changing

color and texture as fast as and effectively as adults and can display almost every adult body pattern, even though leucophores and iridophores have not yet developed and patterns and displays refine during ontogeny (Hanlon and Messenger, 2018). Similarly, feeding behavior in cuttlefish hatchlings is as accurate as in adults (Messenger, 1977), contrary to the basic feeding behavior of *Octopus* and loliginid paralarvae (Villanueva, 1995; Vidal and Salvador, 2019).

These abilities involve advanced development of the visual (binocular depth-detection) and neuromotor (camouflage and swimming coordination) systems (Hanlon and Messenger, 2018). Intricate body patterns are important for camouflage, predator avoidance and efficient predation in the benthos. Thus, the set of character states that defines a juvenile are their relatively large size at eclosion, a well-developed nervous system, and an overall morphology that is similar to the adult conspecific.

The largest coleoid cephalopod hatchlings are benthic/demersal juveniles. The Antarctic incirrate, *Megaleledone setebos*, is likely the largest based on its egg size (41.5 mm, Allcock et al., 2003). Other large juvenile hatchlings are found in subtropical coastal reef areas (e.g. *Sepia latimanus*, 14 mm ML), in temperate to polar waters (e.g. *Eledone moschata*, 10.5 *mm* ML) and in the deep-sea [e.g. *Graneledone boreopacifica*, 28 *mm* ML (Villanueva et al., 2016); *Octopus californicus*, 12 *mm* ML (Khen et al., 2022); *Grimpoteuthis* sp., 13 mm ML (Shea et al., 2018)].

In species that have a planktonic paralarva, the Juvenile phase is reached after the attainment of morphological development required to adopt the full adult habitat (Figure 2C). It is noteworthy that the few studies available for small-egged Octopodidae species, have shown that the morphology of post-settlement juveniles is quite similar to that of large-egged juvenile benthic hatchlings (see Section 3.7) (Promboon et al., 2011; Dan et al., 2022; Roura et al., 2023).

Morphological development continues in the Juvenile phase but the phase is primarily characterized by extremely fast, even exponential growth rates in both the wild and under laboratory conditions (Forsythe and Van Heukelem, 1987). Growth in length is intensified in relation to other body parts and thus, body proportions and shape changes. This is an important developmental process because variation in a wide variety of morphological and physiological traits are highly correlated with variation in organism size (Kubodera and Okutani, 1977; Hernandéz-Garcia et al, 1998; Pélabon et al., 2014). These morphological changes in body proportions and growth have been well documented in many families.

High growth rates are possible because of high feeding rates, capacity for exceptional food conversion efficiencies and the allocation of energy to gonad production is reserved until late in the life cycle (Boyle and Rodhouse, 2005). Individual growth rates are subject to considerable plasticity both in laboratory conditions and in wild populations (e.g. Forsythe and Van Heukelem, 1987; Semmens and Moltschaniwskyj, 2000), which causes large variability in size-at-age estimation in wild populations (Boyle and Rodhouse, 2005).

In addition to fast growth, juveniles develop species-specific morphological features. For example, body proportions change and morphological features such as hooks are formed in onychoteuthids and pterygioteuthids (Kubodera and Okutani, 1977; Young and Harman, 1988). Photophores develop in many families (Young, 1975a; Sweeney et al., 1992; Roper and Jereb, 2010). In *Pterygioteuthis microlampas* the end of the Juvenile phase is marked by the development of arm hooks at 9-11 mm gladius length (Young and Harman, 1988).

The development of these species-specific features, particularly chromatophores and photophores, play key roles in the recognition of and visual communication with conspecifics (Hanlon and Messenger, 2018). In Thysanoteuthidae, the development of the anal photophore and the relative length of arms are important in the formation of pairs of same-sized juvenile males and females (<100 mm ML), that are believed to remain together during their lifetimes, a unique social organization among cephalopods (Nigmatullin and Arkhipkin, 1998; Roper and Jereb, 2010). Also, sexual dimorphism can be expressed in some families as a result of different growth rates and heterochrony between males and females. The Juvenile phase ends with the acquisition of the adult morphology, even though body proportions are different from the adults.

The Juvenile phase is absent in some families. This is particularly evident in the Chiroteuthidae (except in *Planctoteuthis* spp.), when the doratopsis paralarval clubs are resorbed to give place to the adult clubs, indicating the transition from the Paralarval phase to the Subadult phase (Young, 1991; Bitondo, 2016) (Figure 2E).

3.4 Subadult phase

Definition: The Subadult phase begins with the attainment of all diagnostic morphological features used to define the species other than those relating to sex and size, and ends with competency for spawning (Table 1).

The Subadult phase or "adolescent" phase as coined by Nesis (2002), is the life phase where the definite adult morphology exists but individuals are not full grown and not competent to spawn. Although for many families there is uncertainty in the differentiation of juveniles from subadults, the subadults have the definitive adult morphology, that is, ontogenetic development is completed. The definitive species features are found in the patterns, shapes and relative sizes of subadults, which have the appearance of adults but are smaller than adults and are not competent to spawn (e.g. Roper and Young, 1975; Young and Harman, 1988; Arkhipkin, 1992).

The main developmental process in this phase is gonadal growth and mass increase that progress until the attainment of the adult body proportions and full competence to spawn. By the end of the Subadult phase somatic growth is virtually completed and the body proportions of the adults achieved or nearly so (Table 1).

The onset of maturation (e.g. microscopic development of sexual cells) in the Subadult phase marks the end of the logarithmic growth phase in coleoid cephalopods (Forsythe and Van Heukelem, 1987). Both feeding and food conversion rates slow

TABLE 3 Paralarval phase duration and associated event marking its end.

Paralarval phase duration	Family	Species	Duration (hours, days)	T (°C)	Size from hatching to end of Paralarval phase (mm ML)	Endpoint event	References
Brief (hours to days)	Sepiolidae	*Euprymna hyllebergi*	6-8 h	28.2	2.0 to 7.0	Settlement**	Nabhitabhata and Nishiguchi (2014)
		Sepiella inermis	12 h to 5 d	28	3.3-4.3 to 6.5	Settlement*	Nabhitabhata (1997)
		Sepiella japonica	10-15 d	26	4.0 to 8.0	Settlement*	Zheng et al. (2014)
		Sepiola atlantica	6 h	14.4	1.9-2.4	Settlement*	Jones and Richardson (2010)
Short (days to a month)	Idiosepiidae	*Idiosepius*	16 d	–		Adhesive behavior	Boletzky et al. (2005)
	Octopodidae	*Amphioctopus aegina*	20-25 d	30	2.7 to 6.3	Settlement*	Promboon et al. (2011); Nabhitabhata (2014)
		Octopus joubini	21 d	24	2.5 to 3.0-4.0	Settlement*	Forsythe and Toll (1991)
		Octopus sinensis	33 d	24.7	2.1 to 6.3	Settlement**	Itami et al. (1963)
			15-23 d	24.2	2.3 to 5.6-7.4	Settlement*	Dan et al. (2021)
Intermediate (a few months)	Loliginidae	*Doryteuthis opalescens*	35-60 d	16	2.3-2.8 to 6.0-15.0	School formation	Vidal et al. (2018)
		Doryteuthis pealeii	50-60 d	13-19	1.8 to 4.0-6.0	School formation	Hanlon et al. (1987)
		Loligo forbesii	40-50 d	12-15	3.4-4.9 to 5.3-9.0	School formation	Hanlon et al. (1989)
		Sepioteuthis lessoniana	10 d	28	5.4-11.0	School formation	Segawa (1987)
			30-60 d	24.5-25.5	5.0-6.0 to 12.0-30.0	School formation	Sugimoto and Ikeda, (2012)
	Octopodidae	*Octopus vulgaris*	47-54 d	21	2.0 to 8.6	Settlement*	Villanueva, (1995)
			40 d	22.5	–	Settlement*	Iglesias et al. (2004)
			52-60 d	21.5	2.2 to 6.5	Settlement*	Carrasco et al. (2006)
			45-60 d	18.1–20.5	1.5 to 4.8-5.7	Settlement*	Roura et al. (2023)
Long (many months)	Octopodidae	*Robsonella fontaniana*	72 d	11	2.2 to 5.7	Settlement*	Uriarte et al. (2010)
	Enteroctopodidae	*Enteroctopus dofleini*	100-117 d	10.8	5.3-5.5 to 13.5	Settlement*	Okubo (1979)
			150-180 d	11	–	Settlement*	Snyder, (1986b); Snyder, (1986a)
		Enteroctopus megalocyathus	90-114 d	12	–	Settlement*	Uriarte and Farías (2014)

