BIODIVERSITY OF SENSORY SYSTEMS IN AQUATIC VERTEBRATES

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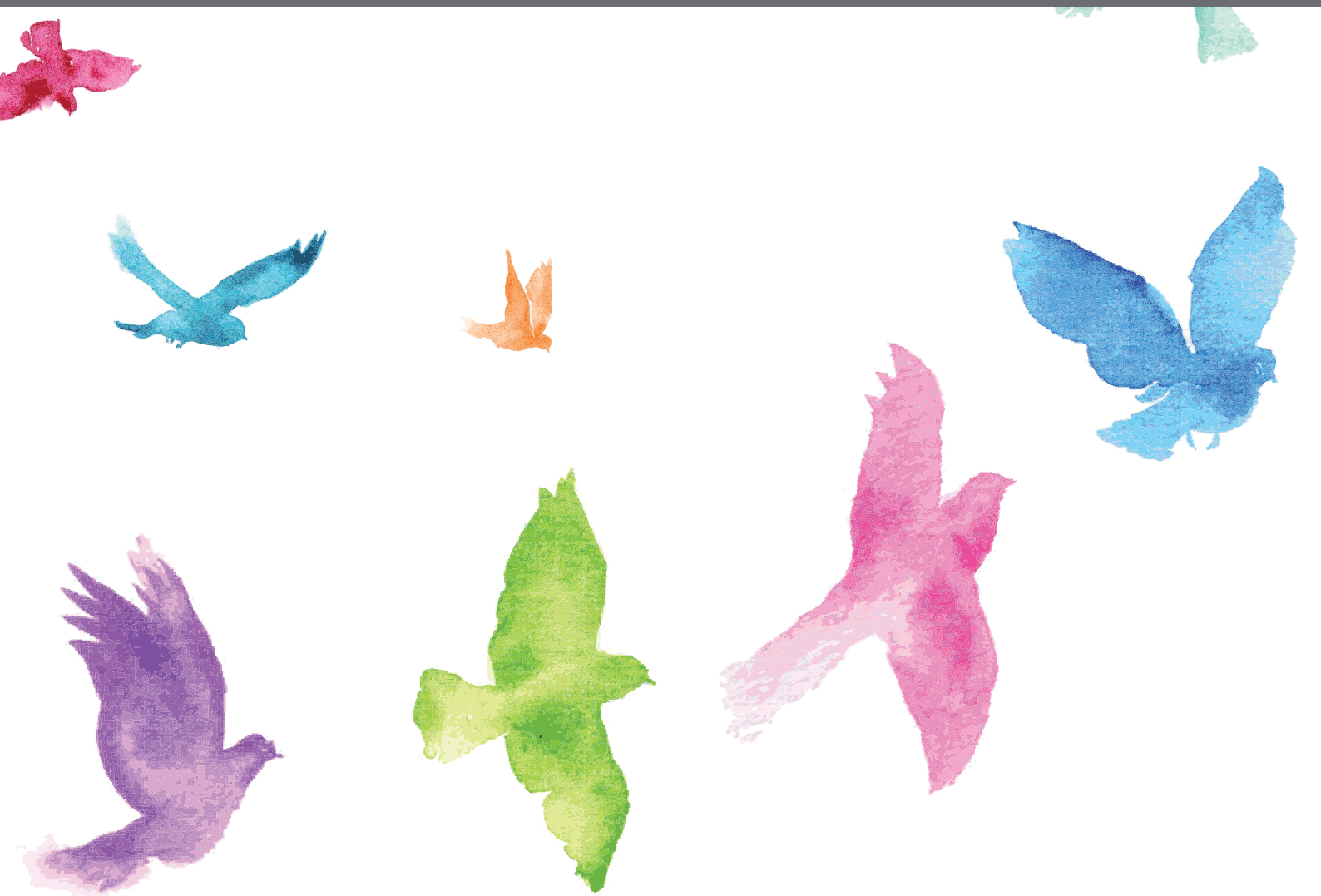

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BIODIVERSITY OF SENSORY SYSTEMS IN AQUATIC VERTEBRATES

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EDITORIAL
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Editorial: Biodiversity of Sensory Systems in Aquatic Vertebrates

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Keywords: biodiversity, sensory systems, aquatic vertebrates, photobiology, chemoreception, audition, somatosensation, climate change

Editorial on the Research Topic

Biodiversity of Sensory Systems in Aquatic Vertebrates

INTRODUCTION

Many sensory systems are more commonly known than others, but all are critical for survival. These include those senses typically described by Aristotle around 300–400 Before the Common Era (BCE), such as sight (vision), hearing (audition), touch (somatosensation), smell (olfaction), and taste (gustation). However, many years of scientific endeavor have shown that these five senses represent only a part of the sensory abilities that are now known throughout the aquatic animal kingdom. The extended repertoire of senses includes the ability for vestibular control (equilibrioception), the sensation of temperature (thermoreception), postural awareness (proprioception), the monitoring of pain (nociception), the use of sonar (echolocation), and the detection of weak electric (electroreception) and magnetic (magnetoreception) fields.

The papers presented in this Research Topic were greatly welcomed and consist of a collection of exciting and well-received articles that incorporated new knowledge on almost all of the known senses in a range of aquatic vertebrates, such as the sarcopterygian lungfishes, both freshwater and marine teleosts, elasmobranchs, marine reptiles, and cetaceans (marine mammals). The papers target many of the known senses in aquatic vertebrates, but are biased toward vision, which reflects the number of active research programs that concentrate on this sensory modality.

RESEARCH TOPIC CONTRIBUTIONS AND THEIR WIDER BIOLOGICAL CONTEXTS

Kremers et al. extend the breadth of knowledge on the vocal and echolocating abilities in dolphins by examining other aspects of audition, such as context relatedness, and the social function of vocalizations and socio-sexual recognition. However, they also present an excellent review of anatomical, physiological, and behavioral data on vision, electroreception, magnetoreception, somatosensation and chemoreception (olfaction and taste), and emphasize the degree by which dolphins, and other cetaceans, utilize a diverse array of senses (Kremers et al.). These same authors (Kremers et al.) also present novel experimental approaches to determine the little-known chemoreceptive abilities of dolphins by examining their spontaneous behavioral responses to chemical stimuli (Kremers et al.). Collectively, these studies reveal that dolphins may discriminate both odors and flavors, as well as many other critical stimuli. However, it should be emphasized that further research is required, especially with respect to the perception of chemical and auditory

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cues via water and air, as well as the influence of natural environmental changes and/or human-induced climate-related effects as discussed below (Kelley et al.).

Fasick and Robinson reveal that the eyes of cetaceans (marine mammals) are a great example of an evolving visual system that has become adapted to the visual demands of foraging at different depths within the water column. While most terrestrial mammals have dichromatic color vision that is based on the presence of two classes of cone photoreceptors [containing short-wavelength-sensitive (SWS) and long-wavelength-sensitive (LWS) photopigments] (Davies et al., 2012), all cetaceans studied thus far lack cone-based color vision: having lost the SWS photopigment and would be considered LWS or L-cone monochromats (Newman and Robinson, 2005). Among the dolphins, porpoises, and beaked whales, the absorbance spectra of rod visual photopigments are shown to be spectrally-tuned to the available radiance spectra at foraging depths, with an inverse relationship between the wavelength of maximum sensitivity of the rod photopigment and depth (Fasick and Robinson, 2000). The average common diving depth of most marine mammals is around 300 m (Ponganis, 2011), reaching an average maximum depth of 1,300 m (Ponganis, 2011). However, some species of whale (e.g., the sperm whale, *Physeter macrocephalus*) commonly dive to 1,200 m, but may reach as far as 3,000 m from the surface (Watkins et al., 1993). Others, such as the two elephant seals *Mirounga angustirostris* and *M. leonina* can dive to depths of over 1,500 m (Leboeuf et al., 1988; Delong and Stewart, 1991; Hindell et al., 1991). Due to the filtering of particular wavelengths of light (as well as a reduction in light intensity) with increasing depth (Loew and McFarland, 1990), namely shorter and longer wavelengths, the predominant wavelengths of down-welling sunlight that penetrate to deeper regions of the water column are around 480 nm. As such, rod photopigments in cetaceans (as well as most deep-sea fishes) have adapted to this attenuated light environment and have become spectrally-tuned to shorter wavelengths (Fasick and Robinson, 2000). This also holds true for the spectral tuning of the conserved LWS cone visual photopigments (Newman and Robinson, 2005). It appears that the melanopsins (encoded by one or more *OPN4* genes) in monochromatic cetacean species are, incidentally, also spectrally-tuned to 480 nm, and are ideally placed to detect the remaining down-welling sunlight (and the bioluminescence that is emitted by over 80% of organisms) that occur at these greater depths. The spectral tuning of melanopsin to 470–480 nm is also common for terrestrial vertebrates (Davies et al., 2010, 2014), where its main functional role is to detect blue-enriched light at dusk and dawn for the photoentrainment of circadian rhythms, such as the sleep-wake cycle (LeGates et al., 2014). This may also be true for monochromatic cetacean species, where melanopsin may be detecting either (or both) down-welling sunlight and/or bioluminescence for photoentrainment (or the maintenance) of daily biological oscillations of the circadian clock. In addition, some cetacean species may possess a mechanism that inhibits relatively rapid deactivation of light-activated melanopsin photopigments. This process would result in prolonged pupil constriction that results in a useful cellular process for the prevention of rod photopigment photobleaching under photopic conditions (Fasick and Robinson); however, further studies are required to determine if this potentially advantageous adaptation is commonplace in all cetacean species, and perhaps even in a broader range of aquatic organisms that also express melanopsin.

The adaptive capacity for vision underwater in biodiverse extant representatives of the lobe-finned fishes (the ancient sarcopterygian lungfishes, *Protopterus dolloi,* and *Lepidosiren paradoxa*) is revealed in the study by Appudurai et al.. Specifically, they showed that the complement of retinal photoreceptor types (one rod and a single cone-type in adult *L. paradox*, compared to one rod and two cone photoreceptor types in juvenile *P. dolloi*) indicates that there are major differences in the capacity to discriminate color in these two "living fossils" and that the visual needs of both species may differ (Appudurai et al.). This is in contrast to the Australian lungfish, *Neoceratodus forsteri,* that possesses three different cone classes in addition to a large rod, thereby optimizing both color sensitivity and wavelength discrimination (photopic vision), and sensitivity to low intensities of light (scotopic vision) (Bailes et al., 2006, 2007).

The relationship between photoreceptor sensitivity, the underwater spectral environment, and the perception of specific visual stimuli is taken one step further by Pauers et al.. In this paper, the authors investigate the co-evolution of spectral sensitivity and body color patterns in Lake Malawi cichlids as a mechanism for enhancing visual communication. They reveal that distinct spectrally-tuned SWS photopigments serve different functions in fishes, and that the communication of "public" signals [i.e., those widely visible to conspecifics and allospecifics alike, such as the advertisement of services by "cleaner" fishes to "client" species for the removal of ectoparasites Grutter, 1999] is found in species with eyes possessing ultraviolet (UV) sensitivity and that the communication of "private" signals [i.e., those restricted to certain species, such as conspecific nuptial coloration for sexual selection (Endler, 1992), but not predators] is found in species with eyes that lack UV-sensitivity. Species with (vertical) barred patterns have SWS peak sensitivity values at wavelengths that are shorter than either of the other patterns (solid and horizontal stripes). Their results indicate that visual sensitivity and color patterns co-evolved in a correlated fashion, and that the ancestral cichlid was likely to be a UV-sensitive (UVS) fish with a barred color pattern that first changed its design from barred to striped, followed by a loss of UV-sensitivity (Pauers et al.). Their work reveals that both the arrangement and contrast of color pattern elements may be just as important as color in mate recognition. However, future work is required to elucidate the cellular mechanisms involved in body coloration more broadly in aquatic vertebrates, where sensing external photo-stimuli (e.g., from local lighting environments or coloration/patterns of predators, prey, or potential mates) may be determined either directly via the skin (for example, see Kelley and Davies, 2016) or indirectly via the eye and other photoreceptive organs such as the pineal gland and/or deep brain structures (e.g., the hypothalamus). These ongoing and future studies will be critical in linking the detection of diverse, rapidly changing photic conditions, and physiological/behavioral activities such as camouflage, mate recognition, and the establishment of complex predator/prey relationships.

In the paper by Anthes et al., it was shown how numerous species of marine fishes display intricate patterns of fluorescence by transforming ambient blue-green light into red light. Based on a series of *a priori* hypotheses regarding adaptive functions, they compare the prevalence of red fluorescence among groups of species based on ecological or biological characteristics, while controlling for shared ancestry. Putative functions of fluorescence include background matching for camouflage in "sit-and-wait" predators, prey localization in species with bright irides, and sexual communication in species showing sexual dimorphic patterns of fluorescence (Anthes et al.). As more ecological data regarding the phylogeny and behavioral ecology of fishes become available, the function of fluorescence and the environmental conditions under which it operates will be better understood.

Ladich and Schulz-Mirbach explore one of the main riddles of fish bioacoustic systems: what selective forces and/or constraints led to the evolution of diversity in the inner ear, including accessory hearing structures, and how is morphological variability linked to hearing abilities? They consider that eco-acoustical constraints are more likely to explain the level of diversity in fish hearing sensitivities rather than to facilitate intraspecific acoustic communication. They also propose that low ambient noise levels may have facilitated the evolution of accessory hearing structures, thereby enabling fish to detect low-level abiotic noise and sounds from con- and hetero-specifics, including both predators and prey (Ladich and Schulz-Mirbach). As more acoustic environments are characterized and assessed with respect to the demands placed on hearing abilities in different species, these relationships will be able to be tested more widely. This should be aided by the ongoing technological advances in bioimaging of the inner ear using magnetic resonance imaging (MRI) and micro-computed tomography (μCT).

Predictions of the roles of vision and olfaction during development in deep-sea grenadier fishes, which occupy some of the deepest regions of the ocean (e.g., 2,000–6,000 m) is the subject of the paper by Lisney et al.. They reveal that at least two species of grenadiers undergo ontogenetic shifts in the relative size of the optic tectum and the olfactory bulbs. Concomitant changes in axonal input (as determined by ultrastructural assessment of nerve axon numbers) are also shown to be associated with the hypertrophy of these sensory brain lobes, suggesting a shift from a reliance on vision to olfaction during ontogeny, in association with a move to a more scavenging lifestyle and a change in diet. This study shows that sensory demands on teleosts in the deep-sea are high and change during development to optimize survival (Lisney et al.). This emphasizes that not one sensory modality operates in isolation, but that the interplay of multiple senses may be important to all aquatic vertebrates that undergo continual growth of both their peripheral and central nervous systems (such as cartilaginous and bony fishes), especially those that live in extreme environments or naturally move from one environment to another during development. It is predicted that many further investigations will demonstrate the vital importance of integrating different modes of sensory information for species behavior and ultimate survival, especially in the rapidly emerging fields that link systems biology with functional genomics.

Finally, Kelley et al. examine how anthropogenic threats to the aquatic environment impact the senses or determine the reactive responses of particular species to environmental change. The authors review in detail how different sensory modalities can act to influence genetic and non-genetic (developmental) responses to environmental change, which, in turn, may cause knock-on effects in a range of other biological systems. They propose that sensory systems lie at the forefront of how various species respond to environmental perturbation and that urgent efforts should be made to recognize the important role they play in determining fitness, which is critical for understanding the effects of external processes such as habitat degradation and climate change (Kelley et al.). As many aquatic environments continue to be degraded by human activities, it is critical that the effects of acidification, elevated levels of carbon dioxide, increases in temperature, chemical and noise pollution, and changes in the transmission and detection of sensory signals are monitored (Kunc et al., 2016; Sharma and Chatterjee, 2017; Amoatey and Baawain, 2019; Chapuis et al., 2019; Kelley et al.).

CONCLUDING REMARKS

The evolution of complex, integrated sensory systems has allowed organisms to sense and respond to continuously changing local and global environmental stimuli. Together, the interplay of these vital biological systems promotes survival via optimized sexual selection strategies, the establishment of hierarchical predator/prey relationships, and the detection and evasion of toxic conditions. This Research Topic consists of a plethora of in-depth original and review articles that provides an overview of different sensory modalities that function in many aquatic vertebrates. Due to the large network of researchers in the field of photobiology, it is not surprising, perhaps, that many papers presented herein focus on vision (or light detection in general), which is regarded as one of the most important and specialized sensory systems to evolve. Nonetheless, other significant senses are discussed in detail, such as chemoreception and audition. In particular, this special assembly of publications highlights the significant increase in recent years of human-induced noise and light pollution, as well as contaminating chemical outflows (e.g., microplastics) into various local and global aquatic systems. Thus, the degree of impairment and species survival will ultimately be dependent upon each physiological level of tolerance and the propensity for compensatory plasticity (or sensory switching) under natural and anthropogenic environmental changes. Such studies are moving toward the forefront of active research in many interdisciplinary scientific fields and will be vital in determining conservation efforts and influencing environmental policy in the foreseeable future. Finally, as hosts of this stimulating Research Topic, sincere gratitude is

offered to all authors for their important contributions to this special issue.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Correlated Evolution of Short Wavelength Sensitive Photoreceptor Sensitivity and Color Pattern in Lake Malawi Cichlids

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For evolutionary ecologists, the "holy grail" of visual ecology is to establish an unambiguous link between photoreceptor sensitivity, the spectral environment, and the perception of specific visual stimuli (e.g., mates, food, predators, etc.). Due to the bright nuptial colors of the males, and the role female mate choice plays in their evolution, the haplochromine cichlid fishes of the African great lakes are favorite research subjects for such investigations. Despite this attention, current evidence is equivocal; while distinct correlations among photoreceptor sensitivity, photic environment, and male coloration exist in Lake Victorian haplochromines, attempts to find such correlations in Lake Malawian cichlids have failed. Lake Malawi haplochromines have a wide variability in their short-wavelength-sensitive photoreceptors, especially compared to their mid- and long-wavelength-sensitive photoreceptors; these cichlids also vary in the degree to which they express one of three basic color patterns (vertical bars, horizontal stripes, and solid patches of colors), each of which is likely used in a different form of communication. Thus, we hypothesize that, in these fishes, spectral sensitivity and color pattern have evolved in a correlated fashion to maximize visual communication; specifically, ultraviolet sensitivity should be found in vertically-barred species to promote "private" communication, while striped species should be less likely to have ultraviolet sensitivity, since their color pattern carries "public" information. Using phylogenetic independent contrasts, we found that barred species had strong sensitivity to ultraviolet wavelengths, but that striped species typically lacked sensitivity to ultraviolet light. Further, the only variable, even when environmental variables were simultaneously considered, that could predict ultraviolet sensitivity was color pattern. We also found that, using models of correlated evolution, color pattern and ultraviolet sensitivity are correlated in Lake Malawi cichlid evolution, with the likely ancestor being a vertically-barred, ultraviolet-sensitive species, the descendants of which lost both ultraviolet sensitivity and a barred color pattern. These results, indicating that communication of "public" and "private" signals is mediated via differing perceptions of color patterns, suggest a functional connection between visual sensitivity and color pattern, a novel finding in Lake Malawi cichlids.

Keywords: Lake Malawi, cichlids, visual ecology, ultraviolet photoreception, correlated evolution, phylogenetic independent contrasts

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INTRODUCTION

For those who study the ecology of vision, the "holy grail" of such studies is to discover an unambiguous link among photoreceptor sensitivity, the wavelengths of light available within the study organism's habitat, and the perception of ecologically relevant visual stimuli, such as food, predators, or mates (Ryan and Rand, 1990; Endler, 1992; van Staaden and Smith, 2011). This is, of course, a convenient "shorthand" view of how vision works in animals, one that overlooks the complex relationships and interactions among photons, opsins, neurons, and the resulting image constructed by the organism's brain (Endler, 1990, 1991; Fernald, 2006). Nonetheless, organisms do face fitness consequences if they fail to detect photons in the proper context (Endler, 1978, 1992; Milner and Goodale, 1995; Land and Nilsson, 2002), so this kind of proximate approach to studying the perception of light is valuable as a first step toward understanding the role color vision plays in ecological circumstances (Endler, 1978, 1990; Endler and Mielke, 2005).

The cichlid fishes of the African great lakes have received much attention from visual ecologists; the bright colors of the males, as well as the presence and importance of visually-based female mate choice, strongly suggest a history of correlated evolution between nuptial coloration and visual sensitivity. While such a connection has been shown in Lake Victorian cichlids (Seehausen et al., 2008), this has not been as clearly demonstrated in the Lake Malawi cichlids. In the most comprehensive such study on Malawian cichlids, Dalton et al. (2010) fail to find any correlation among sensitivity, photic environment, and coloration; indeed, they found that depth does not influence the perceptibility of cichlid hues, suggesting that depth may not have an influence on photoreceptor sensitivity.

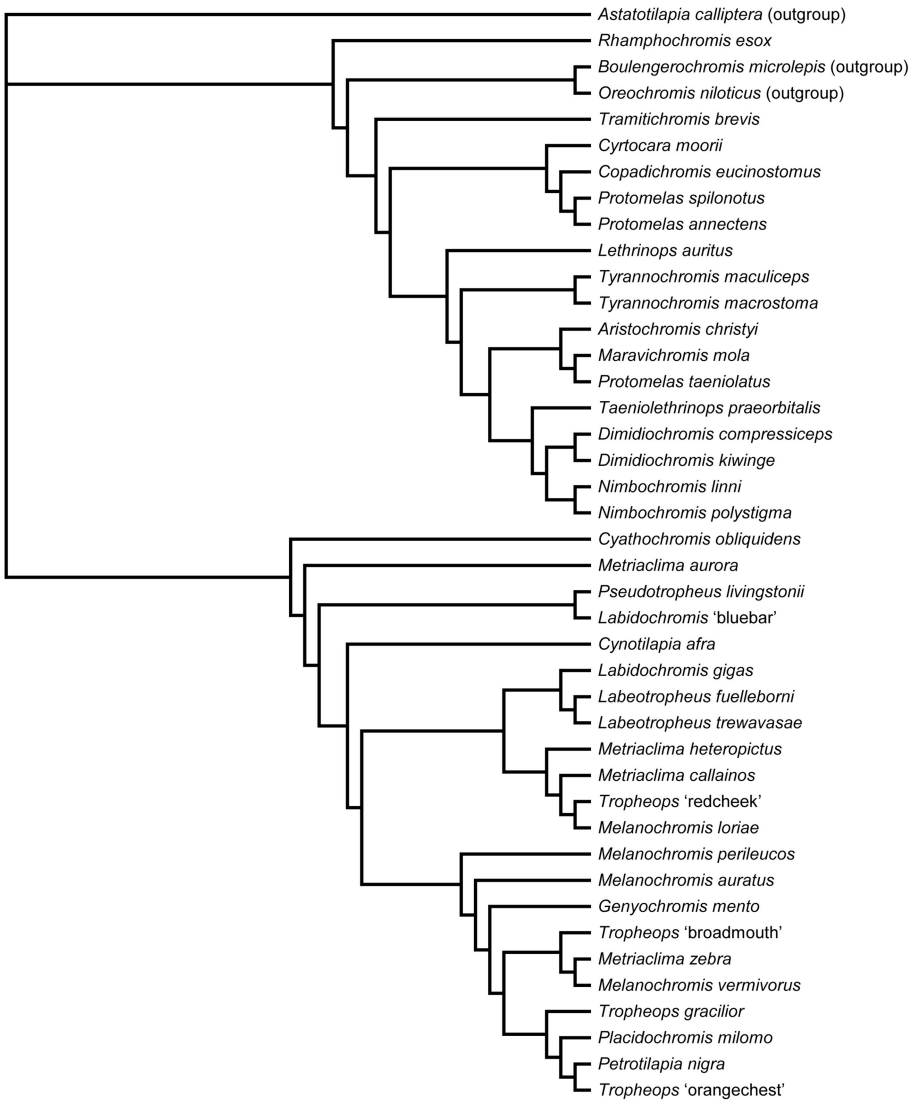

FIGURE 1 | Maximum Likelihood phylogeny of Lake Malawi cichlids based on the ND2 mitochondrial gene.

Within the past 15 years, the existence of ultraviolet (UV) vision in fishes has also attracted the attention of visual ecologists (Losey et al., 1999). While initially thought rare, due in part to the rapid attenuation of UV wavelengths in water, many fishes have UV-sensitive photopigments or UV-reflective color patterns (Losey et al., 2003; Marshall et al., 2003; Jordan et al., 2004a; Siebeck et al., 2010). In fishes, UV vision is known to aid foraging (Browman et al., 1994), species recognition (Cheney and Marshall, 2009; Siebeck et al., 2010), and mate choice Kodric-Brown and Johnson, 2002). The role of UV vision in fish communication is particularly interesting because it is often used as a "private" means of communication; i.e., a range of wavelengths visible to conspecifics, but not to other species, especially predators (Endler, 1992; Cummings et al., 2003).

In Lake Malawi cichlids, there is wide variability in the peak sensitivities of short wavelength sensitive (SWS) photopigments. Some species have UV-sensitive SWS photopigments, while others are violet- or blue-sensitive (Parry et al., 2005). Some of the rock-dwelling species ("mbuna") use their UV sensitivity to aid foraging (Jordan et al., 2004b), and many species have UV-reflective color patterns (Jordan et al., 2004a; Pauers et al., 2004; Parry et al., 2005), though the use of UV vision or UV-reflective color patterns in communication has not been explicitly demonstrated. This variability in SWS sensitivity is in stark contrast to the much smaller variation among the peak sensitivities of the longer wavelength-sensitive photopigments within these same species; the peak sensitivity of the SWS photopigment ranges from 360 to 433 nm, while the peak sensitivity of the longer-wavelength sensitive photopigments ranges from 499 to 548 nm (Parry et al., 2005; Dalton et al., 2010). This relatively broad range in SWS sensitivity strongly suggests that there must be a function associated with the difference in UV- vs. violet- or blue-sensitive SWS photopigments.

Lake Malawi cichlids also display marked differences in their gross color patterns. Many species display vertical bars as a major component of their color patterns, and horizontal stripes are also common (Seehausen et al., 1999). Horizontal stripes are well-understood to be used as camouflage, especially by piscivorous cichlids, but the function of bars, on the other hand, seems related to promoting crypsis in a highly structured habitat (Seehausen et al., 1999). A third color pattern common to Lake Malawi cichlids consists of solid patches of contrasting colors. Interestingly, these "solid" patterns are likely to evolve under conditions similar to those that promote the evolution of vertical bars (Kenward et al., 2004), but are also likely to have evolved in these fishes for the purpose of mate attraction (Seehausen et al., 1999).

No matter the type of gross color pattern present, species that use these patterns to be conspicuous to conspecifics would have a selective advantage if they were simultaneously cryptic to their predators (Endler, 1992; Cummings et al., 2003). Further, predatory fishes would also have a selective advantage if their camouflage markings were visible to their prey, no matter the visual sensitivity of the observer. Thus, we suggest that in Lake Malawi cichlids, SWS sensitivity and color pattern have coevolved to create "private" and "public" bands of communication. Specifically, we hypothesize that cichlids with UV sensitivity are more likely to have color patterns featuring vertical bars, since both are likely components of "private," cryptic signals. Conversely, predatory fish, which rely on clear, obvious camouflage, are more likely to have color patterns with horizontal stripes, and less likely to need "privacy" for this signal, and are thus likely to lack UV-sensitive SWS photoreceptors.

TABLE 1 | Species used in phylogenetic analyses and GenBank accession numbers for ND2 sequences.

Species	Accession number
Aristochromis christyi	EF585282
Copadichromis eucinostomus	EF585268
Cyathochromis obliquidens	GQ422579
Cynotilapia afra	EF585264
Cyrtocara moorii	AY930089
Dimidiochromis compressiceps	EF585267
Dimidiochromis kiwingi	GU946222
Genyochromis mento	AF305297
Labeotropheus fuelleborni	EF585259
Labeotropheus trewavasae	GU946225
Labidochromis 'bluebar'	GQ422573
Labidochromis gigas	EF585276
Lethrinops auritus	U07252
Maravichromis mola	EF585274
Melanochromis auratus	AY930069
Melanochromis perileucos	GQ422574
Melanochromis loriae	JX119227
Melanochromis vermivorous	EF585270
Metriaclima aurora	EF585266
Metriaclima callainos	EF585271
Metriaclima heteropictus	GQ422584
Metriaclima zebra	DQ093114
Nimbochromis linni	EF585279
Nimbochromis polystigma	EF585262
Petrotilapia nigra	EU661721
Placidochromis milomo	GQ422590
Protomelas annectens	EU661718
Protomelas spilonotus	EF585253
Protomelas taeniolatus	GU946232
Pseudotropheus livingstonii	EF585273
Rhamphochromis esox	GU946233
Taeniolethrinops praeorbitalis	GU946236
Tramitochromis brevis	AF305320
Tropheops "broadmouth"	GQ422589
Tropheops gracilior	EF585260
Tropheops "orangechest"	GQ422583
Tropheops "redcheek"	GQ422568
Tyrannochromis macrostoma	EF585257
Tyrannochromis maculiceps	GQ422571
Outgroups	
Astatotilapia calliptera	GU946219
Boulengerochromis microlepis	AF317229
Oreochromis niloticus	AF317237

MATERIALS AND METHODS

Malawi Cichlid Color Pattern, Photoreceptor Sensitivity, and Ecological Data

Data were compiled regarding photopigment sensitivities (Hofmann et al., 2009); male nuptial color patterns (Ribbink et al., 1983; Konings, 2007); diet (Hofmann et al., 2009; Konings and Stauffer, 2012), depth (Ribbink et al., 1983); and the wavelength of the radiance spectra about which quanta are likely to be most abundant (λP50) at two locations in Lake Malawi, Otter Point and Thumbi West (Sabbah et al., 2011). Regarding photopigment sensitivities, Hofmann et al., 2009) report two values: the peak sensitivity of the SWS opsin found in the single cones, but only the stronger of the two sensitivities recorded for the two opsins found in the double cones; we use both values as reported, with the understanding that we are missing information about the way the double-cone system discriminates among wavelengths (Neitz and Neitz, 2011). Using Seehausen et al. (1999) and Konings (2007) as guides, color patterns were classified as barred, striped, or solid. Colors comprising ≥ 50% of the body were considered the main hue of the fish, and all long-wavelength colors (e.g., yellows, oranges, and reds) were classified as "carotenoid" colors. Maximum depths of most species (n =

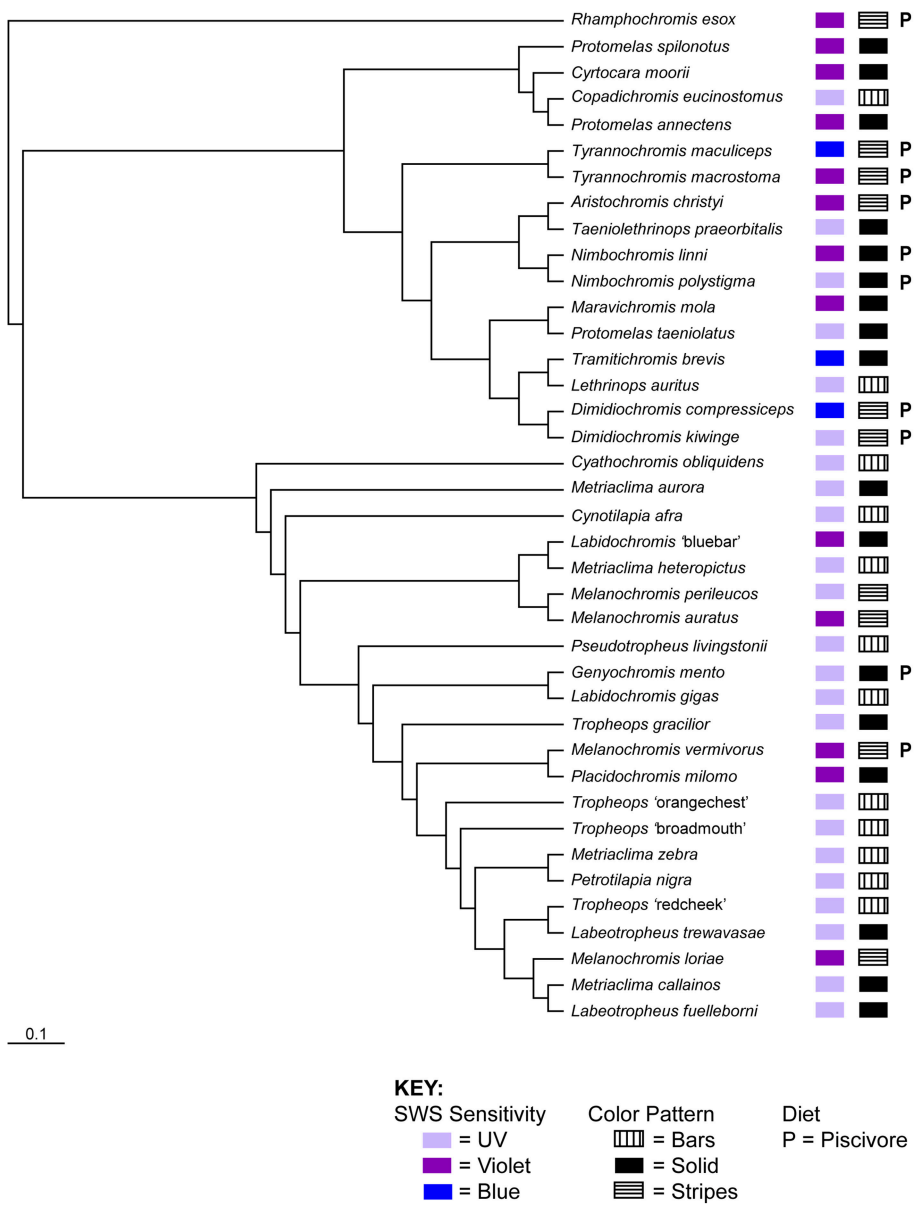

FIGURE 2 | One example of the 1000 trees generated from the phylogeny in Figure 1, with color pattern (vertical bars, solid patches, or horizontal stripes), SWS sensitivity (UV vs. non-UV), and dietary (piscivorous vs. non-piscivorous) characteristics mapped onto the phylogeny for each species.

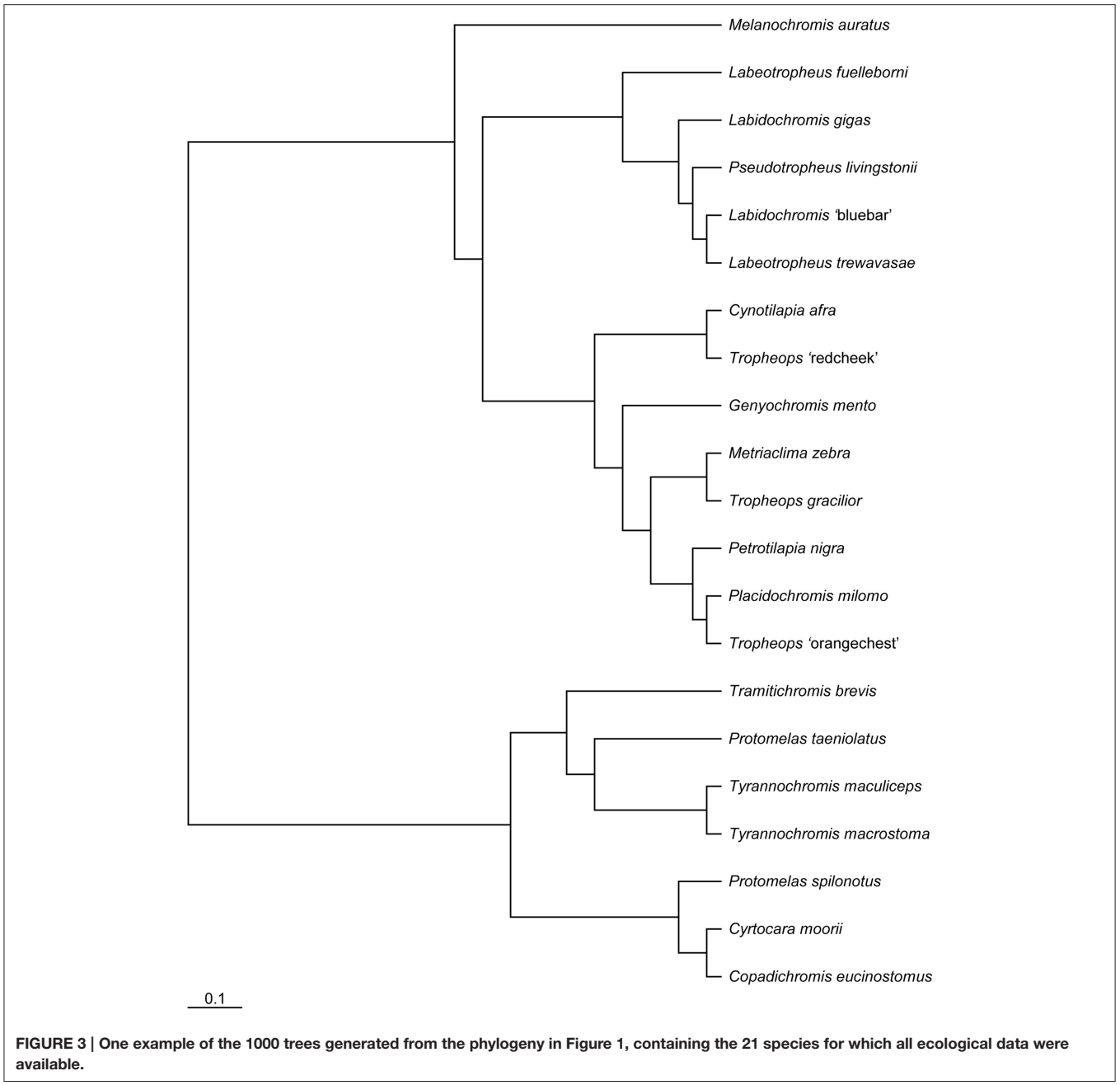

FIGURE 3 | One example of the 1000 trees generated from the phylogeny in Figure 1, containing the 21 species for which all ecological data were available.

27) were obtained from Ribbink et al. (1983). The λP50 of the sidewelling radiance spectra was used to represent spectral habitats, as radiance spectra include radiant, reflective, and transmissive sources (Endler, 1993). The λP50 at the maximum depth of each species were estimated from Sabbah et al. (2011); because these authors recorded spectral data at discrete depths (at 1, 3, 5, 6, 9, 12, and 15 m of depth) that did not always match the distributions reported by Ribbink et al. (1983), the λP50 of the next deepest depth was used (e.g., a species with a maximum depth of 10 m was assigned the λP50 at 12 m). Further, species with maximum depths $\geq$ 15 m ($n = 13$) were assigned the λP50 of 15 m.

Phylogenetic Independent Contrasts

We used RAxML (Stamatikis, 2014) to generate a ML tree (**Figure 1**) using sequences of the mitochondrial ND2 gene downloaded from GenBank (**Table 1**). First, these sequences were aligned using T-Coffee (Notredame et al., 2000), and we then removed poorly aligned regions using stringent conditions in Gblocks (Castresana, 2000). Using the GTRGAMMA model of molecular evolution, we generated support values using 1000 pseudoreplicates. These 1000 trees were then imported into the ape package (Paradis et al., 2015) in *R*, and we began by pruning all outgroups (**Figure 2**). We then calculated a phylogenetic independent contrast between the peak sensitivities of the SWS

TABLE 2 | Phylogenetic Independent Contrast between opsin peak sensitivity and color pattern.

(A) SWS PEAK SENSITIVITY					
Residuals	Minimum	1st Quartile	Median	3rd Quartile	Maximum
	−155.430	−52.920	−24.410	54.500	262.370
Coefficients	Estimate	Std. Error	*t*-value	*Pr*(>|t|)	
Color pattern	31.150	6.421	4.851	0.00002	
Residual standard error: 87.91 on 36 degrees of freedom					
Multiple $R^2 = 0.395$, Adj. $R^2 = 0.379$					
$F_{(1, 36)} = 23.530$, $p = 0.00002$					
Pairwise Comparisons; *p*-values Holm corrected					
	Bars	Solid			
Solid	0.02907	–			
Stripes	0.00032	0.02907			
(B) DOUBLE CONE PEAK SENSITIVITY					
Residuals	Minimum	1st Quartile	Median	3rd Quartile	Maximum
	−139.603	−15.460	5.923	17.916	125.287
Coefficients	Estimate	Std. Error	*t*-value	*Pr*(>|t|)	
Color pattern	−1.182	3.264	−0.362	0.926	
Residual standard error: 44.68 on 36 degrees of freedom					
Multiple $R^2 = 0.0003$, Adj. $R^2 = -0.024$					
$F_{(1, 36)} = 0.131$, $p = 0.719$					
Pairwise Comparisons; *p*-values Holm corrected					
	Bars	Solid			
Solid	1.00	–			
Stripes	1.00	1.00			

photoreceptors and color pattern of all species in the dataset ($n = 39$). We then pruned the phylogeny again, leaving only those species for which we had a complete set of photoreceptor sensitivity and ecological data (**Figure 3**; $n = 21$). Using this pruned phylogeny, two phylogenetic independent ANOVAs were calculated. In the first, SWS peak sensitivity was the dependent variable, and color pattern, diet, maximum depth, irradiance, and body color were independent variables; in the second, the double cone peak sensitivity was used as the dependent variable, with the same set of independent variables.

Correlated Trait Evolution

Following the methodology of Kelley et al. (2013), we attempted to determine whether or not visual sensitivity and color pattern have evolved in a correlated fashion in Lake Malawi cichlids. To begin, we used the geiger package (Harmon et al., 2014) in *R* to calculate λ, an estimate of phylogenetic signal (Pagel, 1999), for both visual sensitivity and color pattern. We modified the phylogeny described above (e.g., outgroups removed and all 39 species of Malawian cichlids included) to create two new trees, one in which branch lengths were set to $\lambda = 0$, indicating no phylogenetic signal; and another in which the branch lengths were set to $\lambda = 1$, indicative of a random, Brownian motion of traits. The fit of these models was compared to that of the original phylogeny using likelihood ratio (LR) tests.

We then generated another ML tree from the original ND2 sequence data, and generated support values using 1000 pseudoreplicates using the GTRGAMMA model of molecular evolution. We then used these 1000 trees in our analyses of correlated trait evolution. To simplify these analyses, we recoded our traits as discrete, binary traits. For visual sensitivity, we classified fish as either UV sensitive (e.g., SWS peak sensitivity $<$ 400 nm) or non-UV (SWS peak sensitivity $\geq$ 400 nm). For color pattern, we had to simplify our three classes (bars, solid, and stripes) into two, bars and stripes. To do this, we examined photographs of juvenile, female, and immature/subordinate males (using Konings, 2007), in order to better distinguish the underlying melanin patterns of solid-colored fishes; even in species in which dominant, territorial males prominently display solid patches of color, other life history stages display a fundamental melanin-based pattern of vertical bars or horizontal stripes (c.f., Baerends and Baerends-van Roon, 1950; Voss, 1980; Seehausen et al., 1999). Thus, we were able to reclassify solid-colored fishes as either having stripes (e.g., *Nimbochromis*) or bars (e.g., *Labeotropheus*).

The 1000 trees and the data matrix of binary visual sensitivity and binary color patterns were imported into BayesTraits (Meade and Pagel, 2014). We used the maximum likelihood (ML) model of evolution to compare both independent (i.e., a model in which discrete character states are assumed to evolve independently) and dependent (i.e., a model in which the evolution of one character depends upon the evolution of the other) models of character evolution.

We originally assumed that, based on O'Quin et al. (2010), a non-UV-sensitive, striped cichlid was the ancestor of the extant Lake Malawi flock. As such, we coded both a lack of UV

sensitivity and a striped color pattern as 0s in our data matrix, while UV sensitivity and a barred color pattern were coded as derived traits and were assigned values of 1. We then used the ML models in BayesTraits to evaluate likely ancestral states by running the dependent and independent models in three ways: "unfossilized" (that is, with no *a priori* information given to the program regarding ancestral state), "fossilized" at state 0,0 (i.e., fixing stripes and non-UV visual sensitivity as the likely ancestral states), and "fossilized" at state 1,1 (i.e., bars and UV-sensitivity as the likely ancestral state). We then compared these models using LR tests. Finally, we investigated the direction of significant evolutionary transitions by sequentially restricting all eight possible changes in character state of the preferred (as indicated by the LR test) model to zero and comparing these to the original, "unfossilized" model.

Photography and Spectrophotometry

The methods used in this study have been described in detail in Pauers et al. (2004) and are only summarized here. The fish were anesthetized with a weak dose of MS-222 and then placed in an ice bath. Upon removal from the ice bath, the fish was placed on a black cloth and illuminated from its dorsal surface with a Newport 100 W ozone-free xenon lamp, a 385 nm LED flashlight, and a 15 W blacklight. Using a quartz lens attached to an Oriel Instaspec IV CCD, measurements of reflected wavelengths were taken at two points on the fish; these two points were chosen after examining the shapes of reflectance spectra from several other points to represent regions of high contrast within each species' color pattern. After these measurements, the fish was also photographed under both full spectrum and UV only illumination. The spectral data were converted to actual reflectances by dividing them by measurements taken from a Spectralon white standard. The reflectances were then used to calculate quantal catches for each type of photoreceptor in typical ultraviolet- (e.g., peak sensitivities = 368, 488, 533 nm) and violet- (e.g., peak sensitivities = 410, 482, 529 nm) sensitive visual systems. In order to display the differences in sensitivity between these visual systems, the quantal catches of each photoreceptor found in both the model ultraviolet- and violet-sensitive visual systems were plotted against reflected wavelengths.

These methods strictly followed the Guidelines for the Use of Animals in Research published by the Association for the Study of Animal Behaviour and the Animal Behavior Society, and were approved by the University of Wisconsin Colleges Animal Care Committee (protocol # 1020143) and were additionally approved by the senior staff of the Milwaukee Public Museum (protocol on file with Dr. Ellen J. Censky and available upon request).

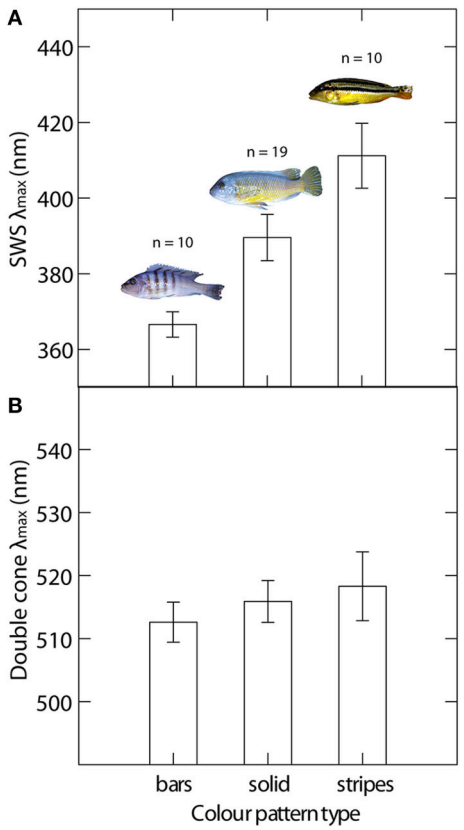

FIGURE 4 | The relationship between color pattern and photoreceptor sensitivity of Lake Malawi cichlids. (A) SWS photoreceptor sensitivity; **(B)** double cone maximal sensitivity. Inset pictures show examples of each color pattern type (bars, *Metriaclima zebra*; solid, *Labeotropheus* c.f. *fuelleborni* "Katale"; stripes, *Melanochromis auratus*). Error bars = standard error.

RESULTS

Phylogenetic Independent Contrasts

Species with a barred color pattern have the shortest SWS photoreceptor peak sensitivity, while those with horizontal stripes have the longest, and solid-colored species have intermediate sensitivities [$F_{(1,\ 36)} = 25.53$, $p < 0.0001$; **Table 2A**, **Figure 4A**]. There is no such relationship between color pattern and the peak sensitivity of the double cone in cichlid retinae. While striped fish have the longest double cone peak sensitivity, and barred species the shortest, this difference is non-significant [$F_{(1,\ 36)} = 0.1312$, $p = 0.719$; **Table 2B**, **Figure 4B**].

The ANOVA results indicate that of the five independent variables, only color pattern predicts the peak sensitivity of the SWS photoreceptor expressed in the single cones of cichlid retinae (**Table 3A**). The effects of body color, maximum depth, radiance at maximum depth, and habitat type are all non-significant. In the case of the double cone photopigments, none of the variables, including pattern, predict peak sensitivity (**Table 3B**).

Correlated Trait Evolution

The value of λ calculated for color pattern was significantly different from zero, but not from one, suggesting that evolutionary changes in color pattern occur gradually. Visual sensitivity, on the other hand, was found not to be significantly different from either zero or one, suggesting that the evolution of visual systems in these fishes is neither purely phylogenetic nor entirely random (**Table 4**).

TABLE 3 | Phylogenetic Independent Contrasts performed on both (A) SWS opsin peak sensitivity and (B) double cone opsin peak sensitivity vs. environmental and color pattern characteristics.

(A) MODEL 1. SWS PEAK SENSITIVITY					
Residuals	Minimum	1st Quartile	Median	3rd Quartile	Maximum
	−161.314	−23.625	3.578	34.990	154.906
Coefficients	Estimate	Std. Error	*t*-value	*p*(>|t|)	
Color pattern	25.314	10.211	2.479	0.0255	
Diet	−18.095	34.768	−0.520	0.6103	
Maximum depth	−0.232	0.351	−0.661	0.5184	
Irradiance at maximum depth	−2.095	1.018	−2.059	0.0573	
Body color	4.044	17.263	0.234	0.8180	
Residual standard error: 85.36 on 15 degrees of freedom					
Multiple $R^2 = 0.5091$, Adj. $R^2 = 0.3455$					
$F_{(5,\ 15)} = 3.112$, $p = 0.0401$					
(B) MODEL 2. DOUBLE CONE PEAK SENSITIVITY					
Residuals	Minimum	1st Quartile	Median	3rd Quartile	Maximum
	−109.251	−23.078	2.589	16.460	43.273
Coefficients	Estimate	Std. Error	*t*-value	*p*(>|t|)	
Color pattern	8.0873	4.7440	1.705	0.109	
Diet	−1.5978	16.1532	−0.099	0.923	
Maximum depth	−0.2151	0.1631	−1.319	0.207	
Irradiance at maximum depth	−0.0459	0.4727	−0.097	0.924	
Body color	−0.7253	8.0206	−0.090	0.929	
Residual standard error: 39.66 on 15 degrees of freedom					
Multiple $R^2 = 0.2591$, Adj. $R^2 = 0.0122$					
$F_{(5,\ 15)} = 1.049$, $p = 0.4255$					

TABLE 4 | Estimates of phylogenetic signal (λ) between simplified color pattern (e.g., stripes vs. bars) and visual system (e.g., UV and non-UV).

Trait	**λ**	**LL λ**	**LL λ = 0**	***p***	**LL λ = 1**	***p***
Color pattern	0.544	−24.31	−27.03	0.02	−23.53	0.21
Visual system	0.448	−26.09	−26.46	0.39	−25.99	0.66

LL, log likelihood.

There was a significant difference between the dependent and independent models of trait evolution, and the dependent model was favored by our analyses, having a much lower BIC than the independent model (**Table 5**). Additionally, since this model indicated that the likely ancestral state was a UV-sensitive fish with a barred color pattern, we reran the dependent model two different ways: One in which a UV-sensitive, barred fish was the ancestor; and one in which a non-UV-sensitive, horizontally-striped fish was the ancestor. All three of these models had very similar log-likelihoods, so the differences among them, based on LR, were not significant (data not shown), but since the original, "unfossilized" model had the most favorable BIC, that is the most likely (**Table 5**).

The eight possible transitions among the four possible pairs of visual and color pattern characteristics are shown in **Figure 5**. Of these eight possible transitions, only rates q34 (the transition from vertical bars to horizontal stripes in UV sensitive fish) and q31 (the loss of UV sensitivity in striped fish) are statistically significant. These results further support the possibility that the ancestor of the Lake Malawi cichlid radiation had a UV-sensitive visual system.

Perception of Color Patterns by UV-Sensitive and Non-UV Visual Systems

To better illustrate how different cichlid visual systems would perceive different color patterns, we present the spectrophotometric data and quantal catches of representative barred, solid, and striped species in **Figures 6**–**11**. Two barred species, *Metriaclima zebra* and *Pseudotropheus flavus*, are shown in **Figures 6**, **7**, respectively. The quantal catches of these species, both of which are taken from a black bar marking, as well as the respective background color, are greatly different between UV- and violet-sensitive species. While a UV-sensitive species would detect a great deal of contrast between these patches, a non-UV-sensitive species would not (**Figures 6C,D**, **7C,D**). Striped species, represented by *Dimidiochromis compressiceps* (**Figure 8**) and *Melanochromis auratus* (**Figure 9**), on the other

TABLE 5 | Model selection based on likelihood ratio tests and Bayesian Information Criteria (BIC).

Model	Log Likelihood	BIC
Independent, complete model	−50.565	45.868
Dependent, complete model	−44.335	−21.854
Likelihood ratio test between the above models: 9.848_{1df}, $p = 0.002$		
Dependent, fossilized at 0,0 (non-UV, striped ancestor)	−45.833	−18.858
Likelihood ratio test: Dependent, complete vs. Dependent, 0,0 models: 2.995_{1df}, $p = 0.080$		
Dependent, fossilized at 1,1 (UV, barred ancestor)	−45.505	−19.514
Likelihood ratio test: Dependent, complete vs. Dependent, 1,1 models: 2.340_{1df}, $p = 0.130$		

The preferred model will have the lowest value for the BIC.

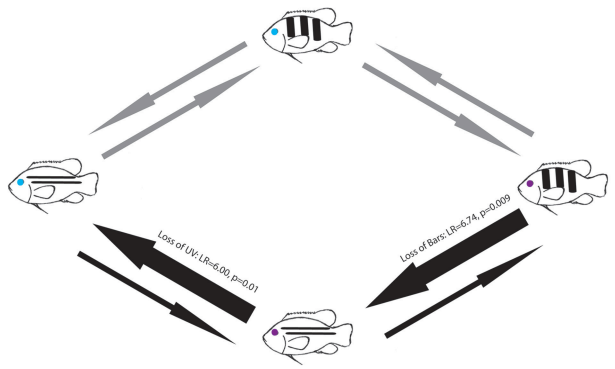

FIGURE 5 | Evolutionary transitions among SWS sensitivity and color pattern character states in Lake Malawi cichlids. Black arrows: likely transitions; statistically significant transitions are indicated by the thick arrows and *p*-values indicated above arrows. Gray arrows: highly unlikely transitions. Violet eyes: UV-sensitive; blue eyes: non-UV-sensitive. Notice that the likely ancestral state of the Lake Malawi cichlids is a UV-sensitive, barred species, descendants of which would subsequently lose both a barred color pattern and UV sensitivity during the radiation of these fishes.

hand, would likely appear the same to either type of visual system, as indicated by the closely overlapping quantal catches in **Figures 8C,D**, **9C,D**.

The solid-colored species, *Labeotropheus* c.f. *fuelleborni* "Katale" and *Iodotropheus sprengerae*, have a surprising amount of ultraviolet reflectivity, especially in their carotenoid-based colors (**Figures 10A**, **11A**, respectively). As such, the major difference between how UV- and non-UV-sensitive species would perceive these patterns would likely be in the amount of contrast between the patches we selected for analysis.

To simulate how a UV-sensitive visual system would perceive the colors of the fishes detailed above, we offer **Figure 12** as a heuristic. In the left column of this figure are pictures of each species taken under full-spectrum lighting, while the right column has photographs of the same individuals taken moments later under UV light. As predicted by our hypothesis, the patterns of barred and solid-patterned species look quite different under UV illumination, while those with horizontal stripes look very similar no matter the illuminant.

DISCUSSION

There is some evidence indicating that differently-tuned SWS opsins serve different functions in fishes. For example, among coral reef fishes, UV-sensitive visual systems are important in species recognition (Côté and Cheney, 2005; Cheney and Marshall, 2009; Siebeck et al., 2010), and are particularly sensitive to species-specific within-color pattern contrast (Losey, 2003; Siebeck et al., 2010); also, UV-sensitive species are better able to discriminate between helpful "cleaner" species and their harmful mimics, largely due to distinctive UV reflectivity in the mimics' color patterns (Côté and Cheney, 2005; Cheney and Marshall, 2009). Lake Malawi cichlids have been documented to use particular color patterns for communication (Pauers et al., 2012); however, our results are the first demonstration of a relationship between gross color pattern and photoreceptor sensitivity. Species with barred patterns have SWS peak sensitivity values at wavelengths that are shorter than either of the other patterns; indeed, these sensitivities tend to fall in the UV spectrum. Further, only color pattern type could successfully predict SWS peak sensitivity; no other variable, including depth and irradiance, had statistically significant relationships with SWS sensitivity. Most interestingly, our results indicate that visual sensitivity and color pattern did evolve in a correlated fashion; the ancestral cichlid was likely a UV sensitive fish with a barred color pattern that first changed its pattern from barred to striped, followed by a loss of UV sensitivity.

The peak sensitivity of the mid/long wavelength sensitive double cone was not correlated with color pattern type among species, nor did any variable successfully predict the peak sensitivity of this photoreceptor. Further, double cone peak sensitivity varied little across all species, so these fishes are all receiving similar information with these cones. While the double cone system may provide wavelength discrimination by means of two distinct opsins, each with its own peak sensitivity, which work in an opponent fashion (Neitz and Neitz, 2011), we are unable to determine the ability of these double cones to provide such discrimination, as only the stronger opsin sensitivity was reported by Hofmann et al. (2009).

Our discovery of correlated evolution of visual sensitivity and color pattern is of particular interest for several reasons. First, this suggests a functional connection between visual sensitivity and color pattern, a novel finding in Lake Malawi cichlids (Dalton

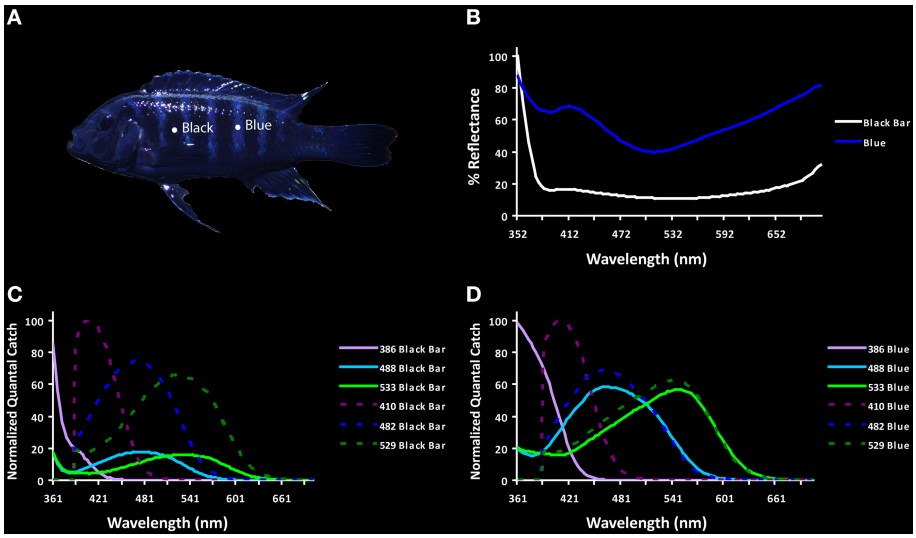

FIGURE 6 | ***Metriaclima zebra*****.** Each figure in Figures 6–11 is arranged in the same fashion. There are four panels in each figure: **(A)** is a photograph of the fish under full-spectrum lighting (including UV illumination), indicating two points at which reflectances were measured; **(B)** illustrates the reflected wavelengths at the points indicated in **(A)**; **(C,D)** are comparisons of the quantal catches of the reflectances by the photoreceptors found in two different classes of Malawi cichlid retinae: UV sensitive (solid lines) and non-UV sensitive (dashed lines). Photopigment sensitivities were taken from Hofmann et al. (2009).

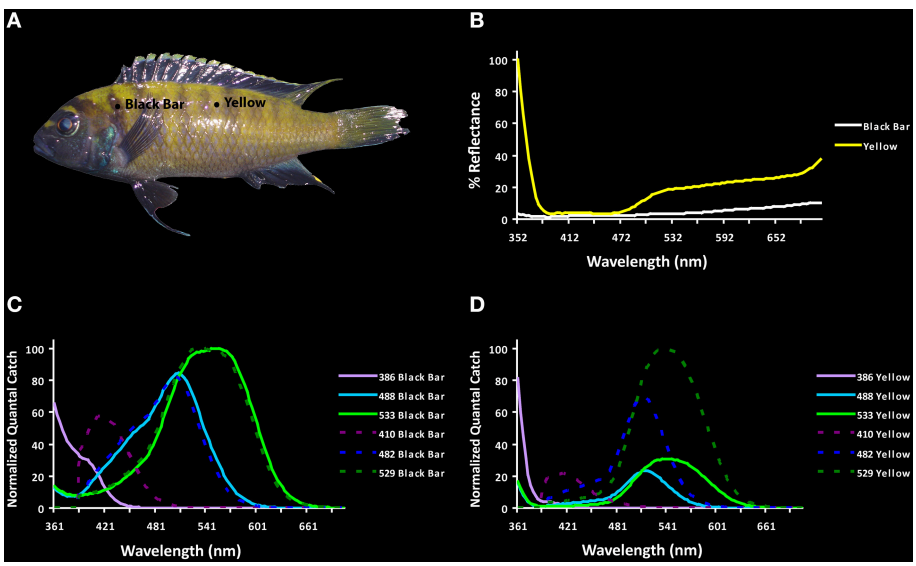

FIGURE 7 | ***Pseudotropheus flavus*****.**

et al., 2010). Secondly, in order for a loss of UV sensitivity to occur, it appears that a horizontally-striped color pattern must evolve first. This indicates that visual sensitivity responds to the change in color pattern, suggesting that visual sensitivity may adapt to maximize the efficacy of visual communication via color patterns. Correspondingly, the values of λ for both visual sensitivity and color pattern were less than one, suggesting that traits are less similar among closely-related species than expected; thus, our analysis explains the diversity in visual sensitivity and color pattern seen within the Lake Malawi cichlid flock. Finally, and perhaps most interestingly, the direction of this evolution, from a barred, UV-sensitive ancestor, to a striped, non-UV-sensitive descendant, is different from what had previously been found in Lake Malawi cichlids. In a recently published phylogenetic analysis of visual sensitivity in African cichlids (O'Quin et al., 2010) found that the ancestor of Lake Malawi cichlids most likely had to have a long-wavelength sensitive visual system, and likely lacked UV sensitivity. This difference between our result and theirs could be partially explained by the different types of data we used; O'Quin et al. (2010) used

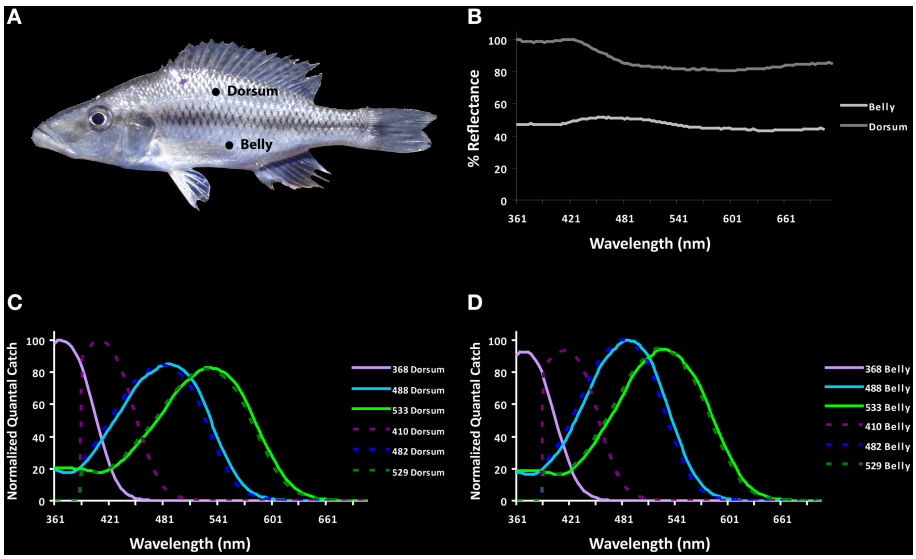

FIGURE 8 | ***Dimidiochromis compressiceps*.**

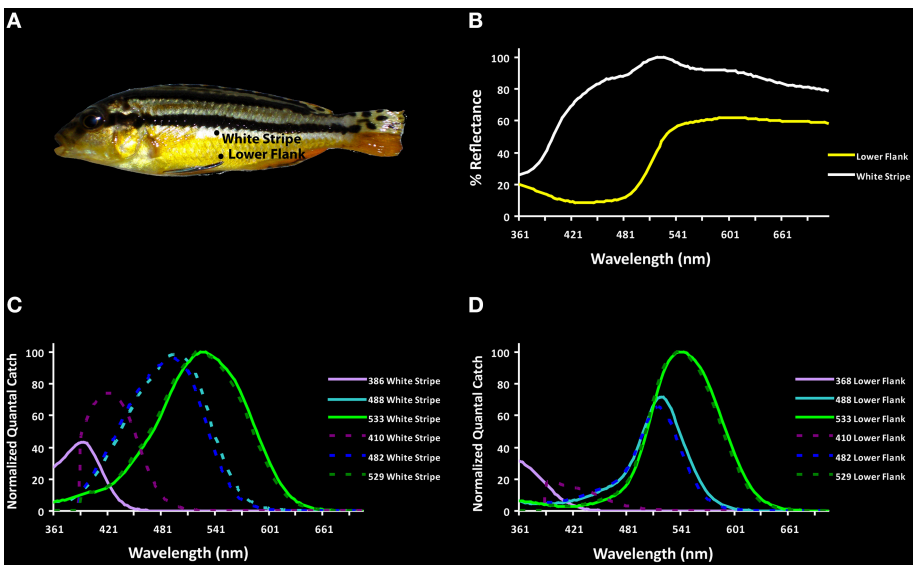

FIGURE 9 | ***Melanochromis auratus*.**

continuous data, while we used categorical, but a recent paper by Hunt and Peichl (2014) provides support for our findings. While losses of UV sensitivity throughout evolutionary and phylogenetic transitions are fairly common, the evolution of UV sensitivity from non-UV-sensitive ancestors is quite rare; only within the birds has UV sensitivity reappeared once lost (Ödeen and Håstad, 2013; Hunt and Peichl, 2014). Thus, the evolution of UV-sensitive cichlids from a non-UV ancestor is rather unlikely.

In an interesting recent study, York et al. (2015) also found that opsin evolution is likely related to the evolution of a different sexually-selected characteristic in Lake Malawi cichlids. Among the sand-dwelling cichlids, some species of which were included in the present analyses, males build bowers of sand that are used to attract females. These authors found that, between species that build the two fundamental types of bower, "pits" and "castles," pit-building species had a longer-shifted SWS photoreceptor sensitivity, while castle builders had a shorter-shifted SWS photoreceptor, often with UV sensitivity. Further, there were no differences between pit- and castle building species in the sensitivities of the longer-shifted opsins found in the double cones of these fishes. York et al. (2015) suspect that this may be

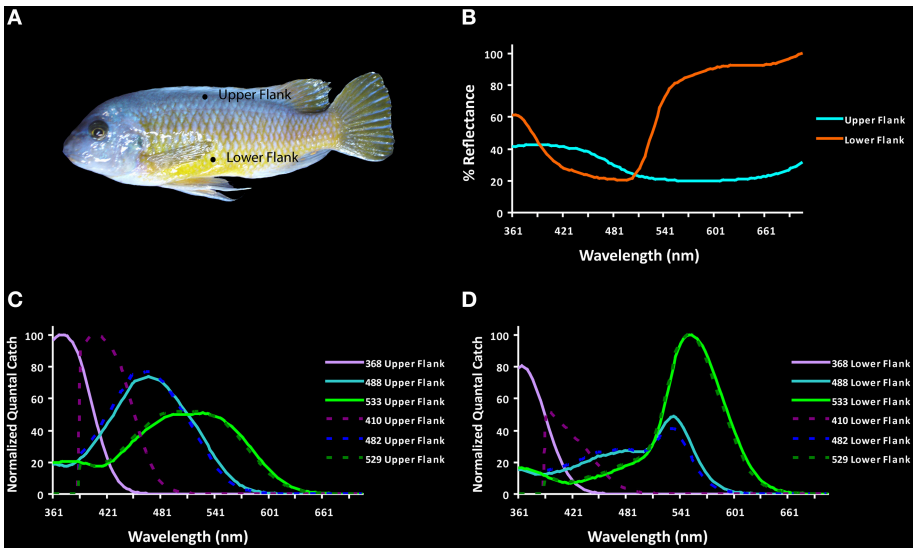

FIGURE 10 | ***Labeotropheus* c.f. *fuelleborni* "Katale."**

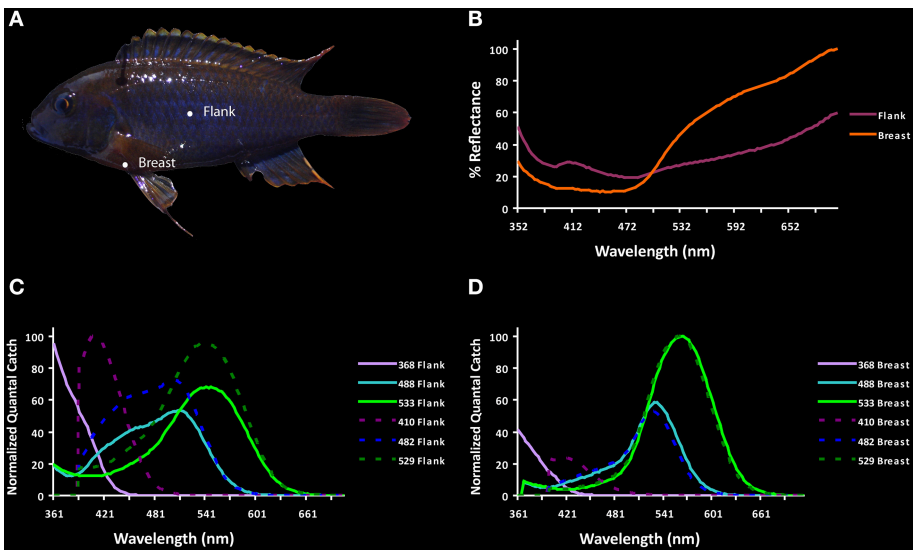

FIGURE 11 | ***Iodotropheus sprengerae*.**

due at least in part to depth, as pit bowers are more common in deeper waters and castles more common in shallow. While these authors did not perform a phylogenetically-corrected analysis between opsin sensitivities and bower type, it is nonetheless interesting that they recovered a similar relationship between SWS opsin sensitivity and the form of a sexually-selected signal in Lake Malawi cichlids (York et al., 2015); it would be further tantalizing to determine whether or not evolutionary changes in SWS sensitivity follow innovations in the shape of bowers in the sand-dwelling Malawian cichlids, similar to how we have found that a loss of UV sensitivity follows the loss of a vertically-barred color pattern.