*Settlement to the benthos.
**Settlement to the benthos. Individuals alternate between the plankton and the benthos before becoming fully benthic.
ML, mantle length; T, temperature.

down at larger body size and consequently growth rates slow down. There are, however, exceptions to this pattern; argonauts and cirrate octopods continue feeding and growing over a wide range of sizes (Villanueva, 1992; Laptikhovsky and Salman, 2003). If sexual dimorphism was not yet expressed in the Juvenile phase, it will become evident in the Subadult phase due to the different growth rates between males and females and the development of the reproductive system (Nesis, 1985, 1995; Boyle and Rodhouse, 2005).

The set of conditions that determine the onset of sexual maturation are complex, not fully understood, entangling many intrinsic and extrinsic factors (Arkhipkin, 1992; Boyle and Rodhouse, 2005; Hoving et al., 2014). Environmental factors are known to trigger the onset of sexual maturation and indirectly reduce feeding and growth rates (e.g. temperature, nutrition, photoperiod). In addition, transition from somatic to gonadal growth is hormonally mediated by the optic gland secretions, which seems to be controlled by photopheriod (O'Dor and Wells, 1978; Arnold, 1984).

Maturation is characterized by the development, packaging, and storage of eggs and sperm. Females can store sperm and delay fertilization and production of eggs. The degree of maturation is based on descriptive scales (e.g., Lipínski, 1979; Arkhipkin, 1992). Full sexual maturity is attained when males and females are competent to spawn (= spawning competency).

Spawning competency is reached when males have mature spermatophores in Needham's sac or terminal organ and females have visible mature eggs in oviducts (Arkhipkin, 1992; Boyle and Rodhouse, 2005). Spermatophores are transferred from males to females by the hectocolylized modified arm or by the penis. The end of the Subadult phase is marked by attainment of the full adult body proportions and competency to spawn.

Not all species will have a Subadult phase if the acquisition of the adult morphology and diagnostic features takes place simultaneous with the attainment of sexual maturity. This is the case of the life cycle of *Leachia pacifica* that transition from the Juvenile to the Adult phase (Young, 1975b).

3.5 Adult phase

Definition: The Adult phase begins with the attainment of full sexual maturity as determined by the presence of mature eggs in oviduct (s) and mature spermatophores in Needham's sac or terminal organ and ends when gametes are spent (i.e. senescence) (Table 1).

The Adult phase of life is marked by storage and use of functional gametes and the spawning process. Reproduction is seasonal in most species. Females may spawn all their eggs over a short period of time (terminal spawning) or distinct bouts of spawining in one season (separate batch or repeated spawning), or during different spawning seasons and, some spawn continuously (Rocha et al., 2001). Species with terminal spawing do not grow between batches and are likely to have a short life span and to die soon after spawning, while those with prolonged spawning periods usually feed and grow between spawnings (Mangold, 1987; Rocha et al., 2001).

Encapsulation of eggs is a feature of cephalopods with a great diversity of capsule structure that varies phylogenetically (Boletzky, 1986; Boletzky, 1998). Among oegopsids, eggs are generally small (1-3 mm) and egg masses are still poorly known. Some species spawn single eggs, others neutraly boyant large egg masses and others "brood" their eggs. In many species spawning is bottom-associated (Nesis, 1995). Myopsid squid eggs are deposited in benthic "mops" where few to multiple eggs (2-10 mm) are organized into capsules (Jereb and Roper, 2010). Sepiidae and Sepiolidae have medium to large eggs (3-10 mm) which are either laid individually or in small clusters in the bottom or in benthic structures (Jereb and Roper, 2005). Cirrate octopods lay large, individual eggs encased in a leathery outer layer on a variety of bottom structures (Boletzky, 1982; Ziegler et al., 2021). In incirrate octopods, both benthic and pelagic, eggs vary greatly in size (1-45 mm) and are laid in batches or individually and brooding takes place until the eggs hatch (Jereb et al., 2016). There is a trade-off between egg size and fecundity. Spawning demands high energy and can occur continually or intermittently for a protracted period of weeks or months and is generally accompanied by an overall physiological deterioration.

The Adult phase of life is generally short, but there are exceptions. A life-span of several years are expected in all cold-water octopod species (Robison et al., 2014) and in nautilids.

After reaching maturity, nautlids lay a few eggs every year (Dunstan et al., 2011a) and can live at least 20 years (Barord and Basil, 2014). There is no information available on the life-span of cirrates.

3.6 Senescent phase

Definition: The Senescence phase begins when gametes are spent and ends with death (Table 1).

The Senescent phase is the final phase of the cephalopod life cycles characterized by overall deterioration of the animal. This phase can last for weeks or months, and is species-specific and temperature dependent. Organ systems degenerate and they cease to function. Declining physiological condition leads to considerable morpho-physiological transformation that characterizes the senescent individual. The morphology of a senescent individual may be quite different from that of the adult, and were even described as a new species (*Chaunoteuthis mollis*, synonym of *Onychoteuthis banksii*, Nesis, 1995).

The physiological processes that trigger senescence are not fully understood but senesence is associated with the optic gland control of gonad maturation, spawning and feeding inhibition (Roumbedakis and Guerra, 2019). These changes are likely triggered by reduction or cessation of feeding leading to weight loss and starvation, re-mobilization of somatic protein that causes muscle breakdown and flaccid tissues, skin lesions, cloudy eyes and retraction of the skin around the eyes, loss of coordination, parasite infection, among others (Chichery and Chichery, 1992; Jackson and Mladenov, 1994; Anderson et al., 2002; Roumbedakis and Guerra, 2019).

In the oegopsids, senescence is reported as degraded musculature, with spent females undergoing a gelatinous degeneration and losing their tentacles (Jackson and Mladenov, 1994; Nesis, 1995; Seibel et al., 2000; Laptikhovsky et al., 2019). In *Illex argentinus*, the mantle becomes thinner and alongated and degeneration of body parts is extreme (Laptikhovsky and Nigmatullin, 1992). Spent females are known to float passively to the surface becoming available for seabirds to forage (Xavier et al., 2014). This is documented in Ancistrocheirinae, Octopoteuthidae, Gonatidae, Histioteuthidae, Cranchiidae among other families (Nesis, 1995).

This phase of life is best-known and described in shallow water species, particularly incirrate female octopods that brood their eggs for weeks to months and even years without feeding (Guerra, 1993; Anderson et al., 2002; Robison et al., 2014; Roumbedakis and Guerra, 2019). Some species are known to use the energy and nutrient reserves of their somatic tissues to fuel the brooding process and this results in loss of muscle mass and physiological condition that contributes to senescence.

In cirrates, sexual maturity likely takes place during most of their lives and egg laying is not related to senescence (Boyle and Rodhouse, 2005). Nautilids are the only cephalopods without optic glands (Arnold, 1984) and senescent individuals have not yet been described.

3.7 Stages in cephalopod life cycles

Each phase of the life cycle is composed of several stages. A stage is defined here as a morphologically distinguishable step in development (Naef, 1928; Nesis, 1979; Eckman, 1996). The number of stages in each life phase and their beginning and end point are species-specific, but stable within a species. Ideally, the complete set of stages within each phase of the life cycle should be known, but this requires an in-depth knowledge of the life cycle and a comprehensive size series that does not exist for most cephalopods. Delineating stages represents an important next step in life cycle research.

The Embryonic stages of inshore and commercially important species are most thoroughly described. Naef (1928), described and illustrated these stages for several species that served as a template for subsequent comparative studies (e.g. Arnold, 1965; Lemaire, 1970; Shigeno et al., 2010; Boletzky et al., 2016; Deryckere et al., 2020). Even so, detailed Embryonic stages of most oegopids and cirrates are still completely unknown. We define the Embryonic stages as: *Distinct development steps that occur within the egg during morphogenesis of the cephalopod body plan.*

The Hatchling stage is also common to all cephalopods, but usage of the term Hatchling has been highly variable, referring to a variety of life stages from newly-hatched individuals to older paralarvae and even juveniles of unknown age (e.g. Vidal et al., 2002; Robin et al., 2014; Kingston et al., 2015; Fernandéz-Gago et al, 2019; Bazarini and Crook, 2020). We define the Hatchling stage as: *The first post-embryonic stage of the cephalopod life cycle. It begins after eclosion and ends with complete absorption of yolk reserves and loss of structures required for hatching* (Figure 2B).

During development, and for a short time after eclosion, all cephalopods have an inner yolk sack containing maternal reserves, which are the remnant of the embryonic 'yolk organ' (Boletzky, 2010) and/or transitory morphological structures specialized for hatching (e.g., Hoyle organ) even after the onset of exogenous feeding. Yolk absorption takes place independent of and concomitant with the digestion of captured prey and there is a temporary overlap of two modes of nutrition: endogenous (embryonic nutritional system for digesting yolk, i.e., lecithotrophy) and exogenous (digestion of exogenous prey, i.e., planktotrophy or predation on benthic prey) (Vidal et al., 2002; Boletzky, 2010). This mixotrophic nutrition allows the hatchling to cope with shortages of suitable prey, the transition to competent predation, and generally mitigates failure at first feeding (Vecchione, 1987; Boletzky, 2010). Consequently, the Hatchling stage is characterized by high mortality rates that have been well documented in laboratory studies (Vidal et al., 2002, Vidal et al., 2014; Iglesias et al., 2007; Braga et al., 2022).

Individual yolk content at eclosion is highly variable and hatchlings can be premature (Figure 4A), normal or late. Premature hatchlings eclose without complete absorption of the outer yolk sac; normal hatchlings eclose after complete absorption of the outer yolk sack and late hatchlings eclose when nearly all the inner yolk sac has been absorbed (Vidal and Boletzky, 2014). The Hatchling stage is easily recognized in the laboratory by monitoring hatching day and the absorption of the inner yolk sack (Vidal et al., 2002), but more difficult in the wild and must be inferred from the presence of the Hoyle organ (Figure 4B) and/or an inner (Figure 4C) or outer yolk sack (Figure 4A).

The Hatchling stage may last days, weeks or months (Figure 2B). *Sepia officinalis* hatchlings go through a major process of reabsorbing the Hoyle organ, which may take up to seven days while yolk reserves are being absorbed (Cyran et al., 2018). For loliginid squid and small-egged *Octopus* hatchlings, the inner yolk sack can last from a few days to weeks (Vidal et al., 2002; Nande et al., 2017). Juvenile hatchlings from colder and/or deep-waters likely digest yolk for months due to the large size of the inner yolk sack in *Grimpoteuthis* (Shea et al., 2018) and *Graneledone boreopacifica* (Voight and Drazen, 2004).

Some cephalopod species go through a Settlement stage, where the paralarva gradually leaves the plankton and adopts a benthic lifestyle. In the larger marine invertebrate literature, this transition is called "settlement" and that terminology has been adopted in cephalopods (Villanueva and Norman, 2008; Roura et al., 2023). This stage has been only documented under laboratory conditions in a few Sepiolida and small-egged Octopodidae species, but it must be common across all taxa that has planktonic paralarvae and benthic juveniles (Figure 2C).

The Settlement stage is the first stage of the Juvenile phase and marked by a gradual transition from the plankton to the benthos that may involve alternating periods in each environment before a fully benthic lifestyle is adopted. For example, in *Euprymna hyllebergi*, the settlement stage lasts up to 25-30 days (at 28°C and 21-25°C, respectively) during which individuals alternate between the plankton during the day and the benthos at night, before becoming fully benthic (Nabhitabhata and Nishiguchi, 2014)

(Table 3). In *Octopus sinensis*, individuals alternated between clinging on the tank walls or sheltering on the bottom during the day with nocturnal swimming in the water column (Dan et al., 2021). While for *O. vulgaris*, the settlement stage lasts around 40 to 60 days (at 18-22°C) and no alternating environments between day and night was reported. In this species the settlement stage comprises of three sub-stages each one with particular morphology and behavior (Roura et al., 2023). It was also reported that the transition between environments in *Macrotritopus* spp. is particularly long and estimated to last from 70 to 140 days (Hanlon et al., 1985). Alternating between the plankton and the benthos during settlement may be an important strategy for finding preferred substrate.

A similar, parallel, stage exists for species that transition from passive plankton drifters to gradually adopt a fully active nektonic lifestyle as juveniles (Roper and Young, 1975). The recognition of this transition to a new life phase is based on development of swimming abilities and strength for individuals to move against surface currents early in life (Bartol et al., 2009; Vidal et al., 2018) for which our understanding is still limited.

Here, we propose the term "Metapelagic stage" to describe the transition from plankton to nekton that has been recognized and described in a few species but unnamed (Sugimoto and Ikeda, 2012; Vidal et al., 2018). The prefix Meta- is Greek and implies a change or shift between two states. Recently, meta- has also been used in self-reference (e.g., metadata is data about data), which also parallels our intent to express that the Metapelagic stage is a transition within the pelagic environment. In species with a Metapelagic stage that form schools (e.g. some loliginid and ommastrephid squids), the transition is easily recognized by advanced swimming control that culminates with the ability to swim in schools (Sugimoto and Ikeda, 2012; Vidal et al., 2018) (Table 3).

The Settlement and the Metapelagic stages are examples of transitional stages based on known life cycles, but still require more studies to be precisely defined in more species. There are several other stages that are common to all cephalopods, including the maturity stages of subadults (Arkhipkin, 1992), and the mating and spawning stages of adults (Puneeta et al., 2015). These stages are discussed elsewhere, and fit easily into this new framework of phases and stages. Clearly there are other stages yet to be described. For example, is the association of argonautoids with gelatinous megaplankton opportunistic, or a long-term association that constitutes a stage (i.e., the Hitchhiking stage)? The description of other intriguing stages of the cephalopod life cycle awaits new research to delineate.

4 Patterns of cephalopod life cycles

Cephalopod life cycles are markedly different from long-lived fish and other marine mollusks. All cephalopods are dioecious, oviparous (except *Ocythöe tuberculate* and *Vitreledonella richardi* which are ovoviviparous). Fertilization is generally described as internal, although the details are unknown for most oegopsids (Hoving et al., 2014). Except for *Nautilus* which can live longer than 20 years (Dunstan et al., 2011a), and some deep-sea and polar species, life spans are short, marked by fast growth, a long early life history, a short adult phase with sexual maturity occurring late in the life cycle, and senescence and death generally, but not always, following reproduction. Cephalopods have flexible reproductive strategies (Rocha et al., 2001; Boyle and Rodhouse, 2005; Ibáñez et al., 2021).