The results presented here are consistent with the hypothesis that stripes and bars represent "public" and "private" information, respectively, and that barred and solid-patterned species maintain this privacy via a UV-sensitive SWS photoreceptor. UV sensitivity could maintain or enhance color contrast among color pattern elements, or could even allow for "private" communication by changing the way a color pattern is perceived. Species that lack UV sensitivity, on the other hand, are likely not able to clearly perceive the fundamental message communicated via barred and "solid" patterns. Further, since the horizontal stripes found in non-UV species likely represent a form of camouflage, such a pattern

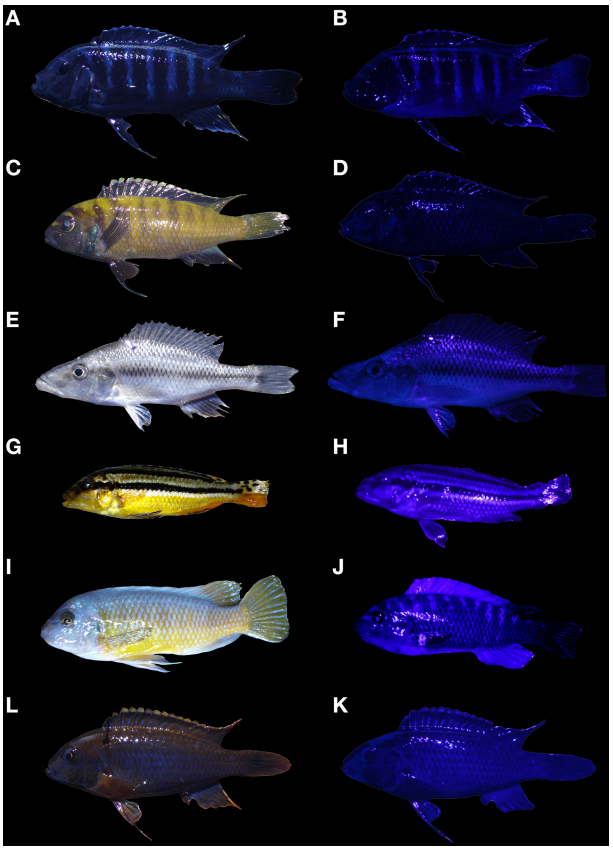

FIGURE 12 | Lake Malawi cichlid color patterns under full-spectrum light and UV lighting. (A,B), *Metriaclima zebra*; **(C,D)**, *Pseudotropheus flavus*; **(E,F)**, *Dimidiochromi scompressiceps*; **(G,H)**, *Melanochromis auratus*; **(I,J)**, *Labeotropheus* c.f *fuelleborni* "Katale;" **(K,L)**, *Iodotropheus sprengerae*. Species displaying different hues were selected to emphasize that the fundamental pattern, and the messages encoded therein, are independent of hue. In the barred and solid species, notice how UV lighting emphasizes the contrast between bars and background (**B**, e.g.,) or among color pattern elements (**J**, e.g.,), sometimes revealing hidden or de-emphasized contrasts (the highly reflective bars and the contrast between peduncle and flank in **J**). In the striped species **(E–H)**, the balance among elements stays the same whether the illumination is full-spectrum or UV, thus suggesting that this pattern is meant to be visible and understood by all visual systems.

would need to be clearly perceived by all species; if the patterns appeared differently to UV-sensitive and UV-insensitive fishes, concealment would be compromised. These results, then, suggest that the overall purpose for animal color patterns consisting of patches of alternating hues, as opposed to patches of alternating brightness, is to provide either "private" signals visible to only certain species (e.g., nuptial color patterns; Endler, 1992); or "public" information, like camouflage or advertisement of services (e.g., cleaner fishes), visible to species of varying visual sensitivities.

Our results also offer an explanation why Dalton et al. (2010) found no close concordance between photoreceptor sensitivity and cichlid body reflectance, as would be predicted if sensory drive processes were responsible for male nuptial coloration (Endler, 1992). Lake Malawi cichlids with a barred color pattern display a wide range of colors across the spectrum including blue (e.g., *Metriaclima zebra*), yellow (e.g., *Pseudotropheus flavus*), and brown bars (e.g., *P. crabro*, *P. livingstonii*, and *P. lombardoi*). The sensory drive hypothesis predicts that species with blue bars should be most sensitive to short wavelengths, while those with yellow or brown bars should be most sensitive to longer wavelengths. The results presented here indicate otherwise; barred cichlids, no matter the colors present in their patterns, have short-shifted visual sensitivities. This calls into question the role that sensory drive processes, based on color alone, may have played in the evolution of male nuptial coloration in the Lake Malawi cichlids. For example, female *Labeotropheus* c.f. *fuelleborni* "Katale" prefer sympatric, conspecific males with high contrast among color pattern elements (Pauers et al., 2004). Actual reflected wavelengths may not matter as long as contrast is maintained, and the contrast defines a distinct pattern that is perceptible by the fishes themselves (Pauers, 2011). This resolves a long-standing conundrum surrounding Lake Malawi cichlids: How is species recognition maintained in these fishes when the putative major cue for mate recognition, male nuptial coloration, is limited to the same color palette across species, and when photopigment sensitivity is also similar among species? Color may certainly play a role, but both the arrangement and contrast of color pattern elements may be just as important.

AUTHOR CONTRIBUTIONS

MP conceived the study described herein, with significant input from JN. MP gathered the data and performed the statistical analyses. SJ constructed the phylogenies used in all analyses, and wrote the phylogenetic methods. MP and JN discussed the results and outlined the manuscript. MP wrote the manuscript, with editorial input from JK, SJ, and JN. JK contributed revisions to several drafts and helped construct and provided the interpretations for **Figures 6–11**. All authors read and approved the final draft for submission.

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REVIEW
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Diversity in Fish Auditory Systems: One of the Riddles of Sensory Biology

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An astonishing diversity of inner ears and accessory hearing structures (AHS) that can enhance hearing has evolved in fishes. Inner ears mainly differ in the size of the otolith end organs, the shape and orientation of the sensory epithelia, and the orientation patterns of ciliary bundles of sensory hair cells. Despite our profound morphological knowledge of inner ear variation, two main questions remain widely unanswered. (i) What selective forces and/or constraints led to the evolution of this inner ear diversity? (ii) How is the morphological variability linked to hearing abilities? Improved hearing is mainly based on the ability of many fish species to transmit oscillations of swim bladder walls or other gas-filled bladders to the inner ears. Swim bladders may be linked to the inner ears via a chain of ossicles (in otophysans), anterior extensions (e.g., some cichlids, squirrelfishes), or the gas bladders may touch the inner ears directly (labyrinth fishes). Studies on catfishes and cichlids demonstrate that larger swim bladders and more pronounced linkages to the inner ears positively affect both auditory sensitivities and the detectable frequency range, but lack of a connection does not exclude hearing enhancement. This diversity of auditory structures and hearing abilities is one of the main riddles in fish bioacoustics research. Hearing enhancement might have evolved to facilitate intraspecific acoustic communication. A comparison of sound-producing species, however, indicates that acoustic communication is widespread in taxa lacking AHS. Eco-acoustical constraints are a more likely explanation for the diversity in fish hearing sensitivities. Low ambient noise levels may have facilitated the evolution of AHS, enabling fish to detect low-level abiotic noise and sounds from con- and heterospecifics, including predators and prey. Aquatic habitats differ in ambient noise regimes, and preliminary data indicate that hearing sensitivities of fishes vary accordingly.

Keywords: inner ears, hearing, accessory auditory structures, weberian ossicles, audiograms, swim bladder

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SOUND DETECTION IN VERTEBRATES

The inner ear is the primary hearing organ in vertebrates. Typically, tetrapods (amphibians, reptiles, birds, mammals) developed thin membranes on the body surface laterally of the inner ears (tympana or eardrums) to pick up sound pressure changes in the air and transmit these pressure fluctuations via 1-3 tiny auditory ossicles to the inner ear fluids (Ladich, 2010). No basal (e.g., lungfishes, *Latimeria*) or derived fish taxon developed a tympanum at the outside of the body or

a middle ear because no net movement exists between the medium (water) and the animal's body (see discussion in Fritzsch, 1992). Because both fish and water have the same density they move synchronously in the sound field (Hawkins, 1986). Thus, fishes cannot detect sound via an outer tympanum similar to tetrapods but need to detect sound in a fundamentally different way. Fishes analyze the movement of their body in the sound field relative to calcium carbonate structures in the otolith end organs of the ear that have a distinctly greater inertia. These calcareous structures (otoconia and/or otoliths) lag behind in movement relative to the fish in the sound field and thereby stimulate the sensory hair cells by deflecting their ciliary bundles. This physically different process, namely detecting the movement of a tiny calcareous stone, means that fish are unable to detect sound pressure but particle motion instead. Particle motion detection differs from pressure detection in several ways. It limits the detectable frequency range to a few hundred hertz, restricts the detectable sound intensities to higher levels, and also shortens distances over which sounds are detectable (Schuijf and Hawkins, 1976; Fay, 1988; Bradbury and Vehrencamp, 2011).

At least one third of all teleost species developed mechanisms for sound pressure detection similar to tetrapods via tympana. Air-filled cavities within the body such as swim bladders or organs for air-breathing undergo volume changes because air is much more compressible than fluids in any sound field. These volume fluctuations will result in oscillations of the walls, which then function similar to tympana as soon as these membranes transmit their oscillations to the inner ears and improve hearing sensitivities (Alexander, 1966). Structures which enhance hearing in fish by enabling sound pressure detection are termed accessory (ancillary, peripheral) hearing structures, hearing enhancements or hearing specializations. These structures function as pressure-to-particle motion transducers (Hawkins, 1986). Fishes possessing such mechanisms have often been termed "hearing specialists" (Ladich and Popper, 2004; Braun and Grande, 2008; Popper and Fay, 2011). So far, no evidence exists that air-filled cavities evolved purely for sound pressure detection, and therefore we have to assume that sound pressure hearing is a by-product of either buoyancy regulation—which is the primary function of the swim bladder—or air-breathing. Nevertheless, it is quite safe to assume that several taxa of modern bony fishes (teleosts) evolved structures which serve only to connect given gas-filled cavities to the inner ears mechanically (e.g., Weberian ossicles).

DIVERSITY IN AUDITORY SYSTEMS IN FISH

Cartilaginous (Chondrichthyes) and bony fishes (Osteichthyes) comprise more than one-half of the approximately 55,000 described vertebrate species (Nelson, 2006). Compared to birds and mammals, fishes possess a high diversity in inner ear morphology and accessory hearing structures. These auditory structures result in a diversity of hearing sensitivities, often within members of the same family. In the non-related families Holocentridae (squirrelfishes), Cichlidae, and Sciaenidae (drums and croakers), some genera possess hearing specializations and improved hearing abilities while others lack such auditory enhancements. The functional significance of this diversity is widely unknown and poses one of the main riddles of sensory biology (Ladich and Popper, 2004; Braun and Grande, 2008; Ladich, 2014a,b, 2016; Schulz-Mirbach and Ladich, 2016).

This review provides an overview of the diversity of fish inner ears and accessory hearing structures as well as auditory sensitivities. We further elucidate whether this structural diversity is correlated with hearing abilities. Finally, we discuss three not mutually exclusive hypotheses explaining why enhanced hearing has evolved in modern bony fishes.

Inner Ears

Basic Inner Ear Structure and Function

Despite the high inner ear diversity among cartilaginous (Chondrichthyes) and bony fishes (Osteichthyes), a basic ear structure can be identified: an upper inner ear consisting of three semicircular canals and the utricle (vestibular system), and a lower inner ear comprising the saccule and the lagena (**Figures 1–2;** Popper, 2011; Popper and Fay, 2011). An endolymphatic duct is present in all fishes (Maisey, 2001). In cartilaginous fishes (**Figure 1A**) this duct is connected to the surface of the head via a small pore (endolymphatic pore), whereas it ends blindly and may be widely reduced in bony fishes (Maisey, 2001; Lisney, 2010).

Each canal and each otolith end organ houses a sensory epithelium. The sensory epithelia in the ampullae of the semicircular canals are termed cristae and are overlain by a gelatinous cupula, whereas those in the otolith end organs (utricle, saccule, lagena) are termed maculae (Popper, 2011).

The maculae of the otolith end organs are overlain by otoconia (except in teleosts) embedded in the otolithic membrane or by a single massive otolith (teleosts), which is connected to the macula via the otolithic membrane (Popper et al., 2005; Casper, 2011; Popper, 2011). In all cartilaginous fishes and some members of the Actinopterygii, a fourth macula, namely the macula neglecta, is present (Casper, 2011). It consists of one or two small patches housing several dozen (e.g., Platt et al., 2004) up to thousands of sensory hair cells (e.g., Corwin, 1981). Similar to the canal cristae, the macula neglecta is overlain by a gelatinous cupula and lacks otoconia or an overlying otolith. The term crista neglecta instead of macula neglecta was therefore suggested by some authors (see Maisey, 2001).

Within the fish's ear, the macula utriculi is mainly oriented horizontally with exception of the lacinia that curves antero-laterally. The macula sacculi and the macula lagenae are both mainly oriented along the vertical plane.

In addition to this differences in the spatial orientation of the whole maculae ciliary bundles of the sensory hair cells are generally arranged in a certain orientation pattern on each macula that is determined according to the position of the eccentrically placed kinocilium within the bundle (Popper, 1976; Hudspeth and Corey, 1977). The orientation pattern of the sensory epithelia of the semicircular canals (=cristae) is similar in all studied vertebrates, and the cristae are thus the most

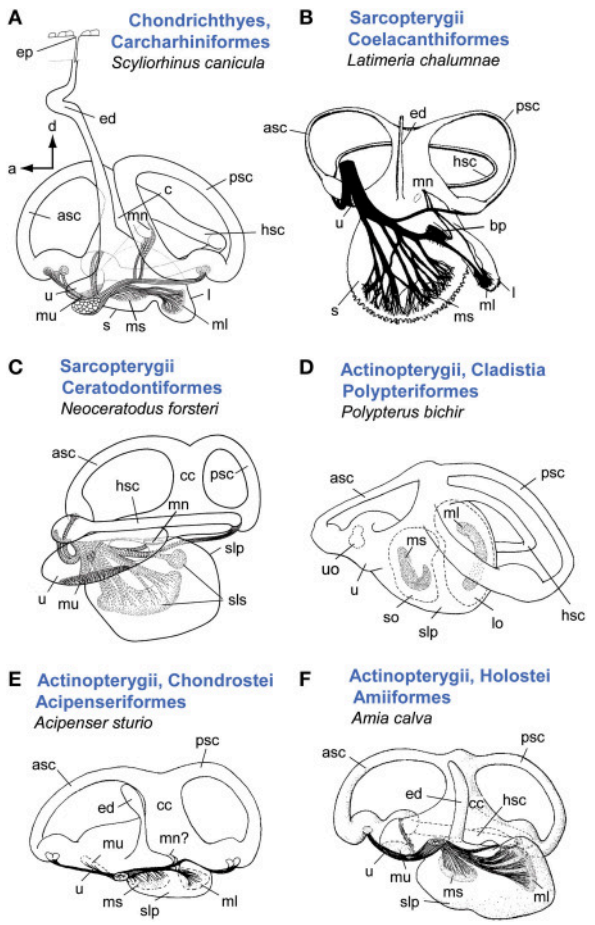

FIGURE 1 | Overview of inner ear diversity in cartilaginous and bony fishes (except teleosts). In sharks **(A)** and *Latimera* **(B)** the saccule and lagena form two sacs, whereas lungfish **(C)** and non-teleost actinopterygians **(D–F)** are characterized by a common pouch for these otolith end organs (sacculolagenar pouch). Note, however, that in other species of chondrichthyes (see especially rays and skates and Holocephali; Lisney, 2010), saccule and lagena may be less well-separated (Evangelista et al., 2010). In cartilaginous fishes the endolymphatic duct connects the inner ear to the head surface and thus to the external medium. In *Latimeria* **(B)**, sturgeon **(E)**, and the bowfin **(F)**, remnants of an endolymphatic duct are visible, but the duct is closed at its dorsal end. A macula neglecta is present in all shown ears except for the bichir **(D)**. Retzius (1881) reported a macula neglecta for sturgeon **(E)**, which was neither confirmed nor refuted in Popper (1978). Note that the macula neglecta in the bowfin is not visible in medial view (see Popper and Northcutt, 1983). In **(A, B, D–F)**, ears are shown in medial view; **C** in lateral view. Illustrations modified from Retzius (1881), Popper (1978), Popper and Northcutt (1983), Fritzsch (1987), Ladich and Popper (2004). a, anterior; asc, anterior semicircular canal; bp, basilar papilla; c, crus; cc, common canal; d, dorsal; ed, endolymphatic duct; hsc, horizontal semicircular canal; l, lagena; lo, lagenar otolith; ml, macula lagenae; mn, macula neglecta; ms, macula sacculi; mu, macula utriculi; psc, posterior semicircular canal; slp, sacculolagenar pouch; sls, saccular and lagenar striolae, so, saccular otolith; u, utricle; uo, utricular otolith. **B** with permission from Nature Publishing Group, **C**, **D** and **A** with permission from John Wiley & Sons, Inc.

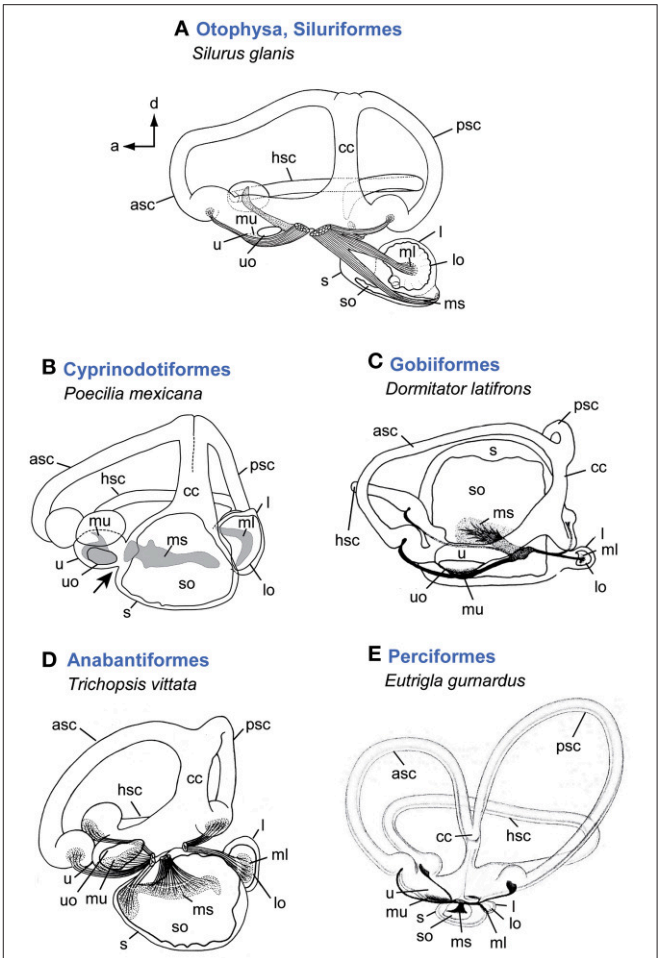

FIGURE 2 | Overview of inner ear diversity in teleost fishes. Otophysan ears have a large round lagena with an asterisk-like otolith and an elongate saccule and a needle-shaped saccular otolith **(A)**. Non-otophysans generally possess a saccule larger than the utricle and lagena **(B–E)**. Cyprinodontiforms **(B)** show a utricle connected anteriorly to the saccule (indicated by black arrow). In gobiiform fishes **(C)** the saccule is distinctly large and semicircular canals run around this end organ rather than having an anterodorsal position. The ear of the anabantiform *Trichopsis vittata* **(D)** represents a gross morphology found in many non-otophysans. Variation regarding the semicircular canals in teleosts is rare compared to the diversity found in the otolith end organs, especially in the saccule and lagena. Some species, however, such as *Eutrigla gurnardus* **(E)** are characterized by distinctly large semicircular canals. All ears are shown in medial view. Illustrations modified from Retzius (1881), Lu and Popper (1998), Ladich and Popper (2001, 2004), and Schulz-Mirbach et al. (2011). For abbreviations see **Figure 1**.

conservative of all sensory epithelia of the inner ear (Mathiesen, 1984). The macula utriculi also shows minimal variation (Platt and Popper, 1981a), indicating that the vestibular part of the inner ear functions similarly in all vertebrates (except perhaps for jawless fishes having just one or two canals; see Ladich and Popper, 2004). The largest diversity in orientation patterns in teleosts occurs on the macula sacculi (Platt and Popper, 1981a; Popper and Coombs, 1982). Different spatial orientation of the whole maculae ("horizontal" vs. "vertical") as well as different orientation groups of ciliary bundles on the same macula are—among others—hypothesized to enable fish to detect sound emanating from different angles in three-dimensional space (for an overview and discussion of sound source localization in fish see Sisneros and Rogers, 2016).

All fish use the vestibular system to gain information about their body position and motion in three-dimensional space (Straka and Baker, 2011). During head and body motion the movements of the endolymphatic fluid in the semicircular canals deforms the gelatinous cupula which leads to deflection of the ciliary bundles of the sensory hair cells. The canals thus detect body rotation (angular acceleration). The utricle is a highly effective transducer for linear acceleration. The mainly horizontally oriented utricular sensory epithelium senses the inertia provoked by the denser overlying otolith (or otoconial mass) and can thus detect static changes in the position of the head or the body relative to the Earth's gravitation vector (Straka and Baker, 2011). In a few fish taxa such as Clupeidae (herring) it is assumed that the utricle serves in hearing beside its function as gravitation sensor (Popper, 2011). Due to its auditory potential the utricle will be treated as part of the auditory structures.

Diversity in Gross Inner Ear Morphology

Diversity in gross features of the inner ear mainly relates to the (1) size of ears compared to overall size of the fish and the brain, (2) amount of surrounding skull bone or cartilage and potential attachment of the membranous labyrinth to the skull, (3) distance between the two ears and presence/absence of a connection between left and right ears, (4) relative position of upper to lower parts of the ear, i.e., position of the utricle relative to saccule and lagena, (5) size and diameter of the semicircular canals, (6) size ratio among the otolith end organs utricle, saccule, and lagena; and (7) whether saccule and lagena form one or two pouches. For a phylogenetic overview of inner ear diversity and accessory hearing structures see **Figures 3**, **4**.

Some deep-sea or cave fishes (teleosts), for example, show exceptionally large ears compared to the brain (Poulson, 1963; Fine et al., 1987), whereas some epipelagic teleost species have extremely small ears and otoliths (Paxton, 2000; Song et al., 2006). Also the amount of encapsulation and attachment of the ear to the skull differs considerably. Certain teleosts such as poeciliids show rather "free" ears with encapsulation limited to the semicircular canals (Schulz-Mirbach et al., 2011), whereas the non-teleost actinopterygian *Amia calva* (Popper and Northcutt, 1983) or elasmobranchs display almost full encapsulation of the ears (Maisey, 2001). In other species, attachment of one or several otolith end organs to the skull is associated with the presence of accessory hearing structures as found in the notopterid *Chitala chitala*, the morid *Antimora rostrata*, or the cichlid *Etroplus maculatus* (Coombs and Popper, 1982; Deng et al., 2011; Schulz-Mirbach et al., 2013). This coupling of an otolith end organ to the bone may play a role for effective sound transmission to the ears via the specialized swim or gas bladder (see discussion in Deng et al., 2011).

A connection between left and right ears is known from the otophysans (saccules communicate via the transverse canal; Wohlfahrt, 1932; von Frisch, 1936) and the coelacanth *Latimeria* (ears are connected at the junction between saccule and lagena to one another via the canalis communicans; Bernstein, 2003). While in otophysans this connection may improve effective sound transmission via the swim bladder and the Weberian apparatus to both ears (von Frisch, 1938; cf. Finneran and Hastings, 2000), it is completely unclear whether the junction between ears plays a role in audition in *Latimeria* due to the lack of physiological data.

Other aspects of diversity relate to the morphology of the semicircular canals (see especially elasmobranchs), the size ratio of semicircular canals to the otolith end organs, or the size ratio among the three otolith end organs. Unlike in Holocephali and bony fishes, Elasmobranchii do not have a connection between the anterior and posterior semicircular canals (common crus); instead the anterior and horizontal canals are connected to each other via a crus (**Figure 1A**; Maisey, 2001; Evangelista et al., 2010). According to Maisey (2001) the posterior canal in elasmobranchs is thus rather a circuit than a semicircular canal. Within this group, species display variability in the presence/absence of the canal ducts that connect the semicircular canals to the otolith end organs and thus may differ in whether the semicircular canals are directly connected to the saccule, in the length of the endolymphatic duct, and in the size of the saccule with respect to the utricle (Evangelista et al., 2010). In teleosts, diversity in semicircular canals is restricted to differences in canal thickness and canal radii. Sea horses (Syngnathidae, Syngnathiformes), for example, display "compact" ears with almost rectangular instead of rounded semicircular canals (Retzius, 1881). Moreover, several unrelated species of flying fishes (*Dactylopterus volitans*, Dactylopteridae, Syngnathiformes; *Exocoetus volitans*, Beloniformes) show distinctly large semicircular canals and extremely small otolith end organs (Retzius, 1881). Large semicircular canals are also present in the angler *Lophius piscatorius* (Lophiiformes) and the gray gurnard *Eutrigla gurnardus* (Perciformes; **Figure 2E;** Retzius, 1881). The functional meaning of these enlarged semicircular canals remains to be studied (see also discussion in Evangelista et al., 2010).

Whereas the upper inner ear (semicircular canals and utricle) is rather conservative across fishes (but see elasmobranchs), diversity is higher in the lower inner ear (saccule and lagena). In Holocephali (Retzius, 1881; de Burlet, 1934), lungfishes (Retzius, 1881; Platt et al., 2004), and non-actinopterygian teleosts (Popper, 1978; Popper and Northcutt, 1983; Mathiesen and Popper, 1987; Lovell et al., 2005) saccule and lagena form one pouch, whereas in the coelacanth *Latimeria* (Fritzsch, 1987, 2003), elasmobranchs (e.g., Retzius, 1881; Ladich and Popper, 2004), and teleosts (e.g., Ladich and Popper, 2004; Popper and Schilt, 2008) these otolith end organs form two interconnected sacs. The saccule is often the largest of the three otolith end organs (**Figures 1A–B**, **2B-E**), with teleost orders including Gobiiformes (**Figure 2C**; e.g., Retzius, 1881; Popper, 1981), Ophidiiformes (e.g., Parmentier et al., 2001, 2002; Kéver et al., 2014), and Batrachoidiformes (e.g., Cohen and Winn, 1967) representing members with one of the largest saccules compared to the tiny utricle and lagena. In these taxa, the semicircular canals run around the large saccule rather than being located dorsally to it. Most otophysans are characterized by having a lagena as large as or larger than the elongate saccule (**Figure 2A**; Popper and Platt, 1983). In ariid catfishes, however, the utricle is distinctly larger than both saccule and lagena (Popper and Tavolga, 1981).

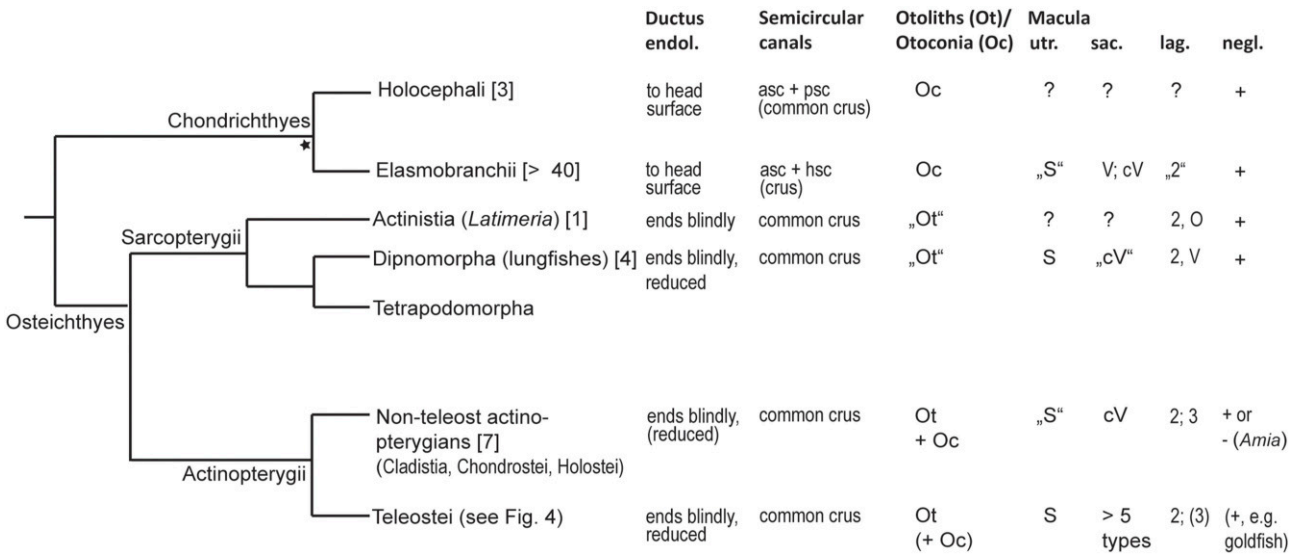

FIGURE 3 | Overview of inner ear characters in cartilaginous and bony fishes. The phylogeny was modified from Betancur et al. (2013), Broughton et al. (2013), and the DeepFin Project (2003-2009), except the split of Chondrichthyes into Holocephali and Elasmobranchii indicated by the asterisk (see Nelson, 2006). For a detailed overview of inner ear characters and species see Schulz-Mirbach and Ladich (2016, Table 2). Numbers given in brackets indicate the number of species studied with respect to inner ear morphology. Ductus endol., ductus endolymphaticus; asc, anterior semicircular canal; psc, posterior semicircular canal; Macula utriculi (utr.): S, standard pattern, i.e., radially oriented ciliary bundles on the cotillus and opposing ciliary bundles in the striola region; Macula sacculi (sac.): cV, curved vertical, i.e., horizontal groups in the anterior portion are due to ciliary bundle orientation that follows the macula curvature. Macula lagenae (lag.): Numbers indicate the number of orientation groups; O, opposing groups of ciliary bundles; V, vertical ciliary bundle orientation. In most species only numbers are given because orientations of ciliary bundles vary to different degrees in a gradual manner along the macula from horizontal, oblique to vertical directions. Macula neglecta (negl.): Note that only presence/absence of this macula type is indicated. Orientation patterns of ciliary bundles are illustrated in **Figure 11**.

Otoconia and Otoliths

In cartilaginous fishes, the maculae (except the macula neglecta) are overlain by numerous tiny otoconia embedded in a gelatinous/fibrous matrix (Tester et al., 1972). These otoconia can be exogenous (sand grains) and enter the ear via the endolymphatic duct (see Casper, 2011) and/or endogenous and can be made of calcite, aragonite, vaterite, or calcium carbonate monohydrate in elasmobranchs or solely of aragonite in chimaeras (Carlström, 1963; Gauldie et al., 1987; Mulligan and Gauldie, 1989; Mulligan et al., 1989).

In lungfishes, the single "otolith" (*Protopterus*, Platt et al., 2004) or the "lapillus" and "sagitta" (*Neoceratodus*, Gauldie et al., 1986a) consist of a firm aggregation of aragonitic and calcitic otoconia (Carlström, 1963; Gauldie et al., 1986a). The *Latimeria* ear apparently contains only one large calcitic-aragonitic "saccular otolith" (Carlström, 1963; Rosauer and Redmond, 1985).

Non-teleost actinopterygians have both otoliths and otoconia that overlie the maculae of the otolith end organs (Carlström, 1963; Popper and Northcutt, 1983; Mathiesen and Popper, 1987; Lychakov, 1995). In sturgeons otoliths and otoconia are made up of vaterite. In bichir, bowfin and gar, however, otoliths are aragonitic whereas otoconia are vateritic (Carlström, 1963; Rosauer and Redmond, 1985).

In teleosts, the maculae of the otolith end organs are each overlain by a single massive calcium carbonate biomineralisate, the otolith that apposes material according to a daily rhythm (Pannella, 1971). The otoliths of the utricle and saccule are composed of aragonite, while the lagenar otolith consists of vaterite. Calcite is only rarely found in otoliths (e.g., Gauldie, 1993; Oliveira and Farina, 1996). The simultaneous presence of otoliths and (aragonitic) otoconia in teleosts has been reported for only a few species (Gauldie et al., 1986b). In contrast to the tiny otoconia in non-teleost fishes, otoliths—especially that of the saccule—possess a species-specific shape (e.g., Nolf, 1985). The effects of different shapes on otolith motion relative to the macula are still widely unknown (Popper et al., 2005). The few experimental and theoretical studies, however, indicate that otolith motion differs depending on its shape and is more complex than just a simple forth and backward movement (Sand and Michelsen, 1978; Krysl et al., 2012).

Macula Diversity: Macula Shape and Orientation Patterns of Ciliary Bundles

Generally, the maculae of the otolith end organs are separated. In lungfishes, the macula sacculi and macula lagenae form a continuum, the so-called sacculolagenar macula in which two regions of high hair cell densities (striolas) are separated by areas of lower hair cell densities (**Figures 1C**, **6C**; Platt et al., 2004). Such a sacculolagenar macula is unique among bony fishes. Holocephali apparently possess a similar joint sacculolagenar macula (de Burlet, 1934; Ladich and Popper, 2004; see also illustration of the holocephalid *Chimaera monstrosa* by Retzius, 1881), but recent detailed studies underpinning this assumption are lacking. Overall, data about orientation patterns of the maculae are scarce for the coelacanth *Latimeria* (Platt, 1994) as

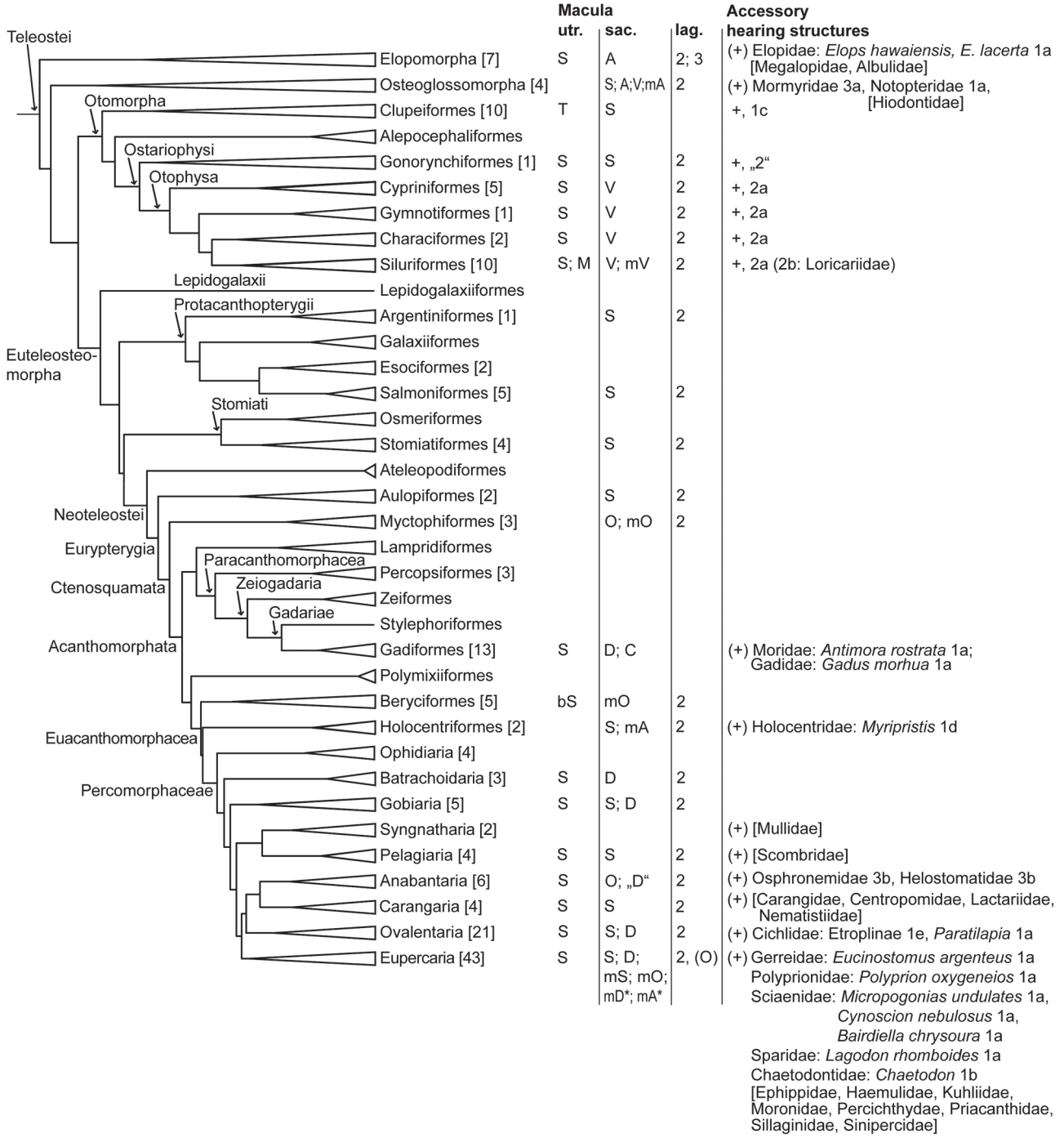

FIGURE 4 | Overview of orientation patterns of ciliary bundles on the maculae and diversity of accessory hearing structures in different teleost groups. The phylogeny was modified from Betancur et al. (2013), Broughton et al. (2013), and the DeepFin Project (2003-2009). For a more detailed overview and list of references see Schulz-Mirbach and Ladich (2016, Table 2). Family names in brackets indicate taxa which possess accessory hearing structure but for which ears and/or hearing abilities have not yet been studied (for details about accessory hearing structures of these taxa see Braun and Grande, 2008). Numbers given in brackets indicate the number of species studied with respect to inner ear morphology. Macula utriculi: S, standard pattern, i.e., radially oriented ciliary bundles on the cotillus and opposing ciliary bundles in the striola region; T, tripartite macula consisting of a macula anterior, macula media and a macula posterior with all three parts showing an opposing pattern of ciliary bundles; M, modified macula shape in *Ariopsis felis,* the macula forms a narrow band around the utricular otolith, no ventral macula part (cotillus) is present; bSt, in Melamphaidae the macula displays a bilobate striola region. Macula sacculi (see also **Figure 8**): S, standard pattern; D, dual pattern; A, alternating pattern; O, opposing pattern; V, 2 vertical groups; modified patterns: mS, in *Arothron hispidus*, 1 vertical group in the anterodorsal part; mA, in *Chitala chitala* (**Figure 7E**) and *Myripristis murdjan* (**Figure 7G**); mD*, as found in *Comephorus baicalensis*, *Cottocomephorus alexandrae*, and *Paracottus knerii* (lake Baikal; Sapozhnikova et al., 2007); mA* as found in *Comephorus dybowski* (lake Baikal, Sapozhnikova et al., 2007); mO, in Myctophidae, Melamphaidae and

(Continued)

FIGURE 4 | Continued
Bairdiella chrysoura (**Figure 7H**); mV, in *Bunocephalus coracoideus* and *Acanthodoras spinosissimus,* 4 vertical groups in the anterior part and 2 vertical groups on the remaining macula; C, complex pattern in *Antimora rostrata* (**Figure 7F**). Macula lagenae: Numbers indicate the number of orientation groups; O, opposing groups of ciliary bundles; V, vertical ciliary bundle orientation. In most species only numbers are given because orientations of ciliary bundles vary to different degrees in a gradual manner along the macula from horizontal, oblique to vertical directions. Types of accessory hearing structures (see **Figure 12** for types 1-3): 1a-b, anterior swim bladder extensions approach or abut skull in region of ear; 1c, anterior swim bladder extension penetrates skull, contacting the utricle; 1d, anterior swim bladder extension penetrates skull, contacting the saccule; 1e, anterior swim bladder extension penetrates skull, complex etropline type; "2," Protoweberian coupling?; 2a-b, otophysic connection via Weberian apparatus; 3a, anterior part of swim bladder extension penetrates skull but is separated from the main swim bladder; 3b, suprabranchial chamber close to ear. For morphological details in *Polyprion oxygeneios* see Caiger et al. (2013). Additional laterophysic connections: 1b-c, 2b.

well as for cartilaginous fishes (Lowenstein et al., 1964; Barber and Emerson, 1980; Lovell et al., 2007). Most studies on macula morphology in Chondrichthyes focused on the macula neglecta in Elasmobranchii (Corwin, 1981, 1989; Myrberg, 2001; Casper, 2011). It therefore remains unclear if cartilaginous fishes show variability not only with regard to gross inner ear morphology (Evangelista et al., 2010) and the macula neglecta but also with regard to the maculae of the otolith end organs. Moreover, to our knowledge data on the macula morphology in Holocephali is completely lacking (see Lisney, 2010).

Macula utriculi

Of the three "otolithic" maculae, the macula utriculi is the most conservative one not only across fishes but also across vertebrates in general (see e.g., **Figures 5B,D,E**; Platt and Popper, 1981a). The macula is bowl shaped displaying (1) the main body—namely the cotillus, which lies on the ventral floor of the utricle—and shows radially oriented ciliary bundles, (2) a striola region in the anterior part, displaying two groups of ciliary bundles with opposing ("face-to-face") orientation and (3) in some taxa an anterolateral element, the lacinia (**Figures 5A,D,E,H,I**; Platt and Popper, 1981a).

Among fishes, some exceptions to this shape and orientation pattern of the macula utriculi are found. In cartilaginous fishes, for example, the studies by Lowenstein et al. (1964) and Barber and Emerson (1980) indicated that ciliary bundles with opposing orientation are interspersed in the radial orientation pattern of ciliary bundles on the cotillus (**Figure 5A**). In addition, the macula utriculi of the lesser spotted dogfish *Scyliorhinus canicula* seems to lack a striola region (Lovell et al., 2007).

Modified maculae utriculi are also found in the non-teleost actinopterygian shovel nose sturgeon (*Scaphirhynchus platorynchus*), which displays a half-moon shaped macula lacking a lacinia (**Figure 5C**; Popper, 1978) or in the ariid catfish *Ariopsis felis*, whose macula utriculi is reduced to a ribbon-like structure lacking a cotillus and which curves around the exceptionally large utricular otolith like an equatorial band (**Figure 5G**; Popper and Tavolga, 1981). Further modifications of the macula utriculi in teleosts relate to the striola region, which is uniquely bilobate in Melamphaidae (deep-sea fishes; **Figure 5I**; Deng et al., 2013). In the cichlid *E. maculatus* the lacinia is exceptionally large and three-dimensionally curved (**Figure 5H**; Schulz-Mirbach et al., 2014).

The most derived macula utriculi characterizes the whole order Clupeiformes (see Platt and Popper, 1981a,b). The unique tripartite macula (**Figure 5F**; Popper and Platt, 1979; Platt and Popper, 1981b; Higgs et al., 2004) is in part (middle and posterior macula) overlain by an also highly modified utricular otolith (Wohlfahrt, 1936; O'Connell, 1955). This otolith has a tetrahedral shape and thin extensions in anterolateral and ventral directions instead of the "stone-like" appearance present in most teleosts (Wohlfahrt, 1936; Assis, 2005).

Macula sacculi

The macula sacculi in cartilaginous fishes is elongate without a distinction into a wider ostial and a narrower caudal macula region that is otherwise typical of many teleost species. Mainly two vertical groups of ciliary bundles are present. In the anterior portion these vertically oriented bundles are brought into a new horizontal orientation by upwards curving of the macula in this region (**Figure 6B**; Lowenstein et al., 1964; Corwin, 1981; Lovell et al., 2007; but see **Figure 6A**; Barber and Emerson, 1980).

A similar transition from a ventral to a more horizontal orientation pattern of ciliary bundles in the anterior macula region is also characteristic in non-teleost actinopterygians (Popper and Fay, 1993). In these fishes, the macula sacculi is hook shaped (*Polypterus bichir*; **Figure 6D**) or has a hook-shaped anterior part (**Figures 6E–H**). In the anterior portion, ciliary bundle orientation follows the curvature of the closest macula margin, thereby creating horizontal groups. In the bowfin *Amia calva* (**Figure 6G**), the anterior portion of the macula sacculi has a distinct 3D curvature bringing the ciliary bundles in a new spatial orientation (Popper and Northcutt, 1983).

In teleosts, five main orientation patterns have been described (**Figure 7**; Popper and Coombs, 1982). Four of them show vertical and "true" horizontal orientation groups and are termed standard, dual, opposing, or alternating patterns; the fifth pattern type is characterized by vertical orientation groups only (Popper and Coombs, 1982). The standard (**Figures 7B,I,J**) and the dual patterns are mainly typical of species that lack accessory auditory structures (Platt and Popper, 1981b; Popper and Coombs, 1982) or in which these structures are not connected to the saccule; the standard pattern of the macula sacculi, for example, is found in clupeiform fishes (**Figure 7B**), whereas a highly modified macula utriculi in these fishes (**Figure 5F**) is associated with the connection of the gas bladder to the utricle (e.g., Denton and Gray, 1979; Platt and Popper, 1981b). In contrast, some species whose accessory auditory structures approach the saccule show a dual pattern such as the cichlid *E. maculatus* (Schulz-Mirbach et al., 2014) or the standard pattern like some sciaenid species (**Figures 7I,J**). In some teleost groups, however, the presence of accessory auditory structures correlates with

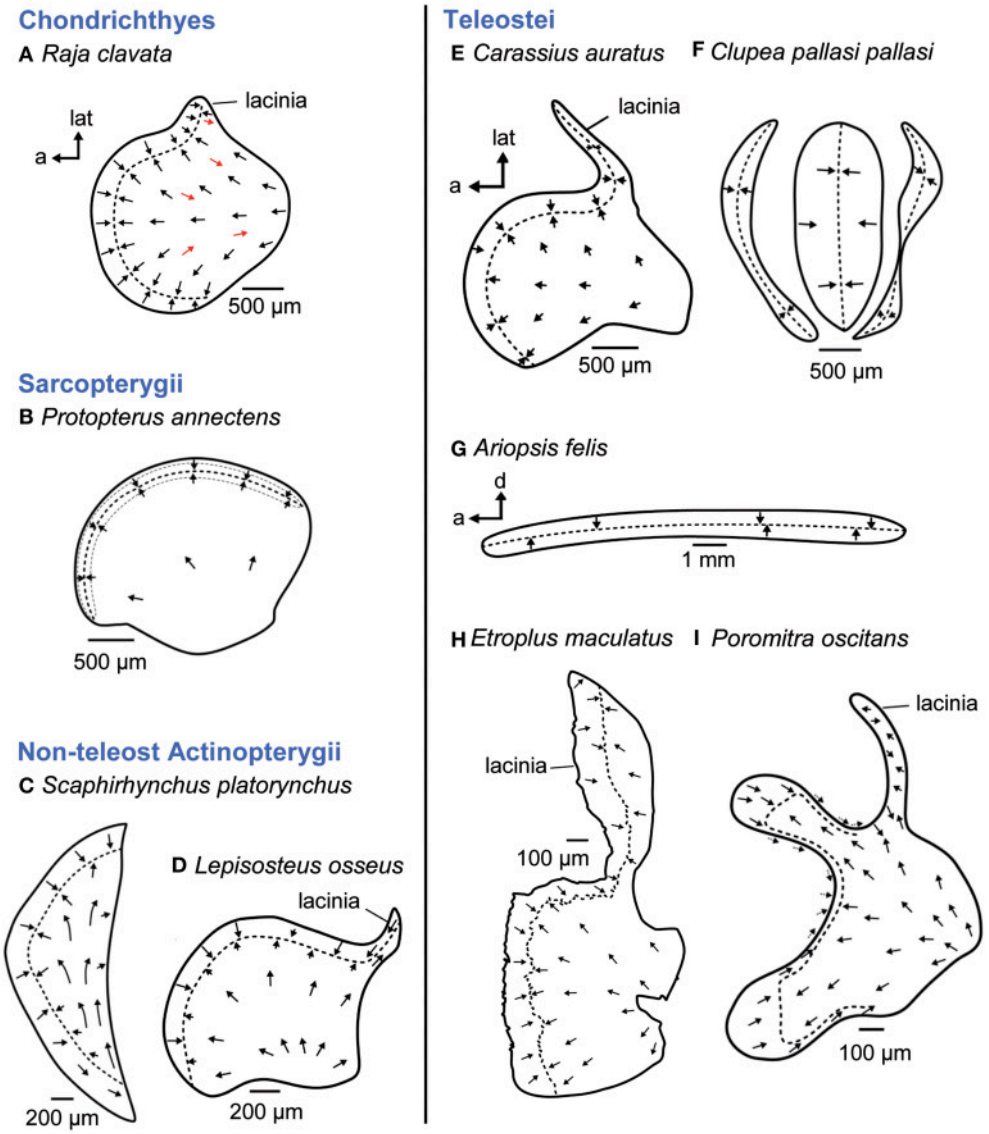

FIGURE 5 | Overview of the diversity of macula shape and the orientation patterns of ciliary bundles on the macula utriculi. Across most fish taxa **(B, D, E)** and even vertebrates in general, the shape and orientation pattern of the macula utriculi is conserved; it has a bowl shape often including a lacinia and the typical radial orientation of the ciliary bundles on the cotillus as well as opposing (face-to-face) orientated ciliary bundles in the striola region (= region around the stippled line). Cartilaginous fishes, however, display some reversely oriented ciliary bundles on the cotillus (**A**, see red arrows), and a half-moon shaped macula lacking a lacinia is present in sturgeons **(C)**. In teleost fishes, the goldfish **(E)** shows the conservative shape and orientation pattern (see also **D**). Modifications thereof are found in clupeiform fishes (**F**, tripartite macula) or ariid catfishes, whose ribbon-like macula lacks the cotillus **(G)**. The cichlid *Etroplus maculatus* **(H)** has a distinctly enlarged lacinia, while enlargement of the striola region results in a bilobate shape in melamphiid fishes **(I)**. The maculae in **(E–H)** stem from species that possess accessory hearing structures. Illustrations modified from Barber and Emerson (1980), Deng et al. (2013), Mathiesen and Popper (1987), Platt (1977), Platt et al. (2004), Popper (1978), Popper and Platt (1979), Popper and Tavolga (1981), and Schulz-Mirbach et al. (2014). a, anterior; d, dorsal; lat, lateral.

modified orientation patterns. Examples include Notopteridae and Mormyridae (both Osteoglossiformes) or otophysans, which have highly modified maculae sacculi, displaying the vertical pattern in mormyrids (**Figure 7A**) and otophysans (**Figure 7C**) or a complex trilobate macula sacculi with a modified alternating pattern in the Clown knifefish *C. chitala* (Notopteridae; **Figure 7E**).

Members of deep-sea fishes (Myctophidae, Bregmacerotidae, Macrouridae, Moridae, Gadidae, Melamphaidae, Opisthoproctidae, Gonostomatidae, Melanocetidae, or Holocentridae) show some of the most remarkable modifications, especially with respect to the maculae (Popper, 1977, 1980; Deng, 2009; Deng et al., 2011, 2013). Several species are marked by complex ("unique") orientation patterns on the macula sacculi (**Figures 7F,G**) and also possess accessory auditory structures such as anterior swim bladder extensions, for example in *A. rostrata* (Deng et al., 2011) and species of the genus *Myripristis* (Nelson, 1955; Popper, 1977).

Given the diversity of orientation patterns on the teleost macula sacculi the question arises what the macula sacculi looked like in the ancestor of the teleosts (Popper and Fay, 1993). Tetrapods have only two "vertical" groups on the macula sacculi and this may also hold true for cartilaginous fishes, non-teleost actinopterygians and lungfishes: the horizontal groups in these fishes are classified to be no "true" horizontal groups because originally vertically oriented ciliary bundles simply follow the curvature of the closest macula margin, gradually leading to an increased horizontal-like orientation (**Figure 8**; Popper and Platt, 1983, Popper and Fay, 1993). Two alternative hypotheses have been discussed (Popper and Platt, 1983). First, the vertical pattern is an ancestral pattern that was retained in otophysans and mormyrids, whereas in the remaining teleosts true horizontal groups evolved at least seven times independently. The second hypothesis assumes that the ancestral teleost condition is the pattern including vertical and horizontal groups and that horizontal groups were lost twice, in otophysans and mormyrids. If the second hypothesis applies—which is the more parsimonious one—the vertical pattern in otophysans and mormyrids may have convergently evolved due to similar selection pressures (Popper and Platt, 1983). The vertical pattern is the constant element in each of the five different orientation patterns on the macula sacculi in teleosts (Popper, 1981), and the vertical pattern is also found in Chondrichthyes, lungfishes, and non-teleost actinopterygians (see above; Popper and Fay, 1977; 1993). Accordingly, it may further be assumed that the vertical pattern on the macula sacculi is the basic vertebrate pattern on this sensory epithelium (Mathiesen and Popper, 1987): it did not experience diversification—including the "invention" of true horizontal groups—before the emergence and diversification of the teleosts.

The five orientation groups can be derived from one another if one either adds two or three horizontal groups to the vertical pattern (resulting in the standard or the alternating pattern) or removing the horizontal groups, leading to the vertical pattern (**Figure 8**). From the standard pattern (1) the dual pattern can be obtained by adding two horizontal groups in the posterior portion and (2) the opposing pattern can be created by bending the anterior macula downwards in ventral direction while ciliary bundles retain their horizontal orientation in this area. Alternatively, the standard pattern can emerge from an alternating pattern when one (the most anterodorsal) horizontal group is lost. Only genetic studies could unravel how orientation groups form during ontogeny, leading to the different orientation patterns. Knowledge about underlying genetic processes of pattern formation is increasing (Duncan and Fritzsch, 2012; Sienknecht et al., 2014) and is likely to shed new light on the evolution of different orientation patterns in different lineages.

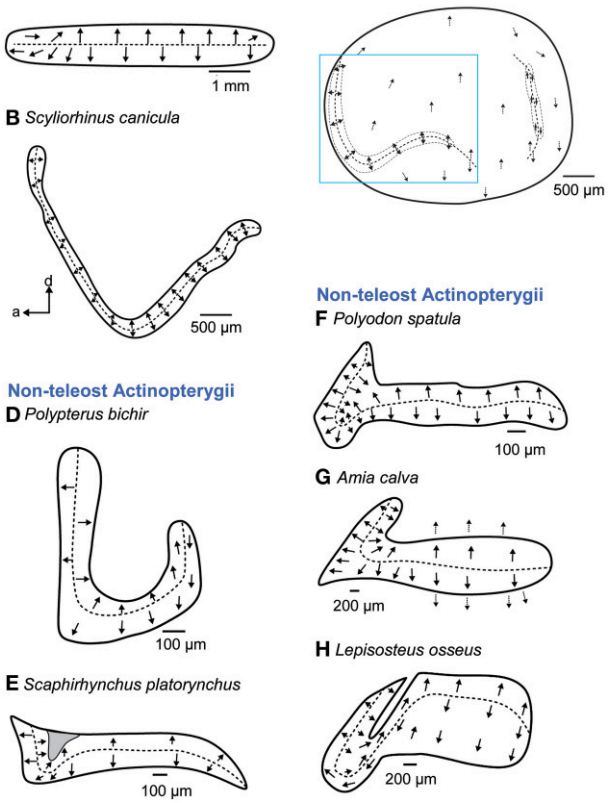

FIGURE 6 | Overview of the diversity of macula shape and the orientation patterns of ciliary bundles on the macula sacculi in non-teleost fishes. The macula sacculi in non-teleost fishes shows two main orientation groups of ciliary bundles which are brought into a horizontal orientation by upwards curving of the anterior macula portion **(B–H)** except in rays **(A)**. The shaded gray area in **(E)** depicts a special area of supporting cells. Arrows around the saccular and lagenar striola regions in **(C)** and around the macula in **(G)** indicate the orientation of ciliary bundles in regions with low densities of sensory hair cells. The rectangle in C indicates the striola region of the macula sacculi. Illustrations modified from Barber and Emerson (1980), Lovell et al. (2005, 2007), Mathiesen and Popper (1987), Platt et al. (2004), Popper (1978), and Popper and Northcutt (1983). a, anterior; d, dorsal.

Macula lagenae

In cartilaginous fishes (**Figures 9A–B**), sarcopterygians (**Figure 9C**; Platt, 1994; Platt et al., 2004), non-teleost actinopterygians (**Figures 9D–H**; Popper, 1978; Popper and Northcutt, 1983; Mathiesen and Popper, 1987; Lovell et al., 2005), and teleosts (**Figures 10A–H**; for an overview see Platt and Popper, 1981b), the macula lagenae is crescent or half-moon shaped and contains two main orientation groups. In rays, these differently orientated ciliary bundles are less strictly organized into two separate groups (**Figure 9A**; Barber and Emerson, 1980; Lowenstein et al., 1964), whereas sharks seem to show two distinct groups on their macula lagenae (**Figure 9B**; Lovell et al., 2007). In contrast to bony fishes, the few studies on the macula lagenae in cartilaginous fishes (Barber and Emerson, 1980; Lovell et al., 2007) indicate that the posterior "orientation group" on the macula shows ciliary bundles oriented in anterodorsal direction while in bony fishes ciliary bundles of the posterior orientation group mainly point in posteroventral direction (compare **Figures 9A,B** with **Figures 9C–E, G–H, 10A,C,E–H**).

Non-teleost actinopterygians show a considerable diversity in the shape of the macula lagenae (**Figures 9D–H**). The macula

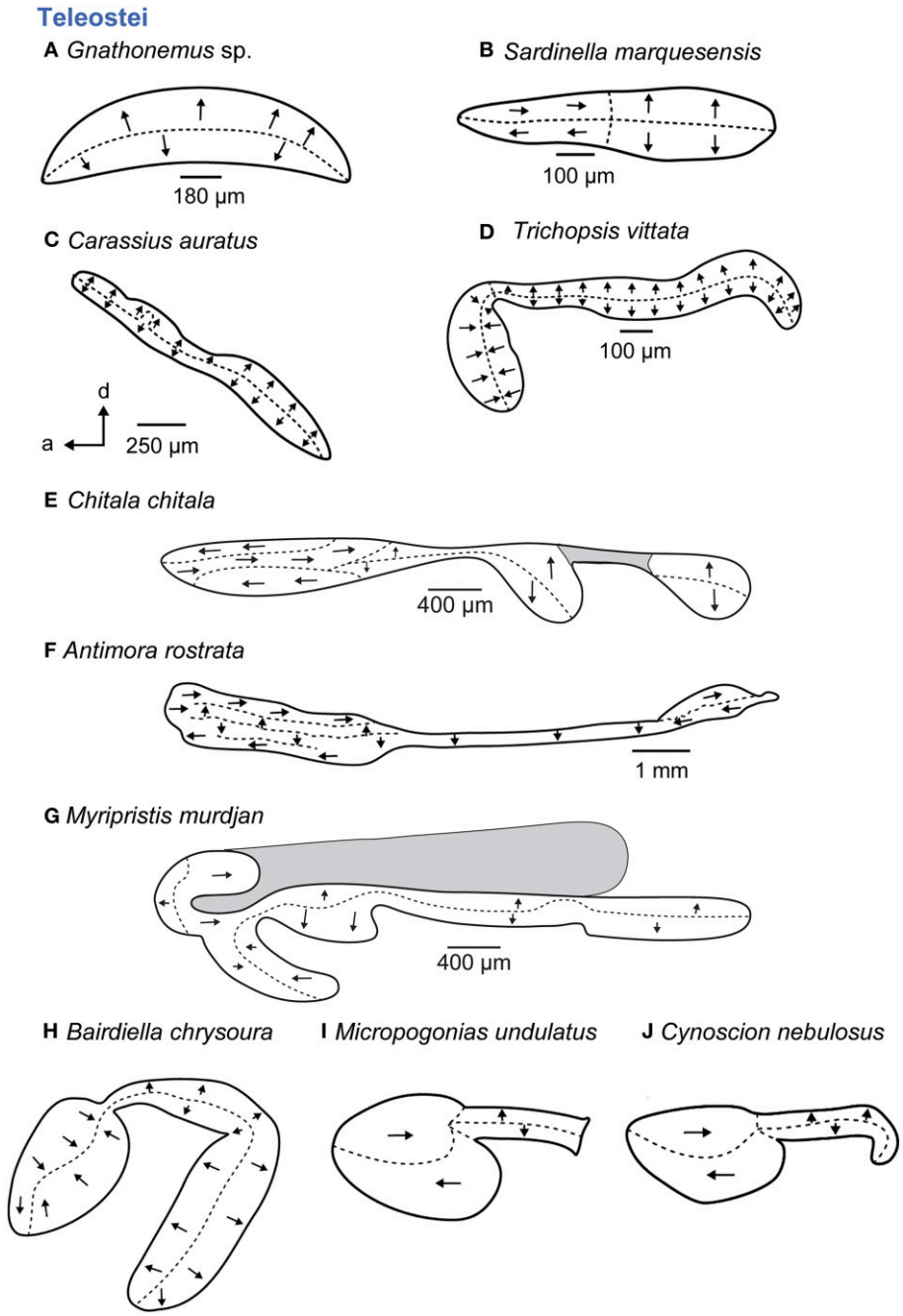

FIGURE 7 | Overview of the diversity of macula shape and the orientation patterns of ciliary bundles on the macula sacculi in teleost fishes. The shaded gray areas in **(E,G)** depict special areas of supporting cells. All maculae stem from species that possess accessory hearing structures **(A–J)**. For the maculae in **(H–J)** no scale bars were given in the original publications (Ramcharitar et al., 2001, 2004). Illustrations modified from Coombs and Popper (1982), Deng et al. (2011), Ladich and Popper (2001), Platt (1977), Popper (1977, 1981), Platt and Popper (1981a), Ramcharitar et al. (2001, 2004), and Platt et al. (2004). a, anterior; d, dorsal.

lagenae is almost as large as or even larger than the macula sacculi (except in *Amia*), which contrasts the condition in many teleost species (Platt and Popper, 1981a; Ladich and Popper, 2004). In addition, *Amia calva* exhibits a striola-like region that resembles that of the utricular maculae (Popper and Northcutt, 1983), and *Lepisosteus osseus* displays three instead of two orientation groups (Mathiesen and Popper, 1987). Three groups are also found in some members of the Elopomorpha (*Anguilla anguilla*; **Figure 10D**; Mathiesen, 1984), especially in some deep-sea elopomorphs (Buran et al., 2005) or the chaetodontid *Chaetodon miliaris* (Popper, 1977); but in these teleosts the third orientation group is restricted to a very narrow band at the posterior margin of the macula lagenae (Mathiesen and Popper, 1987).

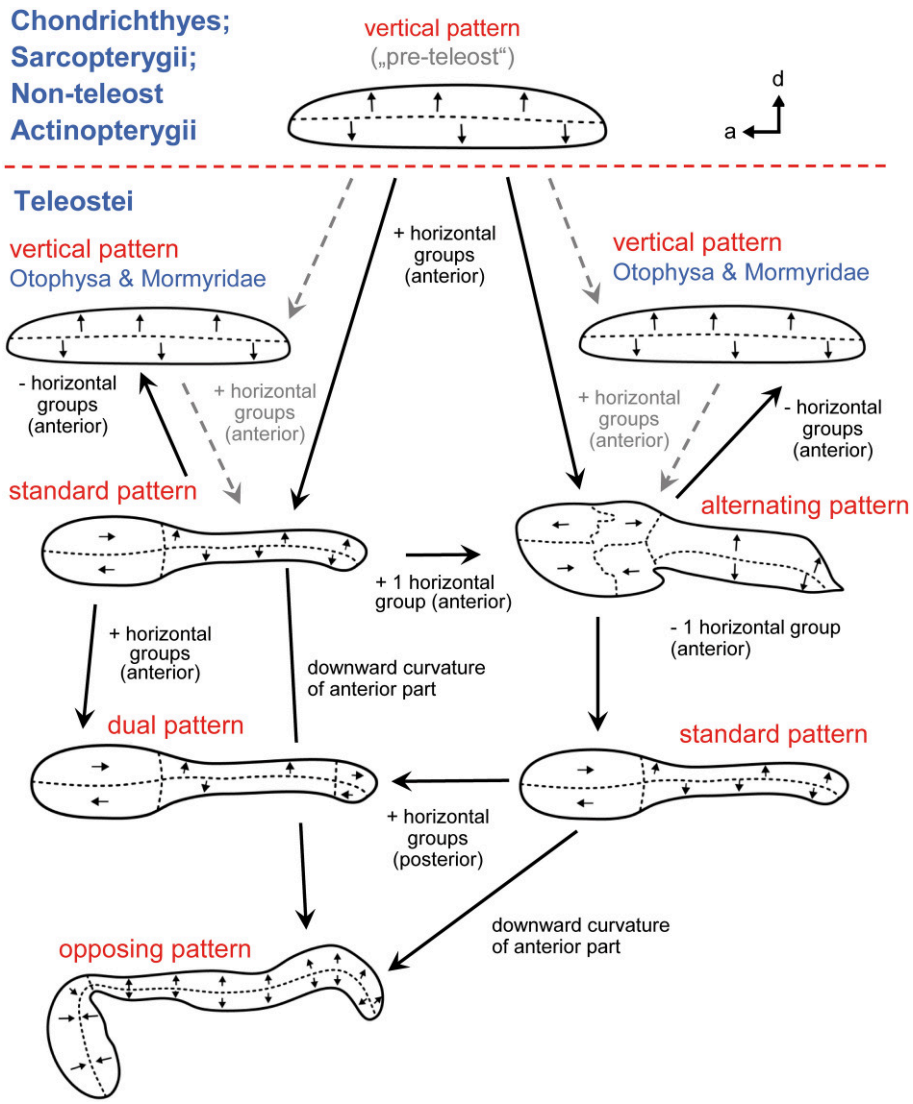

FIGURE 8 | The main ciliary bundle orientation patterns on the macula sacculi in teleosts and how the patterns may be derived from one another (see also Popper and Fay, 1993). Arrow tips point in the direction of the kinocilia, indicating the orientation of the ciliary bundles in the respective area; the dashed lines separate different orientation groups. Addition of two or three horizontally oriented groups of ciliary bundles results in the standard or alternating patterns, respectively. From the standard pattern the dual pattern can be derived by adding horizontal groups in the posterior region; in the opposing pattern the anterior macula portion is ventrally bent while the orientation of the horizontal groups is retained. The standard pattern may also be obtained by removing one horizontal group from the alternating pattern. The vertical patterns in otophysans and mormyrids may be derived by removing the horizontal groups from the standard or the alternating patterns. The five patterns are modified from Popper and Coombs (1982) and Popper and Schilt (2008). a, anterior; d, dorsal.

Some teleost taxa with accessory auditory structures such as mormyrids (Popper, 1981), otophysans (e.g., Popper and Platt, 1983), and the cichlid *E. maculatus* (Schulz-Mirbach et al., 2014) possess a large macula lagenae that may be even larger than the maculae sacculi (Popper et al., 2003). In addition, the maculae lagenae of otophysans tend to be oriented more along the antero-posterior axis than stretching along a dorso-ventral or posteroventral to anterodorsal axis (compare **Figures 10A,C,G** with **Figures 10E,H**).

Macula neglecta

In cartilaginous fishes a macula neglecta is always present. It contains one patch with "randomly" orientated ciliary bundles in benthic species (**Figure 11A**) or two patches with a preferred orientation on each of the patches in more pelagic species (**Figure 11B**; Corwin, 1981; 1989; Myrberg, 2001). In bony fishes, the macula neglecta—if present—is smaller than in cartilaginous fishes (Corwin, 1989). *Latimeria* (Fritzsch, 1987) and lungfishes (maybe except *Neoceratodus*) possess a macula neglecta: it is a single patch in *Protopterus*, with ciliary bundles uniformly oriented along the antero-posterior axis (**Figure 11C**; Platt et al., 2004). In non-teleost actinopterygians and teleosts possessing a macula neglecta, it consists of two patches with a preferred orientation of ciliary bundles on each patch (**Figures 11D–F**; Platt, 1977; Mathiesen, 1984; Mathiesen and Popper, 1987). Thus, if a macula neglecta is present in

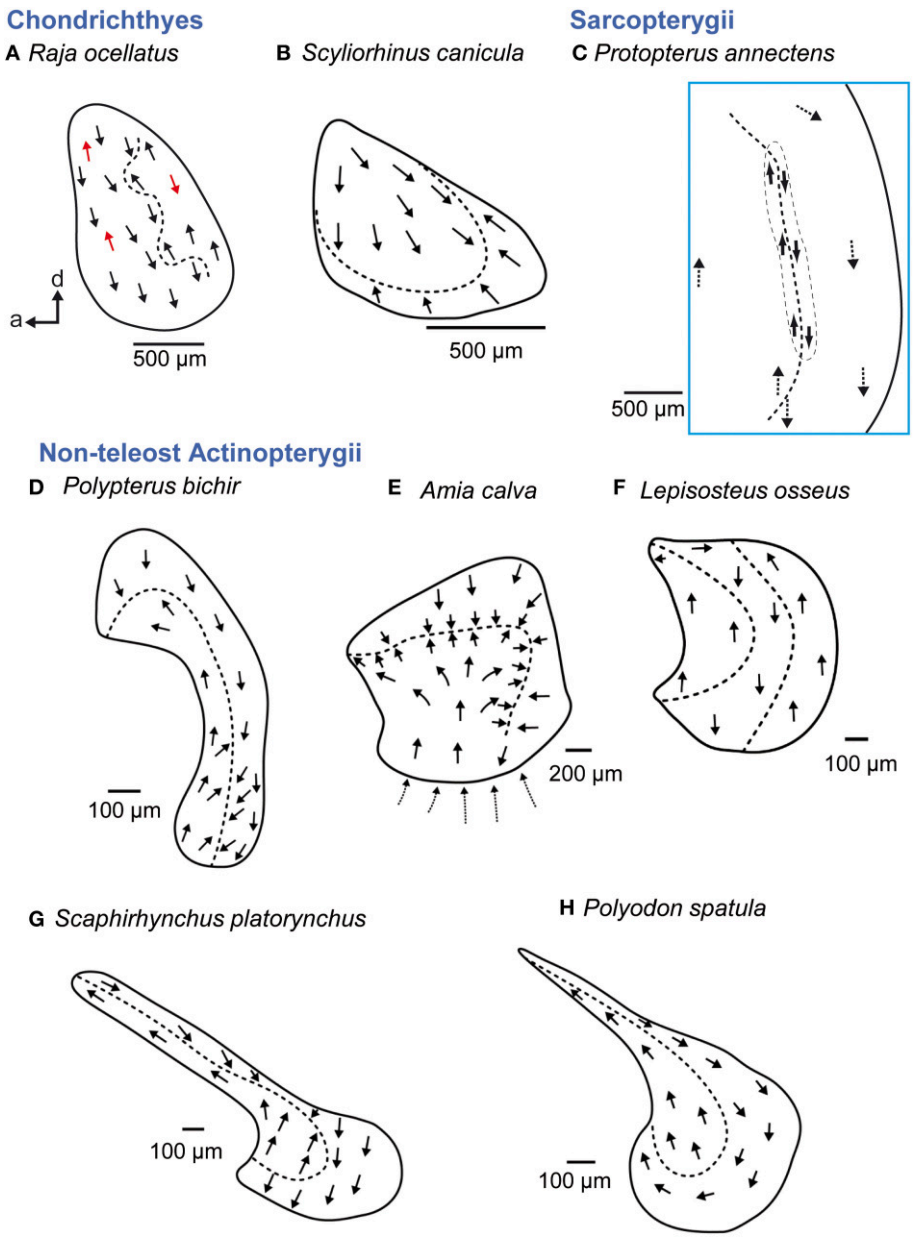

FIGURE 9 | Overview of the diversity of macula shape and the orientation patterns of ciliary bundles on the macula lagenae in non-teleost fishes. The macula lagena is characterized by two main orientation groups of ciliary bundles **(B–E, G–H)** except in rays **(A)**, whose ciliary bundles show opposing directions across the whole macula (indicated by red arrows) or in gar **(F)**, which have three orientation groups on the macula. Arrows around the saccular and lagenar striola regions in **(C)** and around the macula in **(E)** indicate the orientation of ciliary bundles in regions with low densities of sensory hair cells. Illustrations modified from Popper (1978), Barber and Emerson (1980), Popper and Northcutt (1983), Mathiesen and Popper (1987), Platt et al. (2004), and Lovell et al. (2005, 2007). a, anterior; d, dorsal.

Actinopterygii, the macula structure and orientation patterns seem to be constant across different species (Mathiesen and Popper, 1987).

Interestingly, the macula neglecta in elasmobranchs is located in the canal duct of the posterior semicircular canal dorsal to the saccule (e.g., Corwin, 1989; Casper, 2011), whereas it is situated in the posterior part of the utricle near the common crus in holocephalans and actinopterygians (Maisey, 2001).

Accessory Hearing Structures and Auditory Sensitivities

Fishes possess a large variety of gas-filled cavities within the body and the swim bladder is certainly the most widespread among these. Swim bladders primarily help to generate the buoyancy necessary for fishes to hover at particular water depths (Alexander, 1966). Only cartilaginous fishes (sharks, rays, chimaeras) and bottom-dwelling fishes such as flatfish or sculpins lack swim bladders. These groups therefore lack

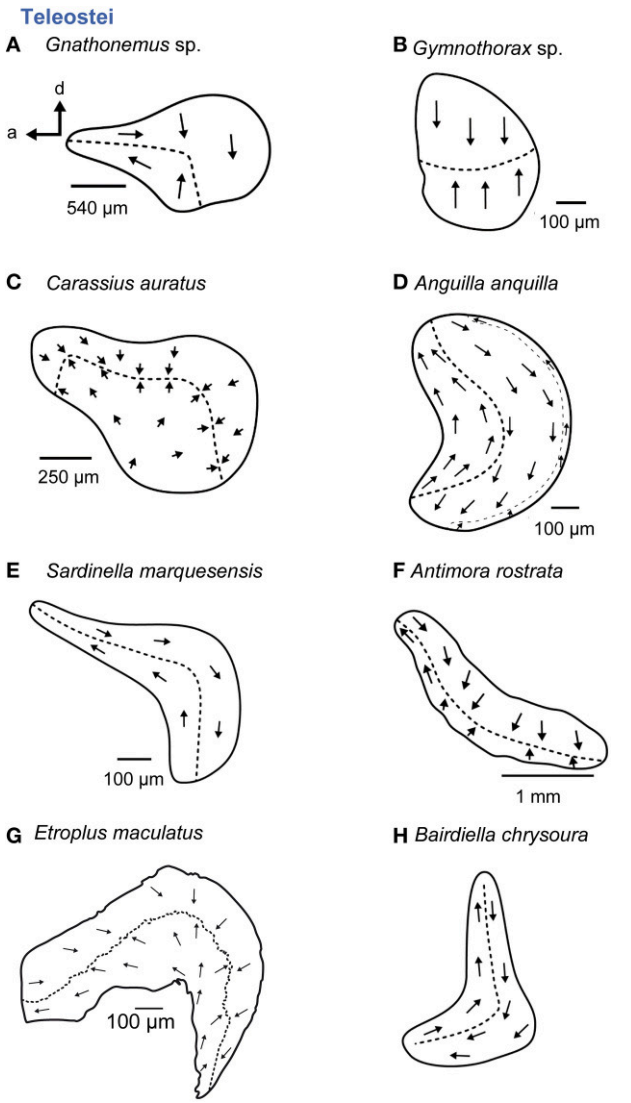

FIGURE 10 | Overview of the diversity of macula shape and the orientation patterns of ciliary bundles on the macula lagenae in teleost fishes. The macula lagena is characterized by two main orientation groups of ciliary bundles **(A–C, E–H)** with exception of the eel **(D)** having three orientation groups on the macula. **(A,C, E–H)** These maculae stem from species that possess accessory auditory structures. For the macula in P no scale bar was given in the original publication (Ramcharitar et al., 2004). Illustrations modified from Platt (1977), Popper (1979; 1981), Platt and Popper (1981a), Mathiesen (1984), Ramcharitar et al. (2004), and Deng et al. (2011) and Schulz-Mirbach et al. (2014). a, anterior; d, dorsal.

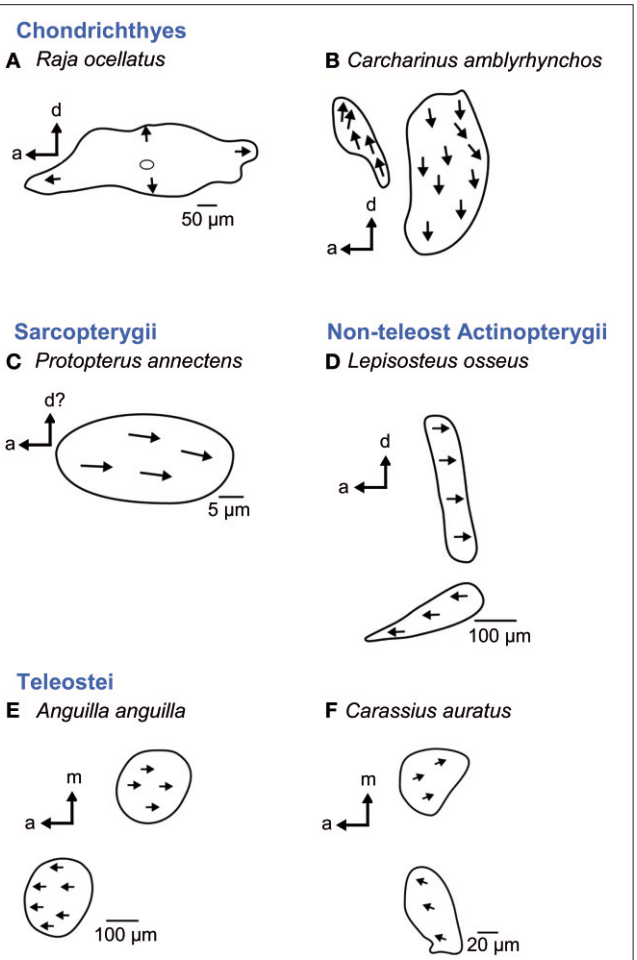

FIGURE 11 | Overview of the diversity of the macula neglecta in cartilaginous (A–B) and bony fishes (C–F). This macula consists of either one patch **(A, C)** or two patches **(B, D–F)**. Ciliary bundles show no preferred orientation **(A)** or are oriented in one direction on the patch **(C)** or on either patch if two patches are present **(B, D–F)**. Note that if a macula neglecta is present in Actinopterygii, the macula structure and orientation patterns seem to be constant across different species **(D–F)**. For the macula in **B** no scale bar was given in the original publication (Corwin, 1989). Illustrations modified from Platt (1977), Mathiesen (1984), Barber et al. (1985), Mathiesen and Popper (1987), Corwin (1989), and Platt et al. (2004). a, anterior; d, dorsal; m, medial.

a pressure-to-particle motion transducer, which limits their hearing sensitivities accordingly (**Figure 12E**). Nevertheless, experimental studies on the lemon shark *Negaprion brevirostris* indicate that sharks may detect sound in parallel in two different ways, giving them more directional information. The non-otolithic channel enables detecting sound directly via loose tissue covering dorsal openings in the skull (parietal fossa) and stimulating the macula neglecta. The otolithic channel enables sound detection indirectly via relative motion between the otoconial mass and ciliary bundles in the saccule (Corwin, 1981, 1989).

All taxa possessing gas-filled cavities may utilize these for hearing (Popper and Fay, 2011). There are three main ways to connect the gas bladder directly to the inner ear (**Figures 12A–C**) and to detect sound pressure. Pressure detection may even take place in the absence of such a direct connection, most likely because tissues between the bladder and inner ear transmit bladder oscillations (e.g., Myrberg and Spires, 1980; see Section Cichlidae; **Figure 12D**).

Otophysan Fishes

The Weberian apparatus connecting the swim bladder to the inner ears characterizes the Otophysa, which comprise four

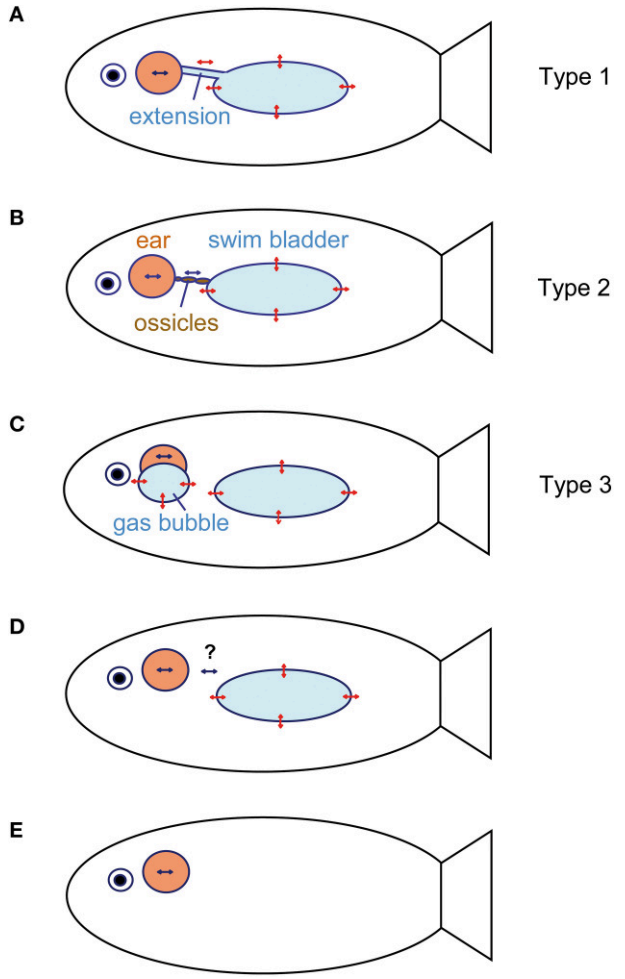

FIGURE 12 | Schematic relationship between the inner ears and accessory hearing structures that enhance hearing in fishes. (A) Anterior swim bladder extensions (see e.g., *Etroplus maculatus*, *Myripristis* sp., or *Chitala chitala*). The extensions may bear an anterior enlargement such as in clupeids. **(B)** Direct connection between the swim bladder and inner ears via a chain of ossicles (Weberian ossicles), which transmits swim bladder vibrations to the ear in otophysans. **(C)** Air-filled cavities directly attached to the inner ears without connection to the swim bladder (mormyrids and labyrinth fishes). **(D)** No connection between a gas bladder (swim bladder, lungs) and inner ear. In the latter, the bladder may (lungfish, damselfish) or may not (toadfish) have an auditory function (see question marks). **(E)** No gas-filled cavity (swim bladder) and subsequently no accessory structure to improve hearing (sharks, flatfishes, sculpins). Double-headed red arrows indicate oscillations of gas bladder walls due to sound pressure fluctuations in a sound field. Blue arrows indicate particle motion within the inner ear endolymph due to the movement of the entire fish in the sound field. This particle motion may be enhanced by additional oscillations of air-filled cavities connected in various ways to the inner ear. Modified from Ladich and Popper (2004) and Ladich (2016).

orders with approximately 8000 species. Otophysans possess a chain of 1-4 Weberian ossicles that function in analogy to the middle ear bones in mammals and transmit vibrations of the anterior swim bladder wall (which can be regarded as an "internal tympanum") to the inner ears (**Figure 12B**). These ossicles were first described by Weber almost 200 years ago, who postulated that they conduct sounds from the swim bladder to the ears (**Figure 13A**; Weber, 1819, 1820).

Several experimental studies which either filled the swim bladder with fluids or removed its gas or which extirpated the tripus—the largest Weberian ossicle—showed a drop in hearing sensitivity, thereby underpinning Weber's hypothesis of sound conduction via the Weberian ossicles (von Frisch and Stetter, 1932; Poggendorf, 1952; Fay and Popper, 1974). Ladich and Wysocki (2003) demonstrated that bilateral extirpation of the tripus in the goldfish *Carassius auratus* resulted in a decline in hearing sensitivity of 7 dB at 100 Hz up to 33 dB at 2 kHz and a loss of detection of frequencies above 2 kHz (**Figure 13B**). Unilateral tripus extirpation did not result in any hearing loss, which is easily explained by the fact that both chains of Weberian ossicles transmit swim bladder oscillations to an unpaired perilymphatic sinus (see **Figure 13A;** Ladich, 2014a).

Otophysans do not exhibit a standard morphology of swim bladders and Weberian ossicles as illustrated by von Frisch and Stetter (1932) (**Figure 13A**) but a large diversity, especially in siluriforms (Chranilov, 1927, 1929; Alexander, 1962, 1964; Chardon, 1968; Lechner and Ladich, 2008). Members of numerous catfish families have large unpaired and free swim bladders and one up to four ossicles. In contrast, several groups have tiny and paired swim bladders located directly behind the cranium (**Figure 13C**). These tiny bladders are surrounded by bony capsules formed by the skull and anterior vertebrae (Chranilov, 1929). The small size of these bladders indicates that they no longer function as buoyancy organs but were most likely retained for hearing purposes (Lechner and Ladich, 2008).

How do these differences in swim bladder size and Weberian ossicle number affect hearing in catfishes? Ladich (1999) observed that members of the families Pimelodidae and Doradidae are more sensitive to sound than a member of the family Callichthyidae with reduced bladders. In order to determine whether this is a common difference between these two catfish groups, Lechner and Ladich (2008) investigated swim bladders, Weberian ossicles and hearing sensitivities in eleven species from eight different catfish families. Representatives of the Ariidae, Pseudopimelodidae, Malapteruridae, Heptapteridae, Mochokidae, and Auchenipteridae possess large, unpaired and free swim bladders and 1–4 ossicles, whereas members of the Loricariidae and Callichthyidae have significantly smaller swim bladders (3–5 vs. 8–13% of fish length), just 1–2 ossicles and thus a significantly shorter ossicular chain. Mean auditory thresholds of six species having large bladders and of all five species having tiny paired bladders revealed significant differences in hearing sensitivity between both groups between 1 and 5 kHz but not at lower frequencies (**Figure 13D**). Moreover, a longer ossicular chain and more ossicles resulted in better hearing at 3 to 5 kHz (for details see Lechner and Ladich, 2008; Ladich, 2016).

Non-Otophysan Fishes

Anterior extensions of the swim bladder directly contacting the auditory region (bullae) of the skull constitute the second type of a direct connection between the bladder and the inner ears (**Figure 12A**). Such extensions are apparently characteristics of three unrelated taxa, namely the order Clupeiformes (herrings),

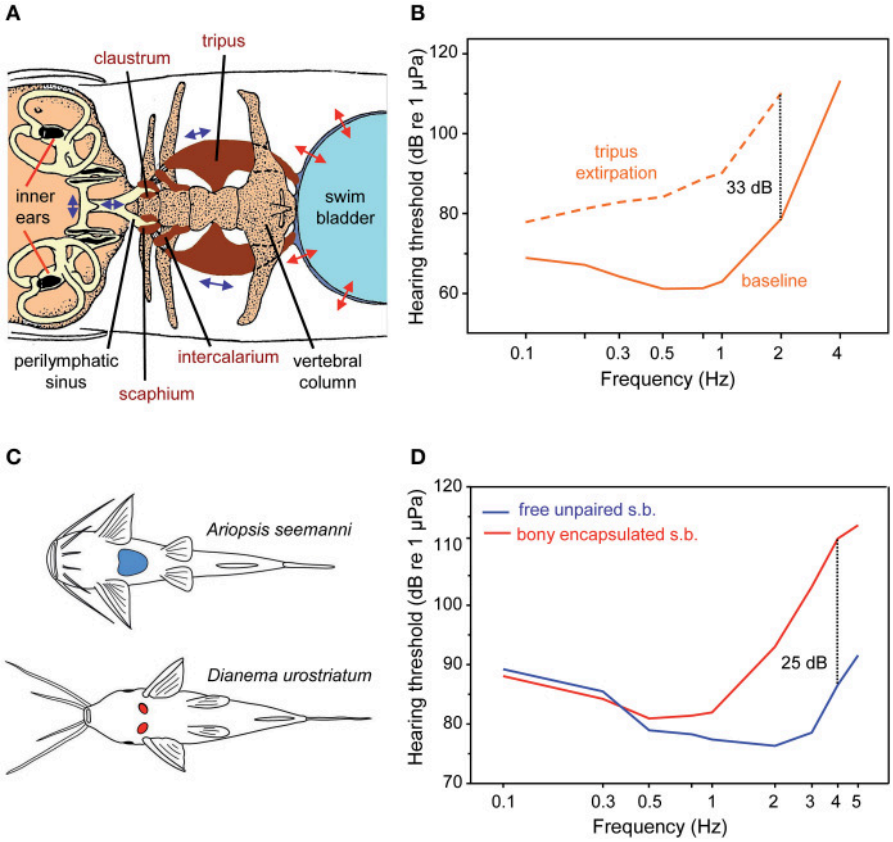

FIGURE 13 | Accessory hearing structures and auditory sensitivity in otophysans. (A) Swim bladder, Weberian ossicles (tripus, intercalarium, scaphium, and claustrum) and inner ears in the minnow *Phoxinus phoxinus* (Otophysa) in dorsal view. The otolith end organs of the inner ears (utricle, saccule, and lagena) and their otoliths (black structures) are shown. Double headed arrows indicate the oscillations of the swim bladder wall, the Weberian ossicles and the fluids within the perilymphatic sinus and the inner ears. Modified after von Frisch and Stetter (1932). **(B)** Mean AEP-sound pressure audiograms of the goldfish *Carassius auratus* before (baseline) and after bilateral extirpation of the tripus (dotted line)—the largest Weberian ossicle—to indicate hearing improvement by the Weberian ossicles. Dotted line: hearing loss at 2 kHz in *C. auratus*. Redrawn after Ladich and Wysocki (2003). **(C)** Diversity in swim bladders in catfishes. Ventral view of swim bladders and ossicles of representatives possessing free, large unpaired swim bladders (blue structure; *Ariopsis seemanni*, family Ariidae) or small, paired and encapsulated swim bladders (red structures; *Dianema urostriatum*, family Callichthyidae). **(D)** Mean AEP-sound pressure audiograms of six catfish species out of six families with free unpaired swim bladders (s.b.) and of five species out of two families with bony encapsulated swim bladders. Dotted line: difference in sensitivity at 4 kHz. Adapted after Lechner and Ladich (2008).

the families Notopteridae (knifefishes; order Osteoglossiformes) and Moridae (deep-sea cods, order Gadiformes; Nelson, 2006; Braun and Grande, 2008). Such linkages are furthermore found in several genera of non-related families such as the genus *Myripristis* (family Holocentridae, order Holocentriformes, Nelson, 1955) or the genus *Etroplus* (family Cichlidae, order Cichliformes, Dehadrai, 1959; Schulz-Mirbach et al., 2012). Families in which only some genera evolved swim bladder extensions and other members lack extensions or possess intermediate stages are particularly interesting for comparative studies.

Osteoglossomorpha: Notopteridae (Knifefishes) and Clupeidae (Herrings)

In notopterids, anterior projections of the swim bladder are attached to the bony auditory bullae, which are thinner than other regions of the skull (Coombs and Popper, 1982). *Chitala chitala* is able to detect sound up to 1000 Hz and has best sensitivities at 500 Hz (67 dB; all threshold values are referenced to 1 µPa in this review), similar to goldfish (Coombs and Popper, 1982).

Clupeiforms possess a quite different connection. The swim bladder extensions widen anteriorly and form large prootic bullae in which the gas is separated only by a bulla membrane from the inner ear fluid. These bullae are additionally in contact with the lateral line, forming a laterophysic connection (Blaxter et al., 1981). Mann et al. (1997, 2001) showed that all clupeiforms detect sounds up to 4 kHz and the members of the subfamily Alosinae (*Alosa sapidissima*, *Brevoortia patronus*) can detect ultrasound with frequencies up to 180 kHz. Note, however, that clupeids are, despite their high-frequency hearing, rather insensitive to sound because their lowest thresholds are about 100 dB and thus at least 40 dB above those of goldfish. Higgs et al. (2004) found that the middle macula of the utricle (see **Figure 5F**) is more

loosely connected to the rest of the utricle in the American shad *A. sapidissima* and presumably vibrates more compared with species that do not detect ultrasound. Wilson et al. (2009) showed experimentally that the gas-filled bullae and their attachment to the lateral line are responsible for ultrasonic hearing in the Gulf menhaden *B. patronus*. The prootic bullae are positioned closer to the body surface in *B. patronus*. Thus, both studies indicate—although in different ways—that anatomical differences between members of the subfamily Alosinae and members of other subfamilies explain why the latter are unable to detect ultrasound. Clupeids demonstrate that small anatomical differences may extend the detectable frequency range considerably.

Cichlidae

Cichlids are a speciose family of freshwater fishes comprising more than 1000 species (**Figure 14B**; McMahan et al., 2013). They exhibit a large diversity in swim bladder size and in the relationship between swim bladder and inner ears (Dehadrai, 1959; Schulz-Mirbach et al., 2012, 2013). Swim bladders can be directly connected to the inner ears in the basal Etroplinae such as the orange chromide *E. maculatus* from India and Sri Lanka in which a bipartite swim bladder extension contacts the upper as well as the lower parts of each inner ear, a condition not observed in any other teleost species studied so far (Schulz-Mirbach et al., 2013). In the Malagasy species *Paratilapia polleni*, the anterior extensions of the swim bladder abut the posterior skull and thus come close to the inner ears but without contacting them directly (Schulz-Mirbach et al., 2012). In species that lack anterior swim bladder extensions, the bladders may be normal sized like in the jewel cichlid *Hemichromis guttatus* or may be reduced (vestigial) in some rheophilic representatives such as *Steatocranus tinanti* (**Figure 14A**).

The structural diversity in swim bladders is paralleled by differences in hearing abilities between species. As expected for species whose swim bladder directly contacts or comes close to the inner ears, hearing sensitivities are significantly better than in taxa lacking such accessory auditory structures (Schulz-Mirbach et al., 2012). *Etroplus maculatus* and *P. polleni* responded to frequencies up to 3 kHz and showed the lowest thresholds of approximately 70 dB at 0.5 kHz (**Figure 14A**). Species lacking a close swim bladder-inner ear relationship are less sensitive, clearly depending on swim bladder size. In *H. guttatus* and *S. tinanti*, auditory sensitivity decreases steeply above 0.3 kHz. This results in sensitivity differences of 20–40 dB between species. *S. tinanti*, having the smallest swim bladder, did not respond to sounds above 0.7 kHz (**Figure 14A**).

The relationship between swim bladder morphology and hearing sensitivity in cichlids allows several conclusions. Those species which have a large bladder but no connection to the inner ears display intermediate hearing abilities. They can detect frequencies up to 3 kHz, similar to *E. maculatus*, but the absolute sensitivity is low and similar to *S. tinanti*. This indicates that the large swim bladder in *H. guttatus* contributes to high-frequency hearing despite the lack of a direct connection to the inner ears.

Considering the hearing abilities in *H. guttatus* the question arises of whether swim bladders without connection to the ears affect hearing and enable fish to detect sound pressure?

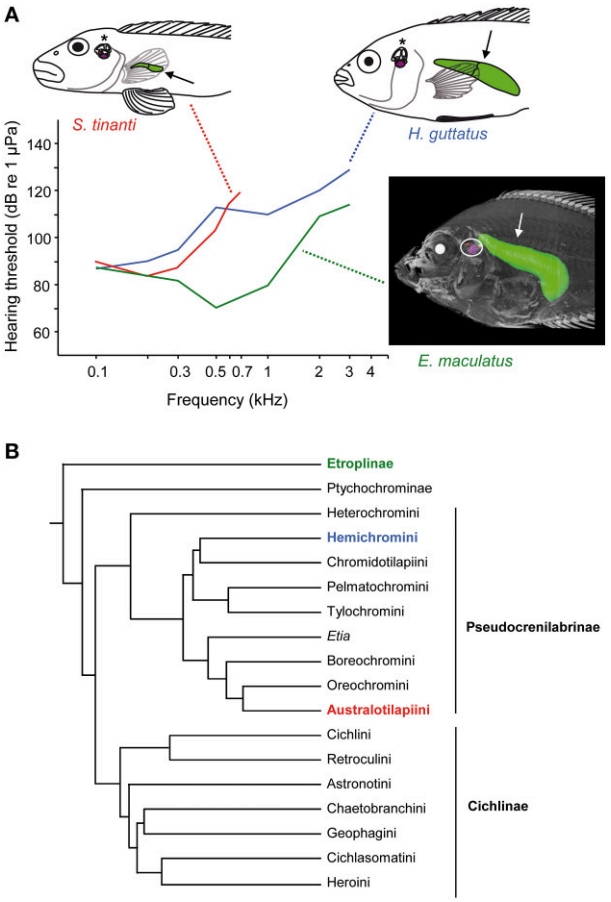

FIGURE 14 | Diversity in swim bladder morphology, auditory sensitivities and the phylogeny of cichlids. (A) Lateral view of three cichlid species including their swim bladders (arrows) and inner ears (asterisks, white circle). *Steatocranus tinanti* and *Hemichromis guttatus* both lack anterior swim bladder extensions but differ widely in swim bladder size (see line drawings). In contrast, *E. maculatus* possesses anterior swim bladder extensions that directly contact the inner ears (see 3D reconstruction based on microCT imaging). Mean AEP-audiograms are shown for all three species. Note that only sound pressure thresholds are given due to the similarity of sound pressure and particle acceleration thresholds in the study by Schulz-Mirbach et al. (2012). Modified from Schulz-Mirbach et al. (2012). **(B)** The illustrated phylogeny is modified from McMahan et al. (2013). Identical colors used in species names, audiogram and the phylogeny indicate the subfamily or tribe to which the studied species belong.

According to our current data the answer to the latter question must be "yes" although the experimental design does not enable differentiating between particle motion and pressure hearing. Sound detection up to 3 kHz can be explained only when a species is sound pressure sensitive. Prior studies in other taxa demonstrated that fishes can detect sound pressure in the absence of a clear connection (**Figure 12D**). This has been shown in the genus *Stegastes* (family Pomacentridae, damselfishes; Myrberg and Spires, 1980), *Gadus* (family Gadidae, cods; Sand and Enger, 1973), and recently in the African lungfish *Protopterus* (family Protopteridae, lungfishes, Christensen et al., 2015). It is assumed that in these families bladder wall oscillations

are transmitted to the inner ears via the interjacent tissue (Hawkins, 1986). This, however, is not a general rule. Yan et al. (2000) demonstrated that, in three spot gourami *Trichopodus trichopterus* (formerly *Trichogaster trichopterus*) and in the oyster toadfish *Opsanus tau*, removal of gas from the swim bladder did not affect hearing; this indicates that the bladder plays no role in audition.

Holocentridae (Squirrelfishes)

Holocentrids represent the second family in which the diversity in hearing sensitivities can be correlated to differences in swim bladder structures. Swim bladder morphology and presence/absence of auditory bullae led to the classification into the two subfamilies Myripristinae with a sophisticated swim bladder inner ear connection and Holocentrinae possessing short anterior swim bladder extensions (*Holocentrus*) or lacking any anterior extensions (e.g., *Sargocentron;* Nelson, 1955). This classification is also confirmed by recent phylogenetic analyses based on nuclear and mitochondrial DNA (**Figure 15C**; Dornburg et al., 2012).

The functional morphological comparison within the family is, however, somewhat limited because morphological and physiological data were, with one exception, gained in different species using different techniques for threshold determination (Nelson, 1955; Tavolga and Wodinsky, 1963; Coombs and Popper, 1979; Hawkins, 1986).

Coombs and Popper (1979) determined that the shoulderbar soldierfish *Myripristis kuntee* detects sounds up to 3 kHz, whereas the Hawaiian squirrelfish *Sargocentron xantherythrum* (formerly *Adioryx xantherythrus*) detects frequencies only up to 800 Hz at much higher sound levels (**Figure 15B**). This difference is paralleled by differences between genera in swim bladder morphology (note that the swim bladder morphology of *M. kuntee* and *S. xantherythrum* is unknown). Nelson (1955) showed that the brick soldierfish *Myripristis amaena* (formerly *M. argyromus*) has an anterior swim bladder extension that extends forward and covers the auditory bullae (**Figure 15A**). In contrast, the bladder of the tinsel soldierfish *Sargocentron suborbitalis* (formerly *Holocentrus suborbitalis*) is not attached to the skull. *Holocentrus adscensionis* represents an intermediate stage in terms of the swim bladder attachment and hearing ability (i.e., in the frequency range detectable but not in absolute thresholds; Tavolga and Wodinsky, 1963). In the genus *Sargocentron*, differences in hearing occur because *S. vexillarius* is much less sensitive than *S. xantherythrum*. This could be due to differences in swim bladder morphology (which is unknown in both species) or different methodologies to measure hearing (Hawkins, 1981). Nevertheless, a shorter distance between the swim bladder and the inner ears in holocentrids results in improved hearing sensitivities. The effects of bladder size on hearing cannot be analyzed because of insufficient data.

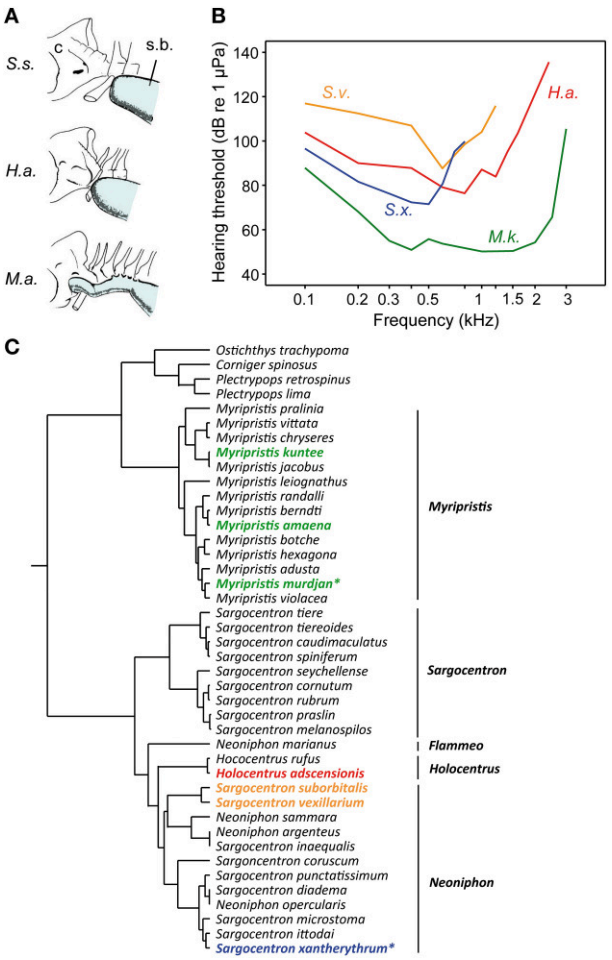

FIGURE 15 | Diversity in swim bladders, hearing thresholds and the phylogeny of squirrelfishes (family Holocentridae). (A) Relationship between posterior cranium **(C)** and the anterior portion of the swim bladder (s.b.) in three species. Anatomical drawings from Nelson (1955) modified by Hawkins (1986). From above *Sargocentron suborbitalis* (*S.s.*), *Holocentrus adscensionis* (*H.a.*), and *Myripristis amaena* (*M.a*). **(B)** Hearing thresholds of *Myripristis kuntee* (*M.k.)* and *Sargocentron xantherythrum* (*S.x.*) in terms of sound pressure levels are depicted from Coombs and Popper (1979), those of *Sargocentron vexillarium* (*S.v.)* and of *H. adscensionis* (*H.a.*) from Tavolga and Wodinsky (1963). **(C)** The illustrated phylogeny is modified from Dornburg et al. (2012). Identical colors used in audiograms and the phylogeny indicate the subfamily or tribe to which the studied species belong. Asterisks highlight those species of which the macula sacculi and macula lagenae have been studied by Popper (1977).

Sciaenidae (Drums and Croakers)

Numerous morphological and physiological studies have been conducted on the auditory systems in sciaenids (order Perciformes) and revealed a large diversity in swim bladder structures and hearing sensitivities, but the situation is less straightforward than that in cichlids and holocentrids (Ramcharitar et al., 2004, 2006; Horodysky et al., 2008; Wysocki et al., 2009). Ramcharitar et al. (2006) showed that the swim bladder in the weakfish *Cynoscion regalis* has anterior horns that terminate close to the ears and that this species detects sound frequencies up to 2 kHz. The spot *Leiostomus xanthurus*, on the other hand, has no extensions and detects frequencies only up to 700 Hz. Surprisingly, both species do not differ in absolute sensitivity, which is rather low (90 dB). In contrast, the silver perch *Bairdiella chrysoura* has an anterior swim

bladder chamber that surrounds the otic capsule and hears up to 4 kHz at thresholds close to that of goldfish (74 dB at 600 Hz; Ramcharitar et al., 2004). Furthermore, Horodysky et al. (2008) reported no significant difference in hearing thresholds in species with (*Cynoscion regalis*, *Cynoscion nebulosus*, *Micropogonias undulatus*) and without swim bladder specializations (*Sciaenops ocellatus*, *Leiostomus xanthurus*; for a comparison of audiograms see review by Ladich and Fay, 2013). In summary, the form-function relationship in sciaenids is less consistent than in catfishes, holocentrids and cichlids. This difference may partly be explained by potential differences in techniques applied (maximum frequency measured by Horodysky et al. was 1.2 kHz) or by differences in the attachment of swim bladder extensions to the ears. These factors, however, cannot explain the lack of sensitivity differences within the same studies (Ramcharitar et al., 2006; Horodysky et al., 2008).

Mormyridae (Elephantfishes) and Anabantiformes (Labyrinth fishes).

The weakly electric mormyrids from African freshwaters (order Osteoglossiformes) and the mainly Southeast Asian labyrinth fishes (order Anabantiformes) possess gas bladders attached to the inner ears; these gas bladders are entirely separated from the swim bladder (**Figure 12C**).

The otic (tympanic) gas bladder in mormyrids constitutes an anterior extension of the swim bladder, which became completely separated and improves hearing sensitivity up to 3 kHz (Stipetić, 1939; McCormick and Popper, 1984). Elimination experiments showed that the otic bladder improves hearing in mormyrids by 15–30 dB between 0.5 and 1 kHz, whereas no change in the detectable frequency range was observed (Yan and Curtsinger, 2000; Fletcher and Crawford, 2001).

The non-related labyrinth fishes (order Anabantiformes) have a suprabranchial chamber (labyrinth organ) which derives from the first gill arch and serves in air-breathing (Bader, 1937). This air-filled chamber is in direct contact with the saccule and enhances hearing (Schneider, 1941; Yan, 1998). Schneider (1941) showed that the upper hearing range dropped from 4.5 kHz down to 800 Hz when the suprabranchial organ was filled with water. Yan (1998) observed a decline in sensitivity between 16 dB in the dwarf gourami *Colisa lalia* and up to 32 dB in the blue gourami *T. trichopterus* when deflating the organ.

Does Inner Ear Diversity Correlate with Hearing Abilities?

The Role of the Macula Neglecta in Elasmobranchii

Several physiological studies in elasmobranchs suggest a main auditory role of the macula neglecta together with the macula sacculi (e.g., Corwin, 1981, 1989; Myrberg, 2001; Casper, 2011). This may explain why the macula neglecta is generally larger in elasmobranchs than in bony fishes and larger in pelagic than in more benthic elasmobranch species (cf. Corwin, 1989; Myrberg, 2001). In holocephalans and especially in bony fishes, which possess a macula neglecta, its function remains elusive (Popper, 2011).

Modified Otolith End Organs in Teleosts

In contrast to elasmobranch fishes, in which it is rather clear that the macula neglecta together with the macula sacculi represent the main auditory organs, the role of the otolith end organs in audition and the vestibular sense in bony fishes is less well-understood. The saccule is assumed to be the main auditory organ in modern bony fishes (e.g., von Frisch and Stetter, 1932; Fay and Edds-Walton, 1997; Lu and Xu, 2002; Lu et al., 2002), which is supported by the fact that when connections or close relationships exist between accessory auditory structures and ears, the saccule is generally contacted by these structures. Nonetheless, several studies provide support for an auditory role of the lagena (e.g., Lu et al., 2003) as well as the utricle (e.g., Lu et al., 2004; Maruska and Mensinger, 2015).

Certain modified orientation patterns—mainly on the macula sacculi—may have evolved to enhance hearing together with accessory auditory structures. Apparently, species with accessory auditory structures, which mostly correlate with improved hearing (Ladich and Popper, 2004; Braun and Grande, 2008; Ladich and Fay, 2013; Ladich, 2014a), often display modified orientation patterns on the maculae, mainly on the macula sacculi (Platt and Popper, 1981a). This is evident in the vertical pattern of otophysans and mormyrids (**Figures 7A,C**), the opposing pattern of anabantiform fishes (**Figure 7D**) or "unique" patterns (see *Antimora*; **Figure 7F**) that cannot be assigned to one of the five patterns. Conceivably, the inner ear in such species and accessory auditory structures coevolved to some degree to guarantee fine-tuning between these two units to improve audition.

In some cases, however, accessory structures and modified orientation patterns—deviating from the standard or dual patterns—are present but without distinctly improved hearing compared to species that lack accessory structures. The clown knifefish *C. chitala*, for example, does not show an expanded hearing bandwidth or higher auditory sensitivities (Coombs and Popper, 1982), and the sciaenid species *Micropogonias undulates* and *Cynoscion nebulosus* show a slightly expanded bandwidth but similar auditory sensitivities as species without anterior swim bladder extensions (Horodysky et al., 2008). Moreover, accessory auditory structures and improved auditory abilities do not necessarily correlate with modified (more complex) orientation patterns on the maculae. This is demonstrated for the Hawaiian ladyfish *Elops hawaiensis* (Elopidae; Popper, 1981) and the cichlid *Etroplus maculatus*: they have "standard" patterns on all three macula types (when analyzing artificially flattened maculae (**Figures 5H, 10G**; Schulz-Mirbach et al., 2014). A distinct 3D curvature bringing the ciliary bundles in a new spatial orientation without modifications of the orientation patterns in 2D is present in *E. maculatus*. The anterior arm of its macula lagenae and the lacinia of the macula utriculi are strongly curved. The wider range of directions of ciliary bundles based on the 3D curvature—a condition also found in the macula sacculi of the silver perch *Bairdiella chrysoura* (Sciaenidae; **Figure 7H**)—might translate into a wider range of directional stimuli being detectible, and thus may play a role in localizing sound sources (Schulz-Mirbach et al., 2014). Finally, species such as the cod *Gadus morhua* that lack a direct morphological connection between the

swim bladder and the inner ears (see Hawkins, 1986) and that display a dual pattern on the macula sacculi (Dale, 1976) were shown to be pressure sensitive (Chapman and Hawkins, 1973).

Though we have a solid knowledge about the diversity of inner ear morphology (otoliths, gross ear anatomy, sensory epithelia) and accessory auditory structures in fishes (see chapters above), our understanding of the ontogenetic development of ears and accessory auditory structures, as well as of the underlying genetic basis and molecular mechanisms for formation of sensory epithelia, is restricted to a few model organisms such as the otophysan *Danio rerio* or the batrachoidid plainfin midshipman *Porichthys notatus*. This hardly covers the tremendous morphological diversity in fishes (see e.g., Baxendale and Whitfield, 2014; Alderks and Sisneros, 2013). Another issue is to unravel the linkage between ear morphology and ear function. In most species, data about hearing abilities still only refer to hearing bandwidth and auditory sensitivities (see Fay, 1988; Ladich and Fay, 2013). To date it remains elusive how certain inner ear modifications are correlated to certain aspects of auditory abilities. Moreover, only few studies successfully disentangle the detection of the amounts of particle motion and sound pressure in fishes (e.g., Myrberg and Spires, 1980; Christensen et al., 2015).

WHY HEARING ENHANCEMENT IN FISHES?

As illustrated above, fishes, especially teleosts, exhibit a considerable variation in the auditory system including inner ears, accessory hearing structures and auditory sensitivities (Popper, 2011; Schulz-Mirbach and Ladich, 2016; Ladich, 2016). So far, we do not know why mechanisms to detect sound pressure, have evolved in taxonomically unrelated species or only in a few genera within entire families. Testable hypotheses have seldom been posed and the evolution of this diversity remains a field of much theoretical consideration (Ladich, 2014a,b; Lugli, 2015a,b).

Accessory hearing structures improve auditory sensitivities in several ways. They may e.g., expand the distance, the frequency range or sound level range (or other auditory abilities) over which fishes are able to detect sound. Accessory hearing structures do not necessarily improve all auditory abilities at the same time (Fay, 1988; Ladich and Fay, 2013). Comparison of baseline audiograms (recorded under quiet lab conditions) reveal that expansion of the detectable frequency range is not always paralleled by an enhanced absolute sensitivity, i.e., lower sound levels necessary to get a response either behaviorally or physiologically. Clupeids are able to detect ultrasound up to 180 kHz but their sensitivity to low level sounds is low in contrast to otophysans (Ladich and Fay, 2013). Thus, the diversity in hearing enhancement even in closely related taxa may help to fulfil different auditory tasks or similar tasks at different sound frequencies or levels.

In general, accessory hearing structures enable fish to detect acoustic information at frequencies and/or sound levels which would not be possible without these structures as demonstrated in numerous elimination experiments (Ladich and Wysocki, 2003). In order to detect such low level or high frequency sound, it is important that the relevant sound is not masked by ambient (background noise of different origin) noise at the sound frequencies but that the sound is loud enough so that there exists a reasonable signal to noise ratio (Fay, 1974). Relevant acoustic information for fish includes abiotic noise (e.g., water falls, coastal surf, reef noise) as well as biotic sound. The latter includes vocalizations from con- and hetero-specifics produced for intraspecific communication but also unintentional sound such as feeding or swimming noise. All of this constitutes the auditory scene (or soundscape, (Fay, 2009)) and provides important information for migration, reproductive activities as well as predator avoidance or prey detection. It needs to be mentioned that such acoustic information may be important for all fish species independent of their hearing abilities and that we have still limited knowledge of what fish hear besides conspecific sounds in vocalizing species (Fay, 2011).

The evolution of the detection of low level or high frequency sounds (or both) as compared to limited hearing in non-specialized taxa may be advantageous in many ways. In the following, we discuss potential factors responsible for the evolution of hearing enhancement in fishes, review current data and formulate as far as possible testable hypotheses.

The detection of high frequency sounds may be advantageous in detecting sound sources in shallow water. Low frequencies with long wavelengths do not propagate in shallow water due to the cut-off frequency phenomenon (Fine and Lenhardt, 1983; Rogers and Cox, 1988). High frequency hearing may have evolved to detect conspecifics or predators at larger distances in such habitats. In order to prove this notion it needs to be shown that fish with hearing specialization communicate or eavesdrop important acoustic information at higher distances in shallow waters than other taxa. Unfortunately, our knowledge on communication distances in fishes proven by playback experiments in the field is very limited and comparative data between fishes possessing different hearing abilities are entirely missing. Successful playback experiments were seldom carried out in the field. Myrberg et al. (1986) showed that female bicolor damselfish *Stegastes partitus* (see **Figure 12D**) were attracted to speakers playing back male chirp sound over distances of maximally 10 meters. However, communication distances in fish are usually much shorter. Typically, fish respond to conspecifics at distances of a few body lengths after an opponent or mate was detected visually (Ladich and Myrberg, 2006; Amorim et al., 2015). Present data do not provide unambiguous support that shallow water acoustics was an important selective force in the evolution of accessory hearing structures in any taxon.

A further potential factor in the evolution of hearing improvement may have been the detection of predators. Numerous insects evolved ultrasonic hearing abilities to detect echolocation clicks of bats, their main predators (Hoy, 1992). Similarly, certain clupeids such as the American shad *Alosa sapidissima* undergo an escape response when they detect ultrasonic clicks (Mann et al., 1998). Despite a lack of field data, it seems likely that predator avoidance was a main selective pressure driving the evolution of accessory hearing structures (type 1, **Figure 12A**) and ultrasonic hearing in several but not

all clupeids (see Section *Osteoglossomorpha*). Ultrasonic hearing is an ideal candidate to test the predator avoidance hypothesis because the ultrasonic frequency range does not overlap with frequencies potentially used for acoustic communication. Yet, the ability to detect ultrasound has only been demonstrated for some members of the clupeid subfamily Alosinae (Mann et al., 2001, 2005). The observation by Astrup and Mohl (1993) that cod (*Gadus morhua*) detect ultrasound at 38 kHz could not be confirmed by studies. Schack et al. (2008) showed that unconditioned cods do not respond to ultrasound and thus will not react to toothed whale vocalizations. Further support for the predator avoidance hypothesis comes from the observation that many fish species avoid predators using a C-like startle behavior (C-start; Canfield and Eaton, 1990; Canfield and Rose, 1996). This escape response is mediated by the ability to detect sound pressure waves of rapidly approaching predators which coevolved with the addition of hearing to the swim bladder function. Thus, fish with hearing specializations could detect predators earlier and initiate escape responses more effectively.

Interestingly, fish species which lack hearing specialization or have only limited hearing improvements such as *Holocentrus* (**Figure 15B**) can detect vocalizations of dolphins as well and respond accordingly. Gulf toadfish *Opsanus beta* and longspine squirrelfish *Holocentrus rufus* reduced calling in the field when low frequency vocalizations but not ultrasound of bottlenose dolphins were played back (Remage-Healey et al., 2006; Luczkovich and Keusenkothen, 2007). Vasconcelos et al. (2011) demonstrated that the auditory system of the Lusitanian toadfish *Halobatrachus didactylus* detects dolphin sounds. These latter species are vocal and their ability to detect predators overlaps with the frequency range used for acoustic communication.

It was also hypothesized that hearing enhancement may have evolved for optimization of intraspecific acoustic communication (Ladich, 1999, 2000). Fishes show a large variety of mechanisms for producing sounds (sonic organs; for recent reviews see Ladich and Fine, 2006; Ladich and Bass, 2011; Fine and Parmentier, 2015). Sonic organs and sound communication are found in taxa with (mormyrids, catfish, piranhas, some labyrinth fishes) and without accessory hearing structures. Sound-producing taxa lacking accessory hearing structures can either be mainly particle motion sensitive such as toadfishes (Batrachoidiformes), sculpins (Cottiformes), and gobies (Gobiidae, Perciformes), or also display sound pressure sensitivity such as damselfish (Pomacentridae) and cods (Gadidae; Sand and Enger, 1973; Myrberg and Spires, 1980). Comparative studies among labyrinth fishes show that closely related genera may be vocal or non-vocal—such as croaking gouramis (genus *Trichopsis*) and Siamese fighting fish (genus *Betta*)—without differing in inner ear ultrastructure or accessory hearing organs (Ladich and Popper, 2001). The fact that sonic organs and/or sound production often evolved in only a few genera within taxa with hearing specializations (labyrinth fishes, weakly electric mormyrids, or cyprinids; **Figure 4**) raises the question how this can be explained at a phylogenetic level. It is possible that these taxa had vocal ancestors which evolved a particular sonic organ and that the majority of genera lost this sonic mechanism. This explanation is unlikely i.e., in labyrinth fishes because the vocal genera possess different sonic mechanisms (Kratochvil, 1985). Therefore, Ladich (2014b) proposed that vocal organs and sound production evolved under a different selection regime namely territory defense and mate attraction.

Coevolution of vocal communication and hearing enhancement is rather unlikely. In otophysans for example all members of this group share the same basic basic structure for hearing enhancement (Weberian apparatus, **Figures 12B, 13**) whereas vocal groups evolved a large diversity of sonic organs and do not share a common sonic mechanism (Ladich and Fine, 2006). It is therefore unlikely that the ancestor of otophysans was vocal. Acoustic communication as a main driver for the evolution of hearing enhancements is contrasted by the presence of numerous vocal taxa which lack any hearing improvement such as toadfishes (Batrachoidiformes), gobiids (Gobioidei), and sculpins (Cottoidei; Ladich, 2014b),

All potential factors facilitating the evolution of hearing enhancements (shallow water sound propagation, predator avoidance, acoustic communication) are based on the notion that the relevant sound is detectable against the background noise at particular frequencies. This notion requires an analysis of the acoustic conditions of the fish's habitats.

Several comparative studies described that ambient noise conditions vary considerably in the habitats of freshwater and marine fishes. Wysocki et al. (2007) analyzed 12 aquatic habitats in central Europe, Lugli (2010) five different habitats in northern Italy and the Mediterranean, and Speares et al. (2011) different places in creeks in Alabama. All these studies reported differences in spectral levels of 40–60 dB, with highest levels in rapidly moving waters such as creeks, large streams, and rocky shores. Kennedy et al. (2010) recorded the ambient noise at 40 reefs of the Las Perlas archipelago in the Gulf of Panama and compared these to offshore sites while the sea was calm. Spectral profiles between different reefs were rather similar, in contrast to offshore recordings in which spectral noise levels were about 20–30 dB lower, mainly due to lack of vocalizing animals such as shrimp. The diversity in ambient noise spectra raises the question whether the high auditory sensitivities of some species are adapted to low ambient noise conditions. To verify this assumption hearing sensitivities have been measured and compared under quiet laboratory and under ambient noise conditions. Chapman (1973) and Chapman and Hawkins (1973) demonstrated in field experiments that cod hearing is unmasked under calm sea conditions and that hearing sensitivity decreases (thus hearing was masked) when ambient noise levels rose. Amoser and Ladich (2005) measured hearing in two common non-vocal European freshwater fish, the carp *Cyprinus carpio* (family Cyprinidae, an otophysine) and the European perch *Perca fluviatilis* (family Percidae, no specializations) in the presence of ambient noise of four different habitats. Carps were moderately masked by the quiet noise of standing waters but heavily affected by the river noise in their best hearing range (0.5–1 kHz). In contrast, perch were only slightly masked by the highest noise levels presented. This raises the question if the diversity in ambient noise levels affected the evolution of particular hearing sensitivities. Ladich (2014b) argued that hearing evolved in adaptation to the acoustical

conditions in the fishes' habitats ("eco-acoustical constraints hypothesis"). Hence, hearing thresholds are assumed to be as low as possible without being masked by the ambient (background) noise in their environment. If the ambient noise varies in aquatic environments, it would inevitably result in a large variety of hearing abilities in fishes. Low noise levels would then facilitate the evolution of accessory hearing structures and the detection of low-level sound against low level background noise, whereas high ambient noise levels likely render such structures meaningless.

Importantly, the eco-acoustical constraints hypothesis does not explain to which sound sources fishes are listening to. Besides vocalizations from con- and hetero-specifics (e.g., predators), fish may also listen to habitat noise built up of numerous abiotic and biotic sound sources. Pelagic coral reef fish larvae (of the families Trypterigiidae, Pomacentridae, Apogonidae, Gobiidae, Lethrinidae) orient to loudspeakers playing back reef noise (Tolimieri et al., 2000; Simpson et al., 2008; Radford et al., 2011). Our knowledge on the importance of acoustic orientation in prey detection or food finding is still in its infancy, but this factor should not be underestimated. The attractiveness of artificial underwater sounds to fish has been exploited by indigenous people all over the world for hundreds of years (see Wolff, 1966). Rattling coconut shells underwater, for instance, is very attractive for sharks (shark rattle) and was used in the South Seas. It remains to be clarified what triggers the catch success in such non-vocal species, i.e., whether it is attraction to potential food sources or startling. Markl (1972) observed that the red piranha *Pygocentrus nattereri*, a representative of the otophysan order Characiformes, attacked prey producing splashing noise more often than silent prey.

SUMMARY AND CONCLUSIONS

Fishes have evolved an enormous diversity of inner ears and accessory hearing structures. While the accessory hearing structures enhance hearing, the diversity of inner ears remains mostly unexplained. They may be adaptations to various ecological conditions and/or and auditory tasks such as improvement of hearing. The latter may be the case in otophysans, in which major changes in inner ear structure (maculae, needle-like saccular otolith) are associated with the presence of the unique Weberian apparatus.

The occurrence of accessory hearing structures and enhanced hearing in fishes does not reflect the phylogenetic relationships among the groups in which these specializations evolved.

On the contrary, these specialized structures evolved several times independently and are either characteristic of a whole group with a high taxonomic rank (e.g., superorder Otophysa; order Anabantiformes; families Notopteridae, Mormyridae) or appear in only a few species or genera within a (speciose) family such as cichlids, holocentrids, or sciaenids. Hearing enhancement may be a simple by-product of other functions such as air-breathing (labyrinth fishes, lungfishes) or buoyancy (damselfish), or it may have evolved solely for hearing enhancement as seems to be the case in otophysans. This interpretation is supported by the observation that the Weberian ossicles have not been entirely lost in any species even though swim bladders were reduced considerably and certainly lost their function in buoyancy control. This leaves us with the question why certain non-related taxa such as the genera *Etroplus* and *Myripristis*, the family Mormyridae or the order Cypriniformes evolved accessory hearing structures and others did not.

We propose several factors which may explain the evolution of hearing enhancements in fishes. They are based on a limited number of observations but need rigorous testing in order to prove their validiy in some taxa. Ultrasonic hearing in some herrings, for example, most likely evolved to detect echolocating clicks of dolphins. To test this assumption elimination of the accessory hearing structure and analysis of the behavior under field conditions will be necessary. The "eco-acoustical constraints hypothesis" could be tested by recording the ambient noise in the habitats of closely related species which differ considerably in hearing abilities. Representatives of catfishes, cichlids or holocentrids may provide ideal candidates for testing as they cover a large variety of auditory sensitivities (**Figures 13–15**; Ladich, 2014a,b). According to this hypothesis, ambient noise levels and spectra in the habitats of the non-related genera *Myripristis* and *Etroplus* should differ as compared to that of other representatives of the same families. Results of these experiments will help to gain deeper insights into the evolution of hearing specializations and enhanced hearing in fishes.

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All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Sensory Perception in Cetaceans: Part II—Promising Experimental Approaches to Study Chemoreception in Dolphins

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Chemosensory perception in cetaceans remains an intriguing issue as morphological, neuroanatomical and genetic studies draw unclear conclusions, while behavioral data suggest that dolphins may use it for food selection or socio-sexual interactions. Experimental approaches have been scarce due to the practical difficulties of testing chemoreception in wild dolphins. Go/no-go tasks are one elegant way to investigate discrimination abilities; however, they require to train the animals, thus preventing spontaneous responses and hence the expression of preferences. Here, we aimed at testing potential spontaneous responses to chemical stimuli and developed novel procedures. First, we conducted a study to test whether captive dolphins respond to a biologically relevant smell. Therefore, we placed dead fish within an opaque barrel at the border of the pool and counted the number of respirations at proximity as an indicator of investigation. The same dead fishes were presented several times during experiments lasting three consecutive days. From the second day on (i.e., when the odor composition changed), dolphins breathed more often close to the fish-smelling barrel than close to the visually identical but empty control barrel. Second, we conducted a study to test whether dolphins are able to discriminate food flavors. Captive dolphins are commonly provided with ice cubes as a source of enrichment. We took this opportunity to provide ice cubes with different flavors and to compare the reaction to these different flavors as a measure of discrimination. Hence, we used the latency of return to the ice cube begging spot as a measure of discrimination from the previous ice cube flavor. Thus, our method used a non-invasive and easily replicable technique based on the spontaneous begging responses of dolphins toward more or less attractive items bearing biological relevance. The procedures used enabled us to show that dolphins may discriminate odors and flavors respectively.

Keywords: cetaceans, *Tursiops truncatus*, olfaction, gustation, flavor discrimination

INTRODUCTION

Although chemoreception plays an important role not only for terrestrial species, but also for animals with an entire or semi-aquatic lifestyle (Nevitt et al., 1995; Catania, 2006; Davis et al., 2006; Hara, 2006; Endres and Lohmann, 2012), it has drawn little attention in research on cetaceans. In dolphins, few studies have been performed compared to other sensory modalities (see Kremers et al., 2016), and their results are contradictory.

On the one hand, several authors posit that some cetacean species have lost their nasal (Kishida et al., 2007) and oral chemoreception (Jiang et al., 2013) in the course of evolution, as airborne odorants may be considered irrelevant due to their aquatic lifestyle (Thewissen et al., 2011). Firstly, corresponding anatomical structures are rudimentary or absent, at least in adult animals. In the nasal cavity of toothed whales, the cribriform plate of the ethmoid bone and ethmoturbinals are absent (Pihlström, 2008). In their oral cavity, no taste buds were found on the tongue or other body areas of various odontocete species (Kuznetzov, 1990). However, the number and/or age of individuals investigated is usually unknown or very limited. Secondly, central structures devoted to olfaction are rudimentary or absent. The olfactory nerve [cranial nerve (CN) I] seems to vanish during early ontogenesis (Oelschläger and Buhl, 1985). The main and accessory olfactory tracts are completely absent in toothed whales, and absent or considerably reduced in baleen whales (Oelschläger, 2008; Pihlström, 2008). Thirdly, olfactory receptor (OR) and taste receptor genes are mostly pseudogenised or entirely absent in Odontoceti (Kishida et al., 2007; Jiang et al., 2013).

By contrast, numerous studies argue in favor of functional chemoreception in cetaceans. Firstly, chemoreceptive cells were found in the frontal and vestibular sac (close to the blowhole) of harbor porpoises (Behrmann, 1989), perhaps enabling some kind of chemical sensation in this species. Moreover, taste buds were found in younger individuals of the same species that were previously described as not having them when investigating adult individuals (Yamasaki et al., 1978; Behrmann, 1988; Kuznetzov, 1990). Other studies did not describe taste buds, but found marginal and vallate papillae on the tongues of dolphins, known to be potential locations of taste buds (Kastelein and Dubbeldam, 1990; Werth, 2007). Secondly, Odontoceti were found to possess a well-developed olfactory tubercle (Oelschläger and Oelschläger, 2009) and bottlenose dolphins possess prominent olfactory lobes possibly activated by the trigeminal nerve (CN V; Jacobs et al., 1971). This nerve is very well developed in dolphins (Oelschläger, 2008) and necessary for odor location in humans (Kleemann et al., 2009). It was proposed that in dolphins CN V might provide a pathway to transmit impulses from the oral cavity to the brain (Oelschläger and Oelschläger, 2009), called trigeminal chemoreception (Kuznetzov, 1990). Unlike other mammals, where cranial nerve VII innervates the taste buds of the tongue (Purves et al., 2001), this nerve does not seem to be involved in dolphin chemoreception but rather in acoustic signal production (Oelschläger, 2008). However, cranial nerve V is, just as cranial nerve VII, able to excite the gustatory neurons in the nucleus of the solitary tract in the medulla (Purves et al., 2001; Boucher et al., 2003). Thirdly, although OR genes are reported to be functionally reduced by pseudogenization in Odontoceti (Kishida et al., 2007), bottlenose dolphins in particular possess 23 G protein-coupled OR genes that are not pseudogenized, thus potentially functional (SEVENS database of G-protein coupled receptor genes; available at: http://sevens.cbrc.jp/search.php?db=ttru&level=4). Similarly, taste receptor genes were found to be mostly pseudogenized in Odontoceti: in bottlenose dolphins sweet, umami, bitter and sour taste receptor genes were found to be inactivated, whereas salty taste receptor genes are intact and potentially functional (Jiang et al., 2013; Feng et al., 2014; Kishida et al., 2015). Finally, go/no-go behavioral tests with trained bottlenose dolphins showed that they can perceive sour, bitter and salty tastes nearly as well as humans (Nachtigall and Hall, 1984; Friedl et al., 1990; Kuznetzov, 1990). The authors of these behavioral studies noticed that dolphins were able to perceive orally what other mammals perceive by smell wherefore they called this perception "quasi-olfaction" (Kuznetzov, 1990) or "water-borne sense of smell" (Nachtigall, 1986). Taken together, this second set of studies suggests that cetaceans might have, to some extent, access to chemosensory information through the olfactory (Thewissen et al., 2011) and/or taste systems (Watkins and Wartzok, 1985; Pihlström, 2008). As anatomical, neuroanatomical, and molecular evidence draw unclear conclusions, behavioral studies are needed.

Obviously cetaceans are difficult to study, especially in their natural habitat where they are difficult to find and to follow, and controlled experiments are often hardly feasible. Therefore, we present two promising experimental approaches for initial behavioral studies on olfaction and gustation in captive dolphins. Given the complex but sometimes subtle behaviors displayed by dolphins in response to internal or external stimuli, go/no-go tasks are one elegant way to investigate chemoperceptual abilities; however, they require to train the animals, thus preventing spontaneous responses. Therefore, the go/no-go paradigm is not optimal to investigate preferences because it imposes time-consuming training of animals and minimizes the measurement of spontaneously-expressed preferences. As we aimed at testing potential spontaneous responses to chemical stimuli, we developed and tried novel methods. First, we conducted a pioneer experiment to test for odor perception. We assumed that biologically relevant odors should be intriguing for the dolphins, especially when food-related. Therefore, we predicted that, if dolphins were capable of perceiving odors, they would express some "sniffing"-like behavior (i.e., taking more breaths) within the range of the odor source. Second, we investigated the flavor discrimination abilities of dolphins, predicting that, if dolphins were capable of perceiving flavors, they would behave discriminatively in response to control vs. flavored ice-cubes, and that they would discriminate different flavors along either sensory features involving their source (i.e., fish vs. non-fish) or along previous exposure to the stimuli (familiar *vs.* unfamiliar).

STUDY I: OLFACTION

Materials and Methods

Study Subjects and Housing Conditions

In May and June 2013, we studied six captive-born bottlenose dolphins (*Tursiops truncatus*, Montagu, 1821) in the delphinarium of Planète Sauvage (Port-Saint-Père, France): four males (aged 5, 8, 14, and 29 years) and two females (aged 5 and 12 years). The three oldest dolphins had been housed together for more than four years when the study took place and participated also in the study on gustation; the three youngest dolphins arrived in the facility one year before the study took place.

Overall, this outdoor facility consists of four pools, covering 2000 m^2 water surface and containing 7.5 million liters salt water cleaned with ozone (without any chlorine). The diet of the dolphins was composed of frozen-stored fresh herring, capelin, sprat, mackerel, blue whiting and squid. The species composition of the diet changed on a daily basis, but contained at least three different fish species each day. A daily ration of 5–10 kg per individual (depending on its weight) was given throughout the day during eight feeding sessions (approx. 15 min lasting), the first at 9:00 a.m. and the last at 5:00 p.m. These feedings were conducted by the dolphin trainers, using the food as primary reinforcement for medical training (e.g., acceptance of inspection and palpation of all parts of the body or being touched by medical equipment) as well as training for public shows (e.g., jump on command). The trainers gave the food directly into the mouth of the dolphin. During the experiment, dolphins were together as a group and free to move.

Stimuli

One kilogram of mixed fish (herring, capelin, sprat, mackerel, and whiting) and squids (hereafter referred to as "fish") that were defrosted during the night preceding the first day of each one of the three experiments were used as odor source and were actually those destined to feed the dolphins. Mixing species was done to avoid responses biased by potential individual preferences. The fish was placed in a familiar opaque plastic barrel (height: 26 cm; diameter: 17 cm), perforated all around with 40 small holes (diameter: 3 mm), that was familiar to the dolphins (**Figure 1**). The inside surface of the barrel was covered with a black plastic bag to avoid visual cues and any leak into the pool. The barrel was placed uncovered at the edge of the pool, simultaneously with a second, identical but empty control barrel. The two barrels were 8 m away from each other (linear distance) and the position for fish/control barrel was randomly changed between sessions that lasted 10 min. The dolphins never had physical contact with the barrels and their top opening was too high for them to look inside even when raising their heads out of the water. Thus, vision and touch were excluded as conveyors of cues to differentiate both barrels.

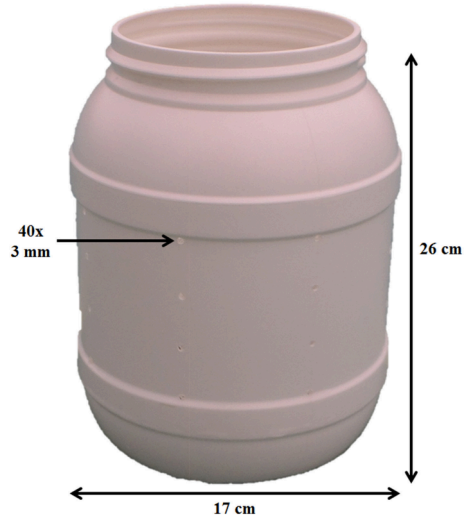

FIGURE 1 | Opaque plastic barrel used in the pilot study on olfaction in dolphins.

Data Collection

The same fish were presented to the group of dolphins on three consecutive days (thus producing an increasingly intense odor of rotten fish) with two sessions per day with the largest possible time interval between experimental and feeding session. Three days made up for an experimental section. Three sections were done in total with a new mixture of fish each time, leading to 18 sessions in total over 9 days (**Table 1**). During a section, the fish was stored at room temperature to facilitate the change in odor composition. This change was also detectable for humans who could discriminate between the fish and the control barrel based on their smell only. During the experimental sessions, that lasted 10 min, each barrel was video-recorded with a Sony HDR-XR155 video camera and neither the experimenter nor another person was close to the pool. An observer blind to the content of the barrels then analyzed the videos.

As no previous studies on olfaction in dolphins were available, we had no information on how a possible reaction of a dolphin toward an odorous stimulus might look like. As the perception of odorants is affected by breathing patterns in other mammals (e.g., Saslow, 2002), we chose to count the number of respirations for each dolphin within a range of 2.5 m around either barrel. Respiration was defined as a visible and audible opening of the blowhole above the water surface. Individuals could be reliably identified based on physical differences (e.g., shape of the dorsal fin).

Data analysis

Statistical analyses were run using R software (version 2.15.0, R Development Core Team, www.r-project.org). We calculated a Respiration Bias Index (RBI) using the following formula: $\frac{(\#Respi_{fish} - \#Respi_{control})}{(\#Respi_{fish} + \#Respi_{control})}$, resulting in RBI values ranging from +1 to −1. Thus, positive RBI values indicate a bias in respiration activity toward the fish barrel (i.e., dolphins breathe more often in the area around the barrel containing fish compared to the control barrel). Accordingly, negative RBI values indicate a bias in respiration activity toward the control barrel (i.e.,

TABLE 1 | Chronological sequence of stimuli presentation (position for fish/control changed randomly between sessions).

	Section 1: 22–24 May 2013		Section 2: 11–13 June 2013		Section 3: 17–19 June 2013	
	position A	position B	position A	position B	position A	position B
Day 1: midday	fish	control	fish	control	control	fish
Day 1: afternoon	fish	control	control	fish	fish	control
Day 2: midday	control	fish	control	fish	fish	control
Day 2: afternoon	control	fish	control	fish	control	fish
Day 3: midday	control	fish	fish	control	fish	control
Day 3: afternoon	fish	control	fish	control	control	fish

dolphins breathe more often in the area around the control barrel compared to the barrel containing fish). This kind of index is common for example in primate laterality studies (Hopkins, 1999). As odor composition changed on a daily basis due to fish decomposition, we tested whether there was a direct relationship between RBI and day with a Wald test on a Linear Mixed Model (ANOVA for repeated measurements; $n = 8$, $\alpha = 0.05$; R-package: lme4). Sections were considered as replicates and therefore treated as random factor. Identity of the dolphins was taken into account by treating individual as random factor in the model.

To further investigate this relationship, we compared the number of respirations between fish and control for each day separately with two-tailed Wilcoxon signed rank tests ($n = 6$, $\alpha = 0.05$). To ensure that dolphins did not differ in respiration activity between the 3 days of the experimental sections, we additionally compared the total number of respirations (i.e., no matter which odor) between all days with two-tailed Wilcoxon signed rank tests ($n = 12$, $\alpha = 0.05$). For the tests, we summed up each individual's values obtained during the sessions of the first, the second, and the third days, respectively, of the three experimental sections. Respiration values in the text and figure are given as mean ± standard error and refer to the session's duration of 10 min.