Commercially important shelf species and laboratory reared species have provided the basis for much of what we know about cephalopod life cycles (Boyle, 1983; Boyle and Rodhouse, 2005; Rosa et al., 2013a; Rosa et al., 2013b). These species are well studied, and provide the opportunity for detailed morphological measurements and behavioral observations which is not yet possible for a majority of open ocean species. Oegopsid data must be pieced together from a variety of indirect methods, including stable isotopes and other elemental analyses of beaks and gladii. These methods have been essential in understanding how deep-sea

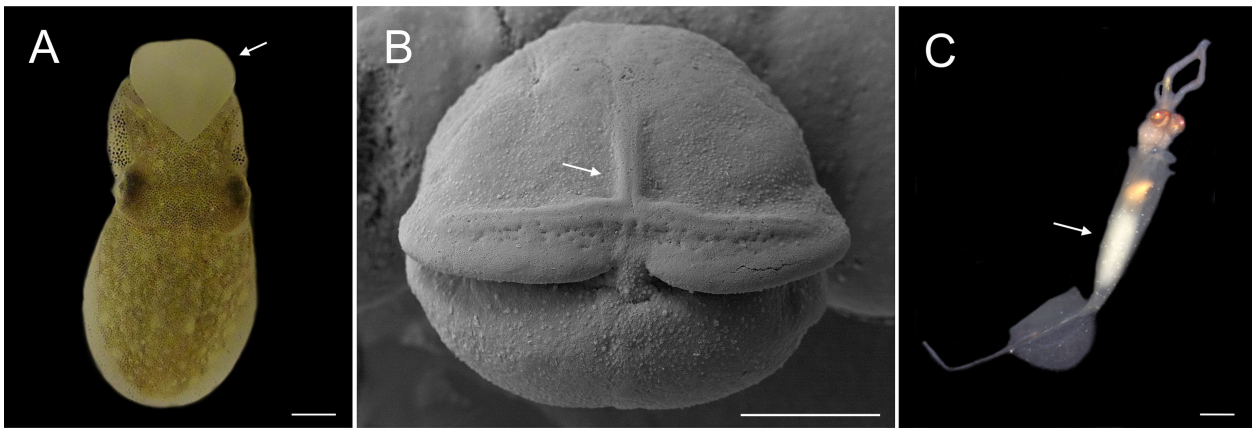

FIGURE 4
Hatchling stage transitory morphological features. **(A)** *Sepia officinalis* premature hatchling with outer yolk sack (arrow), *Photo credit*: C. O´Brien, scale bar= 1 mm. **(B)** *Sepia officinalis* Hoyle organ, anchor-shaped structure on posterior dorsal mantle (arrow), scale bar= 500 µm, *Photo credit:* N. Cyran. **(C)** Mastigoteuthid paralarva with inner yolk sack (arrow), scale bar= 1 mm, *Photo credit*: Danielle Ortiz de Ortiz.

species occupy different water masses or localities throughout their life cycles (e.g., Cherel and Hobson, 2005; Semmens et al., 2007; Zumholz et al., 2007; Lukeneder et al., 2008; Staudinger et al., 2013; Golikov et al., 2018; Golikov et al, 2022b).

Cephalopods exhibit four main life cycle patterns characterized by where each phase of life is lived and whether they have planktonic paralarvae. Life cycles may be fully pelagic, fully benthic or alternating between benthic and pelagic environments (Figure 5). Species with alternating life cycles may hatch as paralarvae or juveniles (Table 4) and the Juvenile, Subadult, and Adult phases may live on bottom, near-bottom or away from the bottom.

Each of these four main patterns have variations categorized primarily on the habitat of the adults. These four patterns do not take into account temporary behavioral variations such as bottom associated spawning in many oceanic squids (Nesis, 1995), bottom resting behaviors in adult *Illex illecebrosus* (Bradbury and Aldrich, 1969; Vecchione and Young, 2018), air-water interface resting behaviors in juvenile *Octopus maya* (Van Heukelem, 1976), or other responses that may move a benthic animal temporarily off bottom (sepiolids, Bello and Biagi, 1995) or a pelagic animal into the air (Ommastrephidae, Muramatsu et al., 2013). How senescence impacts these life cycles is currently unknown because senescent animals are rarely found, likely due to predation. However, senescent specimens either remain in the same habitat as adults (small-egged octopods), float to the surface (Rodhouse et al., 1987; Xavier et al., 2013) or may sink to the bottom (Roper and Vecchione, 1996; Nesis et al., 1998; Hoving et al., 2017). Based on this and the fact that these habitat changes are not under active control of the organism, the Senescent phase was not treated in Figure 5. A fuller description of how the Senescent phase impacts the cephalopod life cycle awaits new research.

Here, we describe and define four life cycle patterns and their variations. Each variation is explained using the known life cycle of a model species.

4.1 Holopelagic

Definition: A life cycle characterized by all phases and stages living in the water column away from the bottom (Table 4).

All holopelagic species known have a Paralarval phase, although *Haliphron* eggs are very large (16 mm) (O'Shea, 2004) and juveniles are often caught in epipelagic waters (Hochberg et al., 1992). It is possible that *Haliphron atlanticus* hatch as juvenile. Some deep-sea species such as the mastigoteuthids, *Spirula spirula* and *Vampyroteuthis infernalis* may have mesopelagic paralarvae and juveniles (Clarke, 1969; Clarke, 1970; Clarke and Lu, 1975; Young and Vecchione, 1999) as they are rarely found in the epipelagic plankton. Many holopelagic adult squids have a spawning stage that will occur near the bottom (Nesis, 1995).

Holopelagic species either occupy a single depth horizon (e.g., Argonautidae) or multiple depth horizons over their life cycle (Figure 5A). The multi-depth variation presented below is particularly synoptic and will likely need to be revised as meso- and bathypelagic life cycles become better known. Currently, this category includes species that occupy deeper depth horizons as they grow and develop (e.g., ontogenetic descent), traditional diel vertical migration, vertical spreading and other depth variations (Roper and Young, 1975; Shea and Vecchione, 2010; Judkins and Vecchione, 2020). Vertical migration patterns are typically described for species that move across hundreds or thousands of meters of vertical space (Roper and Young, 1975). Judkins and Vecchione (2020) identify three patterns of vertical migration based on the depths occupied and whether the patterns are synchronous or asynchronous, and provide evidence that some species may undergo an ontogenic "ascent" where larger individuals are found at shallower depths.

Holopelagic - Single depth: This variation refers to species that occupy a single major depth horizon throughout their entire life cycle. For example, *Argonauta argo* remains in the epipelagic and *Vampyroteuthis infernalis* remains in the mesopelagic during its entire life cycle. This category includes the non-migrating epipelagic, mesopelagic and bathypelagic diel behaviors identified by Judkins and Vecchione (2020) (Figure 5A).

Argonauta argo is a small epipelagic octopod found worldwide in tropical and subtropical open waters (Finn, 2016). They are holoepipelagic non-migrators (Judkins and Vecchione, 2020) and all life phases are found in the epipelagic zone (Figure 5A) (Table 4). Adult females lay very small eggs (1.5 mm) that are attached to the internal axis of the brood case, and are brooded until hatching. There may be up to 86,000 eggs (Laptikhovsky and Salman, 2003) with up to five different developmental stages developing simultaneously (Finn, 2016). Planktonic paralarvae hatch at about 0.7- 1.0 mm ML (Hochberg et al., 1992). Juvenile females and adult males are found 0 – 300 m. Adult females begin to brood eggs at smaller sizes and continue feeding, growing and producing eggs as they age and are found at the surface during the day, dusk, and night (Laptikhovsky and Salman, 2003; Finn, 2016). Senescent individuals have not been described.

Holopelagic - Multi-depth: This variation includes species with life phases that occur in two or more different depth horizons. Most commonly, this category includes species that have epipelagic paralarvae that gradually move into the meso- or bathypelagic waters with ontogeny. These species may move through multiple pelagic depth horizons every day (Figure 5A).

Thysanoteuthis rhombus is a large oegopsid squid found worldwide in tropical and subtropical open waters and has a 1-year life span. Adult females lay large (up to 2m long), pelagic, cylindrical egg masses that drift in the open surface waters and contain 32,000 to 75,000 developing embryos (Roper and Jereb, 2010). Planktonic paralarvae hatch at about 1.5 mm ML. Paralarvae and juveniles are commonly found in the upper 50 -100 m of the epipelagic zone (Figure 5A) (Table 4). The Juvenile phase begins at about 15 mm ML when the body shape is similar to the adult, but arms are proportionally much longer than in the adult (Wakabayashi et al., 2005). Late juveniles form pairs at approximately 100 mm ML, and probably remain together during their lifetimes. Subadults and adults are found at 600 – 800 m during the day, and 0-50 m at night indicating an extensive diel vertical migration (Roper and Jereb, 2010).