RESULTS

There was a linear relationship between the Respiration Bias Index (RBI) and the day of the experiment (mixed LM: $\chi^2 = 3.877$, $P = 0.0489$). The **Figure 2** shows, that the bias of dolphin respiration activity toward the fish barrel increased over the course of the experiment.

On the first day, the number of respirations toward the barrel containing fish vs. the control barrel did not differ significantly (fish: 6.5 ± 1.6; control: 11.8 ± 3.1; $P = 0.063$, $V = 20$). However, the dolphins breathed significantly more often in the area around the fish-smelling than in the control situation both at the second day (fish: 13.5 ± 1.3; control: 5.5 ± 0.5; $P = 0.036$, $V = 0$) and at the third day (fish: 16.8 ± 1.9; control: 4.8 ± 1.0; $P = 0.031$, $V = 0$; **Figure 2**). Overall, the total number of respirations (regardless the odor source close by) did not differ between the 3 days (day 1 vs. 2: $P = 0.824$, $V = 30$; day 2 vs. 3: $P = 0.348$, $V = 22$; day 1 vs. day 3: $P = 0.783$, $V = 35$).

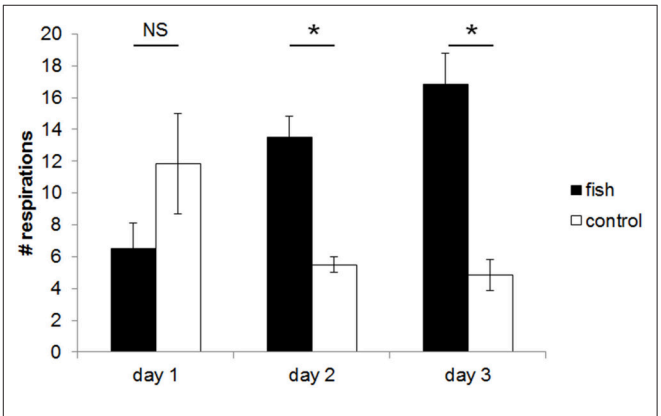

FIGURE 2 | Mean (±SE) number of respirations per 10 min of the dolphins in the area within a radius of 2.5 m around the barrel containing fish or the empty control barrel. The dolphins breathed more often close to the barrel containing fish compared to the control barrel both at day 2 and 3. Statistical difference is indicated by asterisks (*$P \leq 0.05$; NS, not significant; two-tailed Wilcoxon signed rank tests using the sums of each individual's values obtained during the sessions of the 3 days of the three experimental sections).

CONCLUSION

The response of six captive bottlenose dolphins to visually identical but differently smelling devices suggests that this species is capable of perceiving chemosensory stimuli in air (odors). This is, to our knowledge, the first study on spontaneous behavioral responses of dolphins toward a potentially biologically relevant odor. That the dolphins did not discriminate between the fish-smelling and the control device at the first day might be caused by the odor composition. Thus, dolphins may respond to certain molecules whose concentrations increase progressively in the course of decomposition, such as putrescine (Sil et al., 2008). Alternatively, dolphins may be sensitive to some molecules dominant at the second/third day of the experiment due to another interest than foraging. Maybe the dolphins simply responded because of their curiosity to the unfamiliar stimulus, as curiosity seems to be a common personality trait in dolphins (Highfill and Kuczaj, 2007; Kuczaj et al., 2012). For example, the natural curiosity of dolphins and their attraction to novelty may lead them to explore preferentially unknown odors.

STUDY II: GUSTATION

Materials and Methods

Study Subjects

In January and February 2012, we studied four captive-born bottlenose dolphins in the delphinarium of Planète Sauvage: three males (aged 8, 12, and 27 years) and one female (aged 10 years). The dolphins had been housed together for more than three years when the study took place. For information on housing conditions see above.

Stimuli

In the current study, we aimed at testing potential spontaneous preferences for food flavors in a "naturalistic" setting, meaning in conditions where dolphins may express their preferences without going through conditioning procedures. Therefore, we developed and tried an approach enabling to measure spontaneous responses of dolphins to different flavors. It is usual to provide captive dolphins with ice cubes as a source of enrichment (Warne-Reese, 1997). We took this opportunity to provide ice cubes with different flavors and to compare the reaction of the dolphins to these different flavors as a measure of discrimination. Hence, we used the latency of return to the begging spot for ice cubes as a measure of discrimination in the previous ice cube flavor. Informal observations of dolphins' behavior after receiving an ice cube indicated that they hold it in mouth and assess it orally. Thus, our method used a non-invasive and easily replicable technique based on the spontaneous begging responses of dolphins toward more or less attractive items bearing biological relevance.

Ice cubes were equally familiar to all tested dolphins as they were commonly used as part of environmental enrichment in the dolphinarium (1–2 times per week); therefore, all dolphins were accustomed to receive, sense and ingest odor- and tasteless pure water ice cubes. For the present experiment, we produced ice cubes with herring, salmon and shrimp flavors (**Table 2**), originally used for human cooking (salmon/shrimp) or for baiting fish (herring). Herring was familiar to all dolphins through food exposure, whereas salmon and shrimp were not. Semispherical ice cubes (basis diameter: 4 cm; height: 2.5 cm) of 20 mL each were produced with every flavor diluted in plain mineral water (to ensure constant basic composition; Danone "Volvic," Paris, France). Flavorless yellow or purple food colorants (Brauns-Heitmann Ltd. "Crazy Colors," Warburg, Germany) were added to homogenize the visual appearance of the ice cubes for the dolphins and to increase their visibility in the pool for the experimenter. To prevent any flavor-color association by the dolphins, the colors were randomly distributed over ice cubes carrying different flavors. Thus, the ice cubes differed only in terms of flavor, but were visually- and tactually-similar. Ice cube were frozen at $-21.5°$C.

Data Collection

Experimental sessions were performed 1–5 times per day between the feeding sessions (with the largest possible time interval between experimental and feeding session) and lasted on average 8 ± 2 min. Two experimental sessions were separated by at least 60 min. During one experimental session one single flavor was tested. The four stimuli were tested consecutively, meaning we completed all sessions for a given flavor before testing a new flavor: first herring, then salmon, followed by shrimp and last control (the order of the four stimuli was chosen randomly).

All dolphins were together in the pool and when they saw the experimenter coming, they immediately and spontaneously approached her standing at the side of the pool. The dolphins were free to participate, meaning that they received ice cubes only when begging (i.e., when clearly opening their rostrum with the head and eyes over the water surface while being oriented to, and less than 1 m away from, the experimenter; **Figure 3**). This behavior was displayed only in this context and was obviously identifiable. The experimental session started when the experimenter took up her position at the pool (no other person was around the pool) where she was standing with the ice cubes next to her. The experimenter, who was familiar with all dolphins and could reliably identify each individual based on physical differences, never interacted with the dolphins beside of responding to their begging by giving an ice cube. After the display of the begging behavior, the experimenter let the ice cube fall in the open mouth of the dolphin. Begging latency was timed (with a stopwatch) from the moment a given dolphin received an ice cube (contact with the tongue) to the moment it begged for a new one. Begging latency was the only measurable parameter, as other behaviors that occurred between the receipt of an ice cube and the begging of a new one (e.g., playing with the ice cube) were not clearly visible from surface as the dolphins swam around in the pool.

Data Analysis

Statistic calculations were run using R software (version 2.15.0, R Development Core Team, www.r-project.org). As we predicted that all dolphins would react differently to fish (salmon/herring) vs. non-fish (shrimp/control) items or to familiar (herring/control) vs. unfamiliar (salmon/shrimp) food, we compared begging latencies between different flavors by using a Wald test on a Linear Mixed Model, considering the individual as random factor (R-package: lme4). Data have been log-transformed prior to analyses in order to homogenize the variances. Pairwise comparisons were performed with the contrasts method (correction for multiple testing: false discovery rate; R-package: doBy). As dolphins were unrestrained in this experiment, number of ice cubes received differed between individuals and between different tastes. However, this was taken into account by treating individual as random factor in the statistical analysis. Additionally, in order to control that the varied number of received ice cubes per dolphin did not bias the results, the same statistical tests were done with a subset of the data, using only the first eight latencies measured per individual and per taste (as $n = 8$ was the smallest total number of ice cubes delivered; see **Table 3**).

RESULTS

The average latency of the four dolphins to beg for another ice cube differed significantly between the distinctly flavored

TABLE 2 | Flavors and concentrations used to produce ice cubes with fish and non-fish flavors.

	Flavor		
	Herring	**Salmon**	**Shrimp**
Purchaised at	Biomin Holding Ltd., Herzogenburg, Austria	Patiwizz Ltd., Vieillevigne, France	CBV Aroma, Mülheim an der Ruhr, Germany
Form	powder	liquid	liquid
Quantity of flavor/L	6 g	25 mL	2.7 mL

Quantities were chosen in order to obtain a stimulus that resembled as much as possible the quality and intensity of the natural reference products what was assessed by the experimenter through tasting.

FIGURE 3 | A dolphin begging for an ice cube (© B. Schaal).

ice cubes (mixed LM: $\chi^2 = 19.16$, $P = 0.0003$). *Post-hoc* tests indicated that all dolphins took more time to come back after receiving herring- or salmon-flavored ice cubes than after receiving shrimp-flavored or control ice cubes (all dyadic comparisons: $5.04 \leq \chi^2 = 13.84$, $0.001 \leq P \leq 0.037$; **Figure 4**). Both fish-flavored ice cubes triggered similar latencies in the dolphins ($\chi^2 = 0.54$, $P = 0.553$); the same was true for the non-fish tasting ice cubes and the control ice cubes ($\chi^2 = 0.33$, $P = 0.564$). The two familiar flavors (herring and control) elicited different latencies ($\chi^2 = 8.64$, $P = 0.007$); the same was true for the two non-familiar flavors (salmon and shrimp: $\chi^2 = 9.19$, $P = 0.007$).

Even when using a homogeneous subset of the data (i.e., the first 8 latencies), there was still an effect of taste on the begging latency (mixed LM: $\chi^2 = 10.72$, $P = 0.0134$). *Post-hoc* tests indicated, similarly to the analysis with all data, that dolphins came back after significantly shorter latencies after receiving shrimp-flavored ice cubes compared to the other tastes ($6.34 \leq \chi^2 \leq 8.34$, $0.023 \leq P \leq 0.024$), whereas herring, salmon, and control ice cubes triggered similar latencies ($0.00 \leq \chi^2 \leq 0.14$, $0.883 \leq P \leq 0.973$).

CONCLUSION

Using an original method to test spontaneous preferences of dolphins for food flavors, it was possible to show that four captive bottlenose dolphins discriminated visually similar stimuli that differed only by their flavor. Dolphins took more time to beg for a new ice cube after receiving herring-/salmon-flavored ice cubes compared to shrimp-flavored/control ice cubes, indicating that they discriminated between fish and non-fish flavors. Whether stimuli were familiar (herring/control) or unfamiliar (salmon/shrimp) did not seem to influence their response. The prolonged latency after receiving fish-flavored ice cubes can be interpreted differently. One hypothesis is that the dolphins are not interested in fish-flavored ice cubes, wherefore they do not come back fast to beg for a new one. Alternatively, the dolphins might be very well interested in those fish-flavored ice cubes, wherefore they spend a longer time orally assessing these ice cubes. Thus, a prolonged begging latency might reflect a longer time spent "exploring" the flavor by the dolphin. We assume that this second hypothesis is probable because the dolphins could indeed be sporadically observed (when they were close enough to the experimenter) playing with the ice cube in their mouth, however, more experiments are needed to conclude further.

DISCUSSION

The here presented approaches allow the conclusion that dolphins may be able to discriminate odors and flavors respectively. Other behavioral studies on dolphin chemoreception are rare, but show for example that bottlenose dolphins can detect the four basic flavors nearly as well as humans (Friedl et al., 1990). These chemoreceptive abilities might be useful in the context of predation. Odor perception might be used for prey location, as fish, the main prey of dolphins (Spitz et al., 2006), do indeed emit odors (Hirvonen et al., 2000). Although most studies on aquatic species focus on the fact that these odors are used by prey species to detect their respective predator fish, it seems possible that olfactory cues may play an important role as well in the reverse case (i.e., the detection of prey by its predator). There are also reports that some dolphins occasionally feed on already dead prey, precisely they take fish baits, sometimes minutes after baiting (Sumpton et al., 2010). One can therefore wonder whether olfactory cues may contribute to fast localization. Similarly, it has been suggested that some Mysticeti may detect prey by using odors in air although they are produced underwater (Thewissen et al., 2011).

Moreover, chemoreception might play a role in prey evaluation. Free-ranging dolphins do indeed display a feeding selectivity as they preferentially select high-energy density fish species even though they are less abundant than low-energy density fish species (Spitz et al., 2010). One possibility is

TABLE 3 | Average latency to beg for another ice cube (in seconds) and number of given ice cubes for the differently flavored stimuli and for each individual dolphin (mean ± *SE*; *n*).

	Stimulus				
Individual	**Herring**	**Salmon**	**Shrimp**	**Control**	**Overall**
Amtan	120 ± 23	160 ± 32	97 ± 33	73 ± 17	118 ± 14
(♀, 10 years old)	*n* = 17	*n* = 12	*n* = 8	*n* = 8	*n* = 45
Cecil	30 ± 5	30 ± 4	24 ± 4	16 ± 1	24 ± 2
(♂, 27 years old)	*n* = 93	*n* = 109	*n* = 104	*n* = 162	*n* = 468
Mininos	39 ± 5	79 ± 32	26 ± 3	95 ± 22	49 ± 6
(♂, 8 years old)	*n* = 86	*n* = 8	*n* = 9	*n* = 18	*n* = 121
Peos	67 ± 8	43 ± 4	38 ± 4	42 ± 5	45 ± 3
(♂, 12 years old)	*n* = 46	*n* = 70	*n* = 85	*n* = 58	*n* = 259

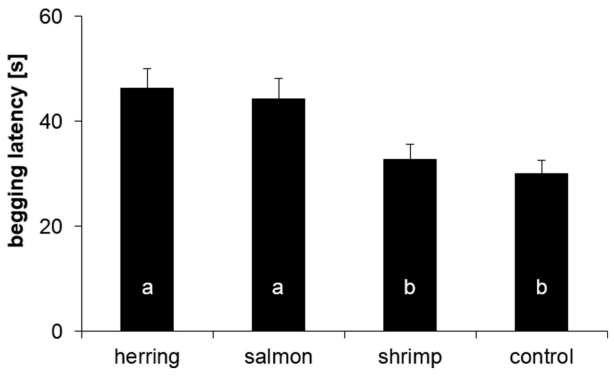

FIGURE 4 | Mean (±SE) latency [s] of the dolphins to come back and beg for a new ice cube after receiving a herring, salmon, shrimp, or control ice cube. Same letters indicate absence of statistical difference ($\alpha = 0.05$).

that dolphins make food choices based on visual or texture differences. Another possibility relates to choices based on taste, odor, or flavor differences. In line with this, salmon and herring, the flavors eliciting longer latencies in our study, are about 1.5–2.5 times more energetic than shrimps (National Nutrient Database for Standard Reference, US National Agricultural Library; available at: http://ndb.nal.usda.gov/). Learned flavor preference may also underlie the choice of dolphins for some foods over others. In the wild, the diet of bottlenose dolphins is primarily composed of fish (94.2% of stomach contents in stranded dolphins), whereas crustaceans are only occasional prey (2.0%; Spitz et al., 2006). This might be caused, aside from differences in pelagic *vs.* benthic preys, by a spontaneous or learned preference for the flavor of fish.

Finally, chemoreceptive abilities may be useful during socio-sexual interactions. Individual recognition or mate detection (e.g., female receptiveness) could be chemically mediated as in many other species. It has been suggested that dolphins may utilize chemosensory cues to gain information about another dolphin's physiological state, for example in reproduction contexts (Norris, 1991; Muraco and Kuczaj, 2015) and they seem to be able to detect the urine and feces of conspecifics (Kuznetzov, 1990). However, further studies are needed to investigate whether and to which extend dolphins actually use chemical cues in different contexts. Proposition on this issue can be found in Kremers et al. (2016).

How dolphins perceive chemical cues remains unclear. Waterborne odors can be carried in water and air what makes them perceptible *via* different perception pathways. Therefore, further investigations are required to shed light on this topic.

In conclusion, our behavioral studies provide results on perception of odors and flavors in dolphins, thus opening new lines of research on cetacean chemoreception. Although the here presented methods are non-invasive, do not require previous training (although these animals were familiar with the barrel and used to receive water ice-cubes) and are easily replicable, we must acknowledge some limitations. First, social facilitation was not controlled in our setting, as dolphins were not tested individually. Second, we used artificial flavors in the gustation experiment and the experimenter was not blind to the stimuli (although we made sure to control the behavior of the experimenter best possible in order to prevent any clue to the dolphins). Moreover, the measured parameter (begging latency) and its interpretation (social influence of collectively assessed animals, habituation) also raise issues wherefore the use of the ice cubes method remains to be validated (e.g., by blindly testing of shortly isolated individuals). Future studies, which should consider these factors, are needed to conclude further.

ETHICAL STATEMENT

The experiments described in this paper were carried out in accordance with the current laws of the country in which they were performed. They complied with the current French laws (Centre National de la Recherche Scientifique) related to animal experimentation and were in accordance with the European directive 86/609/CEE. The research was approved by the "Direction Départementale des Services Vétérinaires" committee of Loire-Atlantique prefecture. No further permit was needed as only behavioral observations were performed. Animal husbandry and veterinary

care were under management of Planète Sauvage, as this study involved animals from a private animal park (no laboratory animals) with whom informed consent has been granted.

AUTHOR CONTRIBUTIONS

All co-authors designed the work. AC, AL, MH, and SC equally coordinated the study on olfaction, including contribution to analysis and interpretation of data. AL, BS, MH, and MT equally coordinated the study on gustation, including contribution to analysis and interpretation of data. DK performed the experiments, supported by MB, on both olfaction and gustation, and analyzed and interpreted the data. DK, AL, and MH prepared the main manuscript text. All co-authors contributed to the manuscript preparation in the form of discussion and critical comments, and reviewed the manuscript.

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REVIEW
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Sensory Perception in Cetaceans: Part I—Current Knowledge about Dolphin Senses As a Representative Species

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A large part of the literature on sensory perception and behavior in dolphins is devoted to its well-developed vocal and echolocation abilities. In this review, we aim to augment current knowledge by examining the literature on dolphins' entire "*Merkwelt*" (which refers to everything a subject perceives, creating a crucial part of the subject's *Umwelt*). We will show that despite extensive knowledge on audition, aspects such as context relatedness, the social function of vocalizations or socio-sexual recognition, remain poorly understood. Therefore, we propose areas for further lines of investigation. Recent studies have shown that the sensory world of dolphins might well be much more diverse than initially thought. Indeed, although underwater and aerial visual systems differ in dolphins, they have both been shown to be important. Much debated electro- and magnetoreception appear to be functional senses according to recent studies. Finally, another neglected area is chemoreception. We will summarize neuroanatomical and physiological data on olfaction and taste, as well as corresponding behavioral evidence. Taken together, we will identify a number of technical and conceptual reasons for why chemosensory data appear contradictory, which is much debated in the literature. In summary, this article aims to provide both an overview of the current knowledge on dolphin perception, but also offer a basis for further discussion and potential new lines of research.

Keywords: cetaceans, Delphinidae, *Tursiops truncatus*, audition, vision, electroreception, magnetoreception, chemoreception

DOLPHIN'S *UMWELT*

Sensory perception is essential for the survival of organisms, be it for the detection of (un)favorable physical conditions, the presence/absence of food or predators, the detection of communication signals or the recognition of social partners. It is crucial for any species to perceive regularities and changes in the properties of their abiotic and biotic environment.

The perception of an organism's local environment is one part of a living being's *Umwelt* (von Uexküll, 1909). The literal translation of *Umwelt* from German to English is "environment," but the typical biological meaning is better described as an organism's "subjective universe" (Chien, 2006). Initially, appropriate sensory receptors have to be able to detect the characteristics of surrounding objects, contexts and conspecifics (von Uexküll, 1909). After being perceived, information concerning the object is further processed through corresponding neural structures and a specific meaning is attributed to each stimulus depending on the context or the subject's internal state. Everything an organism perceives creates its *Merkwelt* (English translation: perceptual world). For the sake of completeness: the other part of its *Umwelt* is the action a living being is taking on its environment according to the meaning that was previously attributed to the perceived stimuli. Everything an organism does creates its *Wirkwelt* (English translation: active world).

Sensory receptors and perceptual processing structures are critical in the perception of the environment, thus a species' body plan determines the *Umwelt* (von Uexküll, 1934). Although several species can share the same environment, each has its own *Umwelt* as sensory abilities may differ from one species to another. Even within the same species, individuals do not necessarily share identical *Umwelten* because of morphoanatomical differences caused by genetic defects or events during ontogeny (e.g., a blind and a seeing person may share the same environment, but not the same *Umwelt*).

It is difficult to determine a species' *Umwelt* from an external point of view because we, as humans, also possess our own *Umwelt*. By simply transferring our perception of reality to another species, we do not respect the subjectivity of a specific organism. Indeed, an object that might be meaningful from the human point of view may be meaningless to another species (Delfour, 2010) either because it does not possess the according receptors to perceive the object's feature or because the object, although it can be perceived, does not have an importance for this species. Therefore, an unbiased study of a species' sensory perception and behavior is necessary.

When it comes to sensory perception, cetaceans are particularly informative because they underwent a drastic change in lifestyle in the course of evolution. This mammalian order is currently considered as having evolved about 47 million years ago (MYA) from a small deer-like ancestor (Thewissen et al., 2009), moving from a terrestrial lifestyle back to an aquatic environment. This evolutionary reversal in habitat caused extensive, yet slow rate changes in anatomy, neuroanatomy, physiology, and behavior (Gatesy et al., 2013). The results of this transformation are overtly seen in the baleen whales (Mysticeti) and the toothed whales (Odontoceti). These two suborders are very different in terms of morphology, feeding ecology and behavior, wherefore knowledge gained about mysticete species can be generalized to odontocete species (and *vice versa*) only with caution, if at all. Therefore, a general "cetacean *Umwelt*" does not exist. A species-specific perspective is required to understand the *Umwelt*. The odontocete family Delphinidae includes the best-studied cetacean species; therefore, they present a suitable model to outline their *Umwelt*. The analysis of the dolphin's perceived environment will begin with a review of some of the sensory abilities of dolphins, namely audition, equilibrioception, vision, somatosensory perception, electroreception, magnetoreception and chemoreception. Each sense is described in a section that comprises anatomical, physiological and behavioral data, followed by propositions for further lines of investigation. Whenever possible, precise data refer to the bottlenose dolphin (*Tursiops truncatus*), but for a broader view other members of the family of Delphinidae are included, as well as information that are true for Delphinidae or Odontoceti in general. For those sensory modalities where little literature is available for dolphins, this review includes other cetacean species.

AUDITION

Current Knowledge on Audition

Most research efforts on dolphin sensory systems over the past 50 years has been devoted to the study of audition (reviewed in Au et al., 2000), namely the ability to detect oscillations of pressure transmitted through air, water or another medium. Hearing in cetaceans has been evaluated mostly by auditory evoked potentials (e.g., Mooney et al., 2015) or behavioral audiograms (e.g., Kastelein et al., 2003).

The sounds that are perceived can originate from prey, predators or conspecifics. Beside echolocation, some delphinids are known to detect their prey by passive listening (Barros, 1993; Gannon et al., 2005), meaning that they use the sounds produced by their prey to locate it. Noise-producing fish make up a large part of the bottlenose dolphin's diet (Barros and Wells, 1998). Indeed, it was suggested that the cetacean ancestor developed high-frequency hearing to locate sound-producing fish already in Eocene and based on this ability echolocation evolved in Oligocene odontocetes enabling the location of silent prey (Fahlke et al., 2011).

Sharks (Heithaus, 2001) and orcas (*Orcinus orca*; Constantine et al., 1998) occasionally attack dolphins. However, not all orcas are hunting mammals (there are also fish-eating orcas) and other cetacean species seem to be able to discriminate between mammal- and fish-eating orcas. The playback of vocalizations of fish-eating orcas elicited an increase in group size in pilot whales (*Globicephala melas*) and a strong attraction toward the sound (Curé et al., 2012), whereas the playback of vocalizations of mammal-eating orcas prompted a clear avoidance response in beaked whales (*Mesoplodon densirostris*; Tyack et al., 2011).

Beside natural sound sources such as prey, predators or conspecifics, cetaceans are also exposed to and disturbed by anthropogenic noises originating from military and seismic survey sonars (e.g., Jepson et al., 2003; Piantadosi and Thalmann, 2004), boat noise (e.g., Buckstaff, 2004), or drilling (e.g., Bailey et al., 2010). After loud noise exposure, several cetacean species show a hearing threshold shift, which can be temporary or permanent, meaning a noise-induced hearing loss (e.g., Mooney et al., 2009a; Finneran and Schlundt, 2010; Mann et al., 2010).

Anatomical Data on Audition

The anatomy of the odontocete ear is exclusively adapted for underwater hearing and differs from that of terrestrial mammals: the outer ear pinna as sound collector is replaced with the lower jaw, and the tympanic membrane as sound transmitter is replaced with a thin and large tympanic bone plate (Hemilä et al., 2010). The primary sound perception pathway is considered to be that the lower jaw receives the sound energy and transmits it through fatty tissue located in the mandibular canal (mandibular fat pad) up to the tympanic plate, with best auditory sensitivity at the middle of the lower jaw (Møhl et al., 1999). The mandibular fat pad is composed of triacylglycerol being similar in density, and thus acoustic impedance, to water (Varanasi and Malins, 1971). Middle and inner ear are located together in the tympano-periotic complex that is surrounded by air cushions, which acoustically isolate the ear from the skull (Cranford et al., 2010). Bone density of the tympano-periotic complex increases rapidly during the first months of life, which possibly reflects the importance of hearing for dolphins (Cozzi et al., 2015).

Physiological Data on Audition

Acoustic impulses are transmitted from the ear to the brain *via* the cochlear nerve, part of the vestibulocochlear nerve (cranial nerve (CN) VIII; **Figure 1**). Dolphins seem to process auditory impulses in at least two brain areas. The primary auditory cortex is believed to be located in the suprasylvian gyrus along the vertex of the hemispheres, lateral and adjacent to the primary visual cortex (Popov et al., 1986). In addition, a recent study found that the auditory cortex also exists in the temporal lobe (Berns et al., 2015). Odontocetes tend to have a 10-octave functional hearing range with peak sensitivity between 40 and 80 kHz (Wartzok and Ketten, 1999). In the bottlenose dolphin, hearing ranges up to 150 kHz, with optimal sensitivity within 10–80 kHz (Houser and Finneran, 2006). Dolphins, like cetaceans in general, have good directional hearing. Generally, the direction from which a sound originates may be determined by the difference in arrival time of a sound to each of two ears. As this interaural time difference is calculated by $\frac{interaural\ distance}{sound\ velocity}$, the increased sound velocity in water (compared to air) leads to a reduction in the interaural time difference (compared to air). Cetaceans can compensate for this effect as they have relatively large heads and therefore a naturally large interaural distance, thus increasing the interaural time difference (Nummela and Thewissen, 2008).

Behavioral Data on Audition

Traditionally, research on audition in dolphins has focused on echolocation and communication. Dolphins produce three different categories of vocalizations: clicks, burst-pulsed sounds and whistles (reviewed in Janik, 2009). Clicks are short broadband signals that can exceed 100 kHz and are mostly used for echolocation. Burst-pulsed sounds consist of highly directional, rapid click trains: for example, the bray calls generated by the bottlenose dolphin (Janik, 2000a), the so-called "squawks," "yelps," and "barks" (Schultz et al., 1995), as well as "moans" or "rasps" (Caldwell and Caldwell, 1967). The distinction between echolocation clicks and burst-pulsed sounds is not always easy. Whistles are tonal,

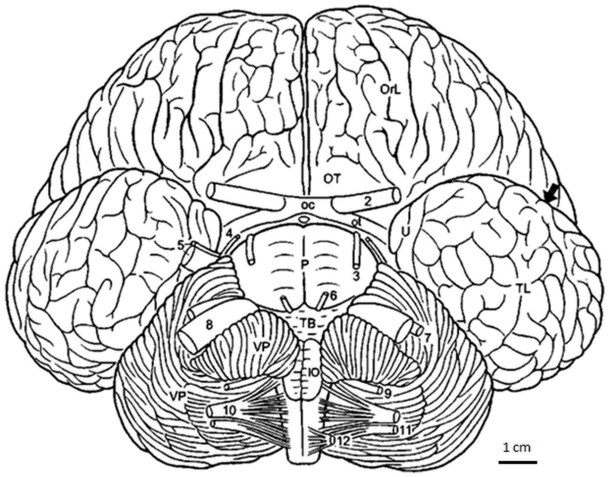

FIGURE 1 | Bottlenose dolphin brain in basal aspect (after Langworthy, 1932, modified after Pilleri and Gihr, 1970; Morgane and Jacobs, 1972). Arrow pointing into sylvian cleft. Ot, optic tract; OT, olfactory tubercle; TL, temporal lobe; U, uncus; VP, ventral paraflocculus; 2-12, cranial nerves; 2, optic nerve; 3, oculomotor nerve; 4, trochlear nerve; 5, trigeminal nerve; 6, abducens nerve; 8, vestibulocochlear nerve; 9, glossopharijngeus nerve; 10, vagus nerve; 11, accessory nerve; 12, hypoglossals nerve. Scale: 1 cm.

frequency-modulated signals with fundamental frequencies lying between 800 Hz (Schultz and Corkeron, 1994) and 28.5 kHz (May-Collado and Wartzok, 2008), and often several harmonics. Whistles and burst-pulsed sounds can be produced simultaneously (Janik, 2009). This corresponds with the generally accepted concept that there are two sites of sound production that can be controlled independently (Dormer, 1979). They are composed of two identical sound producing structures consisting of fatty dorsal bursae within a pair of phonic lips, one in the left and one in the right nasal passage (Cranford et al., 1996). A recent study suggested that the two dolphin brain hemispheres, which sleep independently (Lyamin et al., 2008), may also act independently when it comes to coordinating prey capture and communication with simultaneously emitted echolocation clicks and social sounds (Ridgway et al., 2015).

Echolocation

An important function of sound for odontocetes is echolocation (or biosonar), where they emit short sound pulses (clicks) and listen for returning echoes to generate an auditory representation of their surroundings for navigation and foraging (Madsen and Surlykke, 2013). As shorter wavelengths have a better spatial resolution, and wavelength is inversely proportional to frequency, high frequencies are better suitable for detecting small objects compared to low frequencies. Consistently, species inhabiting acoustically complex inshore and river waters use higher frequencies for echolocation (>100 kHz) than near- and offshore species (<100 kHz) that inhabit low object-density environments (Wartzok and Ketten, 1999). Rapid auditory temporal processing facilitates echolocation and sound location (Mooney et al., 2009b). While echolocating, dolphins are able

to hear (Li et al., 2011), to adjust their hearing (Nachtigall and Supin, 2008), as well as being able to process the heard echoes and vocalize while still echolocating (Ridgway et al., 2012). Furthermore, dolphins possess several mechanisms for gain control (reviewed in Supin and Nachtigall, 2013). Echolocation is so effective in bottlenose dolphins that they can still detect a small object of less than 8 cm at distances of over 110 m (Au and Snyder, 1980) and discriminate objects by using spectrum shape of the echo, as well as its peak and center frequency (DeLong et al., 2006). Bottlenose dolphins start to echolocate at the early age of around 2 months (Carder, 1983), supporting the hypothesis that the structures involved in hearing develop early in life due to the importance of these inputs for survival and development (Cozzi et al., 2015).

Communication

In a habitat where vision is not always possible, acoustic signals provide a good communication channel, even for over long distances. Most delphinids use whistles for communication, but some species use pulsed sounds (e.g., Commerson's dolphin, *Cephalorhynchus commersonii*; Yoshida et al., 2014). Why some delphinid and other odontocete species [e.g., the family of Phocoenidae (porpoises), the pygmy sperm whale, *Kogia breviceps*, and the genus of *Pontoporia*] do not produce whistles, but only pulse sounds was connected to the orca predation risk. It was hypothesized that species with high orca predation risk were subject to a selective pressure favoring vocalizations restricted to sounds that orcas hear poorly or not at all (i.e., below 2 and above 100 kHz; Morisaka and Connor, 2007). On the other side, orcas adapt their vocal behavior to the prey they are hunting: transient orcas that feed on marine mammals (a prey with sensitive underwater hearing) vocalize less and reduce their vocal activity before and during hunting compared to resident orcas that feed on fish (a prey with poor hearing abilities; Deecke et al., 2005).

Most studies on delphinid communication are concerned with whistles because they are thought to play an important role in social interactions (Díaz López, 2011). Whistles have varying numbers of harmonics and delphinids can distinguish between whistles with and without harmonics (Yuen et al., 2007). Whereas the fundamental frequency is relatively omnidirectional, higher order harmonics are directional (Lammers and Au, 2003). Bottlenose dolphins can discriminate tonal sounds that differ in frequency by only 0.2–0.8% (Thompson and Herman, 1975), but they seem to pay more attention to frequency modulation than to the absolute frequency (Ralston and Herman, 1995). The active space (i.e., the transmission range over which a signal can be detected by conspecifics) for bottlenose dolphins' whistles is determined as 10–20 km for frequencies below 12 kHz (Janik, 2000b). However, the active space of a sound depends (among other factors such as its frequency) on bottom substrate and water depth. For example, the same call is perceived at less than 200 m in a shallow sea grass area of 1.6 m depth, but up to more than 6 km in a sandy bottom area of 3.5 m depth (Quintana-Rizzo et al., 2006).

Bottlenose dolphins are known for their production of signature whistles, which are individually distinctive whistles that do not depend on the individual's voice features, but the whistles' frequency contour (reviewed in Janik and Sayigh, 2013). These are used for individual recognition, for example between mother and offspring (Sayigh et al., 1998). A proof of the dolphins' capacity to discriminate even complex frequency modulations is their call matching which has been experimentally tested in wild dolphins and it seems possible that dolphins use these copies (i.e., mimicking sounds) as referential vocal labels in order to address each other (King and Janik, 2013). Furthermore, dolphins seem to be capable of remembering the signature whistles of other individuals for at least 20 years (Bruck, 2013).

Perspectives on Audition

Although there is a multitude of studies concerning the vocal communication of delphinids, many questions remain unresolved that often due to technical and methodological constraints, such as individually assigned recordings and unlimited access to the animals. The latter concern greatly influences those studies with direct observation of free-ranging cetaceans, which are not always easy to detect and to follow. Technical assistance is necessary to identify the vocalizing individual as it is not possible to visually assign a vocalization to its emitter (i.e., dolphins do not open their mouths to vocalize and or systematically produce any other visible correlate of vocalizing). Although such approaches do exist, they are often expensive and/or possibly disturbing to the animals, as the device is generally secured to the body (e.g., Johnson and Tyack, 2003; Blomqvist and Amundin, 2004). For instance, most studies only tag one or a few individuals, which may result in limited data that does not appropriately address a particular research question. This technique might not be suitable for studies that want to test the social function of vocalizations or communication rules during vocal exchanges as this is difficult, if not impossible, when only one group member is tagged.

Alternatively, individuals can be temporarily restrained to record individually assigned vocalizations (e.g., Watwood et al., 2005), but this particular context does not allow for a broad range of vocalizations to be recorded and further studied as stress strongly influences the pattern and content of vocalizations. For example, bottlenose dolphins are thought to encode their level of stress *via* the whistle rate (Caldwell et al., 1990) and an alteration of acoustic parameters, while keeping the overall frequency modulation pattern constant (Esch et al., 2009). Another, less invasive technique is triangulation, where the location of the sound source is determined by recording with two or more hydrophones and then calculating the origin of the sound. Therefore, simultaneous visual information is necessary to identify the individual that is present at the location calculated as the sound source, making this technique sometimes difficult to apply to free-ranging dolphins.

With regards to echolocation, there is an approach that might yield some insightful results: the "echolocation visualization and interface system," which can visualize echolocation signals and be used as an "acoustically operated 'touch screen"' (Amundin et al., 2008). The echolocation signals of dolphins are recorded with hydrophones when those clicks are aimed at

a semitransparent screen. Subsequently, the recorded acoustic signals are translated into a corresponding visual image that is projected on the location where the echolocation signals contact the screen: this leads to immediate visual feedback (Amundin et al., 2008). Another approach is eavesdropping on echolocation signals of conspecifics (reviewed in Gregg et al., 2007). In a behavioral experiment, a bottlenose dolphin was able to correctly choose an object in a matching-to-sample task by eavesdropping on the echoes produced by the echolocation signals of a conspecific (Xitco and Roitblat, 1996). However, the ecological implications of this passive echolocation remain unknown.

EQUILIBRIOCEPTION

Current Knowledge on Equilibrioception

Equilibrioception is the sense of balance, which provides information about the body's movement. Due to physical differences, more three-dimensional movements are possible in water compared to land, which lead to an increased importance of equilibrioception for aquatic species.

Anatomical Data on Equilibrioception

In vertebrates in general, the sensory organ of balance is the vestibular system in the inner ears. Linear movement and gravity are detected by the two otolith organs, one in the utricle and the other in the saccule, which are located in the vestibule (Rabbitt et al., 2004). Rotational movements are detected by the three membranous semicircular ducts, which are enclosed by the three bony semicircular canals (anterior, posterior, and lateral; Graf, 1988). The otolith organ seems to be well developed and fully functional in cetaceans, with a thicker membrane compared to other mammals (Solntseva, 2001). The semicircular canal system of cetaceans is smaller relative to body size when compared to other mammals. In bottlenose dolphins, the mean radius of curvature of the three canals is 1.1 mm (Spoor and Thewissen, 2008). For comparison: in greater kudus (*Tragelaphus strepsiceros*), an Artiodactyla species with a similar body mass, the mean radius of curvature of the three canals is 3.5 mm (Spoor and Thewissen, 2008). Furthermore, the cetacean lateral canal is the largest and the posterior canal the smallest of the three semicircular canals (reviewed in Spoor and Thewissen, 2008). In bottlenose dolphins, the relative size of the anterior, posterior and lateral canal is 34, 28, and 38%, respectively. This is in contrast to other mammals where the lateral canal is the smallest if the three canals.

The size reduction concerns only the semicircular canals, and not the entire inner ear of cetaceans, as their cochlea is similar in size relative to body mass when compared to other mammals (Spoor et al., 2002). Is was proposed that this size reduction is due to the dominant auditory function of the inner ear and thus a limited space for the vestibular system (Boenninghaus, 1903). Another explanation for the comparatively small semicircular canal system in cetaceans concerns its sensitivity: the smaller the semicircular canal system, the less sensitive it is. What seems disadvantageous at first sight might be favorable for species with increased head movements, as is the case for cetaceans due to their swimming movement and fused cervical vertebrae (leading to a mostly immobile neck that no longer compensates for body movement to stabilize the head). Here, reduced sensitivity of the sense of balance might help to avoid an overstimulation of the semicircular canal system, which would otherwise lead to disorienting effects (Spoor et al., 2002). Consistent with this hypothesis is the fact that size reduction is more pronounced in Odontoceti, which are more agile compared to Mysticeti (Spoor and Thewissen, 2008).

Physiological Data on Equilibrioception

It is presumed that equilibrioception in dolphins works physiologically similar to other species: impulses from the vestibular system are transmitted to the brain *via* the vestibular nerve, part of the vestibulocochlear nerve (CN VIII). The information is then processed in the vestibular brain stem nuclei, which transmit neural signals to motor nuclei in order to generate reflexive movements of the eyes and/or other body parts in order to stabilize the body (Sipla and Spoor, 2008).

VISION

Current Knowledge on Vision

Another important sense to perceive the environment is vision, which constitutes the ability to detect variations in the intensity and wavelength of light. When light passes through water it is differently absorbed, refracted and scattered depending on the wavelength of the light, as well as the concentration and type of dissolved material in the water. In shallow waters, longer wavelengths of the light spectrum are common, whereas only shorter wavelengths can penetrate well into deeper layers of water (Wartzok and Ketten, 1999). In general, light decreases with depth.

Anatomical and Physiological Data on Vision

As in all cetaceans, dolphin eyes are located laterally (directed ventronasally), allowing a panoramic vision with a 120–130° visual field, and can be moved independently from each other (Mass and Supin, 2009). Several anatomical structures inside the eyes protect them from mechanical damage (e.g., a thickened cornea to resist water pressure) or cooling (Mass and Supin, 2009). Furthermore, a secretion produced by the Harderian gland protects the eyes from the high concentration of salt in marine water (Dawson et al., 1972, 1987). Bottlenose dolphins have good underwater and in-air vision with a visual acuity of 12.6 min of visual angle from a distance of 2.5 m (Herman et al., 1975), probably due to their asymmetric double-slit pupils (Rivamonte, 2009) and excellent distance estimation (Mobley and Helweg, 1990). Under low-light conditions this pupil is round and roughly U-shaped in bright light conditions (Mass and Supin, 2009). In general, the lens of the cetacean eye is very strong and more similar to those of fish compared to the lens of terrestrial mammals (Wartzok and Ketten, 1999).

In cetaceans, visual sensitivity is maximized by a high density of photoreceptors (400,000 cells/mm^2 in bottlenose dolphins; Dral, 1977), as well as a *tapetum lucidum* (i.e., a reflective layer behind the retina that increases the amount of light absorbed

by the photoreceptors; Dawson, 1980). Both rod and cone photoreceptors have been described in the retina of bottlenose dolphins (Perez et al., 1972), with absorption maxima of 488 and 524 nm for the rod and cone pigments, respectively, which are both short-wavelength shifted compared to many terrestrial mammals (Fasick et al., 1998). However, bottlenose dolphins only possess long/middle-wavelength-sensitive L-cones but no short-wavelength-sensitive S-cones, thus they are thought to lack the common dichromatic vision typical of many terrestrial mammals and may, therefore, be colorblind (Simons, 1977; Fasick et al., 1998; Peichl et al., 2001). However, under mesopic conditions, where both rods and cones are active, bottlenose dolphins (as well as other so-called monochromatic cetaceans) might exhibit "conditional dichromacy" and a rudimentary form of color vision (Davies et al., 2012), as hypothesized for wobbegong sharks (Theiss et al., 2012).

The dolphin retina possesses (partly giant) ganglion cells (Perez et al., 1972) with a density of up to 670 cells/mm^2 (Mass and Supin, 1995). The retinal ganglion cells receive visual information from the photoreceptors via inter-retinal neurons and transmit them through the optic nerve (CN II). The optic nerve has a low fiber density (50,000 fibers/mm^2 compared to >220,000 fibers/mm^2 in monkeys), which in bottlenose dolphin comprise of 150,000–180,000 optic fibers in total (Mass and Supin, 2009).

Visual impulses are transmitted by the optic nerve to the midbrain, the thalamus and the cerebral cortex (superior colliculus, lateral geniculate nucleus and primary visual cortex) where they are processed further (Glezer et al., 1995).

Behavioral Data on Vision

Tested in a visual-matching task, the patterns of perceptual similarities for two-dimensional forms of dolphins was found to be similar to those of chimpanzees and humans (Tomonaga et al., 2014). Contrarily to the previously mentioned hypothesis of color blindness in dolphins (Peichl et al., 2001), a behavioral experiment showed that a bottlenose dolphin had two peaks in spectral sensitivity and that it could discriminate between two wavelengths with equal brightness (Griebel and Schmid, 2002). These findings are consistent with the "conditional dichromacy" hypothesis (Davies et al., 2012) mentioned above. The debate on the evolution and underlying mechanisms of cetacean color vision is still ongoing (Griebel and Peichl, 2003; Meredith et al., 2013).

Delphinids use their good sense of sight in a variety of contexts, from social interactions to prey capture. In short-range communication, visual displays are known to play an important role for delphinids. Postures are thought to signal intent and demeanor of the signal emitter (Dudzinski, 1996). The S-posture, in which the dolphin's body is bent into an S-shape (head pointing down, pectoral fins stretched out), is often associated with aggressive behaviors that include sexual interactions and disciplinary behavior toward juveniles (Dudzinski, 1996; Bojanowski, 2002). The S-posture is consistent with aggressive behaviors in other cetaceans too (e.g., humpback whales, *Megaptera novaeangliae*), which might be comparable with the arched head and neck position known in many terrestrial mammals during displays of aggression (Dudzinski, 1996). The dolphin's head-to-head posture is often accompanied by jaw claps, hits, tail hits and "squawks" (burst-pulsed sounds) that are thought to express irritation or anger (Dudzinski, 1996). Jaw claps and head jerks are also described by Connor et al. (2000) as aggressive behaviors. Furthermore, these authors describe a distinct posture, in which the dolphin arches the head and flukes down, which may be used to threaten another dolphin.

Vision also mediates non-aggressive interactions. Affiliation between individuals is, among others, expressed by proximity and synchronous movements (Connor et al., 2000). Another visual display occurs in reproductive contexts; for example, when dolphins present their genital region to sexually attract a mating partner (Tyack, 2000).

There is some evidence that dolphins use pointing gestures (Xitco et al., 2001) and that complex behaviors such as foraging techniques are taught by action imitation that in turn require observation and good vision (Bender et al., 2009; Abramson et al., 2013).

Beside conspecifics, cetaceans use vision for the inspection of their surroundings, both in water and air. A common behavior of several cetacean species is spyhopping, i.e., surfacing vertically and lifting the head out of the water (e.g., Ford, 1984; Whitehead and Weilgart, 1991; Jensen et al., 2013), that seems to serve the inspection of objects above water (Madsen and Herman, 1980). When inspecting objects or humans, familiarity of the object/human to dolphins influences their behavior: dolphins show a visual laterality, using the left eye when looking at familiar objects and the right eye when looking at unfamiliar objects (Blois-Heulin et al., 2012). Furthermore, their gaze lasts longer when viewing unfamiliar humans compared to those that are familiar (Thieltges et al., 2011). Dolphins use their accurate vision, for example, when catching fish in air after they have hit them firmly with their fluke (Wells et al., 1987). Some foraging behaviors of the bottlenose dolphin were also found to be lateralized, meaning a localization of function or activity on one side of the body in preference to the other (e.g., Silber and Fertl, 1995; Lewis and Schroeder, 2003). It was suggested that the observed right-sided lateralization in dolphins (but also whales) when foraging may be associated with the visual perception of prey (Karenina et al., 2016).

Perspectives on Vision

Color vision is another topic that appears worth of further analyses. Knowing which opsin-based photopigments are expressed in the cetacean retina and their corresponding spectral sensitivities only suggest the potential for color vision. However, behavioral experiments are critical to understanding the functional consequences of the suggested colorblindness or hypothesized "conditional dichromacy" that might exist in many so-called marine mammal monochromats. For example, the normally white ventral side of bottlenose dolphins can be remarkable pink in periods of high sexual activity (personal observation of the authors), which might be used as a reproductive visual cue. How males react to a female whose abdomen is colored pink (either in the field or altered experimentally) is unknown and worthy of further study.

SOMATOSENSORY PERCEPTION

Current Knowledge on Somatosensory Perception

Somatosensory systems comprise the perception of touch (via pressure and strokes), pain (nociception), temperature (thermoreception), and body position (kinesthesis and proprioception). Several different receptor types are involved (including mechanoreceptors, nociceptors, and thermoreceptors) that are located in the dermis, muscles and joints. Aquatic species can perceive water movement through their mechanoreceptors (e.g., Dehnhardlt et al., 1998). The ability to know the relative body position of an organism is crucial for an air-breathing animal that lives in a three-dimensional underwater habitat in order to orient itself toward the surface even when no visual cues are available and to feel whether the blowhole is above the water (to ensure respiration).

Anatomical Data on Somatosensory Perception

The skin of bottlenose dolphins is furrowed by small ridges that are circumferentially oriented in the anterior part of the body (head to dorsal fin) and more obliquely positioned in the posterior part of the body (dorsal fin to caudal fin). The function of these ridges has been implicated in tactile sensing (Shoemaker and Ridgway, 1991), hydrodynamics (Ridgway and Carder, 1993) or both. In the region of the blowhole, large numbers of mechanoreceptors are present that are thought to serve in the perception of pressure changes that occur when the whale/dolphin breaks through the water surface in order to ensure that the blowhole is opened for respiration only after surfacing (Bryden and Molyneux, 1986). Most odontocetes possess vibrissae (i.e., sensory hair), especially on the rostrum of newborns, losing them shortly after birth (Ling, 1977). Thus, adult dolphins possess hairless vibrissal/follicle crypts on the rostrum, except for the Amazon River dolphin (*Inia geoffrensis*) where the presence of rostral sensory hairs continues into adulthood (Dehnhardt and Mauck, 2008). In general, vibrissae are more common in mysticetes (e.g., Drake et al., 2015).

Physiological Data on Somatosensory Perception

Cetacean skin is well innervated and very sensitive to touch (Tyack, 2000). Skin sensitivity was examined by studies using either somatosensory evoked potentials (e.g., Lende and Welke, 1972) or the galvanic skin response (e.g., Kolchin and Bel'kovich, 1973). Dolphins are most sensitive on their heads (corners of the mouth, eyes, snout, melon, and the area around the blowhole), reaching a sensitivity comparable to human fingertips or lips (Ridgway and Carder, 1990). Somatosensory information is processed in the postcruciate gyrus of the cerebral cortex (Supin et al., 2001).

Behavioral Data on Somatosensory Perception

Dolphins are able to perceive pressures as small as 10 mg/mm^2 around the blowhole and the eyes (Kolchin and Bel'kovich, 1973). Besides the surrounding water, somatic stimuli can originate from objects in the environment. Rubbing occurs in both captive and free-ranging cetaceans. Delphinids were found rubbing their bodies on particular substrates (e.g., pebbles, sand, or along rocky edges; Smith et al., 1992; Whitehead et al., 2004; Rossi-Santos and Wedekin, 2006), which may possibly have a role in pleasure, hygiene (Dudzinski et al., 2012), or might even be a result of play behavior (Kuczaj et al., 2006).

Touch is also an important short-range communication signal utilized during play, sexual, maternal and social contexts, and involves the entire body (Dudzinski et al., 2009a). Tactile contacts between dolphin conspecifics can be observed during aggressive interactions (including biting etc.), but are also common in affiliative contexts (Paulos et al., 2008; Dudzinski et al., 2009b, 2010, 2012). Affiliation between individuals is expressed by proximity and physical contact (Connor et al., 2000), which includes contact swimming, gentle stroking with the pectoral fin or rubbing against another individual. Sakai et al. (2006) reported that flipper rubbing in wild Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) is an affiliative behavior, which could be a quantitative measure of social relationships among individuals. Tamaki et al. (2006) reported that flipper rubbing might contribute to restore friendly relationships between former opponents or reduce conflicts. Thus, flipper rubbing may be the cetacean equivalent of primate grooming (Tamaki et al., 2006; Connor, 2007). People working with delphinids in captivity suggest that petting is appreciated by the animals and, therefore, could be used as a reinforcer in training (Dudzinski et al., 2009a).

Perspectives on Somatosensory Perception

Most odontocetes possess vibrissae, especially on the rostrum, right from birth (Ling, 1977). It would be informative to test whether these perioral hairs serve a tactile function in the context of suckling, when acoustic (echolocation) and visual abilities of young dolphins are not fully developed.

ELECTRORECEPTION

Current Knowledge on Electroreception

Electroreception is the ability to detect an electric field. Electric stimuli can originate from both abiotic and biotic sources. Bioelectric fields are generated for instance by all muscle movement and the water medium provides ideal conditions for conducting electrical currents, although the spread of these stimuli is far less in the marine environment compared to freshwater (Czech-Damal et al., 2013). In active electroreception, the animal itself generates an electric field and senses distortion of this field from objects of varying conductivity present in its habitat; for example, in electric eels (*Electrophorus electricus*; Souza et al., 2007). In passive electroreception, the animal perceives electric fields generated by an object that is located in close vicinity (Czech-Damal et al., 2012); for example, prey detection by elasmobranchs (Kalmijn, 1971) that possess electroreceptors called ampullae of Lorenzini (Murray, 1960).

Behavioral Data on Electroreception

So far, there is only one study on electroreception in cetaceans, which analyzes the behavioral response of a trained Guiana dolphin (*Sotalia guianensis*) toward electrical stimuli. The

dolphin has been found to be sensitive to weak electric currents, such as those emitted by the muscles of prey fish buried in the sediment (Czech-Damal et al., 2012). The electroreceptors are probably situated within the hairless vibrissal crypts on the rostrum (Czech-Damal et al., 2013). As a control, these vibrissal crypts were covered with a plastic shell to prevent contact with seawater. After such treatment, the dolphin could not detect the electric stimuli, whereas a plastic shell that did not impede seawater from contacting the vibrissal crypts did not affect the dolphin's ability to detect electric stimuli (Czech-Damal et al., 2012).

Perspectives on Electroreception

Electroreceptors as found in the Guiana dolphin (Czech-Damal et al., 2012), however, further investigation is essential in other dolphin species to determine their broad functional role in electroreception. Bottlenose dolphins, for example, possess vibrissal crypts on their rostrum, but their involvement in electroreception has not been assessed. Passive electroreception could function as a supplementary sense to echolocation during benthic feeding (Roitblat et al., 1995), which is not uncommon in bottlenose dolphins (Rossbach and Herzing, 1997).

MAGNETORECEPTION

Current Knowledge on Magnetoreception

Magnetoreception is the ability to perceive a magnetic field. The Earth's magnetic field is a dipole field that is generated by the Earth's fluid outer iron core (Wiltschko and Wiltschko, 1995), providing a consistent source of directional information (Winklhofer, 2010). Its intensity ranges from over 60,000 nanoteslas (nT) near the magnetic poles to 30,000 nT at the magnetic equator, but shows minimum values below 26,000 nT at the east coast of South America. In the ocean, the magnetic topography (i.e., variation in the magnetic field) is regular and stable long-term, with "magnetic hills" (i.e., local higher total intensities) and "magnetic valleys" (i.e., local lower intensities) quasi-symmetrically arranged on both sides of the mid-oceanic ridge. There are some anomalies that run parallel on opposite sides of the ridge and some that are found in a perpendicular orientation (reviewed in Walker and Dennis, 2005). Differently magnetized rocks can cause such local anomalies (Wiltschko and Wiltschko, 1995). Besides spatial variation, the geomagnetic field also shows temporal variation caused by solar electromagnetic radiation (leading to regular daily variations) or sun spot activity (leading to irregular fluctuations called magnetic storms; Wiltschko and Wiltschko, 1995).

Physiological Data on Magnetoreception

There are two main mechanisms that underpin the perception of a magnetic field, namely those that are based on induction or are reliant on magnetite (reviewed in Wiltschko and Wiltschko, 1995). Induction-based perception assumes that the electric field, which is generated by the magnetic field, is detected by electroreceptors; it is dependent on the conductivity of the surrounding medium, thus salt water provides a suitable conductive medium. By contrast, magnetite-based perception is mediated by ferromagnetic particles such as magnetite (iron oxide). These miniature magnets align themselves in the magnetic field and are connected to the central nervous system; however, the exact pathways of signal transmission are still unclear (Lohmann and Johnsen, 2000). Magnetite has been found in the dura mater of both bottlenose (Bauer et al., 1985) and short-beaked common dolphins (*Delphinus delphis*), where nerve fibers have been identified adjacent to the surface of the iron oxide particles (Zoeger et al., 1981).

Behavioral Data on Magnetoreception

Magnetoreception is commonly used for navigation, i.e., animal orientation based on the geomagnetic field (reviewed in Wiltschko and Wiltschko, 1995). Observations of free-ranging cetaceans show some evidence of magnetoreception-based navigation. For example, fin whale (*Balaenoptera physalus*) migration routes are correlated with low geomagnetic intensity (Walker et al., 1992) and offshore cetacean live strandings seem to occur where valleys in the geomagnetic field cross the coast (Klinowska, 1985; Kirschvink et al., 1986). However, foraging routes of wild short-beaked common dolphins do not seem to be influenced by the geomagnetic field (Hui, 1994). Generally, there are few studies that test magnetoreception in dolphins. Kuznetsov (1999) reported that neurovegetative responses in dolphins, such as the electrocardiogram, galvanic skin responses and respiration, are altered by changes in the magnetic field. The author interpreted this as "a high sensitivity of the dolphin to changes in the permanent magnetic field (a 'magnetic sense')". However, as this study is only presented as an abstract, it is difficult to evaluate both the data and conclusions of the work. When captive bottlenose dolphins were exposed to a magnetic field created in their pool by an induction coil (magnetic field strength unknown), they did not show any differential response (Bauer et al., 1985). Even during a series of conditioning experiments using two-choice discrimination and go/no-go designs (magnetic field strength: 37 μT), the dolphins did not show any indication of magnetic discrimination (Bauer et al., 1985). However, Bauer et al. (1985) admitted "experiments that constrain the subject in time and place may be putting significant limits on appropriate orientation." In a recent study, we conducted an experiment that neither confined dolphins spatially to a given position (as, for example, during a go/no-go experiment), nor demanded a direct response (as it is the case in conditioning experiments), but rather observed their spontaneous reaction toward magnetized and demagnetized devices. Dolphins approached the device with shorter latency when it contained a strongly magnetized neodymium block (magnetic field strength of 1.2 T) compared to a control demagnetized block that was identical in form and density and, therefore, undistinguishable through echolocation (Kremers et al., 2014). This finding suggests that dolphins may be able to discriminate the two stimuli used in our study based on their magnetic properties.

Perspectives on Magnetoreception

The mechanisms underpinning magnetoreception still need to be studied in dolphins, as no primary magnetoreceptors have

been identified unequivocally (Lohmann and Johnsen, 2000; Winklhofer, 2010). To date, only a magnetite-based system has been proposed in cetaceans (Walker et al., 1992). Since the geomagnetic field is on average 4.5 μT strong (Wiltschko and Wiltschko, 1995), it is not clear whether or not dolphins are sensitivity enough to perceive and use geomagnetic cues for navigation. As such, further studies concerning the magnetic perception threshold, as well as the possible influence of the orientation of the magnetic field, on dolphin behavior awaits to be tested. Finally, it is still unclear whether magnetic fields are attractive or repulsive to dolphins. Such information could be important for the development of repellent devices that could, for example, protect fishing nets from foraging dolphins and simultaneously decrease the dolphins' risk of entanglement in those nets.

CHEMORECEPTION

Compared to the other senses, chemoreception has drawn little empirical attention in marine mammals and its functional status in cetaceans remains unknown. The different modalities of chemoreception (i.e., olfaction, vomerolfaction, gustation, and trigeminal sensation) are sometimes difficult to tell apart in aquatic animals due to less clear physiochemical selectivity of stimuli conveyed by water (Hemilä and Reuter, 2008). Moreover, it is possible that chemoreceptors of aquatic mammals are found on unexpected body parts compared to terrestrial mammals. Cetaceans might possess chemoreceptors allowing them to sense all types of substances carried in either water or air (Hemilä and Reuter, 2008). However, chemoreceptive structures known from terrestrial mammals may be modified, displaced, reduced or absent in extant cetaceans due to evolutionary adaptation to an aquatic environment. The latter appears to be the case for the vomeronasal organ and related accessory olfactory tracts (Thewissen, 2009), although a recent study found some evidence for the potential presence of a vomeronasal organ in a neonate gray whale (*Eschrichtius robustus*; Kienle et al., 2015). Accordingly, the following sections will only focus on olfaction and gustation.

Current Knowledge on Olfaction

Olfaction is traditionally defined as the ability to detect airborne volatile compounds, *viz*. compounds having a molecular weight below 400 Dalton (Hemilä and Reuter, 2008; Mollo et al., 2014). In terrestrial mammals, odorants have to dissolve in the mucus covering the olfactory epithelium inside the nasal cavity, where they adhere to binding proteins that in turn activate olfactory receptors (ORs) located on the cilia of sensory neurons that transmit impulses to the brain *via* the olfactory nerve (CN I). However, olfactorily active stimuli can be conveyed by water, as shown, for example, in the fetuses of mammalian terrestrial species that react to acute chemical stimulations and detect the chemosensory qualities of their amniotic environment (Schaal and Orgeur, 1992). Thus, even when the detected chemicals are waterborne, the modality has to be considered as olfaction if the neural transmission pathway involves CN I (e.g., Hara, 1994). The important point here is that olfaction can be fully functional under aquatic conditions. For instance, several marine (Davis et al., 2006; DeBose et al., 2008) and freshwater (Hara, 2006) fish species are known to use odorants as social or foraging cues and display a specific behavior called "sniffing" or "coughing" to drive water into the olfactory sacs, thus increasing the supply to the olfactory epithelium (Nevitt, 1991). In addition, the olfactory modality might not necessarily require receptor cells that are exclusively located within the nasal cavity.

Anatomical Data on Olfaction

The nasal cavity is not considered to be involved in olfaction in odontocetes as it accommodates parts of their echolocation system and because the cribriform plate of the ethmoid bone, as well as the ethmoturbinals, are absent (Breathnach, 1960). The main and accessory olfactory tracts are absent in toothed whales and considerably reduced or absent in baleen whales (Breathnach, 1960; Oelschläger, 2008). Furthermore, CN I appears to vanish during early ontogeny in both of these species (Oelschläger and Buhl, 1985).

By contrast, other studies imply that cetaceans may possess neural structures involved in olfaction. Chemoreceptor cells were found in the nasal cavity (frontal and vestibular sacs) of harbor porpoises (*Phocoena phocoena*; Behrmann, 1989), perhaps enabling some kind of odor sensation. Odontoceti were found to possess a well-developed olfactory tubercle (Oelschläger and Oelschläger, 2009). In bowhead whales (*Balaena mysticetus*), a complex olfactory bulb and olfactory tracts are present and more than half of the OR genes are intact, suggesting a potentially functional sense of smell (Thewissen et al., 2011; Kishida et al., 2015a). However, OR genes are reported to be functionally reduced by pseudogenization in Odontoceti (Kishida et al., 2007). Bottlenose dolphins possess only two class I and ten class II OR genes that are intact, as well as a single vomeronasal receptor type 1 gene (Kishida et al., 2015b).

Current Knowledge on Gustation

Gustation is the ability to detect waterborne compounds such as hydrophilic substances (i.e., organic acids, amino acids, or nucleotides), but also traces of all sorts of miscible or hydrophobic compounds (Hemilä and Reuter, 2008), that are ingested with prey or during social interactions. Gustation provides information about water or food materials already in the mouth, through taste bud receptor cells that are located on the tongue, palate, epiglottis, esophagus and duodenum (Purves et al., 2001).

Anatomical Data on Gustation

No taste buds were found on the tongue or other areas in the oral cavity of various odontocete species (Kuznetzov, 1990). However, the number and age of individuals investigated are often unknown or very limited; therefore, these findings remain unconvincing. Nevertheless, several authors have suggested that cetaceans in general and odontocetes in particular should exhibit taste sensation (e.g., Pfeiffer et al., 2001). Taste buds were indeed found in younger individuals of the same species that were previously described as absent in adults (Yamasaki et al., 1978; Behrmann, 1988; Kuznetzov, 1990). Other studies did not

describe taste buds, but found marginal vallate papillae on the tongue of dolphins, known to be potential locations of taste buds (Kastelein and Dubbeldam, 1990; Werth, 2007), as well as cells that resemble von Ebner's glands (also called gustatory glands) that might be important for chemoreception (Ferrando et al., 2010).

Physiological Data on Gustation

It was proposed that in dolphins the well-developed trigeminal nerve (CN V; Oelschläger, 2008) might provide a pathway to transmit impulses from the oral cavity to the brain, called trigeminal chemoreception (Kuznetzov, 1990). In mammals, CN V innervates the oral and nasal cavities, as well as the eyes, and responds especially to chemical irritants (Silver and Finger, 2009). Unlike other mammals, where CN VII innervates the lingual taste buds (Purves et al., 2001), this nerve does not seem to be involved in dolphin chemoreception, but rather in acoustic signal production (Oelschläger, 2008). However, CN V, just like CN VII, is able to excite gustatory neurons in the nucleus of the solitary tract in the brainstem of other mammals (Purves et al., 2001; Boucher et al., 2003), so it might be involved in taste perception in cetaceans.

Taste receptor genes are reported to be mostly pseudogenized in Odontoceti: in bottlenose dolphins, sweet, umami, bitter and sour taste receptor genes are non-functional, whereas salty taste receptor genes are intact and potentially have functional roles in gustation (Jiang et al., 2013; Feng et al., 2014; Kishida et al., 2015b). Recent molecular findings suggest that this reduction of gustatory abilities in cetaceans occurred between the Artiodactyla-Cetacea and the Odontoceti-Mysticeti evolutionary divisions (Kishida et al., 2015b).

Behavioral Data on Gustation

Behavioral studies have shown that bottlenose dolphins can perceive the sour and bitter tastes of citric acid and quinine sulfate/hydrochloride dehydrate solutions, respectively, nearly as well as humans (Nachtigall and Hall, 1984; Friedl et al., 1990; Kuznetzov, 1990). Moreover, they were able to detect the salty taste of sodium chloride solution (Friedl et al., 1990; Kuznetzov, 1990). Studies that test the perception of sweet stimuli are contradictory, stating that dolphins are able to perceive the sweet taste of sucrose solution (Friedl et al., 1990) or not (Kuznetzov, 1990). In addition to these simple tastants, dolphins were able to detect complex tastants, such as those conveyed in conspecific urine and feces (Kuznetzov, 1990). Kuznetzov (1990) proposed the term "quasi-olfaction" to describe the chemical sense in dolphins that combines characteristics of both gustation and olfaction. Recent behavioral studies suggest that dolphins are able to detect airborne odors and discriminate between different flavors (Kremers et al., 2016).

Perspectives on Chemoreception

Generally, the air above the ocean is chemically less rich compared to air above land, wherefore the chemical environment for air-breathing aquatic species is less diverse compared to terrestrial species: indeed, this lack of chemical variation might have been one of the main drivers that led to the evolution of more specialized sensory abilities in aquatic species. Water birds, for example, show adaption to their aquatic environment in their olfactory receptor complement compared to land birds, with OR families 2/13, 51, and 52 (that were correlated with aquatic adaptations) being expanded (Khan et al., 2015). Just as other marine species are able to detect chemical compounds and exploit them as source of information (e.g., from conspecifics, prey, and predators), it is not unreasonable to hypothesize that dolphins might use a similar sensory system, either by detecting airborne molecules when they surface or waterborne cues. Indeed, fish emit chemical cues that are perceived by other fish and used for the detection of conspecifics, prey and predators, as well as analyzing the chemical profile of water to direct locomotion (Hara, 1994; Hirvonen et al., 2000). Although it is widely accepted that dolphins use echolocation to locate prey, it may be possible that dolphins also use chemical cues to identify and assess the quality of prey. These questions have not been investigated so far, which is probably due to technical issues and the availability of animals for testing.

Extrapolations from anatomical data in determining actual sensory capacities may have been over-interpreted and should be revised accordingly. For example, the reduced size of chemoreceptive organs in some cetaceans do not exclude that a given species may exhibit specialized sensitivity to particular chemical cues (Pihlström et al., 2005; Nummela et al., 2013). As the chemical senses in dolphins are not yet sufficiently delineated, all chemosensory modalities (i.e., olfaction, gustation, and trigeminal sensation) are potentially involved. Behavioral studies might be a good approach to investigate the functional status of chemoreception in dolphins as anatomical and genetic studies have yielded conflicted and controversial results. Similarly, it was proposed that sweet taste perception in hummingbirds (who lack the specific corresponding taste receptor genes) is enabled through unrelated taste receptors that have undergone a change in function (Baldwin et al., 2014). Thus, the simple absence or pseudogenization of taste or olfactory receptor genes should not be the sole basis for proposing firm conclusions that relate to the chemosensory abilities of a particular species: appropriately conducted and controlled behavioral studies should be included.

In general, go/no-go tasks are an elegant way to investigate perceptual abilities as the behaviors displayed by dolphins in response to internal (e.g., pleasure/liking, aversion, interest, fright etc.) or external stimuli can be subtle. However, these experiments require that dolphins are trained, thus preventing the investigation of spontaneous responses or preferences. Similar to experiments conducted to test gustatory stimuli, detection thresholds for airborne stimuli could be determined using go/no-go tasks. Furthermore, dolphins could be trained to react to the presence of an odor with a certain response (e.g., choosing one of two proposed symbols or buttons) and to the absence of another, using positive reinforcement.

Although dolphinariums provide a good opportunity to train dolphins involving several research tasks, there are a number of constraints: for example, training sessions and actual presentations for public viewing limit the time available for experimentation. Furthermore, some methods that work well in captivity are not applicable in the field. For instance, in

contrast to captive dolphins, wild dolphins cannot be trained; therefore, other methods may have to be developed for the same research tasks. While chemoreception may be tested in captive dolphins by using odor sources close to the pool or ice cubes (see Kremers et al., 2016), floating dispensers (such as the ones used for chlorine tablets in swimming pools) may be adopted for wild dolphins. For olfactory studies, the substance to be tested is simply placed inside the device. For gustatory studies, the substance is contained within large ice cubes, which themselves are placed within the device. When submerged into water, this allows for a slow release of the tastant through holes located at the bottom of the device. The behavior of the dolphins toward the device, such as their distance to it or their approach latency, can then be analyzed and compared with other tested substances or controls. Given the fact that such observations are made at the surface of the water body, they are often inaccurate due to the shallow angle of the observer; therefore, it might be helpful to use drones (i.e., small unmanned aerial vehicle that are remotely controlled) equipped with cameras to record animal movements from above. An aerial view with an approximately perpendicular angle has the advantage that even animals under the water surface are visible (as long as they are not too deep). Furthermore, the use of drones would permit an increased distance between the boat and the device, thereby reducing the potential disturbance to the test subjects.

It would be informative to test different chemical cues within diverse experimental contexts. As dolphins prefer fish species with a high energy density (Spitz et al., 2010), it would be insightful to investigate if food choices are dependent on chemical cues. Therefore, a test with high vs. low energy density fish would be revealing. Another substance to test is dimethyl sulfide (DMS), which is released by phytoplankton when being grazed on by zooplankton (Dacey and Wakeham, 1986), and used by predators (including at least one other marine mammal, the harbor seal *Phoca vitulina vitulina*) to find their prey (Nevitt et al., 1995; Kowalewsky et al., 2006; Wright et al., 2011). As phytoplankton attracts zooplankton and zooplankton in turn attracts fish, the ability to detect DMS might allow dolphins to find fish. Indeed, prey detection by using chemical cues has already been suggested for bowhead whales (Thewissen et al., 2011).

Another approach would be to test for chemical cues in social and reproductive contexts, as individual recognition or mate detection (e.g., female receptiveness) could be chemically mediated as is common in many other species. Therefore, the use of urine (given the fact that dolphins seem to be able to detect urine and feces; Kuznetzov, 1990) and/or excretions from the urogenital glands could be used as stimuli (e.g., those obtained from known and unknown individuals or receptive and non-receptive females). The idea of odor-mediated sexual behavior was previously suggested for spinner dolphins (*Stenella longirostris*; Norris, 1991) and exploratory behaviors such as "genital inspections" have also been reported (Norris and Dohl, 1980; Herzing, 1996). In a behavioral and endocrinological study of three female bottlenose dolphins, that were observed during three conceptive estrous cycles, Muraco and Kuczaj (2015) found that reproductive behaviors increased with estradiol and luteinizing hormone levels. During estrus, females received more behavioral attention than they initiated, including an investigatory behavior ("genital tracking", as defined by the authors of the corresponding study) and having their genital slit being touched by the rostrum of another dolphin ("goose," as defined by the authors of the corresponding study). Thus, the authors suggested that dolphins might be able to gain information about the physiological state of another dolphin (e.g., during reproduction) by using chemosensory abilities.

Finally, it seems worth investigating whether some repulsive stimuli are inherent or acquired. Chemical stimuli that are potentially involved in eliciting negative responses might be compounds associated with natural predators (e.g., orcas, a natural predator of dolphins), or intensively irritating, tasting or smelling substances such as capsaicin or putrescine. A possible application could be the development of a device to repel dolphins from fishing nets or areas with high boat traffic by using an effectively repulsive substance, thus minimizing adverse human-dolphin interactions.

FURTHER QUESTIONS AND POTENTIALLY PROMISING APPROACHES ON PERCEPTION IN DOLPHINS

As the previous sections have illustrated, there is a huge amount of knowledge on sensory perception in dolphins. Nevertheless, as always in research, each finding raises new questions that require further experimentation. One promising line of research is cross-modal perception, which describes the interaction between two or more different sensory modalities (i.e., the ability to relate information received from one sense with information obtained from another). Probably the best-studied example of cross-modal perception in dolphins concerns their ability to link auditory and visual cues. Dolphins are able to recognize objects visually that were previously inspected by echolocation and vice versa (e.g., Herman et al., 1998; DeLong et al., 2000; Hoffmann-Kuhnt et al., 2008). Furthermore, dynamic information about movement, in addition to stationary objects, can be perceived across those two senses (Kuczaj et al., 2008).

So far, cross-modal perception in dolphins was only investigated with regards to the interaction between audition and vision within the context of object and movement recognition. However, other senses and contexts should also be investigated. Possible valid questions may concern if dolphins are able to link information in the context of: (1) individual recognition of conspecifics between audition (signature whistles), vision (individually distinctive physical features) and/or chemoreception (chemical profile); (2) communication between audition (e.g., jaw claps, tail hits or "squawks") and vision (body postures); (3) prey location between audition (returning echo) and electroreception (electric field generated by prey); and (4) food evaluation between audition (returning echo), somatosensory perception (haptic characteristics of prey) and/or chemoreception (flavor characteristics of prey).

CONCLUSION

Although intensively studied for decades, many facets of dolphin biology remain unknown. Without doubt, this is partly due to the difficulties researchers encounter when studying marine mammals, especially in the field. Generally, hearing is considered to be the most important sensory modality, not only in dolphins but also in odontocetes in general (e.g., Thewissen, 2009), as it is involved in navigation, prey location, and communication. This has led to the majority of studies addressing questions related to hearing, sound production, echolocation and related communicative activities. By contrast, other sensory modalities are considered to be less important (e.g., Marriott et al., 2013) and, therefore, have become physically reduced or may even be absent due to complex trade-offs between different sensory modalities (Nummela et al., 2013). This approach appears biased and runs the risk of distorting knowledge or oversimplifying the degree by which dolphins, and other cetacean, might possess and utilize a potentially diverse array of senses. Therefore, the sensory perception of cetaceans, and in particular the dolphin, should be revisited, especially regarding the study of those modalities that have been largely neglected, namely electroreception, magnetoreception and chemoreception.

AUTHOR CONTRIBUTIONS

DK, AL, and MH prepared the main text of the manuscript. All authors contributed to the preparation of the article, in the form of discussion and critical comments, and reviewed the manuscript.

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Adaptations of Cetacean Retinal Pigments to Aquatic Environments

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The underwater environment places unique constraints on the vision of cetaceans compared to their terrestrial mammalian counterparts. Water absorbs and filters light affecting both the intensity and spectral distribution of light available for vision. Therefore, the aquatic environment restricts the spectral distribution of photons and limits the distance at which objects may be observed. The cetacean eye possesses numerous anatomical, cellular and molecular adaptations to the underwater light environment that increase photon capture in a light-limited environment. These adaptations include a powerful spherical lens, a unique corneal design allowing for acute vision in both air and water, as well as a blue reflective optic tapetum. There are also molecular adaptations that influence the spectral sensitivity of both the rod and cone visual pigments. The spectral sensitivities of cetacean retinae have been a focus of attention over the last two decades. While most terrestrial mammals have dichromatic color vision based on two classes of cone photoreceptors, all cetaceans lack cone based color vision. For example, the Delphinidae (dolphins), Phocoenidae (porpoises), and some members of Ziphidae (beaked whales) possess single rod and cone photoreceptor classes. Recently, rod monochromats were identified in Ziphidae, Physeteroidea, and almost all of the mysticete (baleen) whales. The absorbance spectra of cetacean rod visual pigments are spectrally tuned to the available radiance spectra at foraging depths with an inverse relationship observed between the wavelength of maximum sensitivity of the rod pigment and depth. This also holds true for the spectral tuning of long-wavelength sensitive cone visual pigments in cetaceans. Cetacean melanopsins, the visual pigment expressed in a small subset of ganglion cells in mammalian retinae, have only just recently been examined in the cetacean rod monochromats. Genetic analyses coupled with molecular modeling predict that cetacean melanopsins possess nearly identical absorption spectra compared to their terrestrial mammalian counterparts. However, it appears that the melanopsins from the cetacean rod monochromats may possess a mechanism that inhibits relatively rapid deactivation of the light-activated melanopsin. This mechanism would result in prolonged pupil constriction resulting in a very useful mechanism in the prevention of photobleaching of rod pigments under photopic conditions.

Keywords: cetaceans, visual pigment opsin, melanopsin retinal ganglion cells, aquatic organisms, adaptation, physiological

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Visual pigments are light absorbing molecules comprised of a light-sensitive chromophore covalently attached to an opsin protein. In vertebrates, chromophores consist of an aldehyde of either vitamin A_1 or A_2 (retinal), are bound to opsin through a protonated Schiff base (PSB) linkage, and lie within an amino acid-lined chromophore binding pocket (**Figure 1**). Although, a protonated chromophore attached to an amine via a Schiff's base has an absorbance maximum (λ_{max}) well within the visible spectrum (around 440 nm; **Figure 2**), wavelength modulation of chromophore absorbance spanning the visible region of the spectrum relies almost exclusively on the amino acids lining the chromophore binding pocket and their dipolar electrostatic interactions with the chromophore (Kochendoerfer et al., 1999). Simply put, amino acid side chains that localize the Schiff base proton at the Schiff base result in blue-shifting of the visual pigment, while side chains that delocalize the Schiff base proton result in red-shifting of the visual pigment. Although, one may expect to see an endless number of amino acid substitutions within the retinal binding pocket to account for the diversity of vertebrate rod and cone photoreceptor sensitivities, in fact many amino acid substitutions have been strongly conserved amongst the vertebrate rod and cone photoreceptor classes (see Yokoyama, 2008 for review). As an example, extreme blue-shifting of a visual pigment, as is found in vertebrates possessing ultraviolet (UV) cone visual pigments, results from the deprotonation of the Schiff base resulting in a pigment similar in its absorbance to that of 11-cis retinal free in solution ($\lambda_{max} \sim 380$ nm; **Figure 2**). Deprotonation of the Schiff base in these pigments relies on a key amino acid substitution in both mammals and birds, the former possessing the substitution Tyr86Phe, with the latter possessing the substitution Ser84Cys (Wilkie et al., 2000; Yokoyama et al., 2000; Shi et al., 2001; Cowing et al., 2002; Fasick et al., 2002). Although, cetaceans lack a functional SWS cone, these marine mammals nevertheless have acquired several amino acid substitutions in both their rod and LWS cone visual pigments that influence the absorbance spectra of these pigments when compared to corresponding pigments from terrestrial mammals.

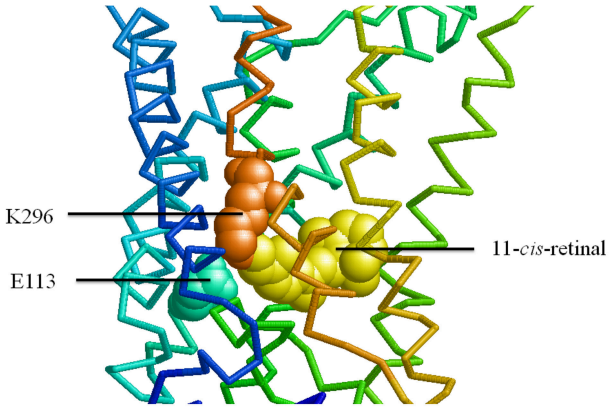

FIGURE 1 | Proximal positions of chromophore, Schiff base linkage, and counterion in rhodopsin. Bovine rhodopsin (Protein Data Bank accession: 1F88; Palczewski et al., 2000) was modeled using RasWin to highlight positions E113 (counterion), K296 (site of Schiff base linkage) and retinal.

CETACEAN VISUAL PIGMENTS

Although, the first published spectral sensitivity measurement of a cetacean retina was made by Dartnall on the humpback whale (maximum sensitivity of 492 nm; Dartnall, 1962), the first comprehensive study of cetacean retinal sensitivity and the supporting visual pigments was conducted by McFarland. He examined 10 species, including both odontocetes (toothed whales) and mysticetes (baleen whales), using a method of partial bleaching to estimate the absorption maxima and homogeneity of visual pigments in the extracts (McFarland, 1971). From this early work, McFarland reported blue-shifted absorption maxima (relative to ungulates) for all species ranging from 497 nm in gray whale to 481 nm in beaked whale. Although, only two mysticete species were examined (gray and humpback whales) both possessed retinal absorbance values greater than 490 nm, while all of the odontocete species studied possessed retinal absorbance values less than 490 nm. In regards to cetaceans, McFarland was the first to report the correlation between diving (foraging) depth and spectral sensitivity of the retinal visual pigments, e.g., the deep-diving pelagic species will have correspondingly more blue-shifted visual pigments compared to shallow-diving species. As McFarland states, selective forces most likely resulted in a balance being struck between the maximization of spectral sensitivity and the degree in which contrast cues may be utilized in visually guided tasks.

Several years after McFarland's work, a series of behavioral studies were conducted on bottlenose dolphin (*Tursiops truncatus*) in order to test spectral discrimination and determine if the dolphin possesses color vision, as well as define the spectral sensitivity of the dolphin retina under both scotopic and photopic light levels (Madsen, 1976). From these studies, Madsen, along with her graduate adviser Louis Herman, was able to demonstrate that the dolphin lacked the ability to discriminate colors and was indeed color-blind (Madsen, 1976). The rod and cone photoreceptor sensitivities were deduced by Madsen by having the dolphin perform light illumination discrimination tasks under either scotopic or photopic light levels where rod and cone photoreceptors typically function, respectively. Interestingly, the dolphin's retinal sensitivity was quite similar under both illumination conditions with scotopic and photopic retinal sensitivity maxima found at 495 and 500 nm, respectively. Both of these values are red-shifted relative to the retinal absorbance maximum observed by McFarland (1971) for the bottlenose dolphin (486 nm).

More recent studies of cetacean visual pigments address the number of different retinal photoreceptor classes as well as the molecular mechanisms of spectral tuning of both the rod and cone photoreceptors. Below we review a series of papers from several laboratories that have contributed to our current understanding of the nature of the retinae of cetaceans as well as other marine mammals.

Fasick et al. used molecular techniques to confirm the absence of color vision in the bottlenose dolphin as well as

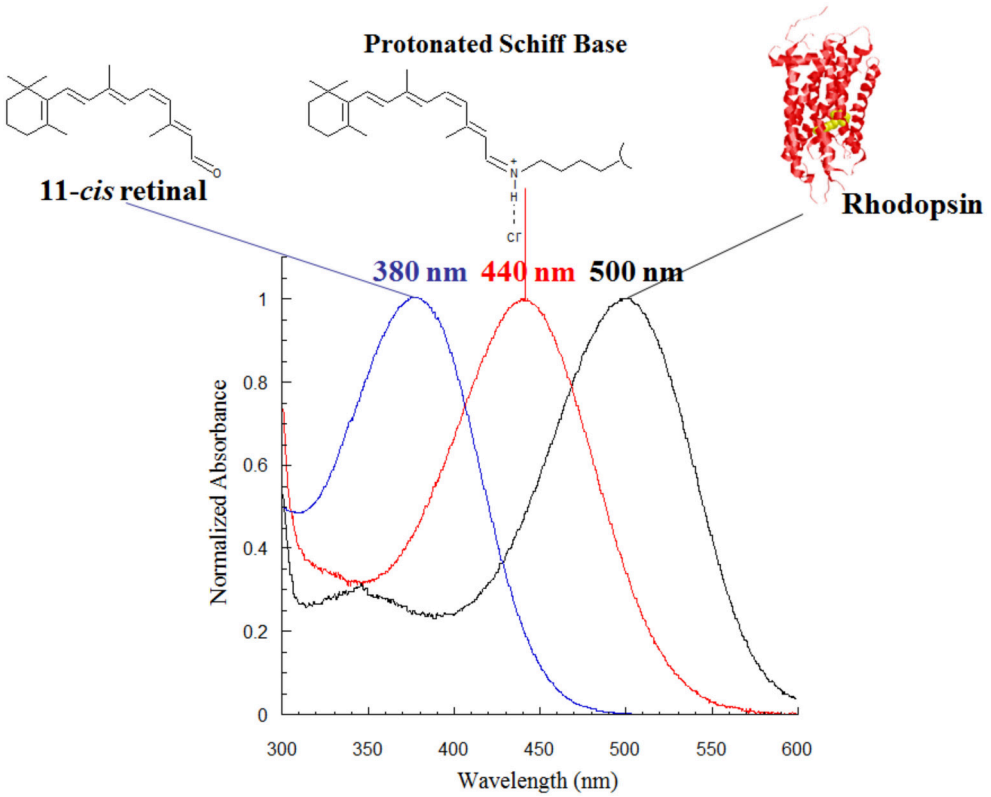

FIGURE 2 | Absorbance spectra of the visual pigment chromophore, 11-*cis* retinal, in three states: free in solution (left); protonated in the presence of chloride ions (middle); surrounded by the bovine RH1 opsin protein (right).

to describe the molecular mechanism underlying the spectral tuning of both the rod and cone photoreceptors from several cetacean species (Fasick et al., 1998; Fasick and Robinson, 1998, 2000). Their approach involved screening a dolphin retinal bacteriophage cDNA library with radiolabeled human homologs of the rod opsin, as well as the short-wavelength (SWS) and long-wavelength sensitive (LWS) cone opsins, open reading frames to determine the number of different photoreceptor classes in the dolphin retina. This approach resulted in the cloning, sequencing and expression of both the rod and LWS cone visual pigments (λ_{max} of 488 nm, and 524 nm respectively), but failed to isolate SWS cone opsin cDNA (Fasick et al., 1998).

Sequence comparisons between the dolphin and bovine rod opsin sequences revealed three likely amino acid substitutions involved in the complete wavelength modulation between the two pigments. The positions of these substitutions were 83, 292, and 299 all of which fell within the transmembrane region of the proposed opsin structure, with positions 292 and 299 positioned approximately one helical turn away from Lys296, the site of the protonated Schiff base linkage with the chromophore (Fasick et al., 1998). A series of bovine mutants were constructed and expressed to determine the degree of wavelength modulation made by individual amino acid substitutions at these positions as well as the combination of all three substitutions together (see **Table 1**). The single bovine mutant Asp-to-Asn at position 83 resulted in a 4 nm blue-shift relative to wildtype bovine rhodopsin (λ_{max} = 499 nm), while the individual Ala-to-Ser mutations in bovine rhodopsin at positions 292 and 299 resulted in a 10 nm blue-shift and a 2 nm red-shift, respectively. When the three amino acid substitutions were included together in a single mutant construct, the triple-mutant resulted in a λ_{max} = 489 nm resulting in a 10 nm blue-shift relative to bovine rhodopsin (Fasick and Robinson, 1998). A subsequent study observed a 12 nm blue-shift in bottlenose dolphin rhodopsin relative to bovine rhodopsin which is in better agreement with the original values from McFarland (1971) and Bischoff et al. (2012). Variations of these three substitutions at positions 83, 292, and 299 are found in a variety of cetacean rhodopsins and appear to correspond with foraging depth (**Figure 3**). Deep-diving odontocetes such as the sperm whale and the beaked whales possess the combination of Asn83, Ser292, and Ala299 with resulting absorbance maxima around 479 nm (Bischoff et al., 2012). By contrast, as outlined in **Figure 4**, the more shallow diving and coastal Balaenidae whales possess the combination of Asn83, Ala292, and Ser299 with resulting absorbance maxima around 493 nm, while the more oceanic Balaenopteridae possess the combination of Asn83, Ser292, and Ser299 with resulting absorbance maxima around 484 nm similar to the delphinidae rhodopsins (Bischoff et al., 2012). Curiously, the pygmy right whale possesses the same amino acid substitutions at positions 83, 292, and 299 as the

TABLE 1 | Comparison of substitutions at positions 83, 292, and 299 in bovine opsin mutants with corresponding absorbance maxima (λ_{max}; from Fasick and Robinson, 2000).

	λ_{max} (nm)
	480 485 490 495 500 505
BOVINE MUTANTS (SUBSTITUTION- λ_{MAX})	
D A S–501	●
D A A (bovine RH1 wildtype-499 nm)	●
N A S–499	●
N A A–495	●
D S S–493	●
N S S–489	●
D S A–487	●
N S A–485	●

sperm and beaked whales, Asn, Ser, Ala, respectively, suggesting the possibility that this baleen whale species is adapted to sensing blue spectral light found at great depths. Very little is known of the foraging ecology of this rare species of whale, but it would be of great interest to explore this hypothesis further.

To address the status of the dolphin SWS cone opsin, Fasick et al. used genomic DNA (gDNA) and PCR amplified the entire dolphin SWS cone opsin gene, subsequently aligning the sequence with the homologous bovine SWS cone opsin gene (Fasick et al., 1998). The sequence analysis of the dolphin SWS cone opsin gene revealed two important features: first, the deduced amino acid sequence resulted in a premature stop codon in exon1; and second, intron 4 was absent with exons 4 and 5 being uninterrupted in the dolphin gene (Fasick et al., 1998). This finding of a dolphin SWS cone opsin pseudogene confirmed the conclusion of Fasick et al. that the bottlenose dolphin lacks any cone photoreceptor form of color vision and the earlier behavioral observations of Madsen and Herman (Madsen, 1976). It is important to note that Fasick et al. failed to isolate retinal cDNA during their initial screening of the retinal cDNA phage library, strongly suggesting the absence of the dolphin SWS cone photoreceptors altogether. This finding turns out to be important as seen in the subsequent discussion of rod monochromacy, and is supported by experimental evidence discussed below.

Two independent groups followed these studies and showed that the scope of SWS cone photoreceptor inactivation was characteristic of the entire cetacean infraorder. Peichl et al. utilized visual pigment-specific antibodies to demonstrate an absence of SWS cone photoreceptors in the retinae of six dolphin species and one porpoise species, as well as in five species of marine carnivore (eared and earless seals; Peichl et al., 2001). The authors suggest that the loss of the SWS cone class from these two distant mammalian orders is due to convergent evolution resulting in an adaptive advantage for this trait (Peichl et al., 2001). These results were confirmed by Levenson and Dizon who examined the SWS cone opsin genes from 16 cetacean species that represented 12 of the 14 extant mysticete and odontocete families (Levenson and Dizon, 2003). Levenson and Dizon also examined SWS cone opsin coding sequences that were PCR amplified from cetacean gDNA and observed, for all species examined, one or more mutations indicating that these sequences were indeed SWS cone opsin pseudogenes.

Although, much work has been done examining the nature of cetacean color vision, or lack thereof, with studies of the SWS cone opsin pseudogenes, very little work has focused on the cetacean LWS cone opsins. The bottlenose dolphin LWS cone visual pigment possesses an absorption maximum (λ_{max}) of 524 nm that is significantly blue-shifted relative to the LWS cone pigments from terrestrial mammals (e.g., bovine LWS cone $\lambda_{max} = 555$ nm; Fasick et al., 1998). Likewise, pilot whale ($\lambda_{max} = 531$ nm) and harbor porpoise ($\lambda_{max} = 522$ nm) share this trait of a blue-shifted LWS cone visual pigment (Newman and Robinson, 2005). Like cetacean rod opsins, cetacean LWS cone opsins have an alanine to serine substitution at position 292 (using the bovine rhodopsin numbering system). Although, positions 83 and 299 do not appear to play a role in wavelength modulation in these LWS cone pigments, all functional cetacean LWS opsins studied to date possess a serine at position 292 replacing an alanine most commonly found in their terrestrial mammalian counterparts.

In 2013 Meredith et al. published a landmark paper reporting that many cetacean species lacked functional LWS cone opsins. These species have an exclusively rod-only retina in the absence of functional expression of both the SWS and LWS cone opsins making them rod monochromats. Why would this occur in species that are often active during daytime hours and frequently observed foraging in the upper regions of the water column, even at the surface as is the case with the North Atlantic right whale? The following section sheds some light on the nature of rod monochromacy in terms of evolution, adaptation, as well as the role that cone photoreceptors may continue to play in rod monochromat whales.

CETACEAN ROD MONOCHROMATS

McFarland first postulated that cetaceans may possess predominantly, if not exclusively, a pure rod retina (McFarland, 1971). As Meredith et al. have shown, this is the case for most of the mysticete species, as well as with Physeteroidea and the Mesoplodont beaked whales, with retinae being exclusively rod-based due to both the SWS and LWS opsin pseudogenes (Meredith et al., 2013). Furthermore, the mutations in the LWS cone opsin gene in Cetacea is an example of convergent evolution as mutations resulting in the cetacean pseudogenes arose at least five different times (Meredith et al., 2013). Based on genomic DNA sequence analyses, the authors placed the timing of rod opsin blue-shifts prior to the formation of the SWS pseudogene with both occurring early in the history of Cetacea (see Meredith et al., 2013 for details on divergence times and appearance of SWS pseudogenes).

Interestingly, the pygmy right whale retains a functional LWS cone opsin gene based on genomic evidence (Meredith

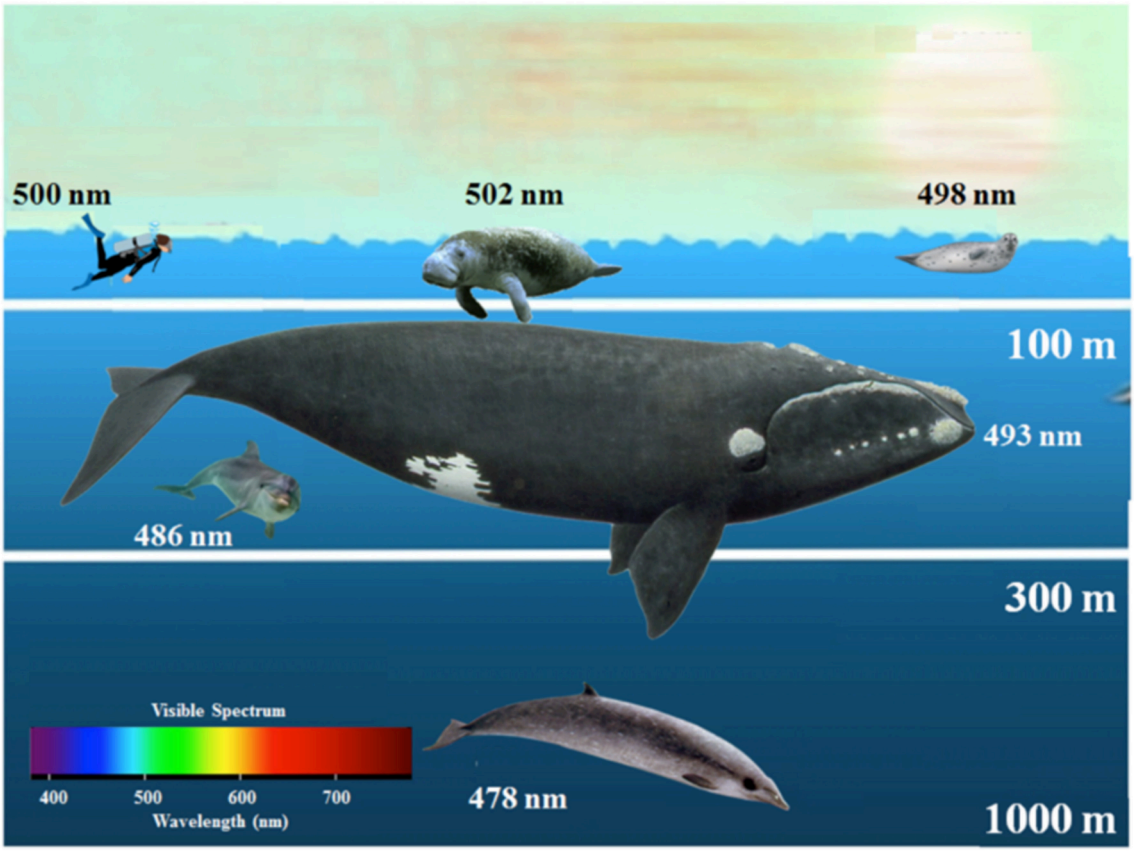

FIGURE 3 | Marine mammal rhodopsin absorbance maxima relative to foraging depth. Diagrammed marine organisms possess the following amino acids at positions 83, 292, and 299: from left to right are: human: D-A-A, manatee; D-A-S, harp seal; D-A-A, from top to bottom are; bowhead whale; N-A-S, bottlenose dolphin; N-S-S, beaked whale; N-S-A. Figure adapted from Fasick (2009).

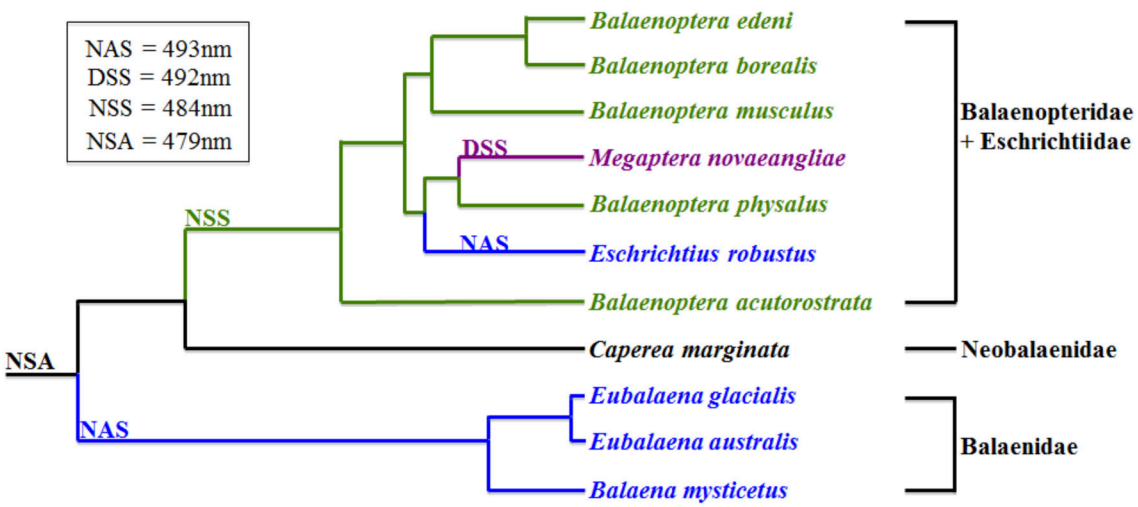

FIGURE 4 | Amino acid substitutions at positions 83, 292, and 299 in 11 mysticete rod opsins. Figure adapted from Bischoff et al. (2012). Phylogenetic tree adapted from McGowen et al. (2009).

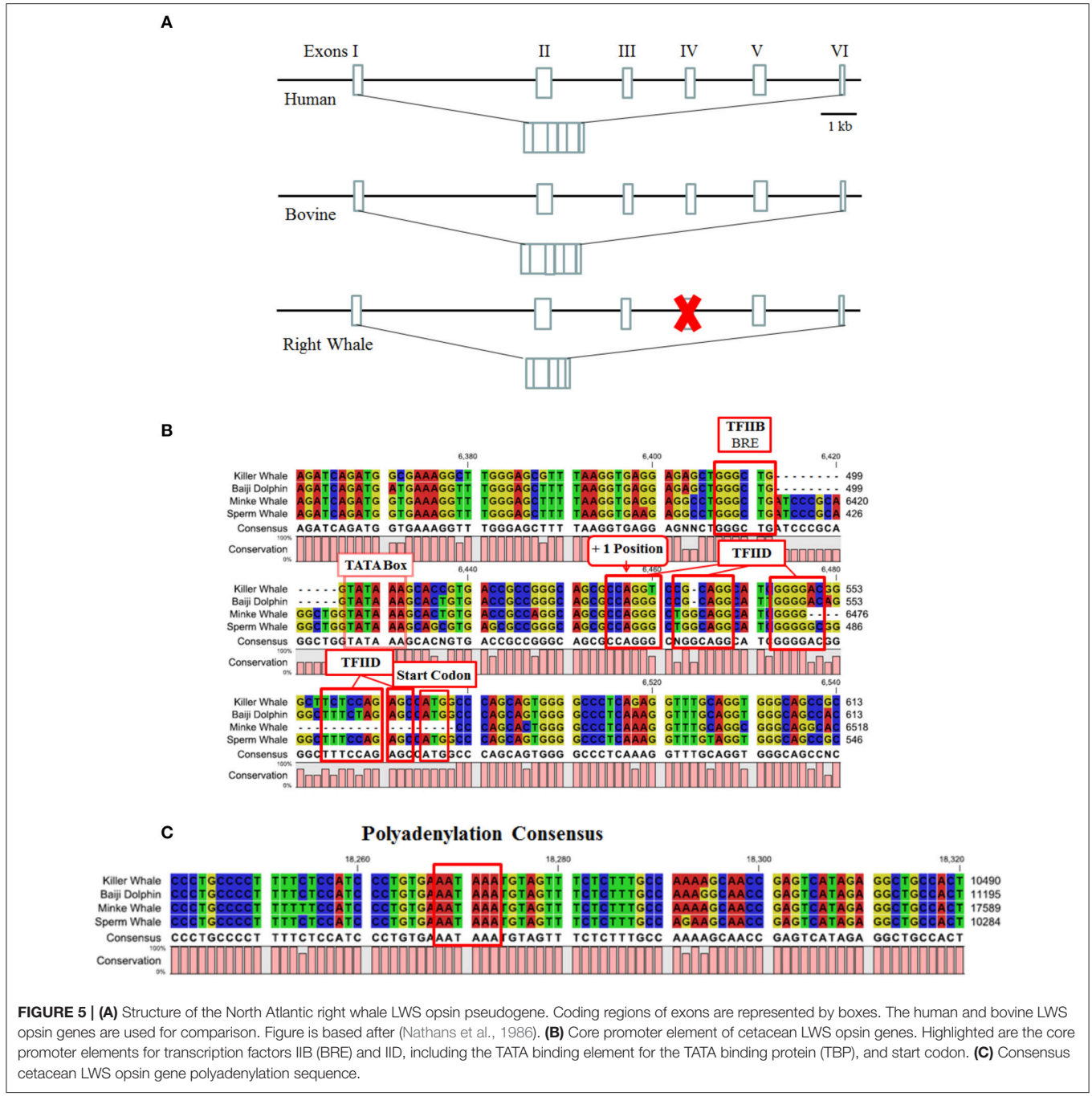

FIGURE 5 | (A) Structure of the North Atlantic right whale LWS opsin pseudogene. Coding regions of exons are represented by boxes. The human and bovine LWS opsin genes are used for comparison. Figure is based after (Nathans et al., 1986). **(B)** Core promoter element of cetacean LWS opsin genes. Highlighted are the core promoter elements for transcription factors IIB (BRE) and IID, including the TATA binding element for the TATA binding protein (TBP), and start codon. **(C)** Consensus cetacean LWS opsin gene polyadenylation sequence.

et al., 2013). As discussed above, the pygmy right whale's rod visual pigment possesses the "deep-diving" set of amino acid substitutions at positions 83, 292, and 299 and a predicted absorbance maximum around 480 nm. These are the same amino acid substitutions found in both the sperm and beaked whales, all of which are deep-divers. Furthermore, many of the Delphinoidea and Ziphiidae species examined by Meredith et al., and which possess functional LWS cones, routinely dive deeper than some of the mysticete species such as right, gray, and minke whales, all of which lack functional LWS cones (e.g., Tyack et al., 2006; Parks et al., 2012). Although the premise that a rod-dominated retina is advantageous in dim light conditions is certainly reasonable, arguments can be made to counter this premise when considering the deep-diving behaviors of some oceanic Delphinoidea and Ziphiidae species, and possibly Neobalaenidae, as well as the common shallow-diving behaviors of several of the Balaenidae, Eschrichtiidae, and Balaenopteridae species.

Although it is clearly evident that many cetacean species lack a functional LWS visual pigment, this does not necessarily

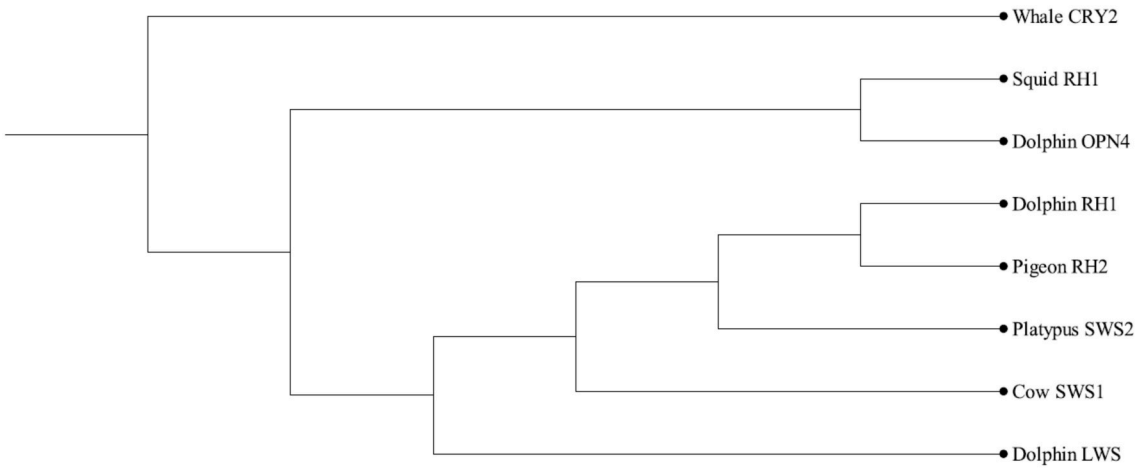

FIGURE 6 | Dendrogram of opsins and melanopsin. *CRY,* cryptochrome (outgroup); *RH,* rhodopsin (Squid rhabdomeric opsin, dolphin rod opsin, pigeon cone opsin); *OPN4,* melanopsin; *SWS*, short wavelength-sensitive; *LWS*, long wavelength-sensitive. After (Sexton et al., 2012).

preclude the anatomical formation of cone photoreceptors, or more correctly, non-photosensitive cone cells (NPCCs). A recent study by Schweikert et al. proposed that cone somata and pedicles may have been conserved in rod monochromats to maintain an alternative rod photoreceptor signaling pathway in the retinae (Schweikert et al., 2016). Preliminary supporting evidence for the presence of NPCCs was first observed in molecular experiments with successful PCR amplification of the LWS cone opsin pseudogene from North Atlantic right whale retinal cDNA. As diagrammed in **Figure 5**, sequence analysis revealed several deleterious mutations in the LWS opsin coding sequence, including the excision of exon 4 with exons 3 and 5 being spliced together and deletion mutations in the core promoter region while other regions in the gene remained intact such as the polyadenylation sequence. Likewise, Emerling and Springer provided evidence of inactivating mutations in several cone phototransduction genes from both minke whale and giant sperm whale (Emerling and Springer, 2015). Further support of the pseudogenization of the LWS, as well as SWS, cone opsin gene resulted from immunohistochemical experiments where anti-opsin immunofluorescence demonstrated the total loss of cone opsin expression in the retina of the closely related species *B. mysticetus* (Schweikert et al., 2016). Taken together, both the genetic and immunohistochemical evidence suggested a loss of functional visual pigment expression, but maintenance of the LWS NPCC, at least in Balaenidae. Direct evidence of cone maintenance resulted from imaging of cone soma and putative cone pedicles by light and electron microscopy, and of rod and cone bipolar cell types by immunofluorescence microscopy in the bowhead whale retina (Schweikert et al., 2016). Schweikert et al. proposed three distinct rod signaling pathways that may increase the signaling speed and sensitivity range of rod-based vision in at least the balaenid whales: (1) rods synapse on rod bipolar cells (scotopic sensitivity); (2) cone soma are synaptic intermediates in a coupling pathway between rod and ON cone bipolar cells (intermediate mesopic-light sensitivity); and (3) rods synapse directly on OFF cone bipolar cells (supporting visual processes such as center-surround receptive fields). Although, Schweikert et al. examined only two balaenid species; it would not be surprising if other cetacean rod monochromats shared a similar retinal design.

This then raises the question of how these rod monochromats are capable of vision during bright-light daytime levels at or near the surface of the water without bleaching the rod photoreceptors? One answer to this question may be found in the pupillary light response (PLR) that is involved in constricting or dilating the pupil. The PLR is regulated by input from a subset of retinal ganglion cells that project to the olivary pretectal nucleus. These ganglion cells not only receive indirect synaptic input from classical rod and cone photoreceptors but also function as photoreceptors themselves. These ganglion cells known as intrinsically photosensitive ganglion cells (iPRGCs) express the pigment OPN4, or melanopsin. Despite the ability of rods and cones to drive the PLR, melanopsin is critical for normal PLR function in allowing maximal pupil constriction in bright and sustained light intensities (Lucas et al., 2003; Xue et al., 2011). In animals lacking cones, as is the case with the rod monochromatic whales, iPRGCs and melanopsin appear critical in the regulation of the PLR. A prolonged PLR under bright illumination might ensure the viability of rod-based vision.

CETACEAN MELANOPSINS (OPN4): WAVELENGTH MODULATION AND INACTIVATION

In the mammalian retina, a small subset of retinal ganglion cells express the photopigment melanopsin and represent a third class of photoreceptors that predominantly mediate non-image forming light functions. In terrestrial mammals, these intrinsically photosensitive retinal ganglion cells (ipRGCs)

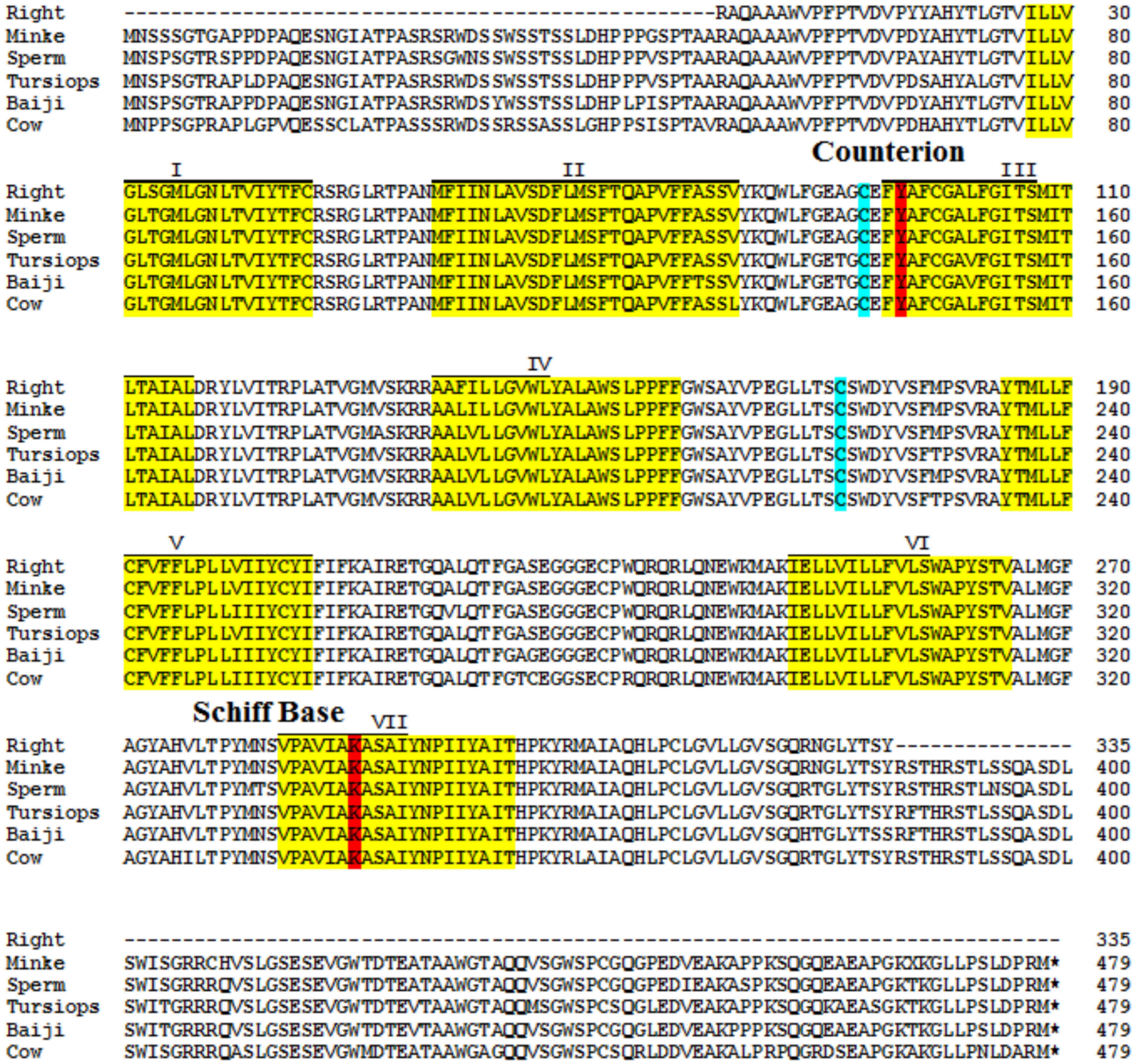

FIGURE 7 | Amino acid alignment of cetacean melanopsins. Transmembrane regions boxed in yellow, Schiff base and Schiff base counterion boxed in red, disulfide bridge cysteines boxed in teal. Figure adapted from Fasick and Samuels (2015).

provide photic information for a number of light-dependent processes, including circadian photoentrainment, pupil constriction, suppression of pineal melatonin, and direct regulation of sleep, mood, and learning (Altimus et al., 2008; Lupi et al., 2008; Tsai et al., 2009; Schmidt et al., 2011a,b; LeGates et al., 2012; Pickard and Sollars, 2012). Recently, it was discovered that ipRGCs are also involved in image forming vision where they contribute to setting contrast sensitivity (Schmidt et al., 2014). ipRGCs differ from the classical rod and cone photoreceptors in both physiology, and the light-activated biochemical cascade. Melanopsin-expressing ipRGCs were identified only 15 years ago (Provencio et al., 1998, 2000). Since then, they have been the subject of intense research primarily in terrestrial dichromatic mammals, such as rodents, with significant advances having been made in elucidating the anatomy and functions of ipRGCs, as well as the rod/cone input to these cells (Lucas et al., 2012; Pickard and Sollars, 2012). However, there is a major gap in our knowledge of the role of ipRGCs in mammals that have adapted to dim light environments and are rod monochromats, such as the cetaceans.

The first vertebrates appeared approximately 500 mya and the vertebrate visual system evolved into both image and non-image forming vision (Gerkema et al., 2013). The

hypothesized nocturnal bottleneck in mammalian evolution influenced the evolution of therian mammalian vision with the loss of at least 3 classes of opsins (*SWS2*, *Rh2,* and *OPN4x*) (Gerkema et al., 2013; Jacobs, 2013). Therefore, the typical mammal has two cone visual pigments (LWS and SWS1); one rod visual pigment (Rh1) and one melanopsin visual pigment (OPN4m). The radiation of mammals into specialized environmental niches has resulted in further adaptations of the mammalian visual system. For instance, the evolution of cone trichromacy occurred in old world primates (Jacobs, 2008; Hofmann and Palczewski, 2015). Other mammals occupy dim light environments and are either cone monochromats or rod monochromats (Meredith et al., 2013; Emerling and Springer, 2015).

Melanopsin exhibits a higher sequence homology to visual pigments from rhabdomeric photoreceptors (Provencio et al., 1998, 2000) than mammalian cone and rod visual pigments (**Figure 6**). Unlike the cetacean rod visual pigments which are spectrally tuned to various underwater photic environments, all melanopsin pigments characterized to date have a fairly uniform absorption maximum centered around 480 nm. Amino acid sequence alignments of cetacean and terrestrial melanopsins reveal very few non-conserved amino acid substitutions that would result in any significant divergence away from this typical absorption maximum (**Figures 7**, **8**). However, there is divergence between the cetacean and terrestrial mammalian melanopsins at the carboxyl tail, specifically at the sites of phosphorylation that are involved in pigment inactivation (Fasick and Samuels, 2015).

The biochemical kinetics of melanopsin activation and deactivation is also in marked contrast to that of both the vertebrate rod and cone visual pigments. As first described by Berson et al. (2002), the OPN4 light response is extremely slow when compared to that of either rods or cones with deactivation being even slower. The "sluggish" light activation of

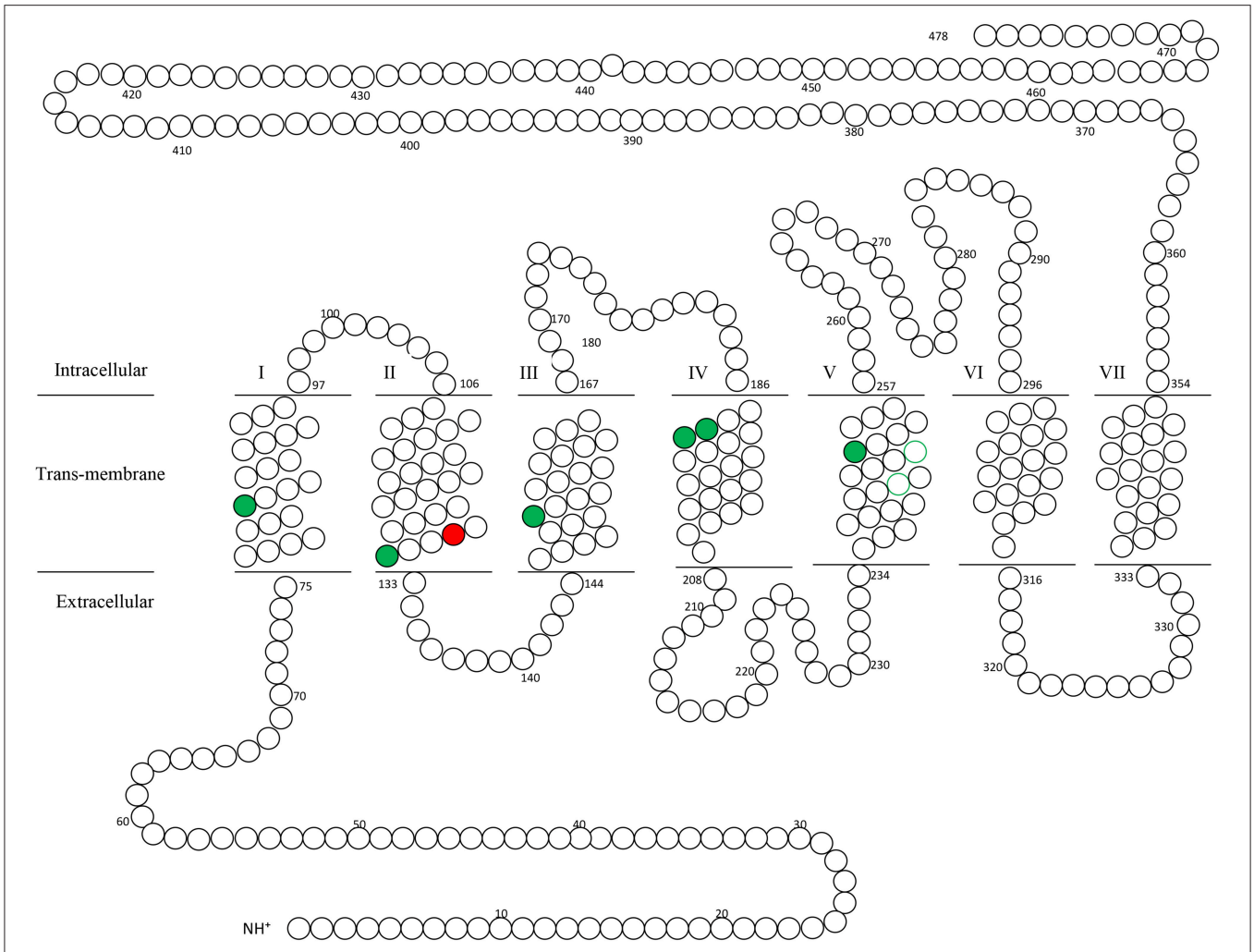

FIGURE 8 | Secondary structure of cetacean melanopsin. Conserved amino acid substitutions between North Atlantic right whale, minke whale, sperm whale, bottlenose dolphin and baiji dolphin are labeled green, while the single nonconserved substitution occurring in baiji dolphin (A129T) is labeled red. Figure adapted from Fasick and Samuels (2015).

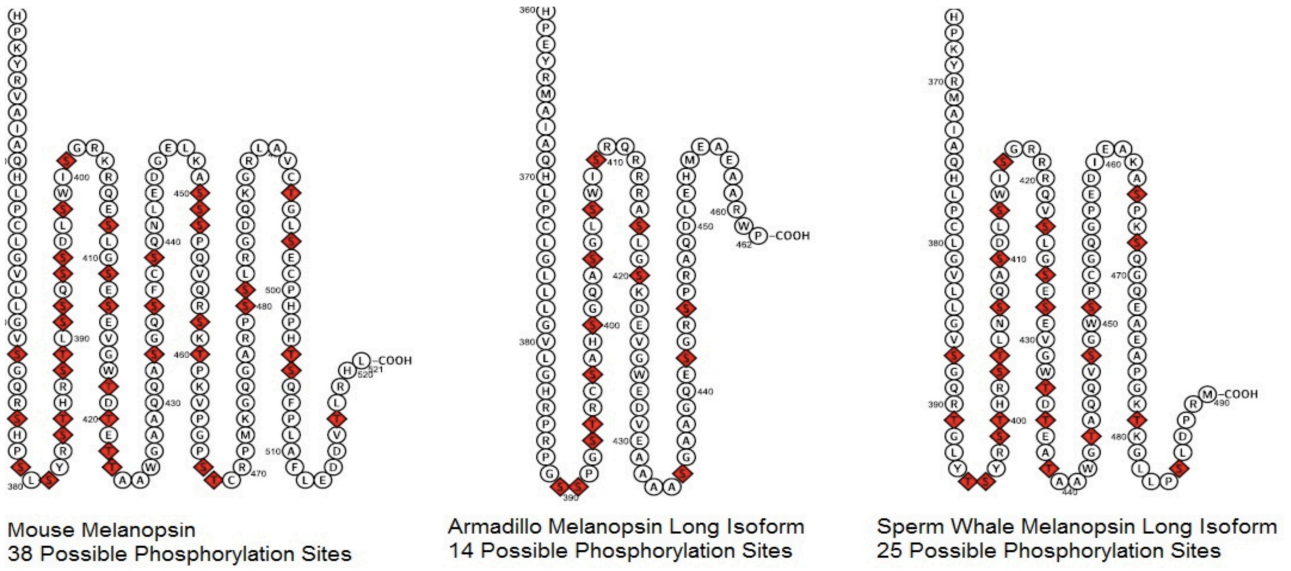

FIGURE 9 | Two dimensional diagram of Melanopsin C-terminus from mouse, armadillo, and sperm whale. Possible phosphorylation sites, serines, and threonines, are indicated in red.

melanopsin postulated by Blasic et al. (2012) is intuitive to this retinal pigment class based on its expression in retinal ganglion cells. Unlike the rod and cone photoreceptors that evolved membranous stacks, as in rods, or invaginations, as is the case in cones, which dramatically increase the membranous surfaces in which visual opsins can integrate, the retinal ganglion cells, by contrast, are composed of a single outer plasma membrane in which integration of melanopsins occurs. This reduction in membrane surface to a single membrane limits the number of melanopsin molecules expressed in each cell. Along with the fact that melanopsin is expressed in only ∼2% of retinal ganglion cells, we begin to appreciate the large number of photons that must strike the retinal surface in order to activate these cells.

Although, significant advances have been made in elucidating the anatomy and functions of ipRGCs as well as the rod/cone input to these cells (Schmidt et al., 2011b; Sexton et al., 2012) our knowledge of the mechanisms underlying the deactivation of ipRGC light responses and the behavioral consequences remain rudimentary. The light response in rods and cones terminates by phosphorylation of the C-terminus of rod and cone opsins by a G-protein receptor kinase (GRK), followed by the binding of visual arrestin. Mouse melanopsin, which has been the subject of much research, has a C-terminus that is 4 times longer than that of rod and cone opsins, containing 171 amino acids with 38 potential phosphorylation sites. Previous investigations have found that in a heterologous expression system the C-terminus phosphorylation of mouse melanopsin regulates the shutoff of the light response (Blasic et al., 2012, 2014). Importantly, this phosphorylation event has significant behavioral consequences as the light-induced pupil constriction is dramatically prolonged in mice expressing a phosphorylation-deficient melanopsin (Somasunda, personel communication). It can be hypothesized then that melanopsin expressed in the retina of cetacean rod monochromats will have a compliment of amino acids in the melanopsin C-terminus that result in slow deactivation kinectics (see **Figure 9**). This slow deactivation of melanopsin will allow these animals to maintain prolonged pupil constriction when they are actually exposed to bright lights thus protecting the rod photoreceptors from photobleaching.

Currently, we know nothing of the role of melanopsin and ipRGCs in cetaceans. Future studies should examine the C-termini of the cetacean melanopsins, specifically the number of phosphorylation sites that presumably are involved with deactivation. It is feasible to hypothesize that the rod monochromat cetacean melanopsin pigments will have slower deactivation kinetics when compared to melanopsins from mammalian rod/cone dichromats. Slower deactivation of these pigments would provide prolonged afferent signaling from ipRGCs to physiologically (and behaviorally) relevant brain nuclei in cetacean rod monochromats.

SUMMARY

Understanding the evolution of vision is a key question in both evolutionary biology and vision science. A combination of morphological, genetic, molecular, functional and physiological data is bringing a new level of insight into a field of inquiry that dates back to Darwin (Lamb, 2013; Nilsson, 2013). In the era of genomics, the genetic sequence of the molecules involved in phototransduction, especially visual pigments, have contributed significant insight into this problem. The typical terrestrial mammal has two cone visual pigments (LWS and SWS1); one rod visual pigment (Rh1) and one melanopsin visual pigment (OPN4m). The radiation of mammals into specialized environmental niches such as the ocean has resulted in further adaptations of the mammalian visual system. Cetaceans are an

example of an evolving visual system that has adapted to the visual pressures of an aquatic environment and foraging at various depths.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Morphology, Characterization and Distribution of Retinal Photoreceptors in the South American (*Lepidosiren paradoxa*) and Spotted African (*Protopterus dolloi*) Lungfishes

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Lungfishes are the closest living relatives of the ancestors to all terrestrial vertebrates and have remained relatively unchanged since the early Lochkovin period (410 mya). Lungfishes, therefore, represent a critical stage in vertebrate evolution and their sensory neurobiology is of considerable interest. This study examines the ultrastructure of the retina of two species of lungfishes: the South American lungfish, *Lepidosiren paradoxa* and the spotted African lungfish, *Protopterus dolloi* in an attempt to assess variations in photoreception in these two ancient groups of sarcopterygian (lobe-finned) fishes. In juvenile *P. dolloi*, the retina contains one rod and two cone photoreceptor types (one containing a red oil droplet), while only one rod and one cone photoreceptor type is present in adult *L. paradoxa*. Both species lack double cones. The large size and inclusion of oil droplets in both species apart from one of the cone photoreceptor types in *P. dolloi* suggests that *L. paradoxa* and *P. dolloi* are adapted for increasing sensitivity. However, the complement of photoreceptor types suggests that there may be a major difference in the capacity to discriminate color (dichromatic and monochromatic photoreception in *P. dolloi* and *L. paradoxa*, respectively). This study suggests that the visual needs of these two species may differ.

Keywords: dipnoi, color vision, photoreceptors, oil droplets, sensitivity, lungfishes

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INTRODUCTION

The South American (*Lepidosiren paradoxa*) and spotted African (*Protopterus dolloi*) lungfishes are dipoan fishes that belong to the order Lepidosireniformes. Lungfishes, including *Neoceratodus forsteri* (order Ceratodontiformes) from Australia diverged from the main vertebrate stock ~410 mya and along with the coelacanth (*Latimeria chalumnae*), encompass the surviving lobe-finned fishes (Sarcopterygii; Bemis et al., 1987; Collin, 2010; Clack et al., 2011). Lungfishes have the ability to breathe dissolved and atmospheric oxygen through gills and "primitive" lungs, respectively.

Linked to humans by being the closest living relatives to the tetrapods (Brinkmann et al., 2004; Amemiya et al., 2013), they are vital to the study of the evolution of vision in terrestrial animals.

The South American lungfish resides in the neotropics of South America and has the most extensive distribution of all lungfish species (Fonesca de Almeida-Val et al., 2011). Its range extends through Argentina, Bolivia, Colombia, Brazil, Paraguay, Venezuela, and French Guiana, and although it is found in the Parana–Paraguay River system, it is preferentially located within the Amazon River basin (Fonesca de Almeida-Val et al., 2011). All African lungfishes are endemic to the river systems of a large part of the African continental landmass, and *P. dolloi* primarily inhabits the Congo River basin.

Previous studies of *N. forsteri*, considered the most basal of all lungfish species (Kemp and Molnar, 1981; Bailes et al., 2006), show that at least some species of lungfish possess a complex tri- or tetra-chromatic color vision resembling that of diurnal vertebrates such as birds and reptiles. This suggests that several of the ocular characteristics lungfishes possess may have evolved in shallow water before the transitioning onto land (Collin, 2010). One such characteristic is the presence of corneal surface microprojections present in terrestrial vertebrates and *N. forsteri* and may have evolved in order to provide clear aerial vision for those lungfishes that aestivate (Collin and Collin, 2001).

Previous anatomical studies have shown that the eyes of lungfishes possess different morphological types of retinal photoreceptor, demonstrating that they have the neural machinery to process color (Walls, 1942; Pfeiffer, 1968; Ali and Anctil, 1973). *N. forsteri* is the only species in which the retina has been studied in detail (Marshall, 1986; Tokita et al., 2005; Bailes et al., 2006). Up to five spectrally distinct types of large retinal photoreceptors have been found in *N. forsteri*: one type of rod (λ_{max} 540 nm) and four types of cones (UVS λ_{max} 366 nm, SWS λ_{max} 479 nm, MWS λ_{max} 558 nm, and LWS λ_{max} 623 nm). UVS cones are only found in the retinas of juvenile *N. forsteri*, suggesting that sensitivity to ultraviolet light is lost during maturation (Bailes et al., 2006; Hart et al., 2008). The possession of four types of cones in juvenile lungfishes implies that they have the potential for tetrachromatic vision, which is perhaps unusual for an animal that has previously been considered predominantly nocturnal or crepuscular (Dean, 1906, 1912; Grigg, 1965; Kemp, 1986). To date, the retinae of the South American and spotted African lungfishes have only been superficially characterized by using light microscopy and both species are thought to possess one type of rod and at least two types of cone, suggesting that these species may also have the capacity for color vision. *P. dolloi* appears to possess both single and double cones, while *L. paradoxa* and *N. forsteri* possess only single cones (Ali and Anctil, 1973; Bailes et al., 2006).

P. dolloi and *L. paradoxa* possess oil droplets in both their cone and rod photoreceptors (Kerr, 1902; Walls, 1942; Pfeiffer, 1968; Ali and Anctil, 1973), whereas *N. forsteri* only possesses colorless oil droplets in their SWS cones, colored yellow pigment in their MWS cones and red oil droplets in their LWS cones (Bailes et al., 2006). Colorless oil droplets are found in marsupial mammals which are generally nocturnal, crepuscular or cathemeral and are thought to improve light gathering ability and thus, overall sensitivity. Colored oil droplets act as miniature spectral filters within each photoreceptor and, by narrowing the spectral sensitivity function of the photoreceptors, are thought to improve color discrimination by reducing overlap with adjacent spectral classes of cone (Vorobyev et al., 1998; Hart et al., 2008). The presence of colored oil droplets in the lungfish retina is surprising because these characteristics are found only in strongly diurnal animals such as birds and reptiles, and the spotted African and South American lungfishes occupy swamps and lakes that consist of lentic (stagnant) water bodies associated with poor visibility, weedy vegetation, low oxygen content, and seasonal drying (Greenwood, 1986; Mlewa et al., 2011).

In most animals with image-forming eyes, the densities of photoreceptor cells are not uniform across the retina (Collin, 1999), and usually reflects key features of their visual behavior in respect to their physical environment, as well as determining the visual acuity or spatial resolving power of their eyes. The areas that exhibit an increase in photoreceptor density in the *N. forsteri* retina vary as the fish matures (Bailes et al., 2006). The highest density of rods in the juvenile and sub-adult *N. forsteri* lie in the temporal retina, implying an increase in retinal sensitivity in the frontal visual field. As the fish matures, this temporal specialization becomes two areas of increased rod density (with the second high density region situated in the central retina in adults), to form a weak horizontal band across the retinal meridian. In general, the increased density of cone photoreceptors is predominantly in the dorso-temporal retina and the ventral-nasal retina in all growth stages of *N. forsteri*, indicating a downwardly directed visual axis (Bailes et al., 2006). The topographic specializations in *L. paradoxa* or *Protopterus* spp. are not yet known.

Both *Protopterus* spp. and *L. paradoxa* undergo aestivation. Aestivation can be described as a "light" state of dormancy, characterized by inactivity and a lowered metabolic rate that can be quickly reversed if the right conditions are met. This process enables these ancient fishes to avoid damage from high temperatures and desiccation during the dry season without leaving the swamps for permanent water, like some teleost fishes (Greenwood, 1986). In their respective burrows, both species of lungfishes remain until the onset of the wet season, which may be for up to 8 months. It is not known if vision plays a significant role in their lifestyle during this aestivating phase, since normal metabolic function and activity is not triggered until the wet season begins.

Consequently, the visual system in lungfishes is something of a conundrum. It appears the habitat and lifestyle of *P. dolloi* and *L. paradoxa* are also not heavily reliant on their visual system. Little is known about the visual behavior of any lungfish species, and it has been stated a number of times that vision does not contribute heavily to their lifestyle, at least in prey capture and navigation (Owen, 1840; Carter and Beadle, 1930; Johnels and Svensson, 1954; Curry-Lindahl, 1956; Pfeiffer, 1968; Greenwood, 1986). The photic habitat of the swamps and rivers of the Amazon and Congo allows for poor visibility, with a substantial amount of sediment and mineral deposits contributing to turbid, and therefore relatively dim, underwater light environments. Combined with their aestivating behavior, where they remain

dormant for most of the year, it would appear that vision does not play an essential role in survival. However, the complex visual apparatus of the lungfish eye, including a well-developed lens and active accommodatory apparatus, the presence of colored oil droplets and the presence of four spectral types of cone photoreceptor in *N. forsteri* (Bailes et al., 2006, 2007), suggests that color vision is more important to lungfishes than previously thought.

This study fills crucial knowledge gaps regarding the visual capabilities of *P. dolloi* and *L. paradoxa* by characterizing specific photoreceptor types based on their size, ultrastructure and topographic distribution. There have only been two published papers specifically focused on the retina of *P. dolloi* (Pfeiffer, 1968) and *L. paradoxa* (Ali and Anctil, 1973) using retinal tissue prepared in paraffin wax for light microscopy. This will be the first report on the retinal photoreceptors of *P. dolloi* and *L. paradoxa* examining resin embedded retinal tissue, enabling the ultrastructure of the photoreceptors to be described at the level of the transmission electron microscope.

The study reveals that based on morphology; there are at least three different photoreceptor types in juvenile *P. dolloi*, and one rod and one cone photoreceptor type in *L. paradoxa*. The topographical distribution of a cone photoreceptor type containing a red oil droplet is also presented for juvenile *P. dolloi*. This suggests that juvenile *P. dolloi* have the potential for dichromatic color vision, and *L. paradoxa* may be the only lungfish species without the potential for color vision. These findings provide novel data that contributes to our understanding of the visual experience of *P. dolloi* and *L. paradoxa*, and provide insight to the role vision plays in their behavioral ecology.

METHODS

Samples

All lungfish eye samples were donated by international collaborators who had obtained the animals through the international pet trade. Eyes from three juvenile *P. dolloi* (total length, TL 20.6, 20.2, and 24 cm; eye codes Pd5L, Pd3R, and Pd4R) were obtained from Dr. Guido Westhoff from the University of Bonn, Germany. Two eyes from an adult *L. paradoxa* (TL 96 cm) were obtained from Prof. Glenn Northcutt from the University of California, San Diego (UCSD), USA. All animals were light adapted and sacrificed during daylight hours. Following enucleation, the lens of *L. paradoxa* was removed. However, due to the small size of the *P. dolloi* eyes, the lens was not removed and the eyes were immersion fixed *in toto*. All samples were preserved in Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) prior to being transported to the University of Western Australia.

Light and Electron Microscopy

All eye samples were processed for both light and electron microscopy to examine the morphology of the photoreceptors within the retina of *P. dolloi* and *L. paradoxa*. Due to the fragility of the photoreceptors, the retina was not removed from the eye cup, but a significant amount of connective tissue surrounding the eye was removed prior to embedding in Procure-Araldite (ProSciTech). However, under some circumstances, the retina of *L. paradoxa* still peeled away from the underlying pigment epithelium, subsequently damaging the photoreceptors. The eyecup was then postfixed for 1 h with 1% osmium tetroxide in 0.15 M phosphate buffer, dehydrated through an alcohol and propylene oxide series and infiltrated with Procure-Araldite (ProSciTech). For light microscopy, semi-thin (1 μm) sections were cut with a glass knife using a LKB Bromma Ultratome NOVA. Semi-thin sections were then deplastinated with a wash in a solution of sodium ethoxide, 70% alcohol and double distilled H_2O and stained with 4% Toluidine blue. Light micrographs were taken using an Olympus camera (Model DP70) mounted on an Olympus compound light microscope (Model BX50F4). For transmission electron microscopy, ultrathin sections (110 nm) were cut using a diamond knife and mounted on a 200 mesh copper grid. Examination of ultrathin sections was done using a JEOL 2100 transmission electron microscope operating at 120 kV and photographed using an 11 megapixel Gatan Orius digital camera.

Assessment of Photoreceptor Dimensions and Morphology

All measurements were conducted from digital images of light and electron micrographs using ImageJ 1.46r (National Institute of Health, USA). Three quarters of the eye were sampled from each individual, and included the nasal/dorsal and nasal/ventral quadrants of the eye. To allow for comparison between photoreceptor cells, the outer segment diameter was measured at the base, and the ellipsoidal diameter was measured at the widest point. Shrinkage of *L. paradoxa* eye tissue could not be determined due to unrecorded pre-fixation dimensions. However, measurements of the *P. dolloi* eye tissue pre-fixation revealed shrinkage (post-fixation) to be 6.3% using the methodology outlined by Bailes et al. (2006). All measurements are quoted as mean ± standard deviation (SD) followed by sample size (n).

Preparation of Retinal Wholemount

In order to analyse the topographic distribution of the large red oil droplet containing cone photoreceptor type, one retina of a juvenile *P. dolloi* (24.3 cm in TL) was dissected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PFA, pH 7.4). A retinal wholemount was prepared according to standard protocols (Stone, 1981; Coimbra et al., 2006). The flattened retina was then rinsed in phosphate buffer, mounted onto a subbed glass slide with the photoreceptor layer facing upwards in 100% glycerol, and sealed. The cones could be distinguished from rods (based on their size and tapered outer segment) by changing the fine focus on the microscope at a magnification of x1000 using a 1.40 numerical aperture (NA) oil immersion objective.

Stereological Analysis and Construction of a Topographic Map

The topographic distribution of the oil droplet containing photoreceptor in *P. dolloi* was assessed using the modified optical fractionator technique detailed in Coimbra et al. (2009). Using

StereoInvestigator software (Microbrightfield Inc., USA) on a Windows 7 PC connected to a compound microscope (Olympus BX50) equipped with a motorized stage (MAC200, Ludle Electronic Products, USA) and digital video camera (MicroFIRE, OPTRONICS), the outline of the retinal wholemount was digitized using a x4 objective (NA 0.13). Using a x100 oil immersion objective (1.40 NA), all red cone photoreceptors in the retinal wholemount were counted. The retinal outline and cell count data were exported as an Extensible Markup Language (.xml) format file and analyzed using the open source statistical program R v2.15.1 (R Foundation for Statistical Computing, 2012) modified with additional packages and a custom script according to Garza-Gisholt et al. (2014) to construct the retinal topographical map.