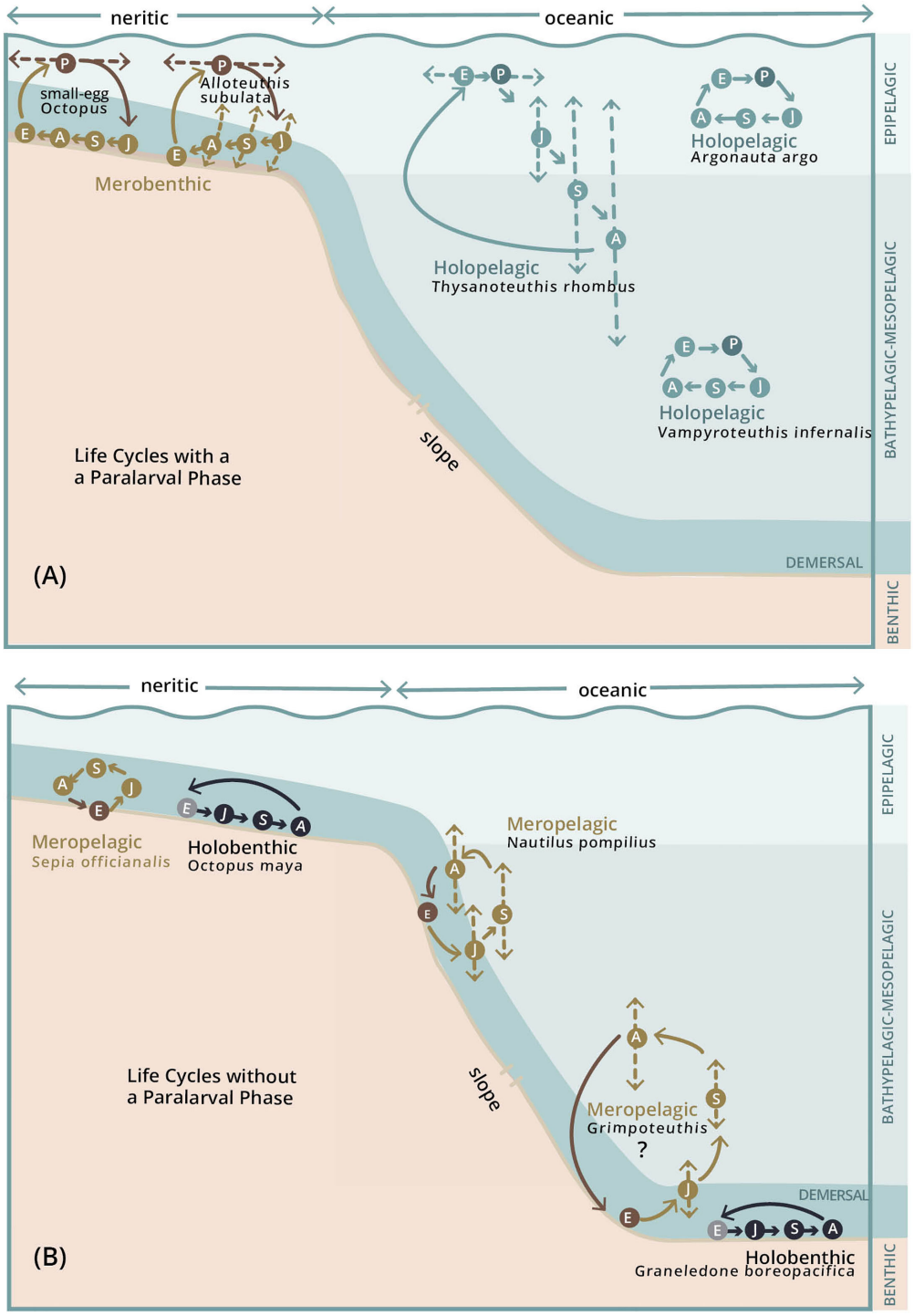

FIGURE 5
Cephalopod life cycle patterns. All known cephalopod life cycles can be distilled into four major categories: **(A)** Life cycles with a Paralarval phase. These species are either Merobenthic (brown circles) or Holopelagic (blue-green circles). E, Embryonic; P, Paralarval; J, Juvenile; S, Subadult; A, Adult Phases. Merobenthic species are primarily found on the shelf, and are characterized by having benthic eggs and planktonic paralarvae (P), and JSA phases that live either near bottom or on bottom. Holopelagic species live all phases of their life cycle in the water column, either entirely within a depth horizon, or moving between multiple depth horizons. **(B)** Life cycles without a Paralarval phase. Species without a Paralarval phase are either Meropelagic (brown circles) or Holobenthic (black circles). Meropelagic life cycles are characterized by having benthic eggs, and JSA that live near-bottom or off-bottom and into the water column. In some of these species, the JSA are highly mobile and may move extensively in the water column as suggested by the dashed arrows. Holobenthic species live all phases of their life cycle on bottom.

TABLE 4 Cephalopod life cycle patterns, main variations and habitat usage by each life phase, except for senescent which is widely reported for only a few species.

Marine environment	Life cycle patterns	Model Species	Habitat usage by life phase				Habitat variation	Vertical distribution	Reference
			Eggs	Paralarvae	Juveniles	Subadults and Adult			
Pelagic	Holopelagic: All phases live in the water column								
		Argonauta argo	Pelagic	Pelagic	Pelagic	Pelagic	Single-depth	Epipelagic (0-300 m)	Laptikhovsky and Salman (2003); Finn (2016)
		Vampyroteuthis infernalis	Pelagic	Pelagic	Pelagic	Pelagic	Single-depth	Bathypelagic (1000-4000 m)	Norman and Finn (2016)
		Thysanoteuthis rhombus	Pelagic	Pelagic	Pelagic	Pelagic	Multiple-depth	Epi-mesopelagic (0-1000 m)	Wakabayashi et al. (2005)
Benthic	Holobenthic: All phases live in contact with the bottom								
		Octopus maya	Benthic	–	Benthic	Benthic	Shallow-water	Sublittoral (0-50 m)	Van Heukelem, (1976); Van Heukelem, (1983); Norman et al., (2016)
		Graneledone boreopacifica	Benthic	–	Benthic	Benthic	Deep-water	Bathyal (1000-3000 m)	Voight and Drazen (2004); Robison et al. (2014)
Benthic-Pelagic	Merobenthic: Phases alternate between pelagic and benthic environments with a Paralarval phase								
(alternating)		*Alloteuthis subulata*	Benthic	Pelagic	Demersal	Demersal	Demersal	Demersal (0-500 m)	Jereb et al. (2010); Roura et al. (2019)
		Octopus vulgaris	Benthic	Pelagic	Benthic	Benthic	Benthic	Sublittoral (0-250 m)	Norman et al. (2016)
	Meropelagic: Phases alternate between pelagic and benthic environments without a Paralarval phase								
		Sepia officinalis	Benthic	–	Demersal	Demersal	Demersal	Demersal (0-200 m)	Reid et al. (2005)
		Nautilus pompilius	Benthic	–	Pelagic	Pelagic	Off-bottom	Pelagic (0-700 m)	Dunstan et al. (2011b); Barord and Basil (2014)
		Grimpoteuthis (?)	Benthic	–	Pelagic	Pelagic	Off-bottom	Pelagic (280-4870 m)	Collins and Villanueva (2006)

4.2 Holobenthic

Definition: A life cycle characterized by all phases and stages living in contact with the bottom (Table 4).

In holobenthic species, benthic eggs hatch to benthic juveniles which grow into benthic adults. There is no Paralarval phase and consequently these species have more restricted distributions. All phases and stages occupy the same water masses, and consequently invading new spaces is difficult (Figure 5B**).** The benthic eggs may be laid directly on the substrate, or on other biotic substrate such as corals and sponges. Two depth variations are named according to water depth which roughly correlates with temperature and impacts development time and the relative robustness of the hatchlings.

Holobenthic - Shallow-water: This variation is generally found in species that occur in shallow shelf waters, including tropical coral reefs.

Octopus maya is a large, muscular octopus species that is endemic to the Gulf of Mexico. Adult females lay festoons of large benthic eggs (11-17 mm) in 2-5 m water (Van Heukelem, 1976), then enter senescence. Juvenile hatchlings (7.0 mm ML) immediately adopt a benthic life style. Maximum size is reached at about 8.5 months, and the total life cycle is 1-2 years (Van Heukelem, 1976; Van Heukelem, 1983; Norman et al., 2016) (Figure 5B) (Table 4).

Holobenthic - Deep-water: This variation is generally found in species that occur in deep-water, where water temperature is very cold and the environment is very stable. Although here it is illustrated with a deep-sea benthic species, shallow polar areas may have similar life cycle patterns.

Graneledone boreopacifica is a boreal deep-sea benthic species found in the North Pacific. Adult females lay small batches of large

eggs and brood them for about 4 years at depth of 1000-2000 m, while they are in their Senescent phase of life (Robison et al., 2014). Premature hatchlings are very large, 23 – 30 mm ML and 55 mm in total length (Voight and Drazen, 2004). Males and females could be distinguished by examination of internal sex organs at hatching. Males had a hectocotylus with a full complement of suckers, suggesting that this species may hatch as a subadult (Voight and Drazen, 2004). Adults are epibenthic on muddy to rocky bottoms (Jorgensen, 2009) and total life span is expected to last several years (Figure 5B) (Table 4).

4.3 Merobenthic

Definition: *A life cycle that alternates between pelagic and benthic environments. Merobenthic species have benthic eggs that hatch as planktonic paralarvae, then settle back to the benthos or near the bottom to live their Juvenile, Subadult and Adult phases* (Table 4).

There are many synonyms for this life cycle pattern in the literature including merobenthic (e.g. Boletzky, 1992; Doubleday et al., 2011; Villanueva et al., 2016), pelago-benthic (e.g. Page, 2009; Nielsen, 2013; Ibáñez et al., 2014) and benthopelagic (Bhaud and Duchêne, 1996; Nixon and Mangold, 1998). Merobenthic is adopted here because it has been used in previous research on octopods and avoids the use of a term that has a well-known meaning when used to describe adult habitats. The prefix "Mero" from Greek means "partial", and benthic refers to the adult habitat, thus Merobenthic is a life cycle that is partially benthic. Merobenthic species are mainly known to inhabit the continental shelf and upper slope.

Merobenthic - Demersal: *Alloteuthis subulata* is found in shallow, coastal waters associated with sandy and muddy bottoms (Figure 5A, Table 4). Adults may form dense aggregations and undergo seasonal migrations (Jereb et al., 2010). Adults mature at a wide size range, but 50% are mature between 70-80 mm ML. Mature females lay small eggs in balloon-shaped capsules that are attached to hard benthic substrates. Embryonic phase lasts 2-3 weeks. Hatchlings are planktonic and emerge at 1.0 – 2.2 mm ML. Paralarvae have coastal dispersal pattern and live in the plankton for about two months before settling into a demersal habitat (Roura et al., 2019). Estimated life span ranges from 6 months to a year.

Merobenthic - Benthic: *Octopus vulgaris* is a sublittoral species, living on rocky, sandy, or muddy bottoms of the Mediterranean Sea and central and north-east Atlantic Ocean, typically in < 100 m. Females lay strings of 100-500K small eggs and attach them to the roof of a sheltered den (Figure 5A) (Table 4). Brooding can take from 1.5 to 5 months depending on water temperature (Iglesias et al., 2004). Hatchlings emerge from the benthic eggs at 1-2 mm ML then swim to the surface waters where they live as planktonic paralarvae. Paralarvae spatio-temporal distribution is strongly associated with upwelling events; and paralarvae display a coastal-oceanic dispersal patterns being carried out to oceanic waters and returning to the shelf close to settlement to the benthos (Roura et al., 2019). In a laboratory setting, the Paralarval phase lasts about 40-60 days (Villanueva, 1995; Iglesias et al., 2007) (Table 3). The Juvenile phase begins with the Settlement stage around day 45 after hatching and lasts until day 90 (at 18-20°C) (Roura et al., 2023). At the end of Settlement stage, juveniles have bodies with sculptural components, horizontal pupils, > 35 suckers per arm and are capable of camouflage. Adults have been reported 0 – 250 m; the total life cycle last about 11.5 – 24 months (Iglesias et al., 2004; Norman et al., 2016). Females go through a conspicuous Senescent phase while brooding the eggs for weeks or months, with loss of muscle mass and considerable morpho-physiological deterioration (Roumbedakis and Guerra, 2019) (see Section 3.6)

4.4 Meropelagic

Definition: *A life cycle that alternates between pelagic and benthic environments. Meropelagic species hatch as benthic juveniles and move off bottom to become demersal or pelagic juveniles, subadults, and adults* (Table 4).

We coin the term Meropelagic as a parallel term to Merobenthic. "Mero" means "partial", and pelagic refers to the adult habitat, thus Merobenthic is a life cycle partially pelagic or demersal. Meropelagic species lay large, benthic eggs that hatch as juveniles. Because there is no Paralarval phase, these species often have a narrow geographic distribution, although cirrate distribution may be broad (Collins and Villanueva, 2006). In all cases, juveniles, subadults and adults can move away from the bottom and into the water column (Figure 5B).

The distance that these taxa live away from the bottom as an adult distinguishes the different variations. The off-bottom category is very broad and can include any species that has a benthic egg, juvenile hatchlings but that moves up into the water column.

Meropelagic - Demersal: *Sepia officinalis* is a neritic demersal species found in the Eastern Atlantic and Mediterranean Sea from subtidal waters to up to 200 m (Reid et al., 2005). In the spring and summer, the species is found inshore; in the fall and winter individuals migrate out to the shelf (Figure 5B) (Table 4). Adult females carry between 150 – 4000 eggs depending on their size. Spawning occurs in shallow waters (13 – 15°C). Eggs are 8-10 mm in diameter and attached to benthic substrates. Juvenile hatchlings emerge at 6 – 8 mm ML after 30 – 90 days of development. The juveniles immediately adopt a benthic life style and live in 50 – 80 m of water, including burying behaviors. Juveniles, subadults and adults are demersal, from 0 – 200 m. The life span is one to two years (Reid et al., 2005).

Meropelagic - Off-bottom: In these species, the adults lay benthic eggs, but the Juvenile, Subadult, and Adult phases occupy pelagic spaces and have a pelagic mode of life that is not dependent on the bottom (e.g., swimming is the primary form of movement). This variation encapsulates many taxa whose life cycle phases are poorly known.

Like other extant nautilids, *Nautilus pompilius* lives on the coral fore reef. Nautilids are slow growing, reaching maturity at 12 – 15 years (Landman et al., 2010). Adult females lay single, encapsulated eggs and attach them to hard structures, likely in shallow waters, 80 – 100 m (Jereb, 2005). Nautilids do not die post-breeding (Saunders, 1984). Rearing studies show that embryos develop for 14 months

and hatch as juveniles (Okubo et al., 1995; Uchiyama and Tanabe, 1996). Juveniles, subadults and adults are mobile and occupy similar habitats over reef slopes (Dunstan et al., 2011b) (Figure 5B) (Table 4). It was suggested that adult nautilids have limited dispersal ability, because 200 km of water deeper than 800 m is sufficient to prevent gene flow (Barord et al., 2023).