RESULTS

Photoreceptor Characterization and Morphology of *L. paradoxa*. The photoreceptors of adult *L. paradoxa* are large and tightly packed. Based on morphological characteristics at both the light and electron microscope levels and dimensions of the photoreceptors (**Table 1**), there is one rod and at least one cone photoreceptor type in adult *L. paradoxa* (**Figure 1A**). Both rods and cones possess oil droplets and were distinguished from each other by their synaptic terminals. Rods maintained spherule synaptic terminals and cones possessed pedicles with an increased number of synaptic ribbons. No double cones were observed. Oil droplets visible in the electron micrographs of photoreceptors appear to contain electron-dense material (**Figure 1B**). All light micrograph images do not contain inclusions within the oil droplets (**Figure 1A**). We believe that the electron dense inclusions in the electron micrographs may be artifacts, but frozen sections were not taken to view these oil droplets using light microscopy.

The rod photoreceptor is large, and possesses a tapered outer segment that is usually shorter in length than the outer segment of the cones (8.0 ± 1.4 μm, $n = 13$, with a basal diameter of 10.1 ± 0.8 μm, $n = 14$; **Figure 1A**). The ellipsoid contains a larger oil droplet than the cones (268.7 ± 51.0 μm^2, $n = 12$ in rods, compared to 82. ± 22.0 μm^2, $n = 7$ in cones), with the majority of mitochondria and the paraboloid located closer to the nucleus (**Figure 1A**). It is more difficult to differentiate rods and cones in *L. paradoxa* in comparison to *N. forsteri* and *P. dolloi*. However, rods can be identified by the presence of incisures in the edges of the outer segment disks and a more cylindrical outer segment.

Like all other species of lungfishes described, the cones are large (13.6 ± 2.8 μm outer segment length; $n = 15$, and 10.5 ± 1.5 μm ellipsoid diameter; $n = 10$ in a 96 cm individual) and contain one oil droplet (mean diameter 82.8 ± 21.9 μm^2, $n = 7$) within the ellipsoid. There are no additional oil droplets embedded within the mitochondria in the ellipsoid region in the cones (**Figure 1B**). The mitochondria are distributed more centrally within the cone ellipsoid, close to the oil droplet (**Figure 1B**) with a paraboloid that consists of an aggregation of granules (**Figure 1B**).

Morphology of Retinal Photoreceptors in *P. dolloi*

The dimensions of photoreceptor types are summarized in **Table 2**. Based on morphology, intracellular characteristics and size, juvenile *P. dolloi* possesses one type of rod and at least two types of cones (one with a red oil droplet [red cone] and one lacking an oil droplet [clear cone]). The rod photoreceptors possessed the typical spherule shape, while cones possessed pedicles with a higher number of synaptic ribbons. There is one example of an additional cone type that contained a yellow pigment within the oil droplet, but there are only 36 cells of this type counted in the retina, so it is unknown if this represents a population of a different, rare cone type or oxidation of the red pigment contained in the red cone oil droplets after enucleation. Like all other lungfish species described, most of the cell types within the retina of a juvenile *P. dolloi* are large (147.2 ± 17.9 μm thick in a 20.6 cm TL individual, $n = 6$, **Figure 2**). No double cones were observed.

The rods are large (16.0 ± 2.1 μm outer segment length; $n = 32$, and 11.7 ± 1.3 μm ellipsoid diameter; $n = 34$, in a 20.6 cm TL individual) and contain one large oil droplet situated within the sclerad section of the ellipsoid of the inner segment amongst the mitochondria (118.4 ± 23.6 μm^2 oil droplet area, $n = 33$), and unlike *L. paradoxa*, a number of smaller oil droplets (between 0 and 5, $n = 12$) are situated closer to the nucleus (**Figure 3A**). The outer segment is cylindrical in shape, and consists of scalloped discs surrounded by a plasma membrane (**Figure 3B**). The presence of rods is confirmed by the presence of incisures in the outer segment discs (**Figure 3C**). There are also unknown inclusions within the rod outer segment (**Figure 3A**). The ellipsoid of the rod is similar to that of *L. paradoxa* and consists of electron-dense, tightly packed mitochondria of various shapes and sizes that become smaller toward the nucleus (outer nuclear layer), and lack discernible internal structure apart from a few remaining cristae. A large paraboloid containing

TABLE 1 | Photoreceptor dimensions of adult *Lepidosiren paradoxa*[a].

	Basal diameter of outer segment (OS) (μm)	Length of OS (μm)	Diameter of ellipsoid (μm)	Length of ellipsoid (μm)	Oil droplet (OD) (Present/Absent)	Size of OD (μm^2)
Rod	10.1 ± 0.8 (14)	8.0 ± 1.4 (13)	18.0 ± 1.8 (13)	27.7 ± 4.1 (10)	Present	268.7 ± 51.0 (12)
Cone	7.5 ± 0.7 (16)	13.6 ± 2.8 (15)	10.5 ± 1.8 (10)	22.7 ± 2.4 (6)	Present	82.8 ± 22.0 (7)

[a] *All values are the mean ± one standard deviation with number of cells sampled in parentheses. All measurements are from one individual 96 cm in TL.*

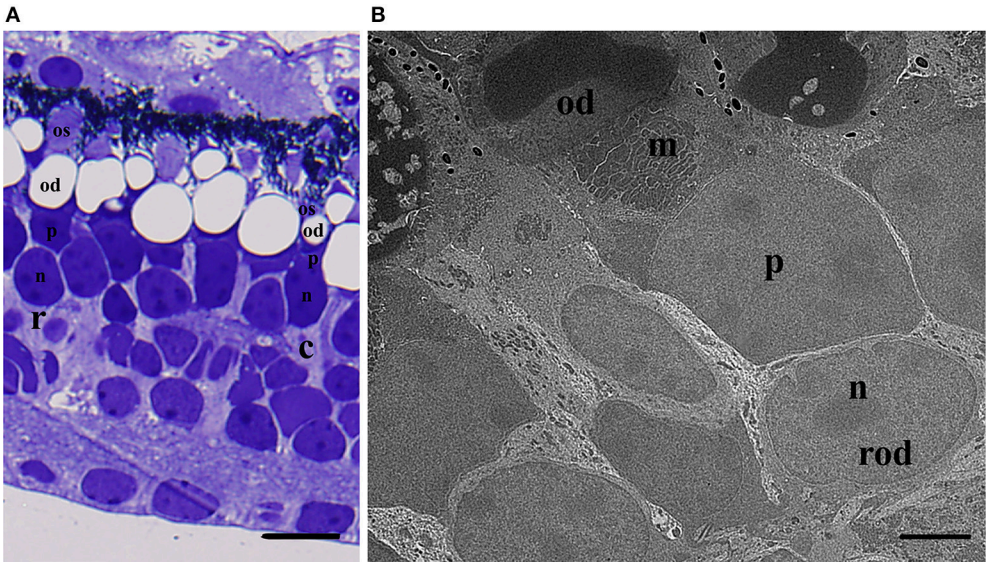

FIGURE 1 | Morphological characteristics of rod and cone photoreceptors in adult *Lepidosiren paradoxa*. (A) Light micrograph of retina in transverse section showing the rod (r) and cone (c) photoreceptor. The rod outer segment (os) is cylindrical, as opposed to the conical, taped cone outer segment. The oil droplet (od) of the rod is much larger than in the cone. Both photoreceptor types contain a paraboloid (p) and nucleus (n) below the oil droplet. Scale bar = 25 μm **(B)** Electron micrograph of a rod. The oil droplet has inclusions that are considered to be artifacts because of their absence in light micrograph. The majority of mitochondria (m) is situated in between the oil droplet (od) and paraboloid (p) and is located centrally, containing electron dense mitochondria of varying shapes and sizes. The nucleus (n) is below the paraboloid, and the cylindrical outer segment (os) is partially obscured. Scale bar = 5 μm.

TABLE 2 | Photoreceptor dimensions and characteristics of juvenile *Protopterus dolloi*[a].

	Basal diameter of outer segment (OS) (μm)	Length of OS (μm)	Diameter of ellipsoid (μm)	Length of ellipsoid (μm)	Oil droplet (OD) (Present/Absent)	Size of OD (μm^2)
Rod	9.2 ± 1.0 (32)	16.0 ± 2.1 (32)	11.7 ± 1.3 (34)	22.2 ± 2.7 (33)	Present	118.4 ± 23.6* (33)
Red cone	4.7 ± 0.8 (15)	6.4 ± 0.7 (14)	7.4 ± 1.8 (14)	12.7 ± 2.0 (12)	Present	39.0 ± 10.3 (13)
Clear cone	3.8 ± 1.0 (13)	5.6 ± 1.4 (13)	9.1 ± 1.0 (15)	12.3 ± 0.7 (14)	Absent	N/A

[a]*All values are the mean ± one standard deviation with number of cells sampled in parentheses. All measurements are from one individual 20.6 cm in TL. *measurements were taken of largest oil droplet in the rod.*

an aggregation of granules lies between the mitochondria and nucleus of the rod, but unlike *L. paradoxa*, there is a space (that may be an artifact) between the mitochondria and nucleus (**Figure 3A**).

Two morphologically different cone types were identified in *P. dolloi* in retinal wholemount, and using light and electron microscopy based on inclusions within the inner segment of the photoreceptors; a cone with a red oil droplet (red cone; **Figure 4A**), and a cone with no oil droplet (clear cone; **Figure 4C**).

The red cone contains a large red oil droplet within the ellipsoid of the inner segment (**Figure 4B**). The outer segment is conical in shape, with a basal diameter of 4.7 ± 0.8 μm ($n = 15$) and length of 6.4 ± 0.7 μm ($n = 14$). The diameter of the ellipsoid is 7.4 ± 1.8 μm ($n = 14$) and is filled with a single red oil droplet (39.0 ± 10.3 μm^2, $n = 13$) in size (**Table 2**). The mitochondria within the ellipsoid have a similar organization to the mitochondria in the rods, where larger mitochondria are concentrated toward the oil droplet and decrease in size closer to the nucleus (**Figure 4B**). In this individual, eleven smaller cones with a small red oil droplet were counted along the nasal edge of the retina. These may be red cones that were damaged by tissue fixation and/or processing or a developmental stage of the red cone receptor type as they resemble the large red-oil droplet bearing cones in all other characteristics.

The cone without an oil droplet has a tapered, conical outer segment with a basal diameter of 3.8 ± 1.0 μm ($n = 13$) and a length of 5.6 ± 1.4 μm ($n = 13$) in an individual of 20.6 cm in TL. The ellipsoid is 9.1 ± 1.0 μm ($n = 15$) in diameter and is filled with mitochondria of varying sizes, the largest of which are positioned closest to the outer segment (**Figure 4C**). Like the rods, the paraboloid of the clear cone consists of granules that sit between the mitochondria and nucleus.

Topographic Distribution of the Red-Oil Droplet Bearing Photoreceptor Type in *P. dolloi*

The photoreceptors of the juvenile *P. dolloi* are tightly packed across the retina. There does not appear to be a geometrically

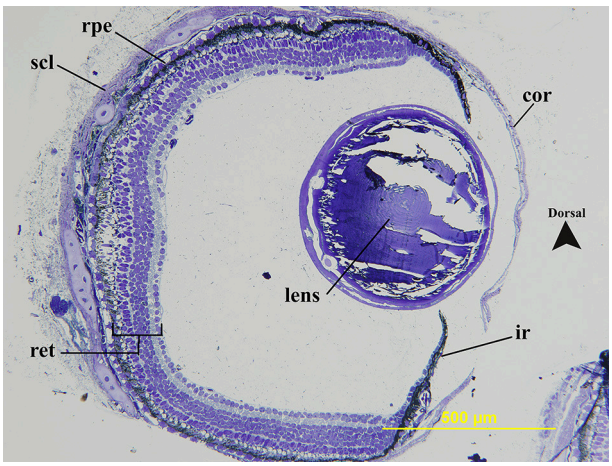

FIGURE 2 | Transverse section of the geometric center of a juvenile *Protopterus dolloi* eye. The sclera (scl) surrounds the back of the eye to become the cornea (cor) anteriorly. The lens is spherical and relatively large in the eyecup and sits more dorsally, with the iris (ir) length being unequal on either side of the lens. The retinal pigment epithelium is darkly pigmented (rpe). All retinal (ret) cell layers are present.

regular photoreceptor mosaic, but no formal analysis of photoreceptor spacing was undertaken. However, the red cones often lie adjacent to each other, in the middle of five other photoreceptors, usually rods (**Figure 4A**). The topographic distribution of red cones in juvenile *P. dolloi* shows regional variation, with a dorso-nasal area centralis in the retina with a peak density of 1.4×10^3 cells mm^{-2} and a total red cone population of 837×10^3 cells in an individual of 20.2 cm in TL (**Figure 5**).

DISCUSSION

This study reveals the presence and ultrastructure of one rod and two cone photoreceptor types in juvenile *P. dolloi*, the topographical distribution of the red cone in a juvenile *P. dolloi*, and the characterization of one rod and one cone type in adult *L. paradoxa*. Like the photoreceptors of *N. forsteri*, the photoreceptors of *P. dolloi* and *L. paradoxa* are large in absolute terms and their large inner segment cross sectional area and outer segment length will aid in maximizing photon capture in dim light. This has been confirmed behaviorally in the cichlid (*Haplochromis sauvagei*) where an increase in photic sensitivity is associated with increased photoreceptor size (Van der Meer, 1994).

Photoreceptor Types in *L. paradoxa*

This study showed *L. paradoxa* possesses one rod and one cone photoreceptor type. This is in accordance with the description by Ali and Anctil (1973) using light microscopy and Zeiss et al. (2011) using immunohistochemistry that identified the presence of LWS cones but no SWS cones. However, no immunostaining for MWS cones was undertaken (Zeiss et al., 2011). During aestivation, *L. paradoxa* remains dormant and does not feed during the dry, winter months that can potentially last for 8 months a year. This is unlike the lifestyle of *P. dolloi*, which make frequent journeys to the top of their burrows during aestivation to breathe, and *N. forsteri* that do not aestivate at all. These disparate lifestyles may have resulted in less dependency on color vision, as *L. paradoxa* is frequently described as the least fish-like, with the least developed retina (Ali and Anctil, 1973), but precisely what the role of color vision is in all lungfish species is still a matter of speculation.

The morphology of the oil droplet bearing rod and cone photoreceptors in *L. paradoxa* closely resembles those of *P. dolloi*, and follows what was described in an ultrastructural study by Ali and Anctil (1973). However, Zeiss et al. (2011) described the rods of *L. paradoxa* as containing an ellipsoid without an oil droplet. This finding is in contrast with Ali and Anctil's (1973) report on rods despite specimens being processed in the same way. In processing the *L. paradoxa* retina for this study, unknown inclusions within the oil droplets of rods and cones appeared in the transmission electron micrographs. This may be an artifact, or may indicate a difference in the composition of the ellipsoid and/or the oil droplet in the photoreceptors in comparison to *P. dolloi*. In order to determine if the inclusions within the inner segment of *L. paradoxa* photoreceptors are oil droplets, the retina can be stained with Oil Red O to show the presence of neutral fats (triglycerides), as was carried out with *N. forsteri* (Bailes et al., 2006).

Of all lungfishes, *N. forsteri* is the only known species that does not possess oil droplets in their rod photoreceptors. Many species of vertebrates contain colorless oil droplets, such as the turtle *Pseudymys scripta* (Kolb and Jones, 1987), the green frog *Rana clamitans* (Hailman, 1976), and the strawberry poison frog *Dendrobates pumilio* (Siddiqi et al., 2004). It is speculated that retinal oil droplets were originally colored, but where species have subsequently become nocturnal these pigments were lost (Hart et al., 2006). This is presumably because the benefits of spectral tuning conferred by colored oil droplets are outweighed by the reduction in absolute sensitivity that would make vision in dim light more difficult. Chickens (*Gallus gallus domesticus*) reared in dim light develop less dense pigmentation in their colored oil droplets compared to those reared in bright light, presumably to maintain absolute sensitivity at the expense of spectral tuning (Hart et al., 2006), so it may be possible that adopting nocturnality provided the selective pressure to lose colored pigments within oil droplets. All oil droplets can potentially act as micro-lenses to increase photon capture (Sivak et al., 1999); although more research is needed to determine what role they play in *P. dolloi* and *L. paradoxa.*

Photoreceptor Types in *P. dolloi*

The photoreceptors of the juvenile *P. dolloi* contain at least one type of rod and two types of cones (red oil droplet containing cones and clear, no oil droplet containing cones) that suggest it has the potential for dichromatic color vision. The photoreceptors are not arranged in a regular mosaic, like *N. forsteri* and *L. paradoxa* (Ali and Anctil, 1973; Bailes et al., 2006). The description of oil droplet containing rods, and cones with and without oil droplets is in accordance with Pfeiffer

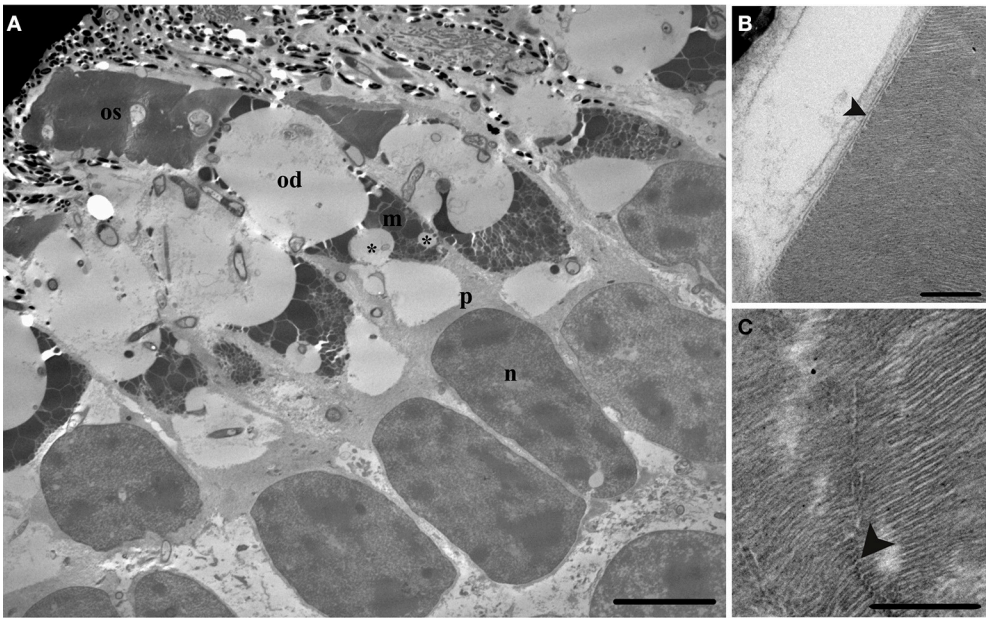

FIGURE 3 | Transmission electron micrographs detailing the ultrastructure of a juvenile *Protopterus dolloi* rod photoreceptor. (A) The rod (rd) is large with one large oil droplet (od) situated within the mitochondria (m) of the ellipsoid, with smaller oil droplets (*) in the mitochondria, closer to the paraboloid (p). The nucleus (n) is elongated, and the outer segment (os) is cylindrical in shape, containing unknown inclusions (arrowhead). Scale bar = 10 μm. **(B)** The outer segment consists of scalloped discs (arrowhead) surrounded by a plasma membrane. Scale bar = 0.5 μm. **(C)** The rods are confirmed by the presence of incisures (arrowhead), which appear as gaps in the discs of the outer segment. Scale bar = 0.25 μm.

(1968). However, our investigation did not reveal double cones. Pfeiffer (1968) described the retina of *P. dolloi* in paraffin wax sections, and reported single cones with oil droplets, and double cones with only one member containing an oil droplet. Our investigation of *P. dolloi* photoreceptors did not show a clear cone next to a cone of a different type that was not another clear cone, rod, or red cone. In fact, there were many occasions where two clear cones were situated next to each other. Ali and Anctil's (1973) description of *L. paradoxa* photoreceptors from tissue processed in paraffin wax did note the presence of pairs of visual cells that may have been unequal double cones. However, they concluded that these instances were random associations of a single cone and rod (Ali and Anctil, 1973). Without a reference image of *P. dolloi* double cones from Pfeiffer (1968), this is difficult to confirm. Double cones are present in many teleost fishes (Stell and Hárosi, 1976; Collin, 1997; Collin and Shand, 2003; Pignatelli et al., 2010), and most terrestrial vertebrates such as birds (Hart, 2001), amphibians (Mariani, 1986), and diurnal reptiles (Detwiler and Laurens, 1920; Sillman et al., 1997), and some marsupials and monotremes (Young and Pettigrew, 1991; Ahnelt and Kolb, 2000; Ebrey and Koutalos, 2001). The function of double cones is still under consideration, but there is evidence that they aid in the detection of motion and discrimination in fine spatial detail rather than chromatic visual tasks in birds (for review see Hart and Hunt, 2007) but are involved in color discrimination in reef teleosts such as *Rhinecanthus aculeatus* (Pignatelli et al., 2010). In dipnoans, *N. forsteri* and *L. paradoxa* do not possess double cones, while double cones are unconfirmed in *Protopterus annectens* and *Protopterus amphibius*. The only species with described double cones is *Protopterus aethiopicus*. *P. aethiopicus* and *P. annectens* are the "youngest" members of the extant lungfishes in the Lepidosirenidae family, and are more closely related to each other than *L. paradoxa, P. dolloi* or *P. amphibius*. The lack of double cones in *N. forsteri*, *L. paradoxa*, and now *P. dolloi*, suggests that they may have evolved after the evolutionary separation from *P. dolloi*. However, until the photoreceptors in *P. annectens* and *P. amphibius* have been described in greater detail, the reason is uncertain because visual specializations or degeneracy in species is greatly dependent on environmental pressures.

The red cones of *P. dolloi* have a similar appearance to the red cones present in *N. forsteri*. Colored oil droplets tend to act as long-pass cut-off filters, selectively transmitting longer wavelengths of light and blocking shorter wavelengths. The spectral location of the cut-off varies, but in general their effect is to narrow the spectral sensitivity function of the cones while simultaneously increasing color discrimination (by increasing contrast between adjacent spectral photoreceptor types; Vorobyev, 2003). Without decreasing the total amount of ambient light entering the eye through a colored lens, as in humans, colored oil droplets allow an animal to utilize almost all the light available for vision without sacrificing clarity (Bowmaker, 1980). In *N. forsteri*, the red cone oil droplet absorbs all wavelengths below about 560 nm and, along with the yellow pigmented cone type, improves the animals' ability

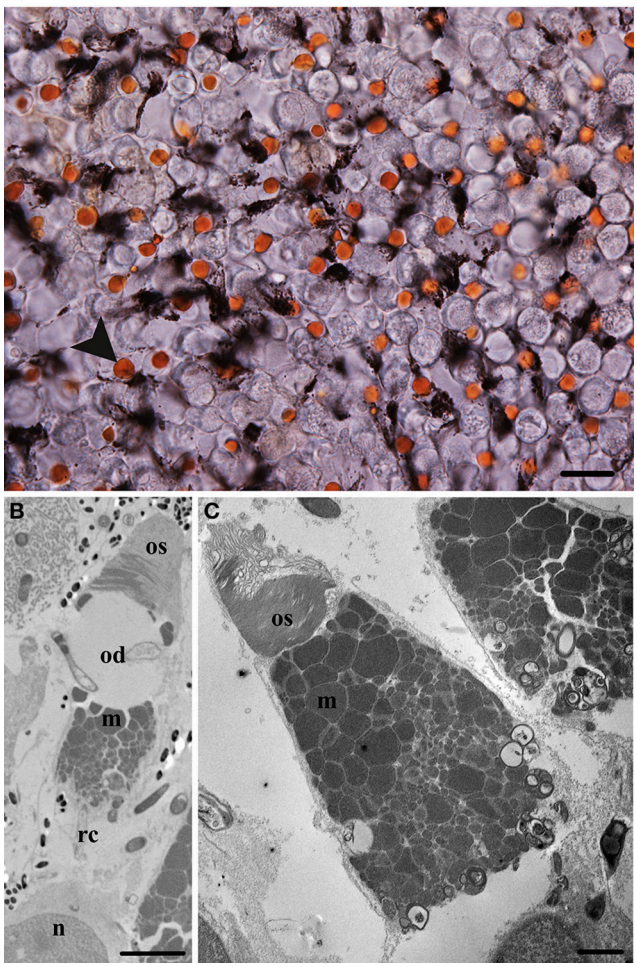

FIGURE 4 | Morphological characteristics of cone photoreceptors in juvenile *Protopterus dolloi*. (A) Retinal wholemount of an unstained *P. dolloi* retina showing the red cones (arrowhead) at the level of the ellipsoid. Scale bar = 20 μm. **(B)** Electron micrograph showing the ultrastructure of a red cone (rc). The outer segment (os) is tapered with an oil droplet (od) located in the ellipsoid region above the majority of mitochondria (m), with the nucleus (n) more vitread. Scale bar = 5 μm **(C)** Electron micrograph of the outer segment (os) and mitochondria (m) containing the ellipsoid of the clear cone. Scale bar = 2 μm.

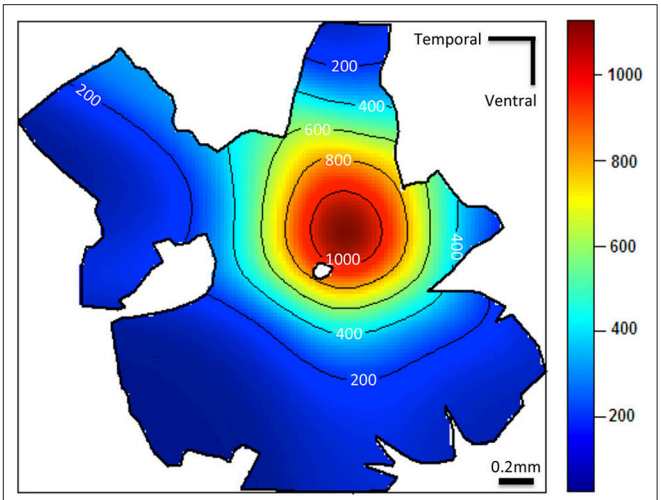

FIGURE 5 | Topographic density map of the red cone photoreceptors (mm^2) in a juvenile *Protopterus dolloi* 20.2 cm in TL. Note that the greatest density of red cones is located centrally in the dorso-nasal region of the retina.

to discriminate colors by a factor of ∼1.3 which may aid their discrimination of foliage, prey items, conspecifics, and potential predators (Hart et al., 2008). However, this assumes that we understand the visual tasks that have driven the evolution of the eye in *N. forsteri*. Until the spectral absorption characteristics of the visual pigments and oil droplets of *P. dolloi* are measured, it is impossible to assess how much the inclusion of red oil droplets in one cone type improves color discrimination. Nevertheless, the presence of red cones in *P. dolloi* suggests that greater discrimination of objects at long wavelengths is an adaptation required for animals living in freshwater rivers and swampland where substantial sediment and mineral deposits reflect light in the red part of the visible spectrum (Hart et al., 2008).

The area centralis of the red cones of *P.* dolloi is located on the central edge of the dorso-nasal quadrant, which provides acute vision within the ventral and lateral visual field where predators would be encountered, especially given the side-to-side movements of the body/head. In the African lungfish *P. aethiopicus* (Curry-Lindahl, 1956; Greenwood, 1987), an acute zone directed forward and into eccentric visual space may aid navigation between tight spaces in the dense vegetation of the swamp (Collin and Pettigrew, 1988). This is similar to the topographic distribution of the cones in *N. forsteri* that indicates a downwardly directed visual axis in juveniles (Bailes et al., 2006). Further study is required to investigate if this increased resolving power in the dorso-nasal region of the visual field is also reflected in other photoreceptor types and within the ganglion cell population thereby providing a better indication of how retinal structures reflects their visual ecology.

CONCLUSION

This study has revealed that the retina of juvenile *P. dolloi* contains one rod and two morphologically distinct cone photoreceptor types, with increased spatial resolving power in the dorso-nasal region of the retina based on the topographical distribution of the red cones. It has also characterized one rod and one cone type in adult *L. paradoxa*. The large size of the photoreceptors and the presence of oil droplets in *P. dolloi* and *L. paradoxa* closely resembles the situation in *N. forsteri,* despite the lack of aestivation in the Australian species, and suggests a visual system that is adapted for high sensitivity, while the presence of one cone photoreceptor type infers that *L. paradoxa* is the only lungfish species described without the potential for color vision. However, further study is needed to establish the importance of color discrimination, and the selective pressures

involved in the specializations of the retina of *P. dolloi* and *L. paradoxa* in order to establish the role vision plays in these species' behavioral ecology.

AUTHOR CONTRIBUTIONS

SC and NH conceived the study; SC, NH, and AA designed the experiments; AA and SC carried out the research and analyzed the results. AA, SC, NH, and IZ wrote the manuscript and all authors were involved in the revision of the manuscript and have agreed to the final content.

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Diversity and Ecological Correlates of Red Fluorescence in Marine Fishes

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Marine environments at depths below −10 to −25 m are almost devoid of ambient red sunlight because water quickly attenuates long wavelengths. This stenospectral light environment presents unique opportunities for organisms that can transform ambient blue-green light into red light by fluorescence. Numerous marine fish species display intricate patterns of fluorescence. Because color vision is a key component of fish sensory ecology, several putative visual functions of red fluorescence have been proposed but are difficult to test experimentally. Here, we follow a comparative approach to assess the consistency between the phylogenetic distribution of red fluorescence with its presumed functions. We collected and analyzed the largest data set of red fluorescence in fishes to date, consisting of confirmed cases in 272 primarily diurnal fish species from 49 out of 90 surveyed fish families and 12 out of 21 surveyed fish orders, contrasted to 393 fish species with confirmed absence of red fluorescence. Based on *a priori* hypotheses on adaptive function, we compare the prevalence of red fluorescence among pre-defined sets of species based on ecological or biological characteristics while controlling for shared ancestry. When comparing between species, we find no evidence that red fluorescence is more prevalent in deep-water species, contrasting with our recent finding that fluorescence brightness increases with depth within species. There is also no evidence for a role in group-driven communication. Phylogenetic patterns are consistent, however, with three other predictions. First, fluorescence with a rather patchy distribution across the body occurred significantly more often among sit-and-wait predators or otherwise sedentary fish than in more mobile species, consistent with background matching for camouflage. Second, small, predatory fishes tended to show red fluorescent irides disproportionally often consistent with a proposed function in prey localization. Finally, sexually dimorphic species showed fluorescent fins more often, as predicted if relevant in sexual communication. From these findings, we derive predictions for experimental investigations of the presumed functions of red fluorescence.

Keywords: fluorescence, animal coloration, color vision, camouflage, prey detection, sexual signaling, visual contrast

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INTRODUCTION

The sea appears blue because water primarily absorbs orange and red light (wavelengths >580 nm) whereas it largely scatters blue light (Jerlov, 1968; Lythgoe, 1979). This generates the strongest, most predictable, and most widespread spectral transition zone in nature. In all reasonably clear aquatic environments the ambient spectrum narrows from broad-spectrum sunlight at the surface to a 400–580 nm range at −20 m and 470–490 m below −100 m (**Figure 1**). Hence, in terms of color vision, the sunlit euphotic zone of marine environments can be subdivided into two zones. The euryspectral zone in the top few meters has an ambient spectrum wider than the core spectral sensitivity of most fish, which often have tuned their peak color sensitivity to the abundantly available blue-green light (450–550 nm) and thus have difficulties perceiving very short (UV) or very long (red) wavelengths (Munz and McFarland, 1973; Partridge, 1990; Losey et al., 2003; Brandley et al., 2013). In the stenospectral zone starting at around −10 m, the ambient spectrum is narrower than the portion of the light spectrum that most fish can perceive (Meadows et al., 2014 and references therein, **Figure 1**).

This phenomenon forms the basis for the prevalent view that long wavelengths ("red" to humans) are irrelevant to most marine fishes. The implicit assumption is that animal coloration is exclusively generated by pigments or optical nanostructures that differentially absorb and reflect parts of the incoming light (Endler, 1990). Such subtractive mechanisms cannot reflect wavelengths that are absent in the ambient spectrum, and pigmentation that appears red at the surface will turn gray at depth.

The recent discovery of red fluorescence among marine fishes (Michiels et al., 2008; Sparks et al., 2014), however, indicates that long wavelengths may be far more relevant to fish visual ecology than commonly accepted. Red fluorescence is well-known from algae and corals, where it may enhance photosynthesis (Schlichter and Fricke, 1990), stimulate symbiotic zooxanthellae (Field et al., 2006), provide photoprotection (Salih et al., 2000; Ben-Zvi et al., 2014), or generate visual contrast (Gruber et al., 2008). Its occurrence in fishes suggests additional visual functions (Gerlach et al., 2014; Meadows et al., 2014; Harant et al., 2016; Michiels et al., in submitted, see also Haddock et al., 2005 for an example in deep see siphonophores). Unlike subtractive color mechanisms, fluorescence is an additive mechanisms enabling emission of wavelengths irrespective of their presence in the environment, allowing red coloration even in stenospectral environments.

Fluorescence is obviously not limited to the red range (>580 nm) of the light spectrum (reviewed in Lagorio et al., 2015), and many marine fishes also exhibit green and yellow fluorescence in the 510–580 nm range (Sparks et al., 2014). We focus, however, on long-wavelength fluorescence from 580 nm (orange) to 750 nm (far red) for two reasons. First, in light environments below −10 m red fluorescent emission is more likely to generate strong color contrasts against the blue-green background (Johnsen, 2012, p. 189, see also Haddock and Dunn, 2015, **Figure 1**). Making use of this free bandwidth would resemble adaptations in vocal communication that focus on

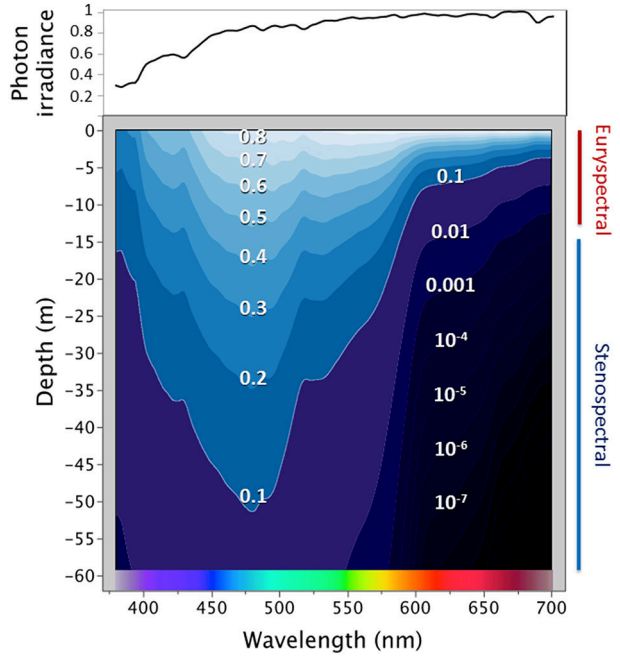

FIGURE 1 | The spectral light environment in tropical marine waters. The top graph shows the ambient spectrum above the water surface (expressed as a proportion relative to the highest irradiance value, Red Sea, March 2013). The lower graph shows how that proportion changes with increasing depth. Each line represents the "iso-brightness" for a given wavelength. Red wavelengths dominate near the surface, but 90% of their irradiance is absorbed within −10 m. For blue light, the same degree of absorbance is only reached at −50 m. Irradiance was measured using a calibrated PhotoResearch PR 670 photospectrometer fitted with a CR-670 cosine receptor between 0 and −25 m depth and is expressed in photons.s^{-1}.m^{-2}.nm^{-1}. These data were used to calculate spectral attenuation coefficients to estimate values down to −60 m (Meadows et al., 2014). Note that the measurements ignore Raman scattering, which is very weak but explains the presence of long wavelengths even in very deep water (Johnsen, 2012, p. 168).

frequencies that are rare in the ambient environmental noise (Slabbekoorn and Peet, 2003; Hart et al., 2015). Second, previous research on reef fish vision has focused on the 350–600 nm range of the color spectrum (Marshall et al., 2006, 2015; Brandley et al., 2013). While fluorescent emission in the near red around 600 nm can be assumed to be detectable by many fishes (Kalb et al., 2015), color patterns in the far red range are only scarcely investigated, prompting novel questions about color perception and private signaling (Gerlach et al., 2014, 2016).

To date, red fluorescent spectral emission has only been characterized for a small number of fish species (Michiels et al., 2008; Wucherer and Michiels, 2012, 2014; Meadows et al., 2014; Sparks et al., 2014; Gerlach et al., 2016). Here, we present the most comprehensive dataset of the phylogenetic distribution of red fluorescence among marine fishes to date. For the first time, we also compare species expressing red fluorescence with confirmed cases of its absence. Furthermore, we provide a quantitative overview of emission spectra and their characteristics within and between fish families. Finally, we use comparative analyses to evaluate whether the phylogenetic distribution of red

fluorescence is concordant with five non-mutually-exclusive hypotheses on ecological function, as described below.

Hypothesis 1: Short-Distance Visual Functions

Just as long wavelengths from the sun disappear rapidly with depth, red fluorescent emission also attenuates rapidly with distance (Lythgoe, 1979). Hence, in a communication context, it is likely to be functional over very short distances only. While communication distances are poorly known for most fish species, spatial resolution as well as visual ranges and reaction distances toward prey items generally increase with body size in marine fish (e.g., Tamura, 1957; O'Brien, 1979; Schmidt and O'Brien, 1982; Li et al., 1985; Aksnes and Giske, 1993). Therefore, we use body length as a proxy for the distance over which communication or predation typically take place, with small species interacting over shorter distances.

Hypothesis 1 predicts that the prevalence of red fluorescence among marine fish species increases with decreasing body length.

Hypothesis 2: Contrast Enhancement at Depth

Near the surface, red fluorescence is too weak to significantly contribute to color contrast compared to reflective mechanisms (Meadows et al., 2014). In the stenospectral zone, however, red fluorescence is the only non-luminescent mechanism by which red hues can be produced. Hence, if used for visual functions, red fluorescent coloration should predominate in the stenospectral zone compared to the euryspectral zone (cf. **Figure 1**). Evidence for this comes from within-species comparisons; red fluorescence was brighter at −20 m than at −5 m in 6 out of 8 tested reef fishes (Meadows et al., 2014). Moreover, individual fish boost their fluorescence when exposed to light environments mimicking the low brightness conditions of deeper water (Harant et al., 2016). Here, we assess whether this hypothesis is also supported when examining patterns *between* species.

Hypothesis 2 predicts that the prevalence of fluorescence among marine fish species increases with the maximum depth at which those species occur.

Hypothesis 3: Camouflage through Background Matching

Red fluorescence seems particularly common in cryptobenthic fishes (Michiels et al., 2008). Indeed, Sparks et al. (2014) describe a phylogenetic concentration of green, orange, and red fluorescence in benthic taxa such as eels, lizardfish, blennies, scorpionfish, gobies, and flatfish. In these, red fluorescence could optimize color matching—and thus camouflage—against a substrate on which sessile organisms such as corals, sponges, and/or algae generate a background of irregular, patchy fluorescence (e.g., Alieva et al., 2008; Michiels et al., 2008, **Figures 2**, **3**). Camouflage constitutes a complex interplay between the benefits of being cryptic, the behavioral and perceptive abilities of the observers, the ambient spectrum, and the background against which the cryptic species is observed (Endler, 1981). For a comparative analysis, the difficulty is to define unbiased criteria to score which species may benefit from camouflage and what characterizes a fluorescent pattern as being cryptic.

With respect to camouflage benefits, we assume that camouflage by background matching is of relevance to all benthic fishes, and particularly so to rarely moving species as represented by sit-and-wait predators such as scorpionfish. In contrast, we consider free-swimming benthopelagic and pelagic fishes as being generally more conspicuous because of their constant movement in the water column. Free-swimming species are more likely to possess alternative camouflage mechanisms based on different optical principles (e.g., Brady et al., 2013). Hence, we categorized species as (1) free-swimming, (2) benthic and frequently moving, and (3) benthic and mostly motionless as in sit-and-wait predators.

With respect to crypsis, we define those fluorescent color patterns as "cryptic" that resemble the patchiness of fluorescence present on hard substrates such as rocks and reefs (see examples in Michiels et al., 2008; Sparks et al., 2014). We therefore separated a category of study species showing "patchy" overall body fluorescence from any other body distribution with e.g., large areas of uniform fluorescence or small, well-defined areas such as the iris (**Figure 2**).

Hypothesis 3 predicts that, among fluorescent fishes, "patchy fluorescence" predominates among benthic fish in general and in motionless foragers in particular.

Hypothesis 4: Prey Detection

Many benthic species including members of the pipefish, goby and triplefin families display striking red fluorescence around their eyes, usually in the irides (Michiels et al., 2008; Sparks et al., 2014). This has raised the idea that red fluorescent irides may induce retro-reflective eyeshine in the eyes of other organisms (prey or predator) facilitating their detection (Bruce, 2009; Meadows et al., 2014; Wucherer and Michiels, 2014). This would be analogous to what has been described for nocturnal flashlight fishes, which produce light through chemiluminescence from a light organ directly below the pupil (Howland et al., 1992). Light emission next to the viewing axis is a crucial feature as this maximizes the retroreflective eyeshine that can be seen in the eyes of other organisms (Jack, 2014). As in *Hypothesis 1*, this is likely to function over short distances only because of attenuation of the fluorescent emission with distance. Hence, such "active photolocation" using fluorescence makes the most sense for small fish that pick individual small prey items that possess eyes (Michiels et al., in submitted). This function of red fluorescence is not expected in species that forage on larger prey with eyes over larger distances and should be absent in species that feed indiscriminately (e.g., filter-feeding, substrate sifting) or visually select organisms that lack eyes (detritivores, corallivores, herbivores).

Hypothesis 4 predicts that red fluorescence is more often near the eye in those fluorescent species that forage on small prey items that have eyes.

Hypothesis 5: Intra-Specific Communication

Fluorescence can be quickly modulated by intra-cellular pigment transport (Wucherer and Michiels, 2012, 2014) and is often

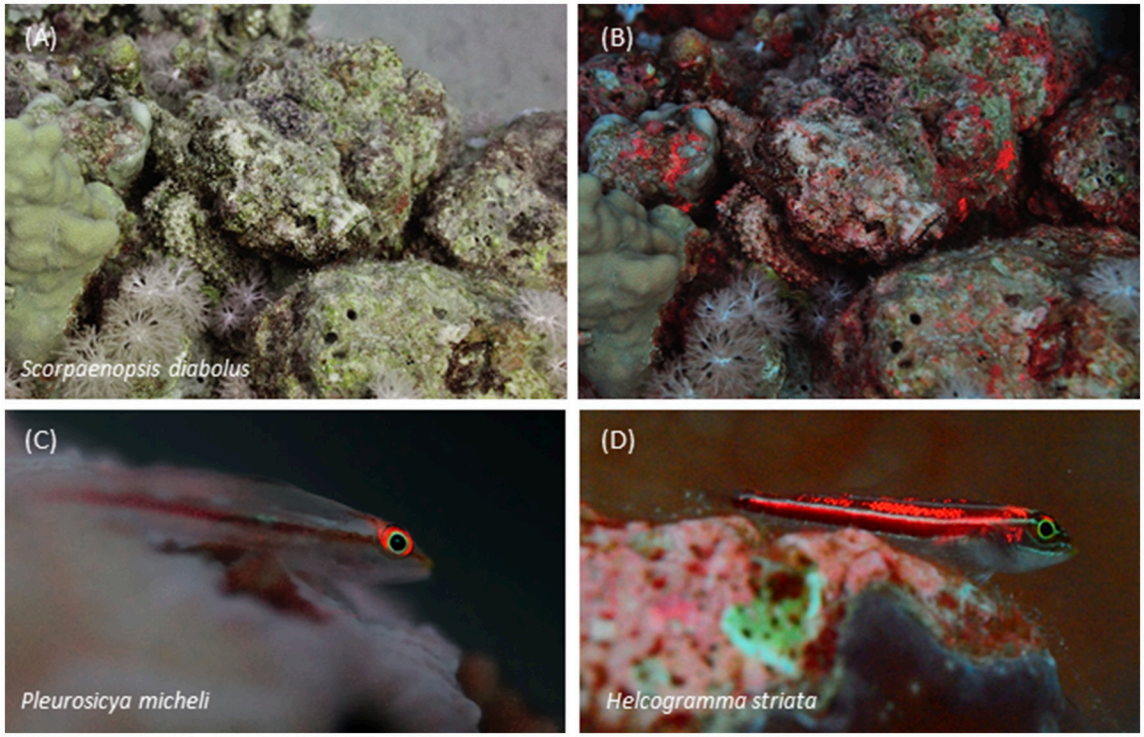

FIGURE 2 | One positive and two negative cases of "patchy fluorescence." The devil scorpionfish **(A,B)** is a typical motionless sit-and-wait predator with patchy fluorescence that appears similar to that of its background. **(C)** and **(D)** exemplify red fluorescent benthic species scored as "negative" for patchy fluorescence. Picture **(A)** taken without filter, the others with a LEE 164 Flame red filter to enhance long wavelengths, all at ∼ −20 m with manual white balance (Photos: Nico K. Michiels).

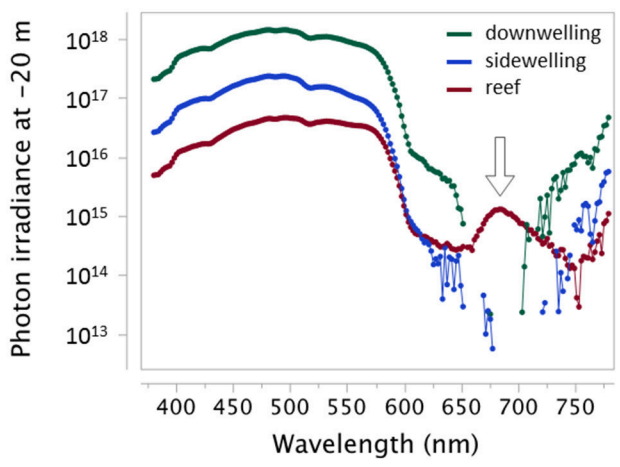

FIGURE 3 | Downwelling light, sidewelling scatter from the open water, and light emitted from a reef in −20 m showing the distinct red fluorescent emission of the reef at this depth (arrow). Measured as photon irradiance (photons.s^{-1}.m^{-2}.nm^{-1}) using a calibrated PhotoResearch PR 670 with CR-670 cosine corrector in El Quseir, Egypt (sunny, midday, March 2013). Raw data shown as connected points. Missing points are wavelengths at which the signal fell below the detection threshold. Note $\log_{10}$ *y*-axis.

associated with signaling structures such as fins (Michiels et al., 2008; Gerlach et al., 2016). This hints at a communication function for fluorescence for courtship, territoriality, or social hierarchies (Sparks et al., 2014). Recent evidence suggests that fluorescent fish perceive (Michiels et al., 2008), and respond to, their own fluorescence. Among wrasses, male-male interactions are mediated by orange-red colors in *Coris julis* (Braun et al., 2014) and by red fluorescence in *Cirrhilabrus solorensis* (Gerlach et al., 2014). Training experiments in the triplefin *Tripterygion delaisi* show that these fish are capable of discriminating between objects in response to weak red fluorescent levels similar to their own (Kalb et al., 2015). Fins are used for signaling by many fishes (overview in Rowland, 1999, see also Ciccotto and Mendelson, 2016a). In contrast to the body, their display angles can be controlled (e.g., pectoral and tail fin), they can be moved even when sitting still (e.g., dorsal fin) and they can be opened or closed to adjust signal size or conceal the signal (e.g., dorsal, caudal, and anal fins).

Hypothesis 5 predicts that red fluorescent fins are more prevalent in fluorescent species that show specific types of intra-specific communication.

To test this hypothesis, we differentiate between two types of intra-specific communication. First, many marine fish live in aggregations of dozens to hundreds of individuals (e.g., Norris

and Schilt, 1988). Compared to species that primarily adopt a solitary lifestyle or form stable pairs, grouping generates more continuous signaling among group members. If red fluorescence played a specific role for such interactions, it should be more prevalent in group-living species.

Second, color signals subject to mate choice are often expressed differently in males and females (reviewed in Wyman et al., 2013, see Kraaijeveld et al., 2007 and Baldauf et al., 2011 for exceptions). Hence, if red fluorescent fin displays are favored by sexual selection, we expect them to be more prevalent among species that exhibit sexual dichromatism.

MATERIALS AND METHODS

Data Sources

We assembled information from multiple sources. Our own sampling campaigns combined direct observation and photographic documentation with qualitative and quantitative measurements of fluorescence spectra from live fish, as detailed below. We complemented these with cases documented in the online supplement of Sparks et al. (2014). The latter do not provide a list of negative cases, but we scored all species labeled as showing green but no red fluorescence as negative for red fluorescence. We further included earlier data from our own projects (Michiels et al., 2008; Wucherer and Michiels, 2012; Meadows et al., 2014) and subjected them to categorizations where possible as detailed below. Our survey did not attempt a balanced coverage across the cartilaginous and bony fish phylogeny and explicitly does not aim at reconstructing the evolutionary history of red fluorescence across fish. Instead, while being taxonomically as broad as possible, sampling focused on benthic, mostly shallow water species, automatically generating limited or no coverage in fish clades with largely pelagic representatives such as the Otocephala (including herrings) or the Protacanthopterygii (including salmon and trout).

Spectrometry and Standardized Documentation of Live Specimens

We measured and documented fluorescence from live fish at five localities:

a. **University of Tübingen, Germany.** We ordered species through the sustainable aquarium trade for spectral measurements in our laboratories in accordance with German animal care legislation (permit ZO 1/12 from the local authority at the Regierungspräsidium Tübingen). These fish served to complement our coverage of fish families and to scrutinize doubtful cases of fluorescence after field observations.
b. **Gulf of Aqaba** (northern Red Sea). We collected fish at the Interuniversity Institute for Marine Sciences (IUI) in Eilat, Israel, in March 2012, under the general IUI collection permit (No.: 2012/38470, Israel Nature and National Parks Protection Authority to Roi Holzman).
c. **North-western Red Sea**. We collected fish from coral reefs in the bays of Sharm Fugani (Mangrove Bay) and Sharm Lassal (Utopia Beach), 15–20 km south of El Quseir, Egypt, in March 2013. Both locations offer protected reefs sloping down to −25 to −30 m. Collection conformed to a 3-year Memorandum of Understanding between the University of Tübingen and the Suez Canal University running 1 Jan 2013–31 Dec 2015.
d. **Mediterranean Sea**. Collections in June 2013 focused on rocky and sandy environments to −30 m at the Station de Recherches Sous-marines et Océanographiques (Stareso) at Calvi, Corsica, France. We collected and registered fish under the station's general sampling permit.
e. **Indopacific Ocean, Indonesia**. We collected fish at a broad range of coral reef habitats up to −30 m depth at Hoga Island in the Wakatobi archipelago off the SE Sulawesi coast, Indonesia in September 2011. Collection was authorized and registered under a general permit of *Operation Wallacea*.

Most field-sampling focused on diurnal species for which field observations using a red filter mask (Michiels et al., 2008) had already indicated the presence or absence of long-wavelength fluorescence. In the Gulf of Aqaba, collection and measurement occurred blind to the presence of fluorescence.

Fish were collected on SCUBA diving with hand nets after partially anesthetizing individuals using clove oil where required (5% clove oil in 5% ethanol and 90% seawater shaken to emulsify). After transportation to the local laboratory in 100 ml Falcon tubes or 4-L zip-lock bags, fish were maintained in aerated seawater for 1 to 8 h before live measurements, followed by their release on site. Species were identified using the general identification literature (e.g., Debelius, 1998; Allen et al., 2002, 2003, 2014) supplemented by monographs (Clark, 1979; Fricke, 1997; Holleman, 2005), Fishbase (Froese and Pauly, 2014) and expert advice for specific fish groups where necessary. Unidentified species were classified at the family or genus level.

Fluorescence Spectrometry and Spectral Analysis

The fish handling routine, the basic spectrometry setup used in the northwestern Red Sea (**Figure 4**), and small deviations for the measurements in the Indopacific and the Mediterranean are detailed in Meadows et al. (2014). The same setup was used in the two other locations, with the following modifications: In the

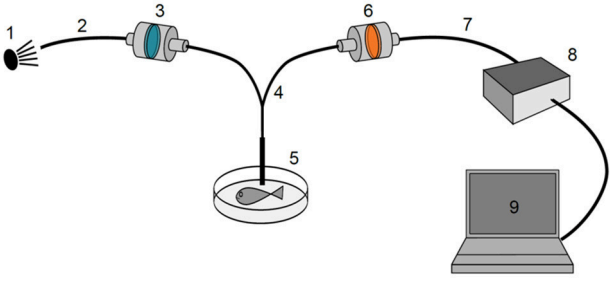

FIGURE 4 | General spectrometry setup. A near-monochromatic green excitation light source (1) was connected (2) via a short-pass or clean-up filter (3) to a bifurcated fiber optics cable (4) that terminated in a hand-held probe (5) close to the measured fish. The emitted light was redirected through a long-pass filter (6–7), analyzed by a high-sensitivity Ocean Optics QE65000 spectrometer (8) and visualized using Ocean Optics SpectraSuite software (9).

Gulf of Aqaba (IUI), we used an Ocean Optics white LED light source (Ocean Optics LLS–Cool White) trimmed to the blue-green range for excitation with a BrightLine HC 533/SP (AHF) short pass filter. The light emitted and/or reflected by the fish was filtered using a stack of four LEE 105 Orange filters in an in-line-filter holder. Measurements at the University of Tübingen used a different bifurcated fiber optics cable (Ocean Optics QR400-7-VIS-BX). Differences between the measuring setups are due to continuous efforts to improve our setup over the years. This precludes direct comparisons of emission intensities and we thus expressed emission intensities on a relative scale as explained below.

Within the excitation range used by Sparks et al. (2014), we used a monochromatic 532 nm light source to excite fluorescent emission that was measured after removing reflected light using a long-pass filter. This single excitation wavelength was chosen, first, to unambiguously differentiate reflection of the excitation light source or short-wavelength fluorescence from red fluorescence with emission peaking at $\geq$580 nm, and second, to allow comparisons among species without confounding by variation in excitation wavelength. Our unpublished fluorescence measurements across complete spectral gradients indicate that peak emission wavelengths (λ_{max}) are nearly insensitive to variation in excitation wavelength, consistent with a general property of most fluorescent pigments due to molecular structure (Johnsen, 2012).

Fluorescence emission spectra were collected from several body parts: Eye, head, operculum, upper and lower flank, and each fin. To obtain summarizing information on the fluorescence characteristics of each study species, we followed a four-step procedure to condense our original spectral readings. First, we identified distinct fluorescence peaks for each measured individual and body part and characterized their peak emission wavelength (λ_{max}) and emission intensity at λ_{max}. Second, we averaged λ_{max} and intensity per body part and emission peak across all specimens of a given species. Third, across all body parts per species, we grouped measurements with the same single emission peak (near-identical λ_{max}), calculated minimum, mean, and maximum λ_{max}, and selected the highest of the averaged intensity values across all body parts. For each investigated species, we thus obtained a single summarizing measurement (λ_{max} and intensity) for one to four distinct fluorescent emission peaks. Fourth, given that intensity readings are sensitive to deviations in measurement procedure, we grouped intensity values into four quantiles *within* each measurement campaign. Given that the distribution of absolute intensity across measurements showed a close match between measurement campaigns (a) through (d), these datasets were combined prior to assigning intensity categories. Assignment to intensity categories was done separately for campaign (e). Only those four resultant intensity categories are reported in this study.

Fluorescence Photography and Image Analysis

All sampled fish were subjected to standardized fluorescence photography (Meadows et al., 2014). While spectrometric measurements are restricted to point information on fluorescence intensities, we performed an independent scoring of the spatial extension of fish fluorescence based on images recorded in the laboratory as well as in the field. First, we allocated fish to one of four categories to describe the spatial extension of long-wavelength fluorescence:

1. No red fluorescence, or fluorescence restricted to minute spots, often originating from contamination by, e.g., ectoparasites or gut content.
2. Fluorescence covers <10% of the body.
3. Fluorescence covers 10 to 50% of the body.
4. Fluorescence covers >50% of the body.

Second, we scored its presence or absence on specific body parts:

1. **Eye fluorescence**: Iris or exposed parts of eyeball or eye socket.
2. **Patchy fluorescence**: Many dots and patches of variable size and shape across the body.
3. **Fin fluorescence**: Fluorescence on any fin or set of fins, and irrespective of sex (note that our current sampling did not differentiate sex-specific fluorescence patterns).

In-situ Observations in the Field

Beyond the study sites mentioned above we also conducted field documentation at sites in the Mediterranean Sea [Corsica (∼200 dives 2009–2015), Elba (5 dives 2012), Croatia (4 dives 2008)], the Red Sea [Gulf of Aqaba (∼30 dives 2011–2012), Marsa Alam (∼200 dives 2007–2015)] and the Indo-Pacific Ocean [Lembeh Strait in Indonesia (∼30 dives 2013), Raja Ampat in West Papua (∼40 dives 2013), Perth in Western Australia (3 dives 2011)]. Fish were scored for the presence of obvious long-wavelength fluorescence perceived as "orange" to "red" by the observer or camera. We only included cases with reliable assessment, requiring conditions that preclude confounding effects by reflective red coloration. Daytime observations were therefore restricted to ≤ -15 m. To facilitate visual or photographic detection, we suppressed the abundant blue-green ambient light with one of several long-pass filters (LEE 105 Orange, LEE 287 Double C.T. Orange, LEE 164 Flame red, LEE 106 Primary red, Nightsea BB62 yellow barrier filter) and documented most species on site using Nikon D300, D700, or D4 digital cameras. To further minimize the likelihood of false positives, observations were cross-checked *in situ* by comparing the putative fluorescent structure with a certified 1.25″ non-fluorescent Spectralon red diffuse reflectance standard (Labsphere) whenever possible, usually in benthic species only. Although we concentrated on species that showed fluorescence under natural illumination, we regularly checked putative cases by highlighting the fluorescence with a blue LED dive light or a blue flashgun. Both were fitted with a short-pass filter to cut out any remaining long-wavelength light (Thorlabs FD2C subtractive dichroic color bandpass filter on Hartenberger Mini Compact blue LED torch; EX-INON Nightsea Excitation filter on Inon Z-240 flashgun). An increase in perceived red emission brightness with increased blue excitation (comparing with and without the blue torch) confirms fluorescence as the origin of the boost in the red signal. Under darker ambient conditions (e.g., shaded substrates, cloud cover),

the blue excitation sources could also highlight fluorescence in shallower water (−5 to −10 m). The patterns of fluorescence were categorized for each species as explained above.

Screening Species in the Aquarium Trade

We visually screened all marine species ($n = 209$) available at one of the largest German wholesale aquarium traders (von Wussow, Pinneberg, Germany) in June 2009. To detect red fluorescence we used blue dive lights (Hartenberger Mini Compact LCD with 7 × 3.5 W 480 nm LEDs) for excitation and three types of LEE filters as barrier filters (LEE 777 Rust, LEE 106 Primary red, LEE 027 Medium Red) with all other light sources in the room switched off. Based on these observations, species were listed as either red fluorescent or non-fluorescent with no further photographic or spectrometric documentation.

Assignment Quality

As an internal control, we compared our observational assessment with spectral measurements of red fluorescence for 91 species for which both types of information were available. Out of 18 species that lacked any fluorescent signal in spectral measurements, two were rated as fluorescent based on observations: In *Coris gaimard,* the spectra were taken on non-fluorescent juveniles, while the adults observed in the field clearly fluoresced in red. In *Nemateleotris decora*, weak fluorescence was visible in the field, but spectral emission peaked in the green-yellow range and thus fell outside the 580 nm cut-off chosen for the current study. Out of the 73 species showing fluorescence in spectral measurements, only the blenny *Atrosalarias fuscus* was categorized as non-fluorescing during field observations. In this species, the fluorescent emission (575 nm) peaked also just outside our cut-off point, and so we rated it as non-red-fluorescent for the current study. Hence, spectral and observational data yielded highly consistent ratings with an error rate of about 3%.

Biological and Ecological Characterization

For all investigated fish, a person blind to the fluorescence rating screened the available literature (primarily Froese and Pauly, 2014, aided by records in comprehensive fish guides, fish ecology books, and individual species papers, see overview in **Supplementary Materials A,B**) to score the following traits (as explained in the Introduction):

(1) Maximum total body length
(2) Maximum recorded depth of occurrence
(3) Substrate association
    a. Benthic: Sit-and-wait predators (mostly motionless)
    b. Benthic: Active foragers
    c. Free-swimming: Benthopelagic or pelagic
(4) Gregariousness
    a. Primarily solitary or pair-living
    b. Primarily in larger social groups
(5) Sexual dichromatism: Sexually dimorphic coloration present or absent
(6) Primary food items
    a. Individually picked microscopic prey with eyes (e.g., microcrustaceans)
    b. Individually picked macroscopic prey with eyes (e.g., crustaceans, fish)
    c. Eyeless prey or indiscriminate feeding (e.g., filter feeding, sediment sifting, digging, herbivory, corallivory, detritivory, etc).

Analyses

Phylogenetic Reconstruction

We used the extensive recent Maximum Likelihood fish phylogeny of Betancur et al. (2013) to generate a family-level tree topology. We pruned this phylogeny to the 71 families also represented in our dataset by removing all non-covered families and maintaining a single terminal node per family. We then manually added the missing 19 families as to reflect established phylogenetic relationships, generating a tree topology that captures the current family-level phylogeny to our best possible knowledge (details in **Supplementary Material D**). Ancestral character estimation for the presence of fluorescence at the family level used maximum likelihood estimation for discrete characters following Pagel (1994). Given the absence of comparable branch length estimates for the manually added taxa, this analysis rests on branch lengths set to unity (Díaz-Uriarte and Garland, 1998; Garland and Ives, 2000). All tree manipulations and analyses were conducted using the packages APE (Paradis et al., 2004) and phytools (Revell, 2012) for R (R Core Team, 2013).

Statistical Analyses

Our statistical analyses evaluate whether the phylogenetic distribution of fluorescence types can be explained by any of five

TABLE 1 | Sample sizes (number of taxa) and evidence type at different taxonomic levels.

	Investigated	Red fluorescence	No red fluorescence
ALL EVIDENCE COMBINED			
Orders	21	12	19
Families	90	49	72
Genera	277	130	189
Species	665	272	393
VISUAL EVALUATION ONLY			
Orders	21	12	19
Families	87	47	70
Genera	229	99	161
Species	480	153	327
SPECTRAL MEASUREMENTS			
Orders	8	7	5
Families	28	16	19
Genera	94	53	51
Species	185	114	71

Species for which we investigated multiple color forms or individuals from different origins count only as a single entry. Note that numbers for orders, families, and genera within each evidence category do not add up because many taxonomic groups contain representatives both with and without red fluorescence.

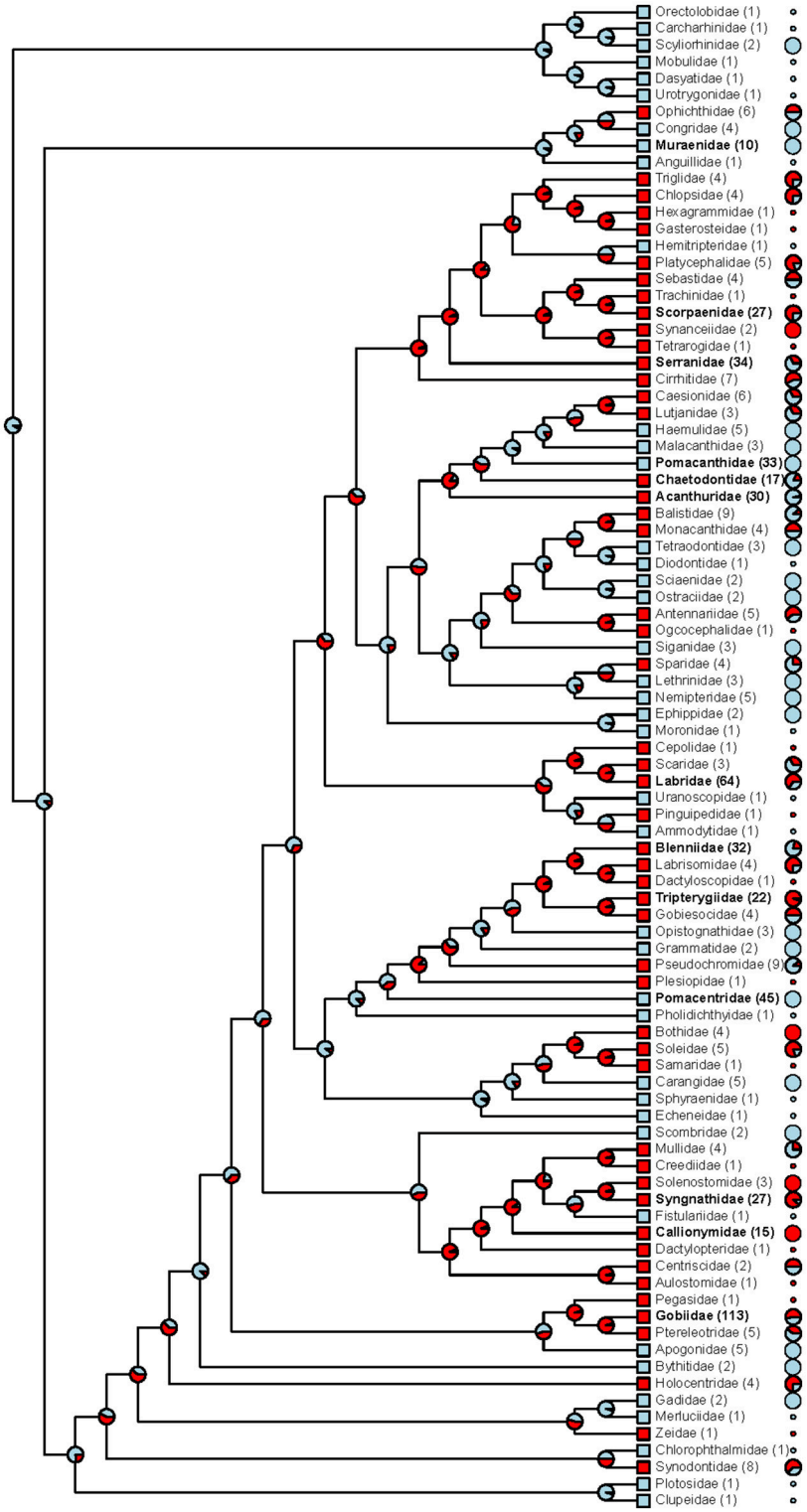

FIGURE 5 | Phylogenetic distribution of red fluorescence among marine fish families. For each family, tip labels show (i) whether fluorescence has been confirmed in at least a single species (red squares vs. blue squares), (ii) the total number of investigated species (in brackets; families with ≥10 species in bold face), and (iii) the proportion of species that exhibit red fluorescence (pie charts; shown for families with ≥ 2 species). At each node, the maximum likelihood for red fluorescence being the ancestral state is indicated by the red pie portion.

hypotheses on putative function as outlined in the Introduction. For each hypothesis, we fitted generalized linear mixed effect models (GLMM) using the glmer function in the lme4 package (Bates et al., 2013) for R. The dependent variable was the presence or absence of the defined fluorescence type as specified in the hypotheses as a binary response, modeled using a logit link function. The predictor(s) as given in each hypothesis were included as fixed effect(s). To account for putative confounding effects of body size, we added the covariate maximum body length (log_{10}-transformed to normalize data distribution, z-transformed to improve model convergence) for hypotheses 2 through 5. Initial full models also contained all possible interaction terms among factors in the fixed model component. Taxonomic ranks (genus, family, and order) were included as nested random factors to take into account trait correlations due to shared ancestry (cf. Luiz et al., 2013; Bridge et al., 2016). This is a compromise given that a fully resolved species-level phylogeny is currently not available for the majority of our study species. We then performed stepwise backward model selection based on the Bayesian information criterion (BIC) and hierarchical likelihood ratio tests (as recommended by Zuur et al., 2009). Only fixed factor effects maintained in the final reduced models are reported, with their statistical significance evaluated using Type III Wald chi^2 tests. We further provide an estimate for the total variation explained by the fixed model component expressed as the marginal R^2 ($R^2_{marg.}$, Nakagawa and Schielzeth, 2013) as implemented in the piecewiseSEM package (Lefcheck, 2016) for R.

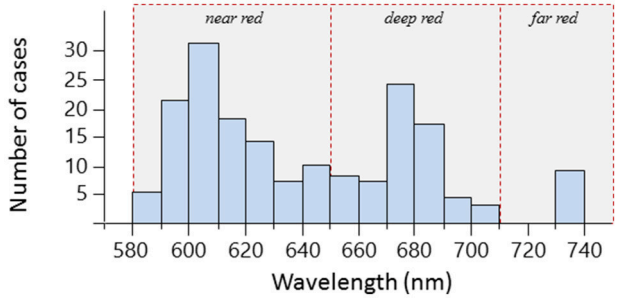

FIGURE 6 | Frequency distributions of peak emission wavelengths (λ_{max}) for 114 fish species. Single species have between one and four emission peaks and can thus be represented multiple times. Hatched red lines delineate our categorization according to peak emission wavelength into near red, deep red, and far red fluorescence.

RESULTS

Red Fluorescence Is Phylogenetically Widespread

We could unambiguously assign the presence or absence of red fluorescence in 665 fish species from 90 families and 21 orders (**Table 1, Supplementary Material A**). Of those, spectral measurements are available for 185 species, documenting red fluorescence in 114 (**Supplementary Material C**). Observational data for the other 480 species revealed red fluorescence in another 153 species. Hence, we document red fluorescence for 272 species (49 families and 12 orders) in total, representing 41.8% of all species in our database. In all other cases fluorescence was absent or hardly detectable.

Mapping the data onto a family-level phylogenetic tree (**Figure 5**) shows that red fluorescence is phylogenetically widespread (at least within the teleost fish), lacks a simple association with phylogenetic history, and has likely been repeatedly acquired or lost.