Cirrates lay single, large, encapsulated eggs in and on deep sea habitats and structures including corals and sponges (Collins and Villanueva, 2006; Boletzky, 2012; Vecchione, 2019). Other information on life cycles is generally lacking and identification is often difficult (Boletzky, 1978-79; Ziegler et al., 2021). Opisthoteuthid cirrates are benthic throughout their life cycle, but other cirrate adults may be collected in deep water pelagic tows. Juvenile hatchlings of *Grimpoteuthis* are morphologically similar to the adults with large functional fins and a large internal yolk reserves (Shea et al., 2018), which suggests a long juvenile Hatchling stage. Two small cirrate hatchlings of *Grimpoteuthis wulkeri* (9 and 11 mm ML) were collected between 1489 and 1997 m depth (i.e., 655 – 147 m off-bottom) (Vecchione et al., 2010), suggesting that after eclosion, they could move up into the water column. Four juvenile cirrates (13-15 mm ML) without an inner yolk sack were collected in deep waters off West Greenland (Golikov et al., 2022a). Because adult cirrates have been collected and observed in the midwater with fins that make them highly mobile, we classify their life cycle here as Meropelagic-Off-bottom. However, the degree to which juveniles, subadults and adults are associated with the benthos or the meso – and bathypelagic water still is an open question (Figure 5B) (Table 4).

5 Discussion

We have provided explicit definitions of cephalopods life phases and stages based on established criteria and in line with the vast marine invertebrates literature (McEdward, 1995; Carrier et al., 2018). Definitions can frame how meaningful problems are conceptualized and how results are interpreted. It is important to build consensus towards a standard terminology and a clear conceptual foundation for analyzing the diversity of developmental patterns and the essence of cephalopod life cycles.

The proposed definitions rest on the foundation that cephalopods are direct developers without a true, metamorphic larva as in other mollusks. At the time of eclosion, the cephalopod body plan exists, and is maintained during ontogeny. We delineate the phases and two stages of the life cycle using morphological criteria. The phases are based on major milestones such as having all morphological features that define a species, whereas the stages represent smaller, incremental morphological changes. Some phases and stages are common to all taxa (e.g., Embryonic phase and stages, Hatchling stage, Adult phase), but others are specific to a subset of taxa (e.g., Paralarval phase, Settlement stage). The time elapse of each phase in the life cycle is variable, likely sex related, strongly dependent on temperature and taxon and species-specific in cephalopods.

Different phases of the life cycle utilize different resources and occupy separate habitats. Physical and biological processes and their stressors impact each phase differently, meaning there are phase-specific growth and survival rates that combined control the duration of each phase, and the probability that an individual will successfully transition to the next phase (Morgan, 1995; Boyle and Rodhouse, 2005; Byrne et al., 2018)

It is critical that phases and stages be clearly delimited to understand how susceptibility to these processes and their stressors vary across the life cycle. Each phase represents an optimal adaptation, varying with body size, for acquiring resources for survival and growth under different ecological conditions (Love and Strathmann, 2018). Successful completion of each phase contributes to the overall balance between population growth and mortality, depending on the vulnerability to intrinsic and extrinsic factors. Phases or stages that are more susceptible to mortality are 'weak links' in the overall connectivity of life cycles phases (Boyle and Rodhouse, 2005).

In theory, if one phase confers a competitive advantage, selection should favor expanding that phase. Conversely, if a phase results in excessive mortality, selection should favor reducing or eliminating that phase. The absence of a phase can be observed in species without a Paralarval phase (Holobenthic and Meropelagic cycles) or without the Juvenile phase (chiroteuthids). Expansion of a phase can be found in species with exceptionally long Embryonic and Senescent phases (e.g., *Graneledone boreopacifica*).

The early life, particularly the embryonic and larval phases of marine invertebrates and fish, are negatively affected by extrinsic stressors and generally have a narrow thermal window when compared to older stages (e.g. Pörtner and Farrell, 2008; Pandori and Sorte, 2019; Onthank et al., 2021). This seems also true for cephalopods (e.g. Rosa et al., 2012; Zakroff et al., 2019), substantiating the notion that the Paralarval phase is a period of intense mortality. In addition to the thermal stressors, predation, starvation and advection should negatively impact paralarval mortality. This impact is inferred by the very large number of eggs produced in species with a Paralarval phase, including most commercially important species (Calow, 1987; Boyle and Rodhouse, 2005). Nevertheless, estimates of mortality due to predation, starvation and advection are scarce (Okutani and Watanabe, 1983; Bigelow, 1992; Roberts and van den Berg, 2002; Vidal et al., 2006; González et al., 2010). Even less is known about the factors impacting survival of the juveniles in Holobenthic, Merobenthic and Meropelagic species. Estimates of early mortality require accurate identification of early stages, accurate measures of their distribution, abundance and growth rates, as well as their main predators and prey. These topics are currently unexamined, and represent essential future studies.

Different stressors may impact the survivorship of particular stages within a phase. The Hatchling stage and the Settlement stage have been associated with intense mortality in laboratory studies due to nutritional transitions, from lecithotrophy to planktotrophy in hatchlings, and from planktotrophy to benthic prey in settlers. These stages represent weak links in the life cycle (Vidal et al., 2014; Roura et al., 2023). Identifying these weak links is particularly important for proper management for commercial species (Rodhouse et al., 2014) and increasingly necessary for recognition of how pollution and climate change will impact species. Climate

change may cause cephalopods to hatch out smaller, grow faster, mature younger and at a smaller size with shorter life spans (Pecl and Jackson, 2008). Evidence also shows that warming oceans have increased the overall number of cephalopod landings (Doubleday et al., 2016). Cephalopods are notable for their potential for quick population-level responses to environmental change (Seibel, 2007; Fuchs et al., 2020). Will cephalopod grow even faster, shortening life phases and size at maturity under warming conditions? Having a baseline understanding of cephalopod life cycles in a broadly-accepted framework will facilitate our understanding of how they respond to rapid change.

Based on stable definitions of life cycle phases and a survey of the literature, we propose four distinct life cycles patterns: Holopelagic, Merobenthic, Meropelagic and Holobenthic. These patterns were distinguishable based on two main factors: the presence of a Paralarval phase and the degree of association with the bottom. Three of the four patterns have variations identified, and additional variations may be found as we learn more about oegopsid and cirrate life cycles in particular.

Reliance on the benthic environment is strongest in Holobenthic species, but the bottom becomes less essential in the Meropelagic where only the eggs are benthic. We estimate approximately 70% of all cephalopod taxa have an Embryonic phase that takes place either directly on the bottom or attached to benthic structures (Jereb and Roper, 2005; Reid et al., 2005; Jereb and Roper, 2010; Norman et al., 2016). About 30% of all cephalopods are Holopelagic, with an Embryonic phase that is entirely disassociated from the bottom. In Holopelagic species, we find a wide array of adaptations for egg development, from large neutrally buoyant egg masses to the release of single eggs [*Brachioteuthis* (Young et al., 1985), Enoploteuthidae (Young and Harman, 1985a), the unique production of a chamber for brooding eggs (Argonauthidae) and brooding (e.g. *Gonatus onyx* (Seibel et al., 2000)), *Japetella diaphana* (Schwarz et al., 2020), and *Bathyteuthis berryi* (Bush et al., 2012)].

Holopelagic and Merobenthic species produce paralarvae (Figure 5A). One of the recognized consequences of planktonic development is dispersal to such an extent that it can influence the distributional range of species (Villanueva et al., 2016), favoring transport into new environments. Paralarvae feeding on the plankton represent a reduction in the maternal sources investments per offspring, as feeding is served in the plankton supporting high growth and survival. The Paralarval phase has these advantages for the life cycle and partially explain why planktotrophic development has persisted in many invertebrate taxa (Levin and Bridges, 1995; Love and Strathmann, 2018). However, advection and turbulence can be efficient in spreading drifting paralarvae and carry them away from suitable habitats increasing mortality. That along with predation and high dependence on environmental conditions for recruitment success to the adult population can result in wide inter-annual fluctuations in abundance (Boyle and Rodhouse, 2005). As expected, the Paralarval phase is increasingly seen as being important in fisheries modelling (Bruggeman et al., 2022).