We found substantial family-level variation in the expression of red fluorescence (**Figure 5**). Even though absolute proportions of fluorescent species are difficult to interpret given that some of our sampling specifically focused on promising candidates for fluorescence, this upward bias applies equally to all target fish families. Out of 13 families with more than 10 sampled species, six showed a predominance of fluorescing species: Gobies (Gobiidae), wrasses (Labridae), triplefins (Tripterygiidae), scorpionfish (Scorpaenidae), pipefish & seahorses (Syngnathidae), and dragonets (Callionymidae). With the exception of the wrasses, all these families exhibit rather cryptic coloration and a mostly secretive lifestyle. In groupers (Serranidae), blennies (Blenniidae), and butterflyfish (Chaetodontidae), red fluorescent species occurred at intermediate frequencies (**Figure 5**). In contrast, and despite explicit search for fluorescent cases, red fluorescence was virtually absent from several particularly colorful reef fishes: Damselfish (Pomacentridae), angelfish (Pomacanthidae), surgeonfish (Acanthuridae), and moray eels (Muraenidae) (but conspicuous yellow fluorescence is present in, e.g., moray eels).

Red Fluorescence Occurs in Distinct Types

Distribution of λ_{max} Values

Out of 665 investigated species, 185 could be subjected to spectrometric measurements. Out of these, emission spectra revealed red fluorescence in 114 species (**Table 1**). Within the investigated 580 to 750 nm range, peak emission wavelengths (λ_{max}) clustered in three distinct groups (**Figure 6**) that we categorize into "near red" (580–650 nm), "deep red" (650–710 nm), and "far red" (>710 nm). In the near red group, most λ_{max} aggregated between 590 and 630 nm, coinciding well with the abrupt start of light attenuation with increasing depth (**Figure 1**). The deep red group clusters around the characteristic emission range of ambient fluorescent light produced by chlorophyll *a* at about 680 nm (**Figure 3**). The far red group contains only few species with λ_{max} at 740 nm, but these come from a diverse array of families with mostly cryptic sit-and-wait predators (see below, **Figure 9**).

Single vs. Multiple Peak Emission

Red fluorescence showed a single λ_{max} in 70 out of 114 species (61%) for which fluorescence spectra are available. Twenty-eight species (25%) showed two, 14 species (12%) three, and the remaining two species (1.7%) four emission peaks (the pipefish, *Corythoichthys nigripectus,* and the triplefin, *Enneapterygius mirabilis*). In species with multiple λ_{max}, these peaks were separated by 48.4 nm on average (range: 10.1–105.5 nm, **Figure 7**). Multiple emission peaks were often associated with

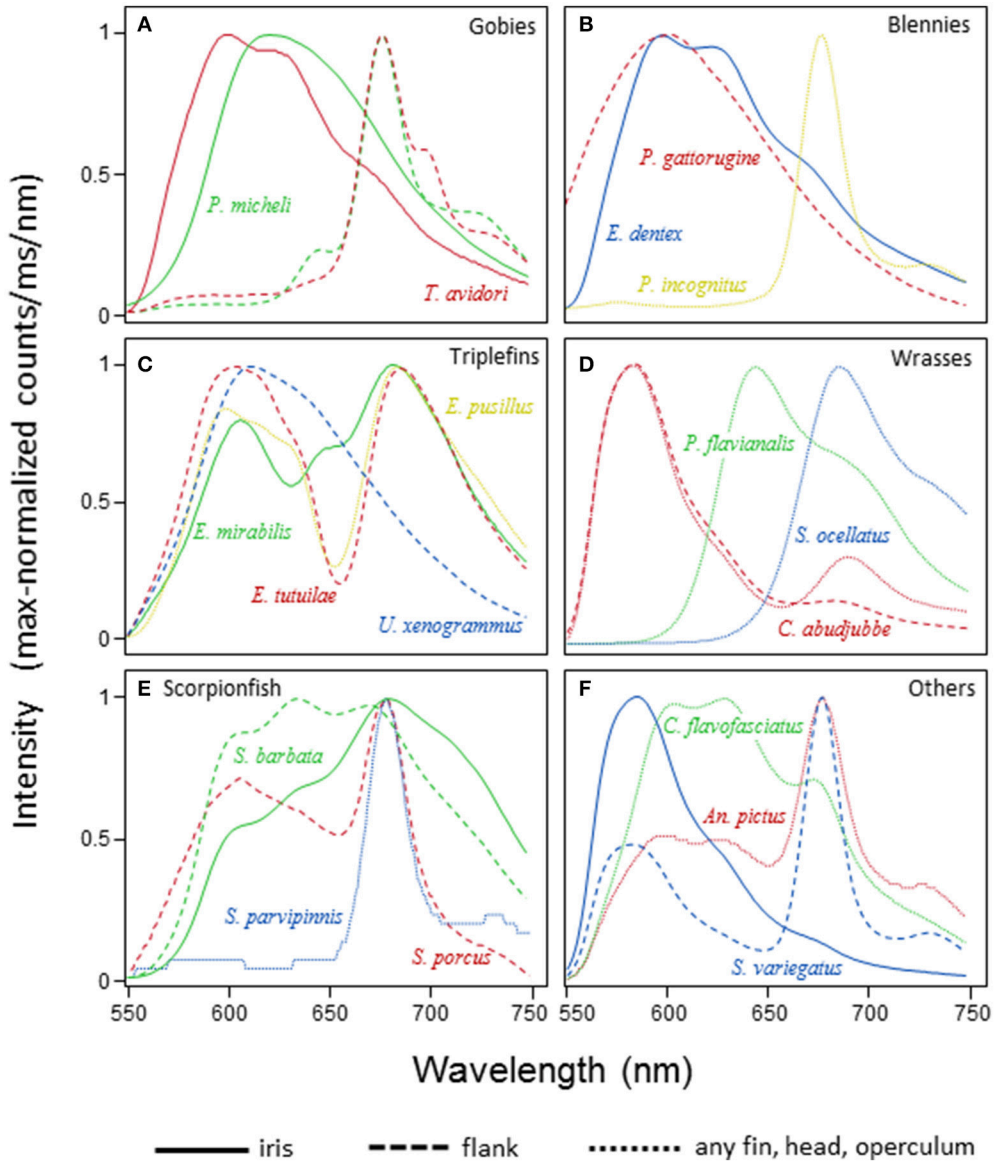

FIGURE 7 | Spectral patterns of red fluorescence. For a selection of families and species, the graphs depict the maximum-normalized shape of fluorescent emission under monochromatic green excitation (see Methods). Within each panel, species are coded by color, body parts by line styles. **(A)** Gobiidae, **(B)** Blenniidae, **(C)** Tripterygiidae, **(D)** Labridae, **(E)** Scorpaenidae, **(F)** Syngnathidae, Antennariidae, and Synodontidae.

different body parts, for example with a near red emission in the iris and a deep red emission on the flank in gobies (**Figure 7A**). Examples of two emission peaks in a single body part are the iris of the blenny *Ecsenius dentex* (**Figure 7B**), fins of the triplefin, *Enneapterygius pusillus* (**Figure 7C**), or the flanks of the wrasse, *Cheilinus abudjubbe* (**Figure 7D**), and the lizardfish, *Synodus variegatus* (**Figure 7F**). Triple emission peaks as on the flank of the scorpionfish, *Scorpaenopsis barbata* (**Figure 7E**) or the caudal fin of the pipefish, *Corythoichthys flavofasciatus* (**Figure 7F**) were rare.

Fluorescent Body Parts and Body Area

The distribution of long-wavelength fluorescence over the fish body shows distinct types (**Figures 7**, **8**). Some species show conspicuous red fluorescence in well-defined, often quite small, uninterrupted areas (e.g., eyes or fin rays), indicative of signaling or prey detection functions (*Hypotheses 4* and *5*) (**Figures 8A–E**). Others show a scattered, irregular, patchy distribution of red fluorescence across the body that we specifically addressed in *Hypothesis 3* (camouflage function). In these cases, it covers 10 to ≥50% of the body area (**Figures 8F,G**).

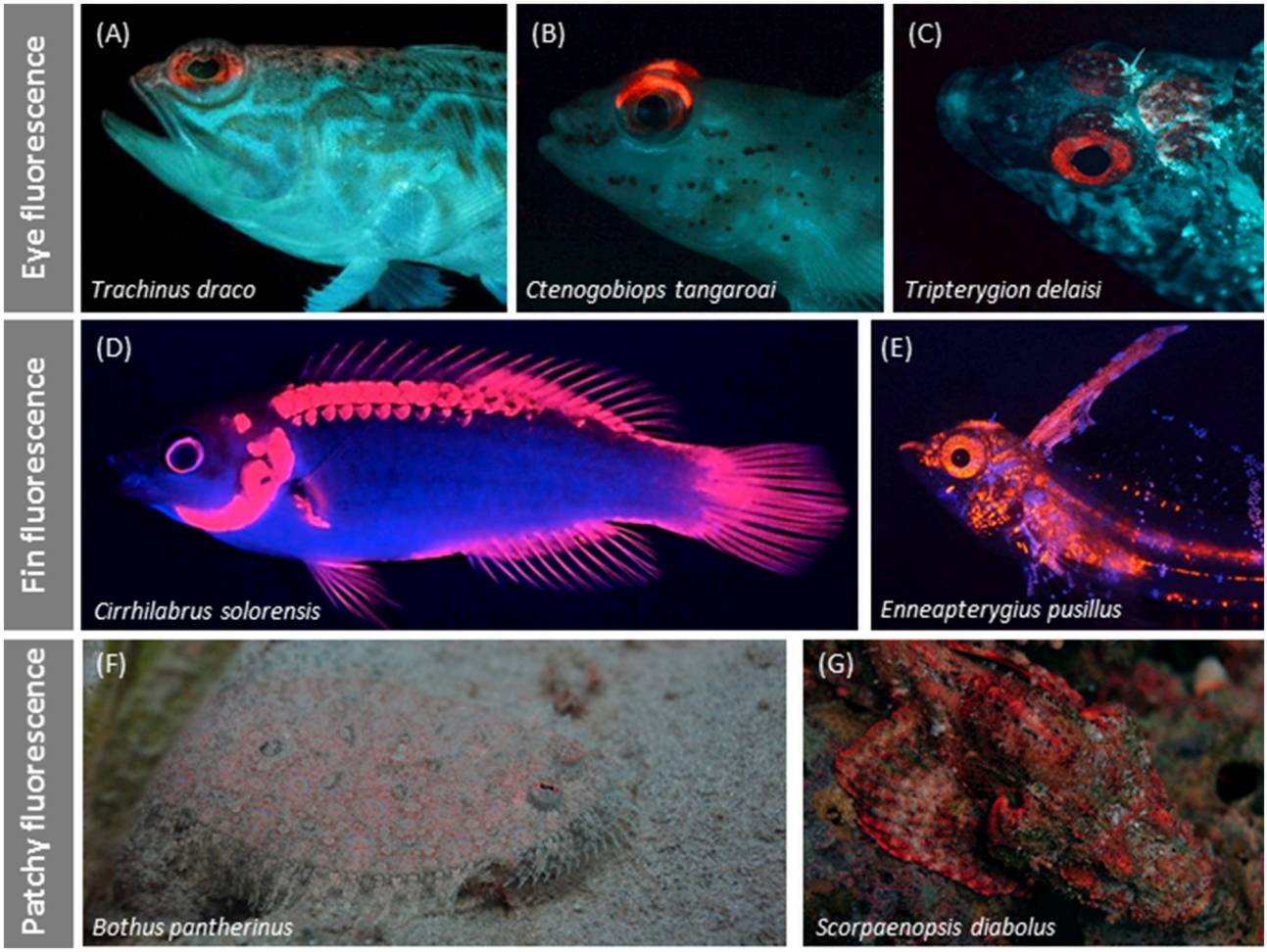

FIGURE 8 | Variation in red fluorescent body patterns. Irides are often the only or clearly most prominent fluorescent structures as in some weevers **(A)**, gobies **(B)**, and triplefins **(C)**. Fin fluorescence may suggest a signaling function as in a fairy wrasse (Gerlach et al., 2014) **(D)** or triplefin **(E)**. Patchy fluorescence characterizes the irregular distribution of red fluorescence over the body as shown by this flatfish **(F)** and scorpionfish **(G)**.

Fluorescence Peaks and Intensities Associate with Fish Families

The distribution of red fluorescent patterns revealed affinities with fish families (**Figure 9**). For example, gobies (Gobiidae) and wrasses (Labridae) show mostly single-peak emissions. These cluster in the near red range in gobies, but are spread across the deep red range in wrasses. In contrast, triplefins (Tripterygiidae) mostly show two to three peaks in the near and deep red range. Finally, several families, including pipefish (Syngnathidae) and scorpionfish (Scorpaenidae), share triple emission peaks, often including a unique emission peak in the far red. Referring to the family-level phylogeny (**Figure 5**) it is clear that these patterns are often shared among unrelated families and, hence, probably have evolved independently.

Functional Correlates of Red Fluorescence

Hypothesis 1: Short-Distance Visual Function

We predicted a higher prevalence of red fluorescence in smaller fish species, using body size as a proxy for interaction distance. Smaller species were indeed significantly more likely to express fluorescence than large species (GLMM length effect $\chi^2 = 14.09$, $P = 0.0002$, $R^2_{\text{marg.}} = 0.064$, $n = 615$ species, **Figure 10A**). Given this relationship, we included body length as a covariate into all further models reported below to correct for its potentially confounding effect.

Hypothesis 2: Contrast Enhancement at Depth

We found no support for the hypothesis that red fluorescence should be more prevalent among species whose habitat extends into greater depths. While controlling for body size, we found the incidence of fluorescence to be independent of maximum depth per species (GLMM depth effect $\chi^2 = 1.42$, $P = 0.23$, full model $R^2_{\text{marg.}} = 0.067$, $n = 615$ species, **Figure 10B**).

Hypothesis 3: Camouflage through Background Matching

While taking the effect of body size into account, we found that patchy fluorescence was rarely expressed in free-swimming

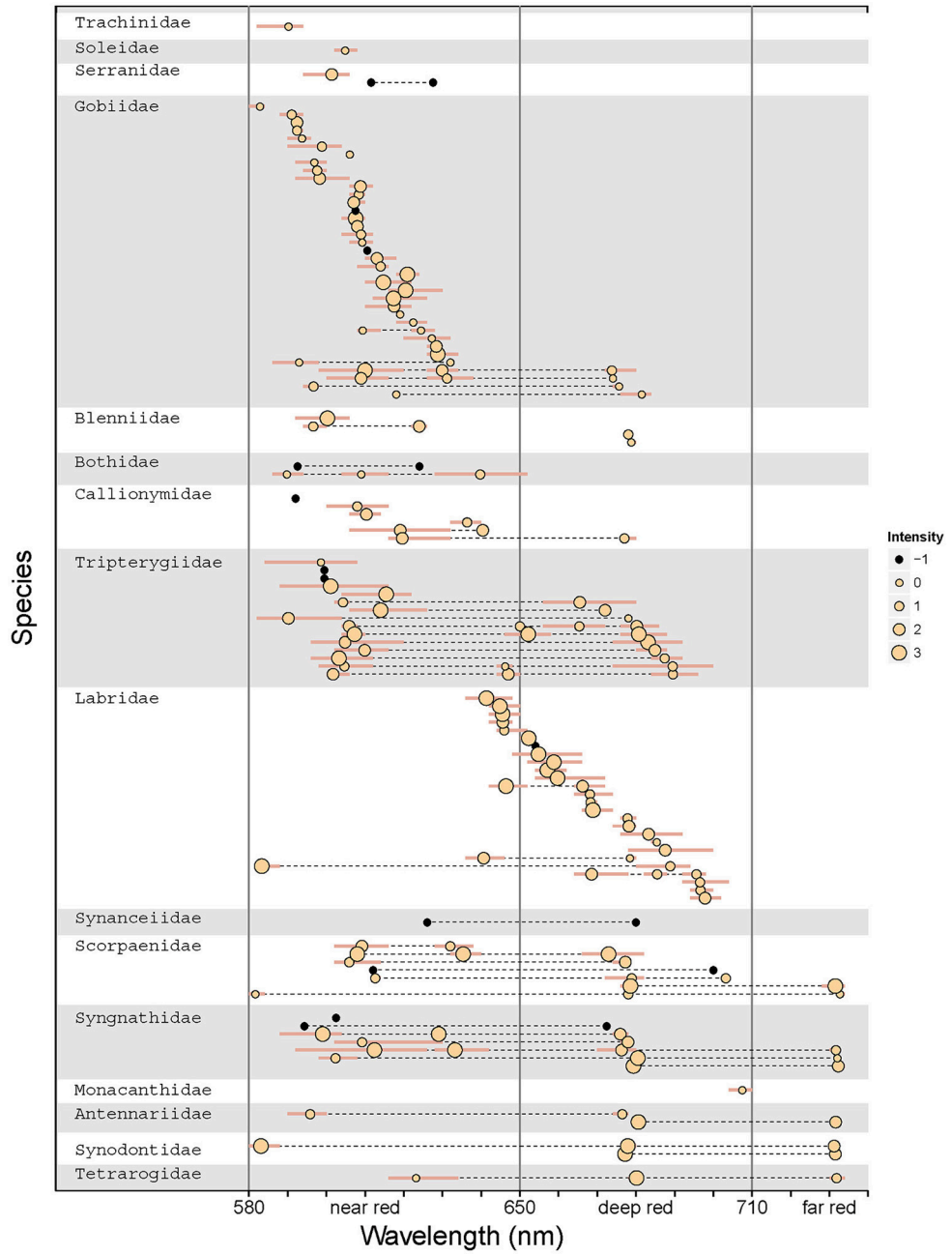

FIGURE 9 | Species- and family-level patterns of fluorescence peak emissions and intensity. The graph indicates the λ_{max} for each emission peak for all 114 measured species, with inter-individual range shown as bars. Multiple λ_{max} values per species are connected by a dotted line. Dot size indicates fluorescence intensity in four intensity quartiles per measurement campaign. Black dots show emission peaks from data sets for which comparable brightness measures were unavailable. Vertical lines separate the data into near red, deep red, and far red fluorescence (cf. **Figure 6**). Families and species are ordered by average peak emission.

species, occurred at intermediate frequencies in rather mobile benthic species, and reached a high average incidence of about 70% in motionless sit-and-wait species (GLMM substrate effect $\chi^2 = 10.97$, df $= 2$, $P = 0.0042$, **Figure 11A**). This pattern is consistent with *Hypothesis 3* proposing that this type of whole-body red fluorescence may contribute to camouflage.

This association showed a significant interaction with body length (GLMM interaction $\chi^2 = 7.37$, df $= 2$, $P = 0.025$, full model $R^2_{marg.} = 0.264$, $n = 187$ species). Among immobile sit-and-wait species, the smaller species were more likely to exhibit patchy fluorescence, while the reverse was true in moving benthic species (**Figure 11B**).

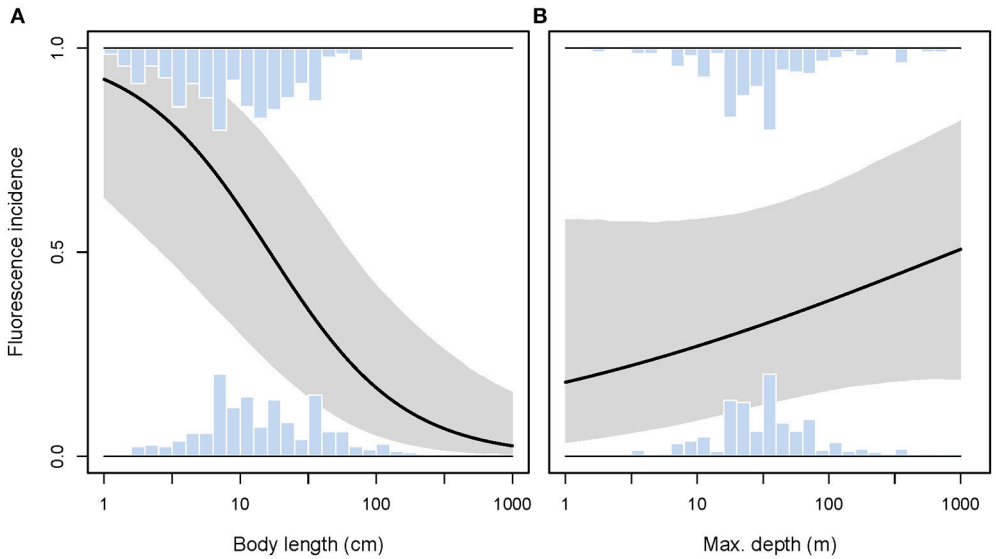

FIGURE 10 | Incidence of red fluorescence in response to maximum body length (A) and maximum depth (B) per species. Marginal histograms display the relative frequency distributions of raw species values. Predicted incidence (black line) and its 95% credibility interval (shaded area) are extracted from binomial models corrected for shared species ancestry. Note $\log_{10}$-scale on *x*-axes.

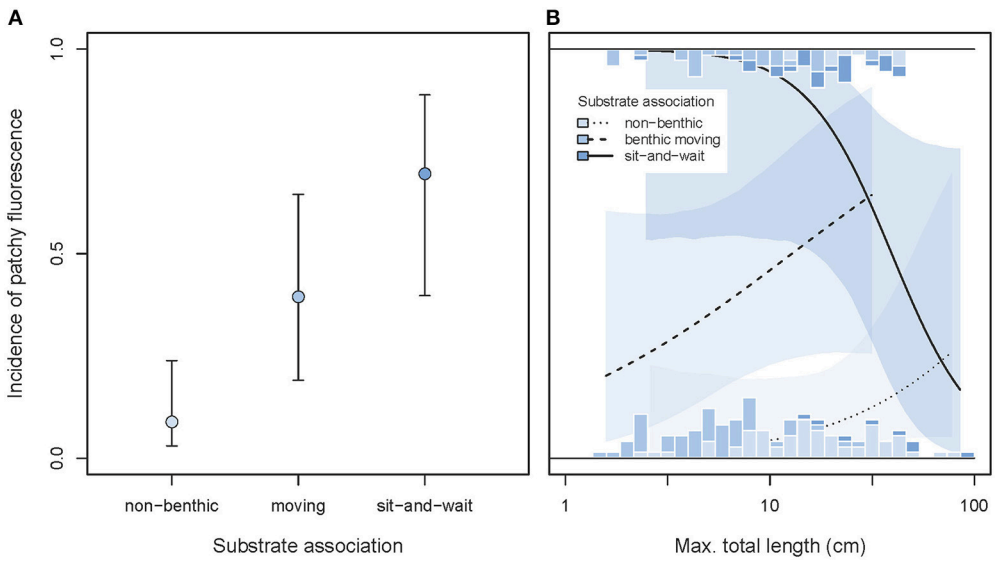

FIGURE 11 | Incidence of patchy whole body fluorescence as predicted by substrate association, maximum body length, and their interaction. **(A)** Shows the overall predicted incidences of patchy fluorescence (±95% credibility intervals), pooling across body size. **(B)** Illustrates the interaction between substrate association and body size (note $\log_{10}$ *x*-scale). Marginal stacked histograms display relative frequency distributions of raw species values. Predicted incidences (lines) and their 95% credibility intervals (shaded areas) are extracted from binomial models taking shared species ancestry into account.

Hypothesis 4: Prey Detection

Taking variation due to body size into account, we found that the incidence of eye fluorescence tended to increase from species feeding on eyeless food items toward species foraging on microscopic, eyed prey (GLMM prey type effect $\chi^2 = 5.44$, df $= 2$, $P = 0.066$, **Figure 12A**). This is consistent with the idea that iris fluorescence may contribute to prey detection when the target exhibits eyes that can directly reflect the incoming light (*Hypothesis 4*).

Body length significantly affected the main prey type effect (GLMM interaction $\chi^2 = 10.76$, df $= 2$, $P = 0.0046$, full model $R^2_{\text{marg.}} = 0.122$, $n = 187$ species). Among fish foraging on small, eyed prey or indiscriminately, eye fluorescence was particularly prominent in the smallest species. The reverse

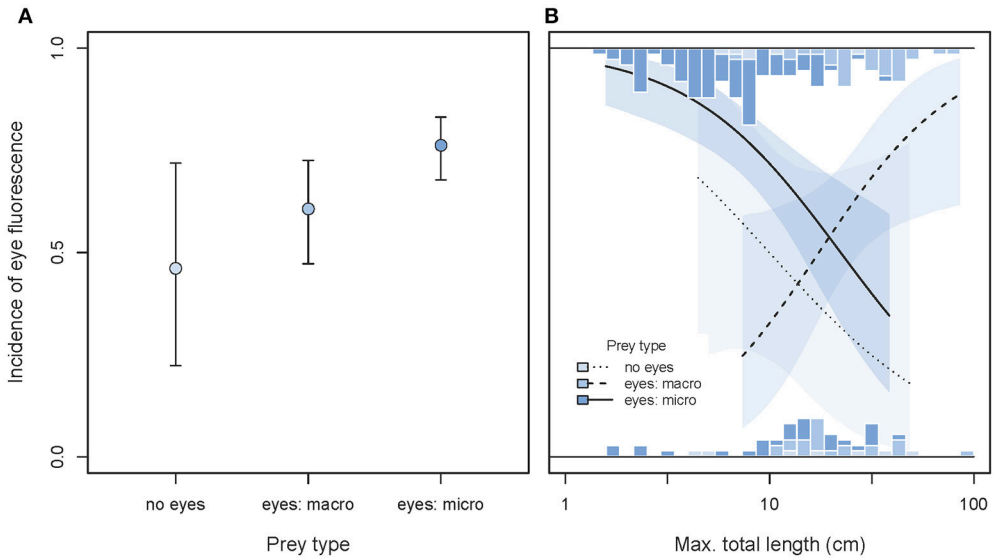

FIGURE 12 | Incidence of eye fluorescence as predicted by prey types, maximum total body length, and their interaction. (A) Shows the overall predicted incidences of eye fluorescence (±95% credibility intervals), pooling across body size. **(B)** Illustrates the interaction between prey types and body length (note $\log_{10}$ *x*-scale). Marginal stacked histograms display the relative frequency distributions of raw species values. Predicted incidences (lines) and their 95% credibility intervals (shaded areas) are extracted from binomial models taking shared species ancestry into account.

was true for fish species foraging on large, eyed prey (**Figure 12B**).

Hypothesis 5: Intra-Specific Communication

The incidence of red fin fluorescence was significantly higher in sexually dimorphic than in sexually monomorphic species (GLMM dimorphism effect $\chi^2 = 6.21$, df $= 1$, $P = 0.013$, full model $R^2_{\text{marg.}} = 0.12$, $n = 187$ species, **Figures 13A,B**), consistent with the idea that fluorescence may play a role in sexual communication. In contrast, no difference in the prevalence of fin fluorescence occurred between solitary/pair vs. group-living species (GLMM sociality effect $\chi^2 = 0.57$, df $= 1$, $P = 0.45$, **Figures 13C,D**), lending no support for a prime function in group-specific social interactions. Both main effects were statistically independent of variation in body size (no significant interaction, **Figures 13 B,D**).

DISCUSSION

Our analyses document substantial variation in the spectral characteristics and body topography of red fluorescence within and between 49 families of marine fishes. We analyzed this variation in the context of five different *a priori* hypotheses and found that (1) small fish were more likely to be red fluorescent, (2) maximum depth of occurrence did not predict the presence of red fluorescence, (3) benthic species in general and motionless sit-and-wait predators in particular were more likely to show fluorescent patterns consistent with camouflage, (4) species predating on small, eyed prey were more likely to possess red fluorescent eyes, albeit with marginal significance, and (5) sexually dimorphic species were more likely to show fin fluorescence. The latter could not be demonstrated for group-living species.

Phylogenetic Dynamics

Red fluorescence is a phylogenetically dynamic trait that has been repeatedly acquired and lost, or at least dramatically changed in expression, across the fish phylogeny, extending and confirming previous analyses on a smaller data set (Sparks et al., 2014). The resolution and phylogenetic coverage of our analysis is insufficient to determine whether red fluorescent pigmentation is ancient within the fish phylogeny, but it clearly appeared early in bony fish evolution. It appears plausible at least that these evolutionary changes are driven by convergent natural selection in independent lineages rather than representing a random, non-functional corollary of other traits. Comparable phylogenetic patterns in color trait evolution have recently been linked to adaptive function also in other fish. For example, the phylogenetically dynamic red and blue body and fin patterns in male darters associate with habitat structure, possibly in the context of predator exposure (Ciccotto and Mendelson, 2016a). In butterflyfish, the evolution of stripe and eyespot patterns was inconsistent with previously assumed predator-avoidance function, but rather co-varied with species ecology (Kelley et al., 2013). Similarly, the evolution of stripe patterns in cichlids (Seehausen and van Alphen, 1999) was phylogenetically highly dynamic and suggested to being driven by ecological specialization beyond phylogenetic affinities.

Interestingly, red fluorescence dominates in cryptic fish groups and seems less prominent in families with conspicuous reflective coloration (see also Sparks et al., 2014)—with the exception of wrasses (Labridae). This suggests that red fluorescence is not primarily used to enhance an already existing

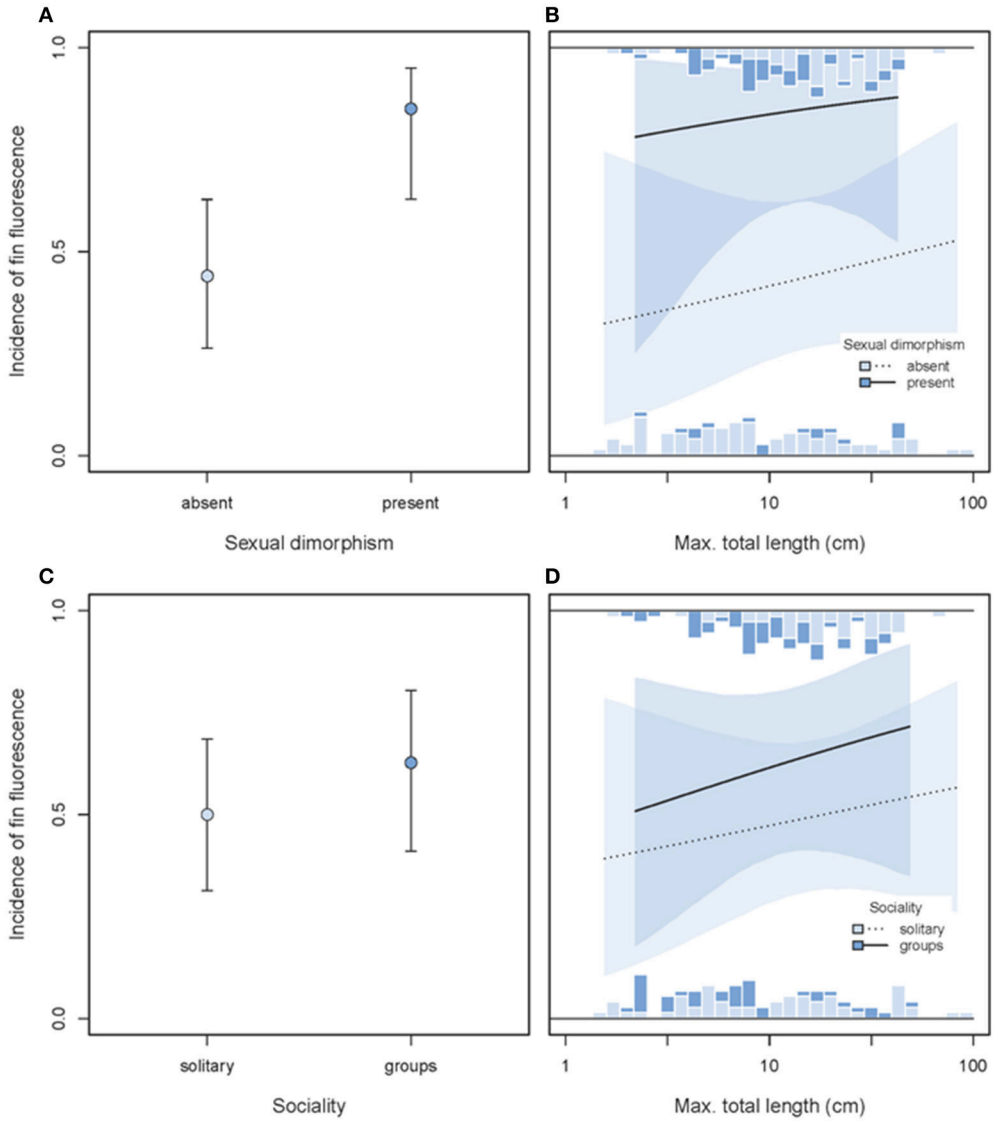

FIGURE 13 | Incidence of fin fluorescence as predicted by sexual dimorphism (A,B) or sociality (C,D) and maximum body length (*x*-axis). In each row, the left panel illustrates the overall predicted incidences of fin fluorescence (±95% credibility intervals), pooled across body size. The right panel illustrates the interaction between sexual dimorphism or social system and body length (note $\log_{10}$-scale). Stacked marginal histograms display the relative frequency distribution of raw species values. Predicted incidences (lines) and their 95% credibility intervals (shaded areas) are extracted from binomial models taking shared species ancestry into account.

reflectance pattern, but rather associates with environmental conditions where red reflectance is not possible, as present in the stenospectral zone.

Variation in Fluorescence Emission Wavelengths

It is striking that the emission peaks (λ_{max}) of fluorescent structures cluster in three distinct ranges in the near, deep, and far red. While this study did not investigate the cellular expression of fluorescence, the pattern suggests the presence of at least three groups of red fluorescent pigments in marine fishes. The actual fluorophores have not yet been characterized, but previous work identified three different fluorescent mechanisms: (i) fluorescent iridophores with fluorescent guanine crystals (Michiels et al., 2008; Wucherer and Michiels, 2014), (ii) fluorescent chromatophores (Wucherer and Michiels, 2012), and (iii) fluorescent scales and fin rays (Michiels et al., 2008). Biochemical analyses are now required to characterize the molecular mechanisms and are likely to reveal cryptic within- and between-family diversity that may be hidden behind similar peak emissions in our study.

Hypothesis 1: Prevalence in Small Fish Confirmed

Our data show that red fluorescence is more common in small species. Assuming that these forage and communicate over

short distances, the pattern is consistent with the idea that red fluorescence is associated with short-range vision measured in centimeters rather than meters. Hence, red fluorescence may well be specific for "small world" functions. Such an effect is less likely for yellow or green fluorescence (e.g., Sparks et al., 2014; Gruber et al., 2016) because the emitted wavelength will travel through water over longer distances. Assuming a function in vision as well, we would predict their prevalence to be independent of body size. For example, the bright yellow tails of some tropical fusiliers (e.g., *Caesio cuning*) and goatfishes (e.g., *Mulloidichthys vanicolensis*) exhibit yellow fluorescence that may enhance the existing reflective signal and serve as a signal for group coherence or as a visual distractor for predators.

Hypothesis 2: Association with Depth Rejected

We could not confirm that species inhabiting greater depths are more likely to express red fluorescence and can think of four non-exclusive explanations. First, light environments poor in long wavelength light are also abundant in shallow water, for example in shaded areas, in turbid water, or at dusk and dawn. Hence, with depth representing just one of several factors favoring red fluorescence, our analysis of gross depth may easily fail to detect this association. Consistent with this idea, the triplefin *T. delaisi* adjusts fluorescence intensity primarily to ambient brightness rather than ambient spectrum (Harant et al., 2016), and may therefore directly respond to diurnal or seasonal changes in overall brightness, irrespective of depth. Second, maximum depths reported in the literature may be rather inaccurate (cf. Bridge et al., 2016) because they are neither systematically assessed nor representative for a species' average depth distribution. Third, our sampling efforts were restricted to depths within reach of regular SCUBA diving, above −30 m. Given substantial within-species, depth-associated variation in fluorescence (Meadows et al., 2014) a general depth effect between species may be obscured. Finally, the benefits of expressing fluorescence may be limited to intermediate depths (e.g., −10 to −100 m) with enough ambient blue-green light to induce fluorescence (unless coupled with a local chemiluminescent source, Douglas et al., 2000). This may be further complicated by the fact that fish may inhabit very different habitats or depths depending on age or season. As for now, however, there is too little information to take such non-linear depth effects into account.

Hypothesis 3: Consistency with Camouflage through Background Color Matching

We statistically confirm an association between a bottom-dwelling, nearly motionless, predatory lifestyle and full-body, patchy fluorescence (Sparks et al., 2014). This supports the suggestion that red fluorescence contributes to camouflage by background matching, where it may complement other camouflaging mechanisms such short-wavelength fluorescence or adjustments in body texture or pigmentation in response to substrate variation. This is particularly likely on complex backgrounds, where sit-and-wait predators are also anatomically very well adapted to blend in with algae, corals, and sponges. The latter generate a background of patchy fluorescence with most emission peaks in the near and deep red. Deep red fluorescence is of particular interest, because chlorophyll produces a distinctive red fluorescent signal around 680 nm. This signal is masked by sunlight in shallow water, but becomes clearly visible at depth (**Figure 3**). Our measurements show that the deep red fluorescence emission of many cryptic species matches this background emission (**Figure 9**). Deep red fluorescence for camouflage, however, only makes sense if relevant predators or prey from which a fluorescent target species aims to hide can perceive these rather long wavelengths. Future research therefore needs to assess the spectral sensitivity of candidate species and empirically test whether fluorescence improves camouflage toward potential prey or predators.

Hypothesis 4: Red Fluorescent Eyes More Common in Micro-Predators

Red eye fluorescence tended to be most prevalent in species that forage on small, eyed prey. This pattern is consistent with the idea that fish use reflective and fluorescent structures near their pupils similar to dragonfish (Douglas et al., 1998, 2000) and flashlight fish (Howland et al., 1992). "Active photolocation" using local emission of wavelengths (such as red) that are otherwise rare or absent from the environment may allow small fish to induce a highly contrasting eyeshine in cryptic prey (or predators) over short distances (Michiels et al., in submitted). The association described here confirms and generalizes earlier field observations (Meadows et al., 2014) and experimental laboratory work on the triplefin, *Tripterygion delaisi*, showing active brightness adjustment in its fluorescent irides (Wucherer and Michiels, 2012; Harant et al., 2016).

Hypothesis 5: Red Fluorescence Associated with Sexual Dimorphism, Not Group Living

Gregariousness did not associate with red fin fluorescence. In contrast, and as the strongest pattern among all hypotheses tested, we found red fin fluorescence strongly linked to sexual color dimorphism. This is consistent with the idea that red fluorescent fins could act as an enhancer of sexual signals in mate choice and male-male competition (e.g., in haremic systems), and generalizes earlier findings that orange and red fin displays are important in within-species intra-sexual communication in wrasses (Braun et al., 2014; Gerlach et al., 2014). The degree to which male fluorescent color displays are indeed preferred by females should be tested in experiments similar to those that revealed phylogenetic associations between male coloration and female preference in darters (Ciccotto and Mendelson, 2016b), and then be matched with measurements of spectral sensitivity to assess co-evolution between color pattern and sensory capacities (Pauers et al., 2016).

Can Marine Fish Perceive Red?

Most of the proposed adaptive functions of red fluorescence require that either the emitting species or relevant bystanders can perceive wavelengths beyond 600 nm. Extreme forms of red sensitivity are restricted to deep sea fishes that emit deep red bioluminescence around 700 nm and possess LWS receptors up to $\lambda_{max} = 590$ nm in *Aristostomias* (Partridge and Douglas, 1995) and even $\lambda_{max} = 671$ nm in *Malacosteus niger* (Douglas et al., 1998). Most marine fish inhabiting the photic zone have a single short wavelength cone and a medium wavelength twin or double cone with λ_{max} in the 500–540 nm range (Losey et al., 2003; Marshall et al., 2006). This arrangement, however, is already sufficient to perceive at least near red fluorescence (600–650 nm). This has for instance been inferred from the degree of overlap between the twin cone sensitivity ($\lambda_{max} = 540$ nm) and fluorescence peaking at 606 nm in the benthic goby *Eviota pellucida* (now *E. atriventris*, Greenfield and Suzuki, 2012) (Michiels et al., 2008). Behavioral evidence confirms this assumption in at least three fish species with "regular" LWS receptors ($\lambda_{max} < 540$ nm). In the triggerfish *Rhinecanthus aculeatus* (LWS $\lambda_{max} = 528$ nm), foraging preferences show a bias to red stimuli > 600 nm (Cheney et al., 2013). The triplefin *T. delaisi* (LWS $\lambda_{max} = 530$ nm, P.-P. Bitton, unpublished data) recognizes its own red fluorescence ($\lambda_{max} = 600$–610 nm) (Kalb et al., 2015), and so does the fairy wrasse *C. solorensis* (LWS $\lambda_{max} = 532$ nm, deep red fluorescence at $\lambda_{max} = 650$ nm (Gerlach et al., 2014, 2016). Only few shallow-water marine fish possess photoreceptors that are more explicitly tuned to perceive red. For example, the wrasse *Thalassoma duperrey* (LWS $\lambda_{max} = 570$ nm) can perceive a red band in its own color pattern under natural light (Barry and Hawryshyn, 1999). Similar long-wavelength photoreceptors have been described for several seahorses and pipefish (LWS $\lambda_{max} = 560$ and 580 nm, Mosk et al., 2007) and the goby *Gobiusculus flavescens* (LWS $\lambda_{max} = 553$ nm) (Utne-Palm and Bowmaker, 2006). As shown by our current study, all these species belong to families that feature a high incidence of red fluorescence.

A Word of Caution

Our current analyses face two limitations. First, solid quantitative information on ecological and biological traits is only available for a handful of marine fish species. For most of our study species, information on, for example, primary food types, foraging style, sexual dichromatism, or maximum depth range could primarily draw from rather rudimentary statements. This adds substantial noise as typical to large comparative analyses. We carefully avoided confounding biases, but noise in the life history data in particular has potential to substantially lower the statistical power to detect associations between red fluorescence and ecological traits. Second, a fully resolved species-level phylogenetic hypothesis for at least a large proportion of the species under investigation does not exist. Hence, we resorted to simplified analyses, first by investigating trait evolution at the family level, and second by controlling for shared ancestry with a rather superficial correlation matrix, taking the taxonomic levels "order," "family," and "genus" into account. We expect that ongoing advances in overall fish phylogeny will soon enable more fine-tuned phylogenetic analyses on red fluorescence and its association with ecology focused on within-family variation as e.g., in gobies, triplefins, or wrasses.

AUTHOR CONTRIBUTIONS

NM, JT, and NA conceived the study. JT and TG acquired most spectrometric measurements and conducted preliminary data analysis. NM contributed most field assessments of fluorescence, supported by JT, TG, and MM. NA was responsible for all data analysis, and drafted the manuscript. MM and NM substantially supported manuscript drafting and data analysis. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00126/full#supplementary-material

Supplementary Material A

Systematic list of fish taxa represented in the current comparative dataset. The Table shows our ratings of red fluorescence (0 = absent, 1 = present), substrate association (1 = benthic moving, 2 = benthic sit-and-wait, 3 = non-benthic), prey type (1 = microscopic prey with eyes, 2 = macroscopic prey with eyes, 3 = prey without eyes), sexual color dimorphism (0 = absent, 1 = present), and sociality (1 = solitary, 2 = group-living). Within fluorescent species, we scored (where possible) the presence of patchy fluorescence, of iris fluorescence, and of fin fluorescence (0 = absent, 1 = present). Where spectrometric measurements were available, we add the intensity quartile (ranging from 1 for the lowest intensity quartile to 4 for the highest intensity quartile) and the number of distinct fluorescent emission peaks. Finally, we list the maximum depth of occurrence (in m) and the maximum total body length (in cm) for each species. Empty cells are due to missing data. For details and sources see the Material and Methods section in the main text.

Supplementary Material B

Major literature sources to complement ecological and biological data to the information available at *fishbase.org*.

Supplementary Material C

Systematic list summarizing all taxa with spectral data for a detailed characterization of red fluorescence. Each row refers to a single distinct fluorescent emission peak and provides information on peak number, the emission category (1 = near red, 2 = deep red, 3 = far red), the wavelength at peak emission (lambda max), absolute emission intensity (in counts per nm per ms), and the intensity quartile at peak emission.

Supplementary Material D

Overview about the origin for the phylogenetic position of fish families as represented in **Figure 5**.

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REVIEW
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Sensory System Responses to Human-Induced Environmental Change

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Sensory input to the central nervous system is the primary means by which animals respond to variation in their physical and biological environments. It is well established that key threats such as habitat destruction, the introduction of non-native species, and climate change are imposing significant pressures on natural ecosystems, yet surprisingly few studies have examined how these threats impact the senses or determine species' responses to environmental change. This review focuses on how anthropogenic impacts on aquatic ecosystems can have a detrimental effect on the sensory systems of aquatic organisms and how these modalities can act to influence genetic and non-genetic (e.g., developmental) responses to environmental change, which in turn can cause knock-on effects in a range of other biological systems. Species often exhibit unique sensory specializations that are suited to their behavioral requirements; at present it is unclear whether and how sensory systems have the capacity to respond to environmental change through genetic adaptation and/or sensory plasticity, and on what timescale this might occur. Sensory systems lie at the forefront of how various species respond to environmental perturbation. As such, determining the important role they play in determining fitness is critical for understanding the effects of external processes such as habitat degradation and climate change. Given the current consensus that human impacts and environmental changes are potentially highly detrimental to the delicate balance of the biome, knowing how organisms respond, and to what degree adaptation is physiologically and behaviorally limited, warrants urgent attention.

Keywords: climate change, sensory ecology, ocean acidification, sensory plasticity, contemporary evolution

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INTRODUCTION

The most important challenge facing biologists today is understanding how animal populations respond to human impacts such as climate change, overexploitation, the introduction of non-native species, and habitat degradation (Sutherland et al., 2013). Environmental disruption is known to cause changes in the abundance and distribution of species (Moritz et al., 2008; Smale and Wernberg, 2013), which often leads to a loss of biodiversity, but populations also express phenotypic responses to environmental change (Hoffmann and Sgrò, 2011; Merilä and Hendry, 2014). Monitoring these phenotypic responses, and determining whether trait changes are based

on genetic or environmental factors (or both), can reveal how altered patterns of selection affect individual fitness and population survival (Charmantier et al., 2008; Gienapp et al., 2008). However, when the relationships between the environment, phenotypic change, and the fitness of an individual organism are considered, a crucial step is missing—the response of sensory systems to environmental disruption (**Figure 1**). Sensory systems provide the fundamental link between the physical and biological environments of an organism and both its physiology and behavior. Sensory systems are, therefore, not only directly (and indirectly) affected by environmental change, but they mediate species-specific responses that determine individual fitness.

Sensory systems are used by organisms to perceive the physical structure and temporal dynamics of the environment, including its chemical and spectral composition, and biophysical information relating to ambient thermal, electrical and magnetic fields. Sensory systems also provide biologically important information, such as the suitability of potential habitats, the identity of conspecifics, and the risk(s) posed by predators (Collin and Marshall, 2003). Sensory systems determine the behavior of an individual, because senses such as vision, olfaction, and audition are essential for acquiring and defending resources, recognizing conspecifics, selecting mates, and avoiding predators. For example, there is a vast amount of literature on the role of chemical cues in affecting the behavior of freshwater fishes, including species recognition (Wong et al., 2005), courtship decisions (Fisher and Rosenthal, 2006), shoaling behavior (Brown and Smith, 1994), and predator avoidance (Brown, 2003). While it is well established that these behaviors are impacted by environmental change, there has been a focus on shifts in behavior rather than the responses of the underlying sensory systems that underpin these behavioral changes.

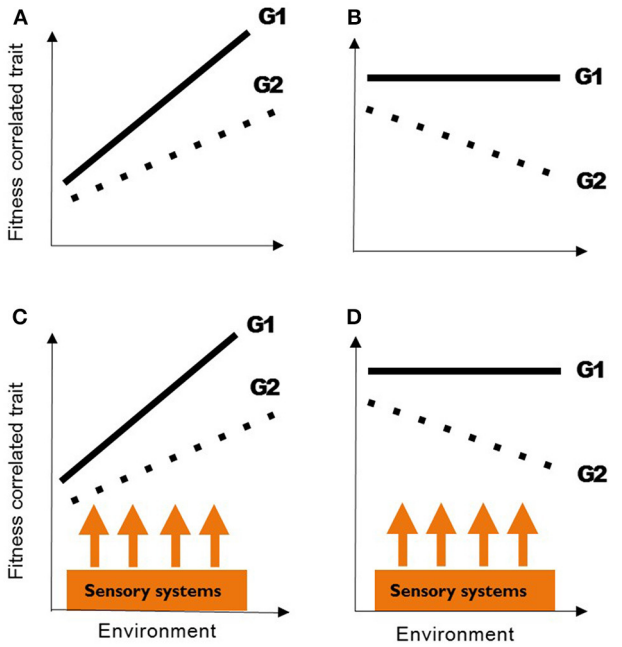

FIGURE 1 | Phenotypic plasticity can result in different phenotypes produced by the same genotype **(A** G1 and G2). If a genotype maintains function under environmental stress, plasticity may allow the same phenotype to be produced [i.e., flat reaction norm; **(B)** G1]. The latter form of phenotypic plasticity is termed "phenotypic buffering" (Reusch, 2014). G1 shows greater phenotypic plasticity and thus has a steeper reaction norm, and higher fitness than G2 **(A)**. Species that respond to environmental change with a decrease in fitness will undergo population decline **(B**: G2), while phenotypic buffering may allow others to persist **(B**: G1). Understanding natural variation in phenotypic traits is therefore important for predicting how populations will respond to human-impacts, such as temperature increases under climate change. Here, we suggest that the key role of sensory systems, which underpin both phenotypic plasticity **(C)** and phenotypic buffering **(D)**, have been overlooked. Figure re-drawn and modified from Reusch (2014).

Altered environmental conditions in aquatic environments might impact sensory systems either directly or indirectly. Pollutants may directly affect the senses; for example, by directly blocking, masking or disrupting sensory reception (such as olfaction), thus leading to a shift in behaviors related to the affected sense. Indeed, sensory structures are, by necessity, directly exposed to the aquatic environment and thus materials in the water column, such as contaminants, may easily interfere with their function (Hara and Thompson, 1978). Altered environmental conditions, such as poor visibility, noise pollution and chemical contaminants, also affect the transmission, detection and reception of sensory information. Sensory systems may be impaired or less reliable if the propagation of sensory signals is impeded; for example, visual signals can be readily diminished or altered when water becomes turbid as a result of human activities (e.g., eutrophication, dredging, terrestrial run-off). Both direct and indirect affects of changed environmental conditions on sensory systems can cause changes in animal behavior. Behavioral changes can occur at different levels, and can be used as indicators of environmental disturbance (Kelley et al., 2018). Sensory systems may respond to environmental disruption via evolutionary genetic change (i.e., sensory adaptation) over a number of generations and/or via behavioral or sensory plasticity. For example, individuals may also display changes in behavior according to the perceived quality of information obtained, switching to information obtained from other senses or sensory cues that may be more reliable (Partan, 2017). Furthermore, since the senses do not act independently, many forms of environmental disturbance will have multisensory affects or disrupt a range of neurological processes, leading to changes in cognition and behavior.

In this review, we examine how environmental change can directly or indirectly (via changes in sensory signal transmission, detection, and reception) disrupt the function of sensory systems. We examine how different types of environmental disturbance are likely to have direct physiological affects on the senses, and whether these impacts result in short or long term sensory disruption. We also consider how environmental change affects signaling environments (e.g., changes in the spectral, chemical, and acoustic environments) and whether there are concomitant changes in behavior, and we ask whether we can predict the outcomes of behavioral change

on individual fitness. An understanding of the link between natural environmental variation and within-species sensory system diversity (i.e., current observed environmental tolerance) is an important prerequisite for predicting the outcomes of human-induced environmental change on species' survival. We, therefore, provide examples of evolutionary adaptation and sensory plasticity in response to natural environmental variation, such as habitat diversity. Our review primarily focuses on fishes, because they are among the best-studied aquatic vertebrates, but we include other examples, where appropriate. Finally, we determine whether there is any evidence that human activities are causing evolutionary changes in sensory systems and whether sensory systems can exhibit phenotypic plasticity. We conclude by suggesting target areas for future research, particularly for sensory modalities that have been less well studied with respect to anthropogenic impacts.

DEGRADATION OF AQUATIC ECOSYSTEMS AND SENSORY DISRUPTION

Coastal aquatic ecosystems are vulnerable to localized activities that cause deterioration in water quality due to eutrophication, sedimentation, and contamination by metals and chemical pollutants from agriculture, pharmaceutical, and manufacturing industries. Coastal habitats are also vulnerable to environmental disruption at a global scale, such as the effects of climate change, including ocean and freshwater acidification and rising water temperatures. Ecotoxicological studies have revealed that contaminants can disrupt olfactory processes and inhibit fish behavior (Atchison et al., 1987; Scott and Sloman, 2004; Tierney et al., 2010). Common pollutants, such as surfactants that are found in household detergents (e.g., linear alkylbenzene sulphonate or LAS) are known to physically damage the gustatory receptors of yellow bullhead catfish (*Ictalurus natalis*), causing a diminished action potential following a few hours of exposure, before there are visible signs of histological tissue damage (Bardach et al., 1965). In whitefish (*Coregonus clupeaformis*), the electrical response of the olfactory bulb is diminished on exposure to the surfactant sodium lauryl sulphonate (SLS) (Hara and Thompson, 1978). The observation that whitefish were attracted to SLS at sublethal doses, but showed no preference at high concentrations, provides further evidence that SLS reduces chemoreceptor function at sublethal doses but largely blocks the response at high doses (Hara and Thompson, 1978).

Subsequent studies with Arctic charr (*Salvelinus alpinus*) have demonstrated the behavioral outcomes of chemosensory disruption; charr that were previously attracted to conspecific odor showed reduced or diminished preferences, depending on the concentration and duration of exposure to LAS (Olsen and Hoglund, 1985). Surfactants can affect shoaling behavior even at very low doses; for example exposure of rainbow trout (*Oncorhynchus mykiss*) to 0.5 μgl^{-1} of 4-nonylphenol (4-NP) for 1 h caused a change in association preference, while higher doses (1–2 μgl^{-1}) caused non-dosed fish to show strong avoidance of treated conspecifics (Ward et al., 2008). In this case, short-term exposure to a chemical pollutant has not inhibited the olfactory sensitivity of this species, but exposure has still resulted in a change in social behavior with important consequences for behaviors such as foraging efficiency and predator defense.

Storm water run-off often contains metal contaminants such as copper and cadmium, and synthetic compounds such as bisphenol A (found in plastics) and polychlorinated biphenyls or PCBs, which are used in the electrical industry. These common contaminants are known to have toxicological effects on the mechanosensory lateral line system of fishes, a sensitive sensory modality that is externally located, and hence directly exposed to compounds in the surrounding environment. The mechanosensory lateral line plays an important role in behaviors such rheotaxis (body orientation relative to water flow), prey capture and shoaling (Montgomery et al., 2014). In zebrafish (*Danio rerio*), for example, the level of damage to the lateral line hair cells (neuromasts) depends on the concentration of dissolved copper, with some loss of hair cells at doses above 20 μg/L and almost complete cell death after 3 h of exposure at 50 μg/L (Linbo et al., 2006). Larvae that were exposed to the lower dose and transferred to uncontaminated water began to regenerate the hair calls within 24 h, while those exposed to the highest concentrations (50 μg/L) displayed permanent lateral line damage (Linbo et al., 2006). Subsequent studies with larval zebra fish have shown that exposure to both copper ($CuSO_4$) and silver ($AgNO_3$) metal salts is associated with a reduction in the number of neuromasts and a failure to orientate into a water current (McNeil et al., 2014). Storm water run-off is often filtered to remove chemical contaminants; this can restore lateral line development in zebrafish, but not in coho salmon (*Oncorhynchus kisutch*), suggesting that species likely differ in sensitivity to contaminants (Young et al., 2018).

An important source of contamination in marine environments is petroleum products such as polycyclic aromatic hydrocarbons (PAHs). A recent comprehensive study examined the effect of PAH concentration on settlement, anti-predator behavior and survival rates of larval coral reef fishes found that all of these traits were altered as a result of PAH exposure (Johansen et al., 2017). The changes in anti-predator behavior included a reduction in shoal size in some species, as well as increased movement between habitats and increased time spent in open areas (Johansen et al., 2017). Since PAHs can affect the development of the peripheral nervous system in teleosts (Irie et al., 2011), it is possible that the central nervous system may also be affected, leading to a change in behaviors associated with higher order cognition (Johansen et al., 2017).

FRESHWATER ACIDIFICATION AND CHEMOSENSORY IMPAIRMENT

The processes underlying the acidification of freshwater ecosystems is relatively well known and occurs because the combustion of fossil fuels releases carbon dioxide, sulfur dioxide, and nitrogen oxides that combine with water to produce highly acidic precipitation (acid rain). A large number of studies have

shown that acidification can affect the ability of freshwater fishes to respond to alarm cues, which are an important cue for predation risk in aquatic environments. Alarm cues are chemicals present in the epidermis of the skin that are released when the skin is damaged (e.g., during a predator attack) and elicit an unlearned anti-predator response in conspecifics (reviewed by Brown, 2003; Wisenden, 2003). For example, the ability of fathead minnows (*Pimephales promelas*), finescale dace (*Phoxinus neogaeus*), pumpkinseed fish (*Lepomis gibbosus*), rainbow trout (*O. mykiss*), and brook charr (*Salvelinus fontinalis*) to detect and respond to olfactory cues from conspecifics (alarm cues) is reduced under low pH conditions (pH 6.0–6.1) (Brown et al., 2002; Leduc et al., 2003, 2004a).

Experimental manipulation of the pH of the skin extract of minnows also results in the loss of an alarm response, suggesting that even weakly acidic conditions result in degradation of the alarm molecule, rather than the loss of olfactory sensitivity of the fish (Brown et al., 2002). A reciprocal transplant experiment, in which juvenile Atlantic salmon (*Salmo salar*) were reared in neutral or acidic conditions and tested under the opposite pH conditions, revealed no long-term effects of acidic conditions on the production or detection of alarm cues (Leduc et al., 2010). Thus, acidic conditions cause a reversible and short-term reduction in olfactory sensitivity, a chemical disruption of the alarm cue under acidic conditions, or a combination of both these effects suggesting that these effects are environmental, rather than physiological or behavioral (Leduc et al., 2010). The chemical composition of alarm cues includes nitrogen oxides such as hypoxanthine-3-N-oxide (Brown et al., 2000), which is converted to 6,8-dioxypurine with a loss of the 3-N-oxide group when treated with acid (Kawashima and Kumashiro, 1969), and may explain the temporary loss of alarm function (Leduc et al., 2013). Irrespective of the mechanisms involved, the loss of alarm functions has significant implications for predator-prey interactions, including the response of populations to introduced novel predators.

The olfactory systems of fishes can be highly sensitive to the chemical signals of conspecifics, and production and detection of these cues plays an important role in reproductive physiology, shoaling behavior, migration, individual recognition, and antipredator responses (Liley, 1982). Nonetheless, there is evidence that acidic conditions can diminish olfactory sensitivity in pink salmon (*Oncorhynchus gorbuscha*) exposed to constant [450 (control), 1,000 or 2,000 μatm] or fluctuating CO_2 conditions (450–200 μatm). Salmon showed diminished growth in both freshwater and seawater, but also showed impaired olfactory responses to alarm cues, common odorants (amino acids), and a reduced response to predation risk (Ou et al., 2015). In particular, fish exposed to elevated CO_2 levels showed corresponding reductions in the electro-olfactogram responses recorded at the olfactory epithelium, suggesting that CO_2 acts to impair olfactory sensitivity (Ou et al., 2015). Indeed, in Atlantic salmon (*S. salar*) exposed to water with a reduced pH, higher concentrations of olfactory cues are required to produce a behavioral response (toward urine and testosterone, which are important mediators of social behavior) that matches the response of non-treated fish (Moore, 1994).

OCEAN ACIDIFICATION AND CHEMOSENSORY IMPAIRMENT

Recent studies examining sensory system responses to environmental change have focused on the projected impacts of climate change in marine environments, and particularly the effects of ocean acidification. Ocean acidification occurs when carbon dioxide, which is emitted from the burning of fossil fuels, enters the ocean, and combines with water to form carbonic acid. This results in the production of hydrogen ions, which react with carbonate ions and aragonite to form bicarbonate (**Figure 2**). This process leads to a decrease in the pH of ocean water, and a reduction in the availability of carbonate, which is required for the survival of coral reefs and invertebrates (Hoegh-Guldberg et al., 2007).

Munday et al. (2009) were the first to demonstrate that ocean acidification can result in loss of olfactory discrimination for preferred settlement sites. Under control conditions (current seawater pH of 8.15), orange clownfish (*Amphiprion percula*) preferred vegetation cues that were consistent with their settlement on tropical islands rather than vegetation cues that contained aversive oils (Munday et al., 2009). When reared under projected acidification conditions (pH 7.8), the preference for tropical vegetation settlement cues was reduced and larvae showed a strong response to vegetation that was previously aversive (Munday et al., 2009). Scanning electron microscopy revealed no difference in the surface structure of the olfactory epithelium in fish reared in low pH conditions, suggesting that the behavioral changes observed were mediated via induced changes in the transmission of chemosensory signals (via the olfactory receptor cells) rather than changes in the gross morphology of the olfactory system (Munday et al., 2009).

Importantly, a subsequent study by Munday et al. (2010) illustrated the fitness consequences of impaired olfactory discrimination, showing that damselfish larvae (*Pomacentrus wardi*) that had been exposed to elevated CO_2 conditions (850 ppm) for 4 days had 5–9 times higher mortality rates than those exposed to current (control) CO_2 conditions (390 ppm). Experiments simulating ocean acidification have also revealed changes in the ability of orange clownfish larvae to recognize and respond to olfactory cues from predators. Both newly hatched and settlement stage clownfish larvae can discriminate between the odors of predators and non-predators, but this ability is diminished in settlement stage fish exposed to CO_2 acidified water (Dixson et al., 2010).

DISRUPTION OF LEARNING PROCESSES: OLFACTORY AND VISUAL CUES

Experience with predator olfactory cues is one of the most fundamental ways in which fishes can learn the identity of novel predators, allowing them to develop an anti-predator response toward cues that were previously unfamiliar or associated with low levels of risk (Brown, 2003; Kelley and Magurran, 2003). Studies with both marine and freshwater fishes have revealed that individuals that have been conditioned by exposing them

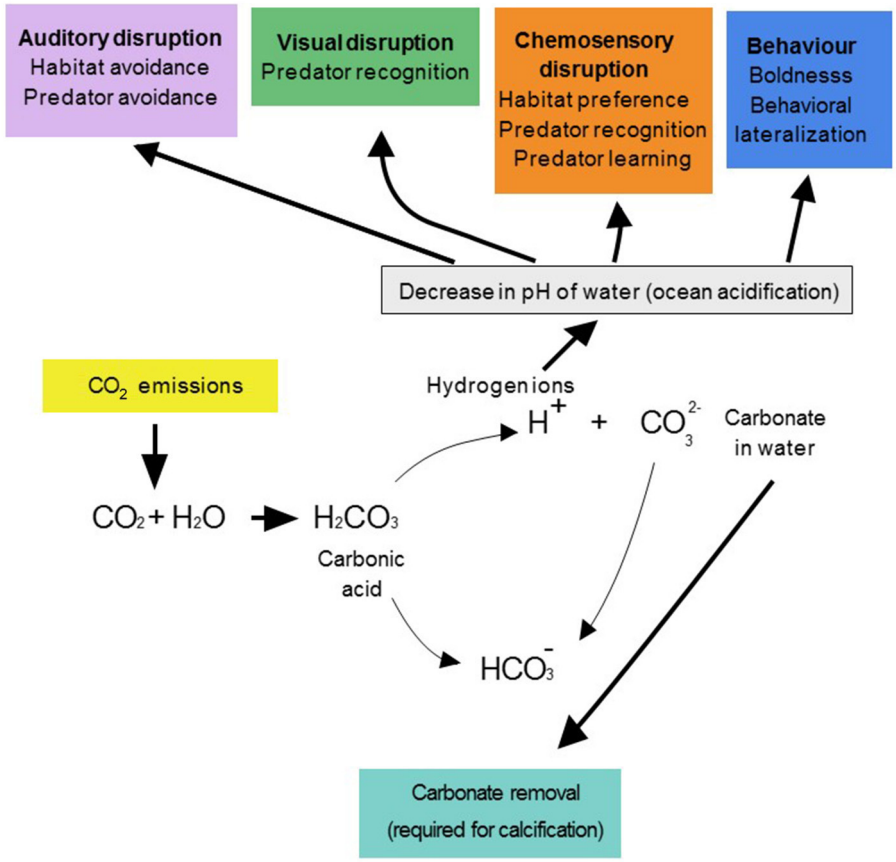

FIGURE 2 | Excess levels of CO_2 from burning fossil fuels enter the ocean and react with water to form carbonic acid. Carbonic acid disassociates into bicarbonate and hydrogen ions that decrease the pH of the water, increasing ocean acidity. Hydrogen ions also combine with carbonate to produce more carbonate, thereby reducing the availability of carbonate required for the production of calcium carbonate, a critical process for shell formation, and coral growth. Ocean acidification has been shown to disrupt behaviors mediated by auditory (purple), visual (green), and chemosensory (orange) modalities.

to chemical cues (e.g., the odor from a novel predator), in conjunction with alarm cues, fail to learn a response to predators under acidic conditions (Leduc et al., 2004b; Smith et al., 2008; Ferrari et al., 2012a). In juvenile rainbow trout (*O. mykiss*), for example, fish learned to recognize a novel predator odor irrespective of the pH of the water, but the learned response was only retained when the pH was unchanged between the learning experience and subsequent chemical cue encounters (Smith et al., 2008). Staged encounters between rainbow trout and largemouth bass (*Micropterus salmoides*) have demonstrated that this disrupted response to alarm cues imposes significant survival costs, even following brief (24 h) rainfall events, which cause a rapid drop in stream pH (0.68 pH units) (Leduc et al., 2009).

Ocean acidification conditions also affect assessment of risk using visual cues. In a similar experiment, juvenile damselfish (*Pomacentrus amboinensis*) were exposed to visual cues from a predator of coral reef fishes i.e., adult spiny damselfish (*Acanthochromis polycanthus*), after being maintained at different CO_2 concentrations (Ferrari et al., 2012b). *Pomacentrus amboinensis* displayed appropriate anti-predator behaviors toward *A. polycanthus* at all treatment concentrations except the highest concentration (850 μatm), where they failed to display the reduction in foraging behavior, activity levels and area use that is typically observed in fishes (Ferrari et al., 2012b). The observation that acidification affects the behavioral assessment of predation risk via both visual and olfactory cues suggests that neural (afferent) pathways are affected, rather than the peripheral sensory organs (Ferrari et al., 2012b). A subsequent study revealed that elevated CO_2 levels reduces the critical flicker fusion threshold of the retina of spiny damselfish, a visual capability that allows animals to track moving stimuli that is likely important for escaping predators (Chung et al., 2014). Treatment with gabazine restored retinal function, again highlighting the importance of the $GABA_A$ receptors (see below) in mediating behavioral functions (Chung et al., 2014).

In the above mentioned studies, it is unclear how the acidification conditions (lowered pH or elevated CO_2) result in the observed olfactory and visual responses. If cognitive function is affected, causing fish to engage in risky behaviors, predation rates may become high (Munday et al., 2010). The only mechanism proposed to date is based on the altered function

of gamma-aminobutyric acid type A (GABA-A) receptors found in the vertebrate brain (Tresguerres and Hamilton, 2017). A study by Nilsson et al. (2012) found that exposure to gabazine (a GABA-A receptor antagonist) restored the loss of olfactory discrimination in orange clownfish (*A. percula)*, and reversed the loss of lateralisation behavior in damselfish (*Neopomacentrus azysron*), that were reared in high CO_2 conditions.

ELEVATED CO_2, AQUATIC CONTAMINANTS, AND $GABA_A$ RECEPTOR FUNCTION

Under conditions of high CO_2, marine fishes regulate their acid balance by accumulating bicarbonate ions (HCO_3^-) and releasing chloride (Cl^-) ions; regulatory changes that lead to a reversal in $GABA_A$ receptor function (inhibition to excitation) and impairment of behavioral processes (Nilsson et al., 2012; Tresguerres and Hamilton, 2017). Altered function of $GABA_A$ receptors, with corresponding effects on fish behavior, has since been reported in a number of studies on both marine and freshwater organisms (reviewed by Tresguerres and Hamilton, 2017) and is caused by a change in intracellular and extracellular HCO_3^- levels in the brain and blood plasma (Heuer et al., 2016). However, beyond this, little is known about distribution and subunit composition of $GABA_A$ receptors in fish, or the function and regulation of other neural pathways involved in HCO_3^- and Cl^- transport (Heuer et al., 2016). For example, glycine receptors, which are responsible for generating motor patterns and spinal reflexes, are also permeable to HCO_3^-. Therefore, these neurotransmitter pathways would most likely be affected by elevated levels of CO_2, with corresponding, and potentially additive, effects on behavioral impairment (Tresguerres and Hamilton, 2017).

Studies addressing other types of human impacts, such as chemical contaminants in aquatic environments (Brodin et al., 2014), have also suggested a role for $GABA_A$ receptors. In Atlantic salmon (*S. salar*), smolt that were exposed to low concentrations of oxazepam (a common anxiolytic pharmaceutical agent and modulator of $GABA_A$ receptors) migrated faster than smolt that were not exposed to this agent (Hellström et al., 2016). Thus, although changes to $GABA_A$ receptor function have helped explain many of the studies reporting an effect of acidification on behavior, there is an urgent need to examine whether other neural mechanisms (including neurotransmitter pathways) are affected, what interspecific differences are present, and whether the effects vary over different stages of development (Tresguerres and Hamilton, 2017).

OCEAN ACIDIFICATION AND AUDITORY IMPAIRMENT

Most marine organisms are known to respond to auditory cues underwater and the acidification of aquatic environments has the ability to disrupt physiological and behavioral traits pertaining to the auditory system. Although there is no information on the impact of acidification on the auditory abilities of marine mammals, the auditory ability of bony fishes is known to be altered from elevated levels of CO_2 (Ashur et al., 2017).