The absence of a Paralarval phase in the Holobenthic and Meropelagic cycles (Figure 5B) suggests a suppression of its dispersal features during the early life stages and life phases might occupy the same or closer habitats. Juveniles are large and similar to the adults, minimizing early life mortality and accelerating the acquisition of the adult morphology (Boletzky, 2003). Alternatively, hatchling juveniles occupy the same habitat as their parents and may be at a disadvantage if the environment has changed and competition with adults for resources has increased (Iwasa et al., 2022). For these species, spawning and embryonic development depend on the ability to find suitable high-quality benthic environments. The microenvironments that these phases occupy may be warmer and highly variable in shallow water species, or colder and stable in deep water species. In contrast, Holopelagic cycles occur in one ecosystem and species are not substantially faced with the hunt for a suitable environment mitigating the harm of turnover habitat between life cycle phases.

The Merobenthic cycles are quite complex as ontogeny progresses between two different ecosystems. Paralarvae adapted for a planktonic lifestyle go through elaborate morphological and behavioral changes to become bottom-dwelling juveniles and find suitable benthic habitat during the Settlement stage to ensure survival of the subsequent life phases. Meropelagic species also alternate between habitats but have larger eggs that hatch as juveniles. As the animal grows through the Subadult and Adult phases, they tend to move up and off of the bottom. *Sepia officianalis* is a Meropelagic demersal species that may only move slightly off bottom, whereas deep sea species may make more use of the meso- and bathypelagic water depths

These observations inevitably raise the question of the ancestral life cycle pattern of cephalopods. Two competing hypotheses exist about the ancestral life cycle in all bilaterians, including cephalopod mollusks. The "larva first" hypothesis suggests that ancient bilaterians had a pelagic larva and a benthic adult. The "intercalation" hypothesis suggests that larval stages have evolved as specializations from an ancestral with direct development (Page, 2009). The question is thus, whether the ancestral cephalopod produced paralarvae or juvenile hatchlings.

The early embryology of nautilids and coleoids is similar to other gastropod mollusks (Shigeno et al., 2010) and coleoids evolved from ancestors with external shells (Kröger et al., 2011). Extant nautilids hatch as juveniles. However, some cephalopod research generally supports the paralarva first hypothesis in coleoids (Fuchs et al., 2020). In this scenario, the Paralarval phase was reduced over evolutionary time, and the derived condition has an increased egg size and produces juvenile hatchlings (Laptikhovsky et al., 2017a; Fuchs et al., 2020). Indeed, other evidences suggest that the origin of the Holobenthic life cycle in benthic octopuses has evolved from a Merobenthic ancestral by the elimination of the Paralarval phase from the life cycle (Boletzky, 1992; Ibáñez et al., 2014). Resolving whether a life cycle with a paralarva or benthic juvenile is the ancestral condition of cephalopods requires more extensive information about the early life phases and stages of many species, as well as a highly-resolved phylogeny.

Life cycle information is still lacking for many species. Indeed, our knowledge of life phases and stages of many species has increased rather slowly for oceanic, deep sea and polar species, but surprisingly also for coastal species from remote and under

sampled areas such as the costs of Africa, India and Indonesia (Jereb and Roper, 2005; Reid et al., 2005; Jereb and Roper, 2010; Norman et al., 2016).

More life cycle oriented research is clearly required (Lipínski, 1998), particularly on defining life stages within phases for which our understanding represent the next level to be accomplished. The Hatchling stage is certainly common across taxa. Other stages belong to particular life cycles, such as the Settlement stage in Merobenthic species (Roura et al., 2023) or the newly proposed Metapelagic stage. Very little work has been directed towards describing important stages within each life cycle phase. This conclusion is strengthened by the observation that only for a few species have been a recent surge of studies defining and delimiting species-specific size or growth stages within the Paralarval and Juvenile phases of squid and octopods based on morphological, behavioral and ecological data (Wakabayashi et al., 2005; Vidal et al., 2018; Franco-Santos and Vidal, 2020; Dan et al., 2022; Roura et al., 2023). Holopelagic juveniles and subadults from many families are generally poorly known (Vecchione, 1987; Sweeney et al., 1992), but expected to be an important part of the diet of midwater fishes (Staudinger et al., 2013) making them an important but understudied part of the marine food web.

Many other life phases are still virtually unknown because of the difficulty of sampling their environments, small catch numbers due to net avoidance and patchy distributions, and collecting deep-sea species alive (Boyle, 1983; Vecchione, 1987; Sweeney et al., 1992; Hoving et al., 2014). Species that are fisheries targets often have large numbers and extensive information but even still there are holes in the life cycle (e.g., Katugin et al., 2013). Species with the most complete information are generally those that can be reared in the laboratory, and have some concurrent information about their wild population. This combination of factors only occurs in some coastal species of the loliginids and octopods, and increasingly model organisms like *Euprymna* spp. (Hanlon et al., 1997; Nyholm and McFall-Ngai, 2021; Jolly et al., 2022). Laboratory-based studies likely represents the shortest path for studying ecology and behavior of many life stages of coastal and oceanic cephalopods. Interestingly, many of the species treated in Boyle (1983) are the same species we use as models here, reinforcing the idea that only a few species are well studied, even 40 years later.

There are certainly more life cycles patterns, variations and arrays of amazing new behaviors to discover as we expand our cephalopod knowledge. In recent years, that has been accomplished by the use of Remotely Operated Vehicles to obtain *in-situ* video recordings from great depths describing fine-scale habitat, coloration, and behaviors never before seen. The first *in situ* observations of *Spirula spirula* showed that it swims oriented nearly vertically with its head upward (Lindsay et al., 2020), while magnapinnid squid were observed coiling the arm/tentacle filaments while trailing parallel just above the seafloor (Osterhage et al., 2020). These studies were quite revealing and reinforce the value and potential of such observations for a proper understanding of the ecology, behavior and life cycle of deep-water cephalopods.

Using the proposed framework of phases, stages and life cycle patterns will help us standardize our language and provide the opportunity to apply the results from field-based disciplines to lab-based disciplines in a synergistic way, thus maximizing our communication ability to learn about this diverse, charismatic group of mollusks. Hoping for a unifying ground, we encourage the cephalopod community to use the definitions and terminology proposed in this paper.

Author contributions

EAGV conceived the idea of this paper, conceptualization and design, wrote the original draft of sections 1, 3 and 5, and produced tables and figures. ES conceptualization and design, wrote the original draft of sections 2, 4 and 5, and produced tables and figures. Both authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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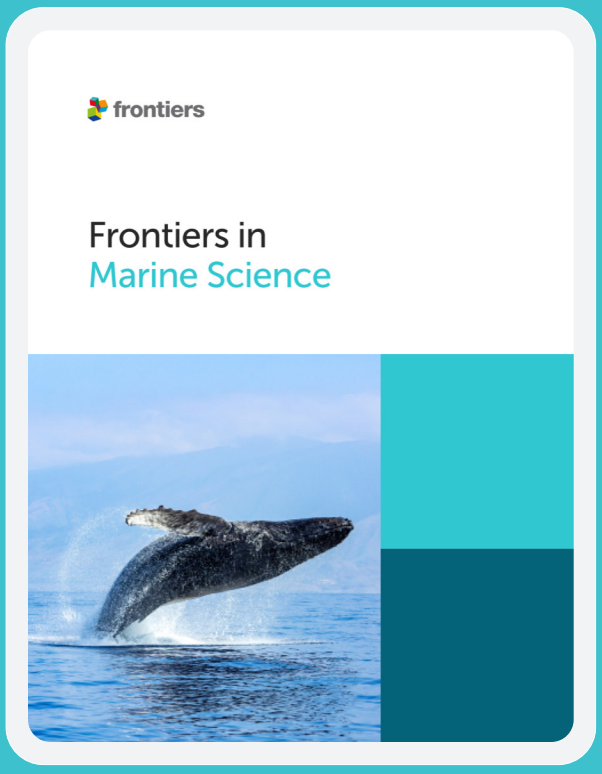

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