The mechanism of sound perception in bony fishes is mediated within the inner ear, which contains dense carbonate earbones, the otoliths. These $CaCO_3$ concretions have an essential function in sound detection (particle acceleration) and as orientation sense organs (Tohse and Mugiya, 2001; Tohse et al., 2004). Given their composition, otoliths are susceptible to either the reduced availability of carbonate ions in seawater at low pH, or to changes in the concentrations of bicarbonate and carbonate ions caused by acid-base regulation in fish exposed to high CO_2 levels (Munday et al., 2011b; Heuer and Grosell, 2014). An increase in otolith size was revealed in a range of species following exposure to as little as 64 μatm of additional CO_2 compared to control levels of CO_2, in species such as sea bass larvae (*Atractoscion nobilis*) (Checkley et al., 2009), clownfish (*A. percula*) larvae (Munday et al., 2011b), juvenile walleye Pollock (*Theragra chalcogramma*) (Hurst et al., 2012), cobia (*Rachycentron canadum*) larvae (Bignami et al., 2013a,b), cod (*Gadus morhua*) larvae (Frommel et al., 2012; Maneja et al., 2013), juvenile sticklebacks (*Gasterosteus aculeatus*) (Schade et al., 2014), mahi-mahi (*Coryphaena hippurus*) larvae (Bignami et al., 2014), juvenile sea bream (*Sparus aurata*) (Réveillac et al., 2015), and mulloway (*Argyrosomus japonicus*) larvae (Rossi et al., 2016b). However, the otoliths of juvenile spiny damselfish (*Acanthochromis polyacanthus*) (Munday et al., 2011a), juvenile clownfish (*A. percula*) (Simpson et al., 2011), Atlantic herring (*Clupea harengus*) larvae (Franke and Clemmesen, 2011), and juvenile scup (*Stenotomus chrysops,* (Perry et al., 2015) showed no size differences at increased levels of CO_2, whereas the size of the otoliths in marine medaka larvae, *Oryzias melastigma,* were even observed to be reduced (Mu et al., 2015). This reveals that the deposition and chemical composition of fish otoliths is dependent on CO_2 levels, and that the effects may be variable (depending on ocean acidification conditions), species-specific, and sensitive to the duration of the study. Although the increase in otolith size in species exposed to high CO_2 levels has not yet been directly linked to behavioral endpoints, variations in the size, shape and symmetry of the otoliths will have a major bearing on the mechanotransduction process and each individual's ability to effectively detect sound (Popper and Lu, 2000; Gagliano et al., 2008). Bignami et al. (2013a) used a computer model to demonstrate that enlarged otoliths of larval cobia (*R. canadum*) in high CO_2 conditions could affect auditory sensitivity, including a 50% increase in hearing range, which may be beneficial for perceiving biologically-relevant cues but detrimental by increasing sensitivity to background noise, hence masking ecologically vital information.

Several behavioral studies have demonstrated impaired acoustic behaviors of reef fish species in low pH conditions. Reef fish larvae use a suite of sensory cues to orient toward and discriminate between potential settlement sites, and ambient reef sounds have been shown to be an important cue for settlement (Montgomery et al., 2001; Leis et al., 2011). Ocean acidification has been shown to alter the ability of larval fishes to use these acoustic habitat cues. For example, clownfish larvae showed a change in their directional response to a predator-rich,

daytime reef recording when reared in elevated CO_2 conditions (Simpson et al., 2011), suggesting an impairment of auditory behavior critical for survival. Similarly, mulloway (*A. japonicus*) and barramundi (*Lates calcarifer*) larvae exposed to high CO_2 levels avoided playback recordings of ambient reef sounds, while individuals reared in normal pH conditions were attracted to these habitat cues (Rossi et al., 2015, 2016b). The perception of soundscapes of reef systems was also shown to be degraded in these fish species as a result of the reduced intensity and frequency of snapping shrimp (*Alpheus novaezelandiae*) sounds (Rossi et al., 2016a,b), thereby, indirectly, influencing reef larval settlement.

Marine invertebrates, like cephalopods, cnidarians, and arthropods, can sense vibrational stimuli through external sensory hairs and/or statocysts. Statocysts generally include gravity and particle acceleration (sound) receptors (Maturana and Sperling, 1963; Budelmann and Williamson, 1994) and a statolith organ, which is analogous in function and structure to a fish otolith. The composition of these statoliths varies among taxa, but many are composed of calcium carbonate (Fabry et al., 2008) and therefore may be affected by ocean acidification, as is the case for the otoliths of bony fishes (see above). However, contrary to fish otoliths, which show increased growth rates under low pH/high CO_2 conditions, at least in some species (Ashur et al., 2017), statolith size is reported to decrease in a range of invertebrate species including squid larvae *Doryteuthis pealeii* (Kaplan et al., 2013; Navarro et al., 2016), Chilean abalone larvae *Concholepas concholepas* (Manriquez et al., 2014) and cuttlefish hatchlings *Sepia officinalis* (Maneja et al., 2011) and even undergoes a change in composition in the squid larvae *Loligo vulgaris* and *Doryteuthis opalescens* (Lacoue-Labarthe et al., 2011; Navarro et al., 2014). At present, there is no information about the effects of statolith size or composition on the acoustic behavior of invertebrates.

In general, the effects of CO_2 on the auditory abilities of marine organisms remain poorly understood, with only a few studies performed in bony fishes (Ashur et al., 2017). There is even less known about the impacts of acidification on mechanotransduction mechanisms in invertebrates and cartilaginous fishes (sharks, rays, skates), reptiles and marine mammals. Only one study has examined the hearing system (otolith size) in freshwater organisms, where Chinook salmon, *O. tshawytscha,* larvae showed incremental decreases in otolith width at reduced water pH (Geen et al., 1985). Although the same physiological impacts on the auditory system that are found in marine environments might be expected to apply to freshwater animals (Ishimatsu et al., 2008), extrapolations must be made with caution, especially as the effects may be species-specific rather than environment-dependent.

NOISE POLLUTION AND HEARING IMPAIRMENT

Human-generated sound, or anthropogenic noise, is a relatively recent addition to the aquatic soundscape, driven by a range of sources, such as shipping, pile driving, seismic surveys, explosions, sonar, deep-sea mining activities, dredging and motor-powered recreational and commercial craft (Hildebrand, 2009). The sounds produced by these activities have been found to elicit behavioral reactions and shifts in many aquatic species, changes in whole populations of organisms and even physical injury (Kunc et al., 2016). The level of noise in the sea has been linked to the global economy (Frisk, 2012), whereby shipping constitutes 90% of the method of trade between different countries, and it is certain to continue to increase as the ocean becomes more industrialized. Many excellent reviews exist on the effects of aquatic noise on marine mammals, bony fishes and invertebrates (Nowacek et al., 2007; Weilgart, 2007; Popper and Hastings, 2009; Slabbekoorn et al., 2010; Hawkins and Popper, 2014; Hawkins et al., 2014a,b; Radford et al., 2014; Whitfield and Becker, 2014; Braun, 2015; Peng et al., 2015; Williams et al., 2015; Zakon, 2015; de Soto, 2016; de Soto and Kight, 2016; Gomez et al., 2016; Kunc et al., 2016; Juanes et al., 2017), so the following information represents only a short synopsis.

The hearing abilities of aquatic organisms vary in their absolute sensitivity and frequency range. Most fishes are sensitive to low frequencies, from 50 Hz to about 5 kHz (Ladich and Fay, 2013), while marine mammals exhibit much larger hearing ranges, between 1 and 150 kHz (Dehnhardt, 2002; Weilgart, 2007). Almost nothing is known about hearing in aquatic invertebrates, although they most likely can only detect very low frequency sounds (Hawkins and Popper, 2014; Hawkins et al., 2014a). Similarly, there is little information on marine reptiles, although sea turtles have a peak sensitivity at about 500 Hz (Willis, 2016). Different sources of anthropogenic noise will affect each taxon differently, depending on the temporal, spatial, and frequency signature of the sound sources. For example, noise from shipping contains mainly low frequency components (<1,000 Hz) (Peng et al., 2015) and may, therefore, be more detrimental to organisms with a peak sensitivity that lies within this range, like bony fishes, and invertebrates.

Anthropogenic noise can have physiological, developmental and behavioral consequences on the hearing systems and acoustic behavior of an individual (Kunc et al., 2016). Several studies have reported damage of sensory hair cells, barotrauma and hearing loss in both freshwater and marine bony fishes (McCauley et al., 2003; Popper et al., 2005; Halvorsen et al., 2012a,b,c; Casper et al., 2013; Smith and Monroe, 2016), damage to the statocysts of several squid species (Guerra et al., 2004; André et al., 2011), as well as hearing loss and inner ear injuries in marine mammals (Ketten et al., 1993; Ketten, 1995; Southall et al., 2007; Weilgart, 2007). Seismic surveys, which blast compressed air to produce pulses of sound that can probe the sea floor for natural resources, have also caused extensive damage to the inner ears of pink snapper (*Pagrus auratus*) (McCauley et al., 2003). The operation of airguns has also been found to elicit behavioral changes in marine fishes, including movement to the bottom of the water column and fast swimming (Fewtrell and McCauley, 2012). Similarly, squid (*Sepiotheuthis australias*) responded to the sounds of airguns with alarm responses, changes in swimming patterns and vertical migration (Fewtrell and McCauley, 2012). McCauley et al. (2017) recently showed that seismic surveys killed zooplankton up to 1.2 km away from the airgun source.

Noise pollution can also cause changes in acoustic behavior when it masks essential information, distracts individuals or alters intraspecific communication (Kunc et al., 2016). Sound plays an important role in communication for aquatic organisms given the sound transmission properties of water and the inevitable constraints of light attenuation, which inhibit visual communication in some environments. The underwater soundscape contains essential information for survival of all taxa including cues to aid in migration, orientation, settlement, predator-prey interactions, and locating reproductive partners. These acoustic cues have the potential to be masked by anthropogenic noise, causing disruption of normal acoustic behaviors (see reviews by Radford et al., 2014; Erbe et al., 2016). For example, the amplitude of acoustic communication signals emitted by killer (*Orcinus orca*) and beluga (*Delphinapterus leucas*) whales has been shown to increase in the presence of ship noise (Scheifele et al., 2005; Holt et al., 2011). The tendency for signal producers to enhance the amplitude of communication signals under noisy conditions has been shown in marine mammals and fishes (Radford et al., 2014), but it is not clear whether sensory systems can be altered (e.g., increased sensitivity) to enhance signal reception. It has been suggested that marine mammals, such as pinnipeds, have evolved low signal-to-noise ratios as an adaptation for sound detection in noisy marine environments (Southall et al., 2000), but it is unclear whether this is the case in fishes. The response of fish larvae to natural reef sounds is also disrupted by boating noise, with implications for settlement and populations in coral reef habitats (Holles et al., 2013). Playback recordings of ship noise even increased the risks of starvation and predation for shore crabs (*Carcinus maenus*) in captivity (Wale et al., 2013), and damselfish (*P. amboinensis*) were more readily captured by their natural predators during exposure to motorboat noise (Simpson et al., 2016). Moreover, anthropogenic noise can also distract individuals: i.e., hermit crabs (*Coenobia clypeatus*) making them more vulnerable to predation (Chan et al., 2010a,b). Interestingly, noise can affect behavior across sensory modalities i.e., cuttlefish (*S. officinalis*), although not reliant on acoustic communication, changed their visual signals (body coloration) when exposed to anthropogenic noise (Kunc et al., 2014). Considering multiple sensory channels may be important to understand the broad implications of anthropogenic sound on aquatic organisms.

DEGRADED OPTICAL HABITATS AND BEHAVIOR

Human activities such as agriculture, forestry, urbanization, and resource extraction often cause a change in water quality, such as increased turbidity, that can have a major effect on behavioral traits and individual fitness. Many aquatic vertebrates rely on vision for basic behavioral tasks such as finding food, selecting mates and avoiding predators (Guthrie, 1986). However, shifts in the optical quality of water can have a critical effect on population survival, species composition and ecosystem biodiversity. Light attenuates with depth depending on the particular absorbance properties of the water and the presence of dissolved organic matter (e.g., "humic substances," such as tannins), phytoplankton, and particulate matter (Kirk, 1994). The nature of the habitat disruption will therefore determine how light is scattered and absorbed. For example, the introduction of suspended particles due to soil erosion increases the scattering of light, while eutrophication increases the algal load of the water and promotes the absorption of light with depth (Kirk, 1994).

Changes in the specific optical properties of the water, can lead to shifts in a particular behavior and this is particularly well known in freshwater ecosystems, where light environments tend to be more dynamic than in oceanic or coastal ecosystems. For example, increased turbidity can decrease the reaction distance of pike (*Esox Lucius*) predators, but increase the escape distance of roach (*Rutilus rutilus*) prey, suggesting that changes in the visual range alter the outcome of predator-prey interactions (Ranaker et al., 2012). Shoaling brings important anti-predator benefits that may be diminished under turbid conditions; shoals are less cohesive and individuals behave more like lone fish when visual contact among shoal members is reduced (Kimbell and Morrell, 2015). Shoaling behavior is also affected by changes in the spectral composition of water. For example, in western rainbowfish (*Melanotaenia australis*), individuals in environments rich in organic matter (which selectively absorbs short wavelength light) shoal further apart than those in water with full spectrum lighting (Kelley et al., 2012). In this study, rainbowfish increased the area and brightness of their coloration, allowing them to compensate for a change in signaling conditions and maintain visual communication among conspecifics (Kelley et al., 2012).

Several studies have revealed how turbidity can affect mate choice, and the signals used to convey male quality. In three-spine sticklebacks (*G. aculeatus*), poor quality males are less likely to curtail their courtship display in the presence of a competitor in turbid water than when in clear water, suggesting that courtship effort is a less reliable indicator of male quality under compromised visual conditions. Female behavior is also affected by turbidity in this species, where females are less attracted to males in turbid water than in clear water, and males compensate for this by heightening their courtship activity in turbid water (Engström-Öst and Candolin, 2006). Although the transmission of visual signals is clearly compromised in these environments, it is not clear whether turbidity causes short term changes in visual sensitivity (but see Plasticity in sensory systems, below) However, the reduced availability of visual cues does not necessarily mean that individuals will become increasingly reliant on other modalities as a compensation mechanism. For example, in broad-nosed pipefish (*Syngnathus typhle*), males spent longer assessing females in clear water than in turbid water, and the presence of olfactory cues did not compensate for the reduced availability of visual cues (Sundin et al., 2010). In addition, the optical properties of the water, such as the presence of humic substances, can also alter the water's chemical attributes. This can significantly disrupt chemical communication and facilitate hybridization between fish species, as has been described in swordtails, *Xiphophorus* spp. (Fisher et al., 2006). Similarly, if turbidity causes a breakdown in the cues used for mate choice, as in the case of African cichlids, sexual selection is relaxed and there is a loss of species diversity (Seehausen et al., 1997). Collectively,

these studies illustrate the complex nature of the relationship between the optical and chemical properties of the water and the role of the light environment in mediating behavior.

ADAPTIVE EVOLUTION OF SENSORY SYSTEMS

Variation in the environmental conditions (i.e., predation risk, habitat structure) can result in strong selection on animal sensory systems (Endler, 1991). Threespine sticklebacks (*G. aculeatus*) that inhabit a diversity of aquatic habitats provide an excellent illustration of evolutionary divergence among populations of a single species. For example, differences in the mechanosensory lateral line system of lake-inhabiting sticklebacks varies depending on whether populations are benthic or limnetic (Wark and Peichel, 2010) and is largely based on heritable (genetic) variation (Wark et al., 2012). Although sticklebacks occupy lake habitats with a variety of photic conditions, there is limited variation in their visual pigment protein (opsin) sequences, with no amino acid differences occurring at functionally relevant tuning sites (Flamarique et al., 2013). However, adaptive divergence of the visual system has occurred between marine and freshwater populations via shifts in the levels of opsin gene expression, which have been linked with changes in photic conditions associated with marine sticklebacks' colonization of freshwater habitats (Rennison et al., 2016). Classic work with Lake Victoria cichlids has revealed how human impacts, such as eutrophication, can alter sensory-mediated processes of selection; turbidity relaxes color-mediated mate choice causing a breakdown in assortative mating and a loss of species diversity (Seehausen et al., 1997). While the genetic basis of senses other than vision is yet to be resolved, it is likely that other sensory systems will exhibit rapid evolutionary responses to human disturbances. Indeed, it is becoming apparent that sensory pollution, such as light and sound, can cause rapid evolutionary change in a variety of physiological and behavioral traits that are ultimately underpinned by sensory system responses (Swaddle et al., 2015).

PLASTICITY IN SENSORY SYSTEMS

It is becoming increasingly apparent that plasticity in animal sensory systems can determine fitness and have important implications for selection (Ronald et al., 2012). The CNS of fishes exhibits indeterminate growth, potentially allowing these taxa a critical advantage when dealing with environmental challenges. For example, fishes often exhibit changes in their retinal morphology across different life history stages (Shand et al., 1999, 2008) and show seasonal shifts in peripheral auditory frequency sensitivity associated with reproductive activities (Sisneros and Bass, 2003). Gymnotiform fishes, such as *Brachyhypopomus gauderio*, exhibit variation in electrocommunication signals that is dependent on sex, body condition and social experience (Salazar and Stoddard, 2008, 2009). While freshwater threespine sticklebacks (*G. aculeatus*) exhibit limited plasticity in the expression of their opsin genes (Flamarique et al., 2013; Rennison et al., 2016), studies with the bluefin killifish, *Lucania goodie*, have revealed rapid (after 1–3 days) switches in opsin expression corresponding with changes in lighting conditions (clear water vs. tea-stained treatments) (Fuller and Claricoates, 2011). Indeed, shifts in opsin expression, along with changes in chromatophore use (vitamin A1:A2 ratios), provide a mechanism for fishes to adapt to developmental stages and life history patterns (Temple et al., 2006; Shand et al., 2008).

There is also some evidence for plasticity in the development of the lateral line system in response to environmental variation. For example, exposure to altered water flows can cause a shift in the abundance of neuromasts (specialized cells for detecting water motion) over particular regions of the body of rainbowfishes (*M. australis*) (Kelley et al., 2017). Understanding the plasticity of sensory systems such as the mechanosensory lateral line is crucial for understanding and managing how fishes respond to physical hydrodynamic changes in the environment, i.e., altered patterns of migration due to dam construction (Goodwin et al., 2014). Given the numerous studies showing disruption of sensory systems and associated behaviors in aquatic systems, we understand surprisingly little of the role of rapid evolutionary change and phenotypic plasticity in facilitating species' responses to human impacts in these environments.

SENSORY SWITCHING UNDER ENVIRONMENTAL CHANGE

Sensory switching, or compensation, occurs when animals rely on alternative sensory modes (compensatory plasticity hypothesis), due to information limitation or sensory masking (e.g., turbidity and background noise), or because the sense organs become damaged. Sensory compensation is thus a form of plasticity that allows individuals to switch between modalities and gain the most information in a given sensory environment. In threespine sticklebacks (*G. aculeatus*), females rely on visual cues to select males in clear water, but switch to olfactory cues under turbid conditions (Heuschele et al., 2009). Furthermore, female preferences for male size are dependent on which sensory cues are available (Heuschele et al., 2009), suggesting that processes such as eutrophication can alter selection on courtship displays (Engström-Öst and Candolin, 2006; Wong et al., 2007). Some fishes migrate between water bodies of varying salinity, providing an opportunity to examine whether individuals rely on different sensory modalities to maintain important behaviors such as shoal cohesion. The Pacific blue-eye (*Pseudomugil signifier*), which occupies habitats ranging in salinity, responds to chemical cues from conspecifics, and forms tighter shoals in freshwater than in saltwater (Herbert-Read et al., 2010).

SUMMARY AND CONCLUSIONS

In this review, we find evidence that different forms of habitat disturbance can disrupt the sensory systems of aquatic vertebrates and invertebrates, with specific types of disruptor affecting species-specific behaviors. This makes it challenging to predict and manage these impacts at the community level,

because species may exhibit different or opposing responses (or show no overt change in behavior), leading to altered ecological interactions, such as predator-prey relationships. Nonetheless, we suggest that significant insights will be gained when the sensory cues underlying a specific fitness-related behavior are known, although this is rarely likely to be the case. In some instances, the effects of human-induced environmental change on sensory systems are relatively well studied, such as the effect of ocean acidification on olfactory discrimination and predator recognition. Importantly, progress in this area has revealed that altered function of the $GABA_A$ receptors in the brain explains at least some of these behavioral impairments. Nonetheless, this research is in its infancy, and it is also possible that other neurotransmitters or neurological pathways are affected but are yet to be discovered (Tresguerres and Hamilton, 2017).

We have shown that many studies on species' responses to human-induced environmental change are focused on behavior. This is because a change in behavior is typically the first observed response to an environmental disruption (Tuomainen and Candolin, 2011), and is probably also the easiest to measure *in situ*. However, neurophysiological studies and knowledge of a species' sensory biology can provide essential information on functional and mechanistic responses at all scales, which can ultimately inform conservation strategies (Cooke et al., 2013). With the development of biosensors to directly monitor animal physiology, it is now easier to assess variation in function and tolerance among individuals, populations and species and more studies of this nature are required to understand the effect of environmental change on sensory physiology. In particular, most of the work has focussed on aquatic vertebrates and there is a clear lack of information concerning the response of invertebrates to human induced environmental change.

Our review of the literature has revealed many areas where knowledge of a species' sensory biology and basic biology are lacking. For example, light is not only essential for visual processes, but also guides behaviors that do not rely on directional monitoring of light, such as photokinesis (locomotory responses to light) and the entrainment of circadian rhythms (Land and Nilsson, 2012). A reduction in water quality that results in reduced light intensity, or a change in spectral composition, could disrupt fundamental biological processes such as sleep, activity patterns, and body colouration (Collin and Hart, 2015). This might be more likely in environments with reduced availability of short wavelength light (e.g., water rich in organic matter), because of the role of non-visual pigments such as melanopsin (with a sensitivity of ∼475–500 mn), but studies are yet to be conducted (Collin and Hart, 2015). Similarly, light pollution has the potential to disrupt basic biological functioning in many organisms, but research tends to focus on particular taxa, such the impacts of artificial light on the movement trajectories of green turtle (*Chelonia mydas*) hatchlings (Thums et al., 2016).

There is a particular need to understand the impacts of human activities on species with sensory modalities that are less well understood. For example, we know little of the potential effects of electromagnetic fields (such as those produced by underwater cables) on the behavior of electroreceptive fishes and sharks (Orr, 2016), although there is evidence that diadromous eels, *Anguilla* spp., that use magnetic fields for migration, display altered swimming patterns when passing over subsea cables (Gill et al., 2012). More research is required on the effects of aquatic pollutants on fish taste buds and chemoreceptive systems (in marine and freshwater systems), particularly in species where olfactory cues play an important part in behaviors such as larval settlement and habitat choice. While ocean noise, such as seismic surveys, is known to affect acoustic communication in cetaceans (e.g., Di Iorio and Clark, 2010), less is known about these effects on other animals, such as teleost fishes. Many sensory systems operate under specific physiochemical thresholds, yet it is unclear how the increased warming of the oceans, for example, will affect these sensory thresholds.

An understanding of animal sensory biology is not only required to understand how species respond to human-induced environmental change, but also has the potential to be an important tool for conservation management. Such knowledge may partly explain the invasion success of exotic species, and hence be used to facilitate management strategies. For example, invasive Western mosquitofish (*Gambusia affinis*) are able to maintain their foraging efficiency across a range of optical water conditions, while native species, such as the New Zealand Inanga (*Galaxias maculatus*), display highly impaired foraging efficiency under turbid conditions (Abrahams et al., 2017). Thus, an understanding of sensory biology, combined with knowledge of the role of the senses in fitness-related behaviors (and plasticity in these traits), can be used to manage human impacts on marine and freshwater organisms.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Ontogenetic Shifts in the Number of Axons in the Olfactory Tract and Optic Nerve in Two Species of Deep-Sea Grenadier Fish (Gadiformes: Macrouridae: *Coryphaenoides*)

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Neuroanatomical studies of the peripheral sense organs and brains of deep-sea fishes are particularly useful for predicting their sensory capabilities and ultimately their behavior. Over the abyssal plane (between 2,000 and 6,000 m), communities of grenadiers (Gadiformes: Macrouridae) play an important ecological role as predator-scavengers. Previous studies suggest that these fishes rely heavily on chemosensation, especially olfaction. Furthermore, at least one species, *Coryphaenoides armatus*, undergoes an ontogenetic shift in the relative size of the optic tectum and the olfactory bulbs, suggesting. a shift from a reliance on vision to olfaction during ontogeny, apparently in association with a shift to a more scavenging lifestyle. Here, we compared the olfactory and visual sensory inputs to the brain in *C. armatus*, and in a second, closely-related species, *Coryphaenoides profundicolus*, by assessing the total number of axons (myelinated and unmyelinated) in the olfactory tract and optic nerve in a range of individuals from both species. In *C. armatus,* the numbers of axons in both tract and nerve increased with body size, with the total number of axons in the olfactory tract being far greater than the number of axons in the optic nerve. These differences became more pronounced in larger animals. In the two smaller *C. profundicolus* individuals (≤315 mm SL), there were more axons in the optic nerve than in the olfactory tract, but the opposite situation was found in larger individuals. As in *C. armatus*, the number of olfactory tract axons also increased with body size in *C. profundicolus*, but in contrast, the number of optic nerve axons decreased in this species. These results suggest that both *C. armatus* and *C. profundicolus* undergo an ontogenetic shift in sensory orientation, with olfaction becoming relatively more important than vision in larger animals. The differences in the ratio of olfactory tract to optic nerve axons in *C. armatus* indicate that olfaction is of

particular importance to larger individuals of this species. In both species, the percentage of myelinated axons in the olfactory tract was relatively low, but we found evidence for interspecific and ontogenetic variation in the percentages of myelinated axons in the optic nerve.

Keywords: axons, brain, deep-sea fish, grenadier, olfaction, ontogenetic shift, sensory system, vision

INTRODUCTION

Grenadiers or rat-tails (Gadiformes, Macrouridae) are a diverse and abundant family of deep-sea, benthopelagic fishes, with just over 400 recognized species (Eschmeyer et al., 2018). These fishes generally have large heads, prominent eyes, and long, tapering bodies (**Figure 1**). Grenadiers have a global distribution, with most species found on the continental shelves and slopes at depths of between 200 and 2,000 m (Marshall, 1979; Cohen et al., 1990; Weitzman, 1997), although some species frequent abyssal depths from 2,000 to below 6,000 m (Gaither et al., 2016; Linley et al., 2016). Given their diversity and numerical abundance, grenadiers often comprise a large proportion of the biomass in deep-sea benthopelagic habitats and probably play an important ecological role as predator-scavengers in these communities (Haedrich, 1997; Drazen et al., 2008; Lee et al., 2008; Gerringer et al., 2017).

Of the abyssal grenadier species, the abyssal grenadier *Coryphaenoides armatus*, which is found at depths of *ca*. 2,000–5,200 m (Gaither et al., 2016), is the most widespread and abundant (Merrett and Haedrich, 1997). Analyses of stomach contents have confirmed that this species is euryphagous, ingesting both living and dead animal material, along with plant debris and even human refuse (Haedrich and Henderson, 1974; Sedberry and Musick, 1978). As well as being an active predator, adult *C. armatus* are heavily reliant on scavenging on carrion including food-falls, such as the carcasses of cephalopods, fishes, and cetaceans (Haedrich and Henderson, 1974; Mauchline and Gordon, 1991; Kemp et al., 2006), for survival (Drazen et al., 2008). Compared to other deep-sea fishes, adult *C. armatus* appear to be particularly well-equipped to detect the location of such food-falls using olfaction as they possess relatively large olfactory bulbs (Wagner, 2001a, 2002). Moreover, *in situ* video recorded by baited cameras on landers (autonomous vehicles deployed on the seafloor) has revealed that *C. armatus* is often the first species to appear when baited cameras are deployed and is presumably attracted to the bait by a very sensitive olfactory system (Wilson and Smith, 1984; Priede et al., 1990, 1994; Armstrong et al., 1992; Wagner, 2003). However, baits are predominantly taken only by large individuals (King et al., 2006), even in locations where trawls have demonstrated that smaller individuals are also present (Henriques et al., 2002; Collins et al., 2005). This suggests that the olfactory sense in smaller individuals of *C. armatus* is not as sensitive as that of larger animals, and/or that these smaller fish may be more dependent on different sources of food that require a greater reliance on sensory modalities other than olfaction. Interestingly, dietary studies show that carrion accounts for a lower proportion of the diet in small *C. armatus* compared to larger individuals, with epibenthic and benthic invertebrates being more numerous in the stomachs of smaller animals (Haedrich and Henderson, 1974; Martin and Christiansen, 1997; Drazen et al., 2008). Moreover, a functional analysis of the feeding apparatus suggests that feeding strategy differs between small and large individuals of *C. armatus* (McLellan, 1977). Smaller individuals have an elongated, shovel-like rostrum that they use to actively dig in muddy substrates for food. In contrast, larger animals, which have a less prominent rostrum, tend to swim above the bottom with their heads orientated downwards. A quantitative comparison of brain morphology across a broad size range also reveals changes in the relative sizes of the olfactory bulbs and the primary visual brain area, the optic tecta, in *C. armatus*, with the optic tectum being relatively larger in smaller animals and the olfactory bulbs being relatively larger in adults (Wagner, 2003).

In fishes, the nervous system grows continuously throughout life (Kaslin et al., 2008). Shifts in the relative size of different sensory brain areas, such as those described for *C. armatus* (Wagner, 2003) have been documented in a range of shallow water fishes and agnathans (lampreys; Cadwallader, 1975; Brandstätter and Kotrschal, 1989, 1990; Kotrschal et al., 1998; Lisney et al., 2007, 2017; Salas et al., 2015). These shifts are correlated with changes in the structure of the peripheral sense organs, and occur in association with ontogenetic shifts in habitat, diet and behavior, and may also coincide with the onset of sexual maturity. Therefore, Wagner's (2003) findings for *C. armatus* provide strong evidence that this species also undergoes a shift in sensory orientation (from vision to olfaction) during ontogeny, in association with changes in diet and feeding behavior.

Wagner's (2003) study on *C. armatus* used the "ellipsoid model" to quantify the volumes of different brain areas. This approach assumes that each brain area approximates the volume of an idealized ellipsoid or half ellipsoid (Huber et al., 1997), where linear measurements of the length, width, and depth of each brain area are translated into volumetric measures. This method has proved useful for quantifying variations in brain morphology in fishes, especially in species that are difficult or impossible to maintain in captivity, or to observe and study in detail in their natural environment, such as large, open water pelagic species (e.g., tunas, billfishes, and oceanic sharks; Lisney and Collin, 2006; Yopak and Lisney, 2012; Yopak et al., 2015) and deep-sea fishes (Collin et al., 2000; Wagner, 2001a,b, 2002, 2003). However, there are caveats associated with this approach. For example, the ellipsoid model can significantly under- or over-estimate the volume of brain areas compared to volumes obtained from other methods, such as stereological approaches on serial

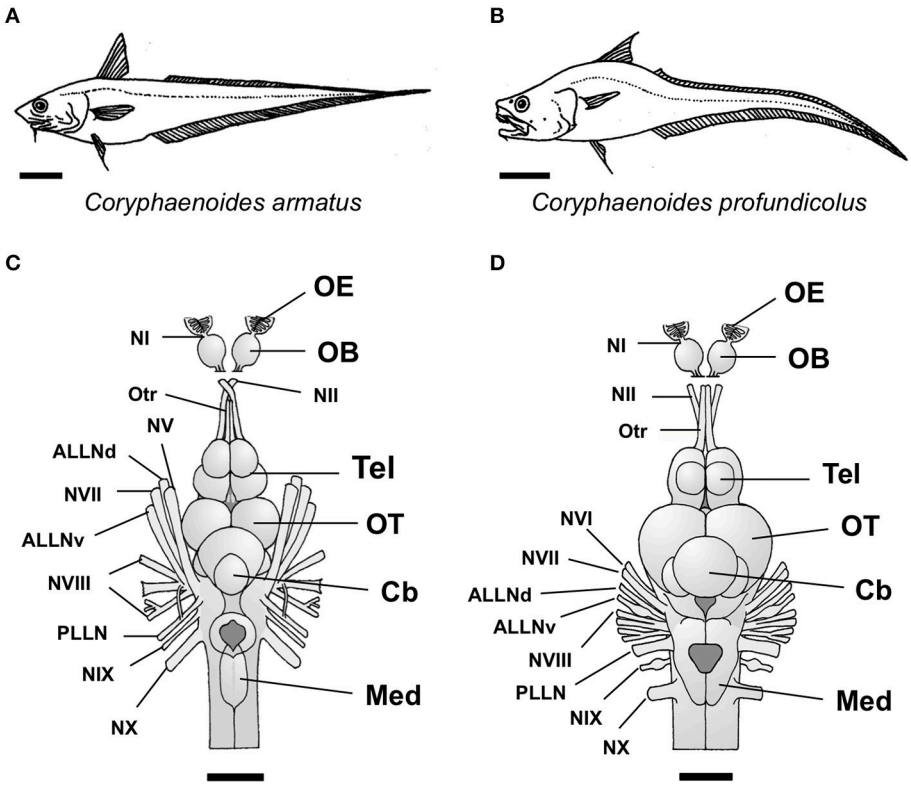

FIGURE 1 | The two species of deep-sea grenadier investigated in this study, the abyssal grenadier *Coryphaenoides armatus* **(A)** and the deep-sea grenadier *C. profundicolus* **(B)**. Dorsal views of the brain and the cranial and sensory nerves from the two largest individuals of these species investigated are presented in **(C)** *C. armatus* (900 mm SL) and **(D)** *C. profundicolus* (890 mm SL). In both species, the olfactory bulbs (OB) are stalked. The olfactory epithelium (OE) is positioned very close to the olfactory bulbs and the olfactory nerve (NI) is very short. The olfactory bulbs are connected to the telencephalon (Tel) by long olfactory tracts (Otr). ALLNd, dorsal root of the anterior lateral line nerve; ALLNv, ventral root of the anterior lateral line nerve; Cb, cerebellum; Med, medulla; NI, olfactory nerve (cranial nerve I); NII, optic nerve (cranial nerve II); NV, cranial nerve V; NVI, cranial nerve VI; NVII, cranial nerve VII; NVIII, cranial nerve VIII; NIX, cranial nerve IX; NX, cranial nerve X; OB, olfactory bulbs; OE, olfactory epithelium; OT, optic tectum; Otr, olfactory tract; PLLN, posterior lateral line nerve; Tel, telencephalon. Scale bars: 10 cm **(A)**; 10 cm **(B)**; 3 cm **(C)**; 2.5 cm **(D)**.

brain sections or the segmentation of brain regions following magnetic resonance imaging (MRI). This may be particularly relevant for multi-lobed brain areas (Ullmann et al., 2010), or those brain areas that enclose a large ventricular space (Yopak and Lisney, 2012). Furthermore, some brain areas, such as the optic tectum are multi-modal, where, in addition to visual input, the optic tectum also receives projections from other sensory modalities, such as the somatosensory and octavolateralis systems (Bodznick, 1991; Butler and Hodos, 2005). Therefore, some caution should be used when interpreting data obtained using the ellipsoid method, and if possible, complementary methods used to assess sensory input to the brain. One such method, which correlates well with differences in sensory orientation and behavior among species, is to compare the number of axons in the sensory (cranial) nerves associated with different senses. For example, in barn owls (*Tyto alba*), which are auditory specialists, there are more axons in their auditory nerve compared to other avian species (Köppl, 1997). In fishes and mammals, visually-oriented species have many more optic nerve axons compared to species that live in dim or turbid conditions (Huber and Rylander, 1992; Wohlert et al., 2016). Furthermore, the star-nosed mole (*Condylura cristata*), which has a unique and highly specialized star-shaped mechanosensory organ on the end of its snout, also possesses more than twice as many trigeminal nerve axons compared to other insectivores (Leitch et al., 2014).

In this study, we investigated olfactory and visual inputs to the brain in *Coryphaenoides armatus*, and in a second, closely-related abyssal grenadier species, the deep-sea grenadier *C. profundicolus*. This was achieved by assessing the number of axons in the olfactory tract and the optic nerve from different sized individuals of both species using transmission electron microscopy (TEM), which allows for the identification of very small axons that cannot be resolved using light microscopy (Vaney and Hughes, 1976). In *C. armatus* and *C. profundicolus*, the olfactory bulbs are stalked, and connected to the telencephalon by long olfactory tracts (**Figure 1**). The olfactory epithelium, which houses the olfactory receptor neurons (ORNs), is positioned very close to the olfactory bulbs (Døving, 1986). The ORNs detect odorants in the water and thus represent the first-order neurons in the olfactory pathway.

Their unmyelinated axons, which comprise the olfactory nerve, project to the olfactory bulb, where they synapse with the second-order neurons (mitral cells) within specialized structures called glomeruli. In species with stalked olfactory bulbs, the olfactory nerves are very short and thus difficult to identify and isolate for neuroanatomical studies. Therefore, we opted to assess axon numbers in the olfactory tract in our specimens. The olfactory tract is primarily comprised of mitral cell axons, which project to the telencephalon and diencephalon, plus a small number of centrifugal fibers originating from the telencephalon that project back to the olfactory bulb (Westerman and Wilson, 1968; Døving, 1986; Hamdani and Døving, 2007). In contrast, the optic nerve contains the axons of the retinal ganglion cells, which are the third-order neurons in the visual pathway, receiving information from the photoreceptors (first-order neurons), via the bipolar cells (second-order neurons). The optic nerve also contains centrifugal fibers that project back to the retina, but the proportion of centrifugal axons within the optic nerve is very low across vertebrates (Itaya, 1980; Dunlop and Beazley, 1984; Brooks et al., 1999) including fishes (Schmidt, 1979; Gerwerzhagen et al., 1982; Collin and Collin, 1988).

For *C. armatus*, we predicted that, in accordance with Wagner's (2003) findings, we would see an increase in the ratio of axons in the olfactory tract compared to the optic nerve as body size increased. In comparison to *C. armatus*, much less is known about the biology of *C. profundicolus*. This species is found at similar depths to *C. armatus* (*ca*. 3,600–4,900 m; Gaither et al., 2016), but is not as abundant or cosmopolitan in its distribution. Nevertheless, in the eastern North Atlantic Ocean, both *C. armatus* and *C. profundicolus* are amongst the most abundant abyssal species and are often caught or observed together in the same locations (Merrett, 1992; Merrett and Fasham, 1998; Milligan et al., 2016). Moreover, *C. profundicolus* is morphologically similar to *C. armatus* (**Figure 1**) and adults this species also have relatively large olfactory bulbs, although not to the same extent as *C. armatus* (Wagner, 2001a, 2002). Therefore, for *C. profundicolus*, we predicted that we would also find a similar increase in the ratio of axons in the olfactory tract compared to the optic nerve with increasing body size.

MATERIALS AND METHODS

Specimens

This study was carried out in accordance with the 5th edition of the National Health and Medical Research Council of Australia's code for the care and use of animals for scientific purposes (National Health Medical Research Council, 1990). The protocol was approved by the University of Western Australia animal ethics committee. Fish were collected on a scientific expedition on board the RRS Challenger in 1995 (Cruise No. 122) in the vicinity of 31–41°N (latitude) and 11–17°W (longitude) over the Madeira Abyssal Plain in the eastern North Atlantic Ocean, using a semi-balloon otter trawling net (OTSB 14) at depths between 100 and 4,000 m. The animals were dead when the nets were retrieved but, due to the advanced trawling techniques, the degree of external damage was minimized, and the time between death and the preservation of nervous material was also optimized. Each specimen was measured (standard length or SL in mm) and then, under a dissecting microscope, the cranium was removed thereby exposing the underlying brain and cranial nerves. Each whole head was preserved for electron microscopy by being immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH, 7.4) for 24 h. The brain (with the cranial nerves still attached) was then carefully removed and stored in either fresh fixative or 0.1 M phosphate buffer until histological processing. Only specimens with suitable ultrastructural preservation were used in this study. In total, four individual *C. armatus* (size range 430–900 mm SL) and five individual *C. profundicolus* (size range 250–860 mm SL) were used in this study.

Olfactory Tract and Optic Nerve Analysis

Small pieces (1–2 mm) of the olfactory tract (close to the telencephalon) and the optic nerve (close to the back of the eye) of each specimen were removed and post-fixed in 1% osmium tetroxide before being dehydrated in an acetone series and embedded in araldite. Both the left and right sides were sampled for each specimen. Blocks were then cut for transmission electron microscopy (TEM) using a Leica ultramicrotome and a glass knife. Selected transverse sections of each tract/nerve were stained with uranyl acetate and lead citrate and examined on a Leo 912 Omega TEM (Carl Zeiss, Oberkochen, Germany). For each section, a series of low magnification (400–630×) electron micrographs of the whole tract/nerve were obtained with the aid of a computerized sampling program that enabled the total tract/nerve area to be photographed (with a 20% overlap). A series of high magnification (5,000–16,000×) photographs were then obtained by sampling at regular intervals throughout the tract/nerve so that any marked increases in axon density (especially within regions containing increased densities of unmyelinated axons) could be identified. Axon counts were made using photographic enlargements by manual counting with the aid of a Zeiss GSZ stereomicroscope (Carl Zeiss, Jena, Germany). In the olfactory tract in both species, three axonal regions could be identified, a central region occupied by high densities of unmyelinated axons surrounded by two regions containing predominantly myelinated axons (**Figure 2B**). Since the mean axon density in the unmyelinated region was appreciably higher than in the myelinated regions (**Table 1**), each of these areas of the olfactory tract was assessed separately. The total number of axons was obtained by multiplying the mean axon density in each region by the total tract area. The total area of each tract/nerve was assessed after the low magnification series of photographic enlargements were all incorporated into a montage and the borders of each tract/nerve traced, making sure to exclude non-axonal regions, such as blood vessels and the nerve sheath. Measurements of the tract/nerve area were performed by scanning these traced outlines into a computer using a scanner (Hewlett Packard Scan Jet IIcx; Palo Alto, CA, USA), and assessing the area using Digitrace software (Imatec, Miesbach, Germany). Axon densities were determined by dividing the total number of axons by the total area of each tract/nerve. The results for the left and right tracts/nerves were pooled and averaged for each individual. In the results section, species averages are presented ± standard deviation.

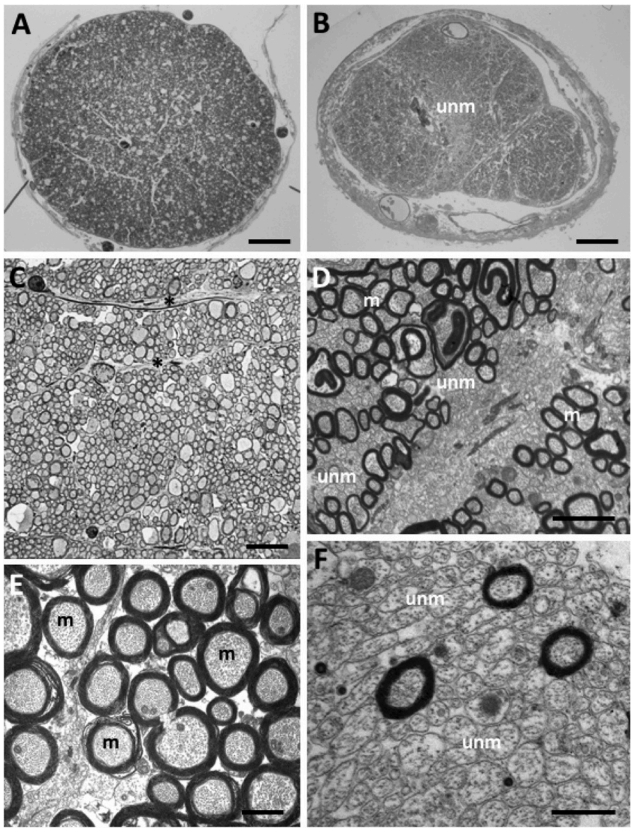

FIGURE 2 | Myelinated and unmyelinated axons in the olfactory tract and optic nerve in *Coryphaenoides armatus* and *C. profundicolus*. **(A)** Light micrograph of the profile of a region of the optic nerve of *C. profundicolus* (390 mm SL) close to the back of the eye. **(B)** Light micrograph of the profile of a region of the olfactory tract in *C. armatus* (430 mm SL) close to the telencephalon, which appears to be divided into three regions including a central region occupied by high densities of unmyelinated axons (unm). **(C)** Low power electron micrograph of the optic nerve of *C. armatus* (900 mm SL) showing dense fascicles of myelinated axons separated by astroglial processes (*). **(D)** Electron micrograph of the olfactory tract of *C. armatus* (530 mm SL) showing a mixture of both myelinated (m) and unmyelinated (unm) axons. **(E)** Electron micrograph of the optic nerve axons in *C. profundicolus* (370 mm SL) showing the high density of myelinated axons (m). **(F)** Close up of the high density of unmyelinated axons (unm) in the central region of the olfactory tract of *C. armatus* (700 mm SL). Scale bars: 0.3 mm **(A)**; 0.2 mm **(B)**; 8.0 μm **(C)**; 2.0 μm **(D)**; 1.5 μm **(E)**; 2.0 μm **(F)**.

RESULTS

In both species, the optic nerve was larger than the olfactory tract (*C. armatus*: average olfactory tract and optic nerve areas 1.97 ± 0.93 mm^2 ($n = 4$) and 2.53 ± 0.96 mm^2 ($n = 4$), respectively; *C. profundicolus*: average olfactory tract and optic nerve areas 0.61 ± 0.39 mm^2 ($n = 4$) and 1.47 ± 0.64 mm^2 ($n = 5$), respectively), and the area of both tract and nerve increased with increasing body size. Representative images of axons in the olfactory tract and optic nerve of both species are presented in **Figure 2**. Both myelinated and unmyelinated axons were identified in the olfactory tract and optic nerve in all specimens (also see **Table 1**). In general, the unmyelinated axons, which were smaller in size, were packed in higher densities than the myelinated axons (**Table 1**). There were also substantial differences in the proportion of myelinated and unmyelinated axons in the olfactory tract and the optic nerve across individuals and between species (**Table 1**; **Figure 3**; see below).

The results of the axon counts in the olfactory tract and the optic nerve in both species are presented in **Table 1**. When comparing the total number of axons across individuals and between species (**Figure 3**), the most noticeable finding is that the total number of axons in the olfactory tract in *C. armatus* far exceeds the numbers counted in the optic nerve in this species, as well as both the olfactory tract and optic nerve in *C. profundicolus*. This is particularly apparent in the larger individuals. For example, in the 900 mm SL individual *C. armatus*, the number of axons in the olfactory tract (541,338) was over four times greater than the total number of optic nerve axons (129,421), and over four and eight times greater than the total number of axons in the olfactory tract (127,600) and optic nerve (66,437), respectively, in the similarly-sized 860 mm SL individual of *C. profundicolus*. On average, the number of olfactory tract axons in *C. armatus* (338,642 ± 166,029; $n = 4$) was three and a half times greater than in the olfactory tract axons in *C. profundicolus* (93,900 ± 23,806; $n = 4$). Despite the substantial differences in the total number of axons in the olfactory tract between the two species, in both *C. armatus* and *C. profundicolus*, the total number of axons in the olfactory tract increased with SL (**Figures 3A,B**). In both species, the percentage of myelinated axons in the olfactory tract was relatively low (**Table 1**; **Figures 3A,B**), averaging 12.6 ± 7.6% ($n = 4$) of the total axon count in *C. armatus* and 11.4 ± 7.4% ($n = 4$) of the total axon count in *C. profundicolus*. However, in *C. armatus*, the percentage of total axons accounted for by myelinated axons decreased as SL increased, while the opposite was true for *C. profundicolus*.

On average, the total number of axons in the optic nerve was higher in *C. armatus* (83,443 ± 36,002; $n = 4$) compared to *C. profundicolus* (77,850 ± 14,213; $n = 5$). In *C. armatus*, the total number of axons in the optic nerve generally increased with increasing SL e.g., from 55,215 in the smallest individual to 129,421 in the largest individual (**Figure 3C**). In contrast, the total number of optic nerve axons in *C. profundicolus* declined steadily with increasing SL e.g., from 100,222 axons in the smallest individual to 66,437 axons in the largest individual (**Figure 3D**). The percentage of myelinated axons in the optic nerve in both species was similar on average *C. armatus*: 63.6 ± 5.7% ($n = 4$); *C. profundicolus*: 60.1 ± 15.6% ($n = 5$) and much greater than in the olfactory tract. However, while in *C. armatus*, the percentage remained relatively similar as SL increased (**Figure 3C**), in *C. profundicolus* the percentage of myelinated axons in the optic nerve increased as SL increased e.g., from 45.3% in the smallest individual to 85.5% in the largest individual (**Figure 3D**).

On average, the number of olfactory tract axons was greater than the number of optic nerve axons in both species (*C. armatus*: 338,642 vs. 83,443 axons; ratio: 4:1; *C. profundicolus*: 94,301 vs. 77,912 axons; ratio: 1.2:1). Given the very large numbers of axons

TABLE 1 | Density and numbers of myelinated and unmyelinated axons in the olfactory tract and optic nerve from different sized individuals of *Coryphaenoides armatus* and *C. profundicolus*.

Species	SL (mm)	Tract/ nerve	Total area of nerve (mm^2)	% of nerve area analyzed	Average axon density (axons $\mu m^2 \pm$ sd)			Numbers of axons		
					Myelinated	Unmyelinated	Total	Myelinated	Unmyelinated	Total
C. armatus	430	Olfact	1.22	2.4	0.266 ± 0.194 ($n = 30$)	0.857 ± 0.543 ($n = 30$)	1.116 ± 0.658 ($n = 30$)	32,574	104,948	136,664
		Optic	1.15	2.5	0.329 ± 0.087 ($n = 30$)	0.169 ± 0.071 ($n = 30$)	0.48 ± 0.142 ($n = 30$)	37,845	19,440	55,215
	530	Olfact	1.25	3.4	0.314 ± 0.159 ($n = 28$)	2.544 ± 2.390 ($n = 28$)	2.859 ± 2.389 ($n = 28$)	39,372	318,984	358,482
		Optic	2.76	0.3	0.197 ± 0.042 ($n = 10$)	0.147 ± 0.044 ($n = 10$)	0.344 ± 0.062 ($n = 10$)	54,298	40,517	94,816
	700	Olfact	2.23	1.8	0.106 ± 0.070 ($n = 29$)	1.301 ± 1.531 ($n = 29$)	1.427 ± 1.534 ($n = 29$)	23,628	289,996	318,082
		Optic	2.83	1.4	0.131 ± 0.046 ($n = 34$)	0.056 ± 0.028 ($n = 34$)	0.192 ± 0.061 ($n = 34$)	37,062	15,843	54,319
	900	Olfact	3.18	1.5	0.128 ± 0.087 ($n = 34$)	1.578 ± 1.910 ($n = 34$)	1.704 ± 1.910 ($n = 34$)	40,664	501,310	541,338
		Optic	3.37	0.3	0.231 ± 0.090 ($n = 10$)	0.149 ± 0.064 ($n = 10$)	0.384 ± 0.138 ($n = 10$)	77,855	50,218	129,421
C. profundicolus	250	Olfact	0.35	17.3	0.132 ± 0.094 ($n = 25$)	2.047 ± 1.938 ($n = 25$)	2.199 ± 1.941 ($n = 25$)	4,675	72,490	77,165
		Optic	1.11	0.9	0.409 ± 0.058 ($n = 10$)	0.494 ± 0.146 ($n = 10$)	0.901 ± 0.142 ($n = 10$)	45,495	54,950	100,445
	315	Olfact	0.38	6.6	0.138 ± 0.079 ($n = 26$)	1.933 ± 1.506 ($n = 26$)	2.013 ± 1.544 ($n = 26$)	5,220	73,112	78,332
		Optic	0.93	1.5	0.467 ± 0.120 ($n = 14$)	0.464 ± 0.260 ($n = 14$)	0.905 ± 0.319 ($n = 14$)	43,211	42,933	86,144
	370	Olfact	0.54	8.9	0.186 ± 0.107 ($n = 23$)	1.549 ± 1.402 ($n = 23$)	1.735 ± 1.388 ($n = 23$)	10,089	84,018	94,107
		Optic	1.10	0.9	0.369 ± 0.064 ($n = 9$)	0.244 ± 0.057 ($n = 9$)	0.634 ± 0.069 ($n = 9$)	40,768	26,958	67,726
	390	Olfact	–	–	–	–	–	–	–	–
		Optic	1.74	1.3	0.242 ± 0.068 ($n = 24$)	0.151 ± 0.089 ($n = 24$)	0.396 ± 0.115 ($n = 24$)	42,050	26,759	68,809
	860	Olfact	1.18	2.2	0.237 ± 0.146 ($n = 27$)	0.841 ± 0.473 ($n = 27$)	1.077 ± 0.545 ($n = 27$)	28,053	99,547	127,600
		Optic	2.48	1.97	0.229 ± 0.062 ($n = 32$)	0.039 ± 0.030 ($n = 32$)	0.268 ± 0.071 ($n = 32$)	56,769	9,668	66,437

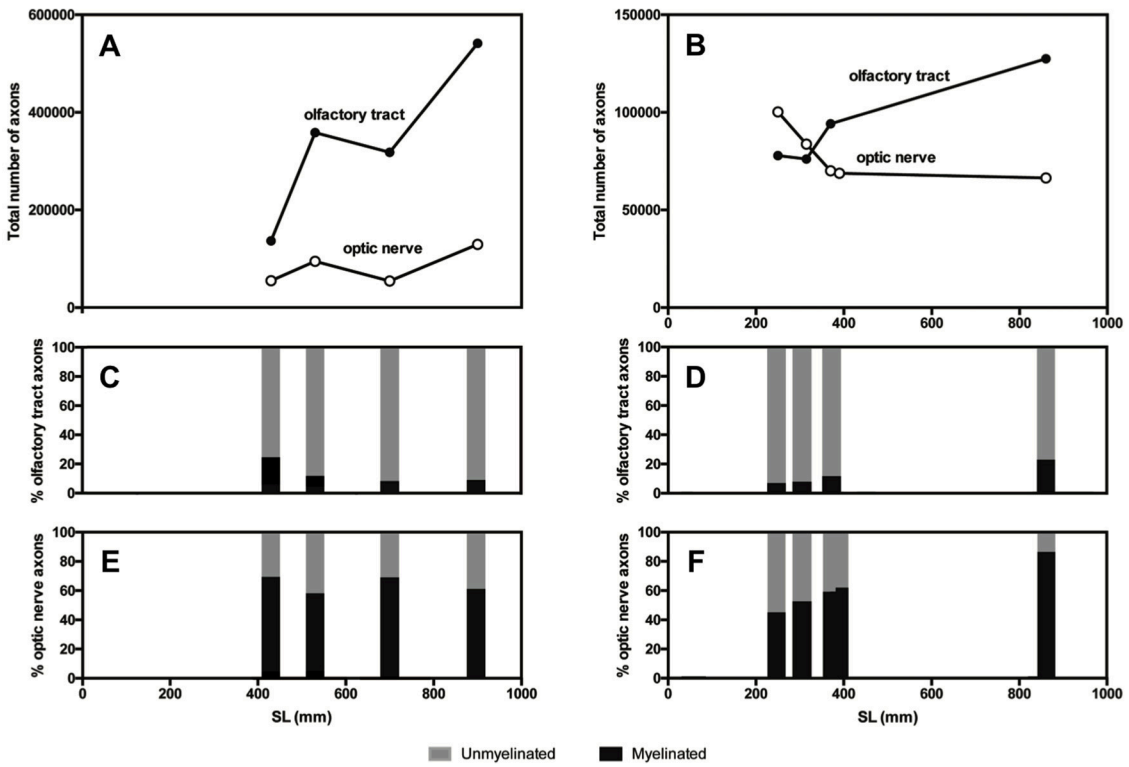

FIGURE 3 | Axons in the olfactory tract and the optic nerve in *Coryphaenoides armatus* and *C. profundicolus*. The total number of axons in the olfactory tract (filled circles) and optic nerve (open circles) from different sized individuals in the two species is presented for **(A)** *C. armatus* and **(B)** *C. profundicolus*. Ontogenetic variation in the percentage of myelinated (black) and unmyelinated (gray) axons in the olfactory tract **(C)** and optic nerve **(E)** from different-sized individual *C. armatus*, and in the olfactory tract **(D)** and optic nerve **(F)** from different-sized individual *C. profundicolus*.

in the olfactory tract in *C. armatus*, the ratios were higher in this species than in *C. profundicolus*. In both species, the ratio of olfactory tract axons to optic nerve axons generally increased with increasing SL (**Figures 3E,F**).

DISCUSSION

Very little is known about the behavior of deep-sea fishes. While it is possible to film benthopelagic and benthic deep-sea animals using baited cameras attached to landers (Bagley et al., 2004; Jamieson et al., 2013), this process is logistically complicated and expensive. Moreover, bringing deep-sea animals to the surface alive is extremely difficult (Bagley et al., 2004; Drazen et al., 2005) making behavioral observations in aquaria all but impossible. Like all animals, deep-sea fishes are reliant on their sensory systems to gather information about their environment and to guide their behavior. Therefore, neuroanatomical studies of the peripheral sense organs and their afferent-recipient sites in the brain have proved a particularly useful approach for making deductions about their sensory capabilities, sensory orientation (i.e., which senses may be of particular overall importance), and behavior (Marshall, 1979; Wagner, 2001a,b). In abyssal food webs, grenadiers, such as the two species of *Coryphaenoides* studied here, occupy the top trophic positions (Drazen et al., 2008; Lee et al., 2008; Gerringer et al., 2017), and so knowledge regarding the sensory biology of these fishes is critical to understanding the biology of these ecologically important animals (Bailey et al., 2007).

Ontogenetic Shifts in Sensory Capability in Coryphaenoides

Previous work by Wagner (2001a, 2002, 2003) on the anatomy of the central nervous system in *C. armatus* resulted in two important conclusions: (1). As adults, the olfactory bulbs are relatively large, indicating that this species is an olfactory specialist, and (2). *C. armatus* undergoes an ontogenetic shift in brain morphology, whereby the relative size of the optic tectum decreases and the relative size of the olfactory bulbs increases as body size increases. This suggests that *C. armatus* undergoes a shift in sensory capability (from vision to olfaction) during ontogeny. Our study supports these findings. Across all of the different-sized individuals we studied, the number of olfactory tract axons was, on average, over four times greater than the number of axons in the optic nerve. Moreover, the ratio of

olfactory tract axons to optic nerve axons increased as body size increased. In other words, the level of olfactory input exceeds the level of visual input to the brain in *C. armatus*, and this difference in olfactory vs. visual input increases ontogenetically. This neuroanatomical evidence for an ontogenetic shift in sensory capability appears to be closely associated with an an ontogenetic shift in diet and feeding behavior. As described previously, small *C. armatus* feed predominantly on epibenthic and benthic invertebrates (Haedrich and Henderson, 1974; Martin and Christiansen, 1997), which they take from or close to the bottom, or dig for in the soft, muddy substrate using their elongated rostrums (McLellan, 1977). Based on our results and those of Wagner (2003), we predict that vision may be more important than olfaction for the identification of these prey, especially epibenthic invertebrates that live on or just above the substrate. As the animals get larger, there is a shift away from benthic invertebrate prey and larger, more pelagic prey, such as cephalopods and fishes become more important. While some of these prey may be actively predated, scavenging on food-falls is particularly important source of food for larger *C. armatus* (Haedrich and Henderson, 1974; Mauchline and Gordon, 1991; Martin and Christiansen, 1997; Kemp et al., 2006). Larger *C. armatus* are also found at deeper depths, a trend seen in other scavenging deep-sea demersal fish (Merrett and Haedrich, 1997; Collins et al., 2005; King et al., 2006). This may be because a larger body size permits, for example, higher swimming speeds, larger energy reserves (and thus greater endurance), and a lower mass-specific metabolic rate (Collins et al., 2005). These factors would give larger individuals a better chance of surviving from meal to meal, allowing them to exploit deep-sea food resources, which although sparse and randomly distributed, tend to be large in size, such as the carcass of a whale or large elasmobranch (Higgs et al., 2014). In contrast, smaller *C. armatus* may occupy a different niche because until they reach a threshold minimum size they cannot compete with their larger conspecifics (Collins et al., 2005). Olfaction appears to become far more important in these larger animals, and their well-developed olfactory system likely helps these fishes to locate sparsely distributed food-falls in the abyss. Indeed, as previously noted, large *C. armatus* are often the first fish to appear at baited cameras (Wilson and Smith, 1984; Priede et al., 1990, 1994; Armstrong et al., 1992; Henriques et al., 2002; Wagner, 2003; Collins et al., 2005; Kemp et al., 2006; King et al., 2006). Wagner's (2003) results indicate that the ontogenetic shift in sensory capability in *C. armatus* occurs at a body size of between 400 and 500 mm SL, which is in agreement with the size range over which the shift in diet occurs in this species (Haedrich and Henderson, 1974; Martin and Christiansen, 1997). Unfortunately, we were not able to perform olfactory tract and optic nerve counts in any individuals smaller than 430 mm SL, but we predict that in smaller animals both the numbers of axons in the olfactory tract and optic nerve, and the ratio of olfactory tract axons to optic nerve axons, will be lower than the values presented for the 430 mm individual presented here.

Our results for *C. profundicolus* suggest that, in adults, olfaction is also important, although probably not to the same extent as in *C. armatus*. Wagner (2001a, 2002) found that, in comparison to other deep-sea fishes, the brain from an 890 mm SL *C. profundicolus* had relatively enlarged olfactory bulbs, and also an enlarged gustatory area, suggesting that chemoreception (olfaction and taste) is important to this species. Our data indicate that, like *C. armatus*, *C. profundicolus* also undergoes an ontogenetic shift in sensory orientation, with olfaction becoming relatively more important than vision in larger animals. Based on our axon counts, this shift appears to occur at a body size between 315 and 370 mm SL. However, the differences in olfactory vs. visual input as assessed by comparing the total number of axons, were not as great as those seen in *C. armatus*, as illustrated by the relatively low ratios of olfactory tract axons to optic nerve axons in *C. profundicolus*. A comparison of the brains of the two species illustrated in **Figure 1** suggests that the optic tectum is relatively larger in *C. profundicolus* compared to *C. armatus*, which implies that vision may be relatively more important in the former. Unfortunately, no information is currently available on the brain morphology of different sized individuals of *C. profundicolus*, so it not known whether this species exhibits an ontogenetic shift in the relative size of the olfactory bulbs and optic tectum, such as that found in *C. armatus*. Moreover, little is known about the biology of *C. profundicolus* in general, especially in comparison to *C. armatus*. Dietary studies in *C. profundicolus* have not identified an ontogenetic shift (Denda et al., 2017), so it is not possible to establish whether the apparent ontogenetic shift from vision to olfaction identified here is correlated with a change in feeding strategy or behavior in this species.

Comparison of Olfactory Tract and Optic Nerve Axon Numbers With Other Species

Little work has been performed to quantify the numbers of axons in the olfactory tract in vertebrates using TEM, so it is difficult to make comparisons between our findings for *C. armatus* and *C. profundicolus* and other species. However, the average number of axons in both species is greater than the number reported for the crucian carp (*Carassius carassius*) (73,246; Westerman and Wilson, 1968). In another freshwater fish, the burbot (*Lota lota*), Döving and Gemne (1965) found that the number of myelinated axons was around 10,000, although these authors did not estimate the number of unmyelinated axons in *L. lota*. Since Westerman and Wilson (1968) found that 89.5% of the olfactory tract axons in *C. carassius* are unmyelinated, if similar proportions are assumed for *L. lota*, the total number of olfactory tract axons in this species could be around 100,000. These values for *C. carassius* and *L. lota* are over five times and three times lower than the highest and the average numbers of total olfactory tract axons that we counted in *Coryphaenoides armatus*, respectively. This supports Wagner's (2001a, 2002, 2003) designation of *C. armatus* as an olfactory specialist. In contrast, the numbers of axons in the olfactory tract in *C. profundicolus* are more similar to those for *Carassius carassius* and *L. lota*, further indicating that the olfactory system in *C. profundicolus* is not as well-developed as in *C. armatus*.

In contrast to the paucity of comparative information on axon numbers in the olfactory tract, quantitative analyses of

the axons in the optic nerve using TEM have been made for a number of vertebrates (Brooks et al., 1999), including fishes. The total axon counts for both *C. armatus* and *C. profundicolus* are lower than those reported for some shallow water fishes, such as the striped mojarra (*Eugerres plumieri*) (200,000 axons; Tapp, 1974) and the goldfish (*Carassius auratus*) (180,000 axons; Easter et al., 1981). This suggests that vision may be relatively less important in the two species of grenadiers studied here compared to *E. plumieri* and *C. auratus*. However, the values we report here are more similar to those reported for minnows (Cyprinidae) (45,000–106,600; Huber and Rylander, 1992), the sandlance (*Limnichthyes fasciatus*) (104,452 axons; Collin and Collin, 1988) and the Australian lungfish (*Neoceratodus forsteri*) (74,100 axons; Bailes et al., 2006). Of these species, those with the lowest number of axons are fishes, like deep-sea grenadiers, that live in light-restricted habitats, such as minnows that live in turbid rivers (45,000–60,800 axons; Huber and Rylander, 1992) and *N. forsteri*. In dim environments, where the amount of light available for vision is limited, visual sensitivity is more important than visual acuity, and animals that live in such environments tend to have relatively low numbers of retinal ganglion cells and therefore retinal ganglion cell axons in the optic nerve. This relationship is consistent across vertebrates and similar examples can be found by comparing axon number in the optic nerve of diurnal and nocturnal birds, e.g., pigeons (*Columbia livia*) have 2.4 million axons (Binggelli and Paule, 1969) while barn owls (*Tyto alba*) have 680,000 axons (Wathey and Pettigrew, 1989) and the optic nerve of primates e.g., tufted capuchins (*Cebus apella*) have 1.09 million axons while Azara's night monkeys (*Aotus azarae*) have 480,000 axons (Finlay et al., 2008).

Ontogenetic Changes in Optic Nerve Axon Numbers

In both *Coryphaenoides armatus* and *C. profundicolus*, the total number of axons in the olfactory tract increased with body size. In *C. armatus*, this was also true for the total number of axons in the optic nerve. A similar situation has been shown to occur in the optic nerve in *Carassius auratus* and *N. forsteri* (Easter et al., 1981; Bailes et al., 2006) and also in the optic nerves of amphibians (Dunlop and Beazley, 1981, 1984). Like fishes, the central nervous system of amphibians exhibits intermediate growth and undergoes widespread and lifelong neurogenesis (Kaslin et al., 2008). It is also well-documented that the retina and the optic tectum grow continuously throughout life in both fishes and amphibians, through the addition of new neurons and tissue stretching (Straznicky and Gaze, 1972; Johns and Easter, 1977; Dunlop and Beazley, 1981, 1984; Easter et al., 1981; Raymond and Easter, 1983; Bakken and Stevens, 2012). Thus, the finding that there is a progressive decrease in the number of optic nerve axons in *Coryphaenoides profundicolus* is unexpected. To the best of our knowledge, this is the first example of a fish in which the optic nerve axons, and therefore their associated retinal ganglion cells, are either not being continually generated throughout life or the retina is undergoing appreciable periods of cell death and therefore axonal loss. Future work on visual development will hopefully confirm the mechanism of this findings in other models known to undergo continual retinal growth, such as the goldfish *Carassius auratus*, the zebrafish *Danio rerio*, the black bream *Acanthopagrus butcheri* (Shand et al., 2000), the bamboo shark *Chiloscyllium punctatum* (Harahush et al., 2014), deep-sea viperfish (*Chauliodus sloani*; Locket, 1980; Fröhlich and Wagner, 1998) and the lungfish *N. forsteri* (Bailes et al., 2006).

Proportions of Myelinated and Unmyelinated Axons

The percentage of myelinated axons in the olfactory tract was relatively low (around 11–12% on average) in *Coryphaenoides armatus* and *C. profundicolus*. Although little information exists on the axonal composition of the olfactory tract in fishes, our values correspond well with those of Westerman and Wilson (1968), who found that 10.5% of the olfactory tract axons in *Carassius carassius* are myelinated.

Regarding the optic nerve, we found that, on average, ∼60% of the axons were myelinated in both *Coryphaenoides. armatus* and *C. profundicolus*. These values are considerably lower than the values reported for some other fishes, such as *E. plumieri* and *Carassius auratus* (≥96% of axons are myelinated; Tapp, 1974; Easter et al., 1981), and *L. fasciatus* (74% of axons are myelinated; Collin and Collin, 1988). However, in another genus of deep-sea fish, *Conocara* sp., only ∼25% of the axons were found to be myelinated (Collin et al., 2000). In *Coryphaenoides armatus*, the percentage of myelinated axons was relatively similar across the different sized individuals, but in *C. profundicolus*, the percentage of myelinated axons increased progressively as body size increased. A similar situation has been described in *N. forsteri*, in which Bailes et al. (2006) found 17% of the optic nerve axons in a small individual to be myelinated, in contrast to 74% in a much larger fish. In some frogs, the proportion of myelinated axons also increases with age, but to a much smaller degree. For example, in moaning frogs (*Heleioporus eyeri*) and African clawed frogs (*Xenopus laevis*), the percentage of myelinated axons increases from 0.3 and 1.5% at metamorphic climax to 2.5 and 11% in adults, respectively (Dunlop and Beazley, 1981, 1984). It is known that the number of myelinated axons can vary along the length of the optic nerve (Cima and Grant, 1982; Playford and Dunlop, 1993), but we consider it highly unlikely that this accounts for the ontogenetic differences we report here, because all of our optic nerve samples were taken from the same location, just behind the eye, in each individual. Overall, it appears that in fishes, the proportions of myelinated and unmyelinated axons in the optic nerve can be highly variable, both ontogenetically within a species and among species. The reasons for this are unclear, but as myelin acts as an electrical insulator and serves to increase the velocity of propagation of nerve impulses along axons, the functional implications are that the speed at which visual information is relayed to the brain may vary among species and/or different size/age classes within a species, which in turn could influence behavioral reaction speeds (Bailey et al., 2007). Alternatively, as fish grow and the optic nerve gets longer (meaning that neural signals must travel increasing distances to reach the brain), increased levels of myelination

could serve to maintain relatively constant conduction velocities (Bakken and Stevens, 2012). Interestingly, in *C. profundicolus*, while the total number of optic nerve axons decreases with body size, the degree of myelination increases with growth, perhaps as some form of compensatory function to maintain or increase the conduction velocity of neural signals along the optic nerve.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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