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

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 $82. \pm 22.0$ 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.*
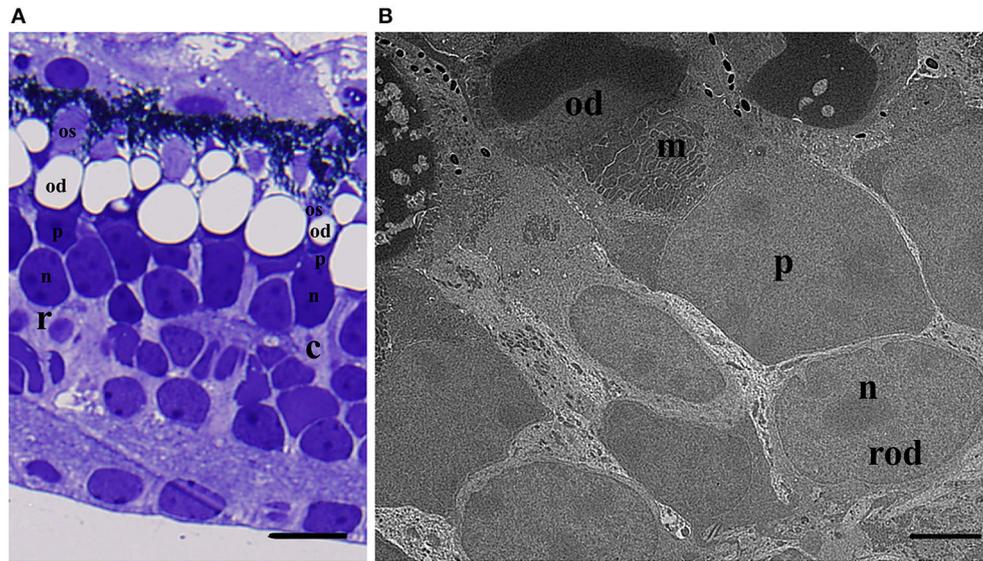
FIGURE 1 | Morphological characteristics of rod and cone photoreceptors in adult *Lepidosiren paradoxa*. (A) Light micrograph of retina in transverse section showing the rod (r) and cone (c) photoreceptor. The rod outer segment (os) is cylindrical, as opposed to the conical, taped cone outer segment. 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**).
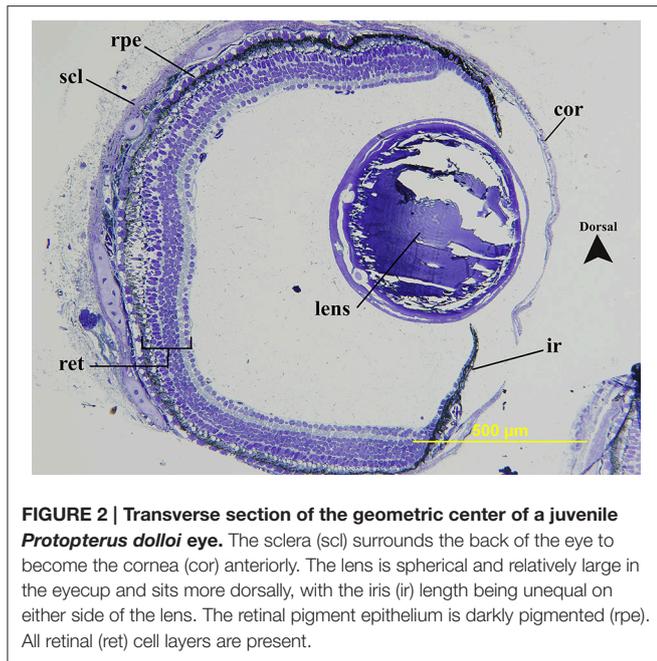
The red cone contains a large red oil droplet within the ellipsoid of the inner segment (**Figure 4B**). The outer segment is conical in shape, with a basal diameter of 4.7 ± 0.8 μm (*n* = 15) and length of 6.4 ± 0.7 μm (*n* = 14). The diameter of the ellipsoid is 7.4 ± 1.8 μm (*n* = 14) and is filled 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
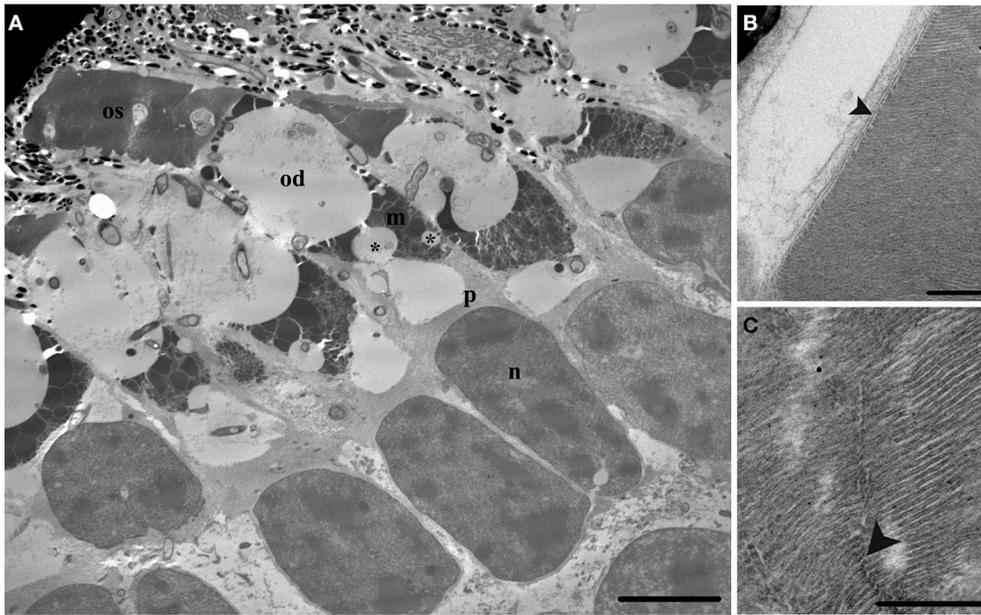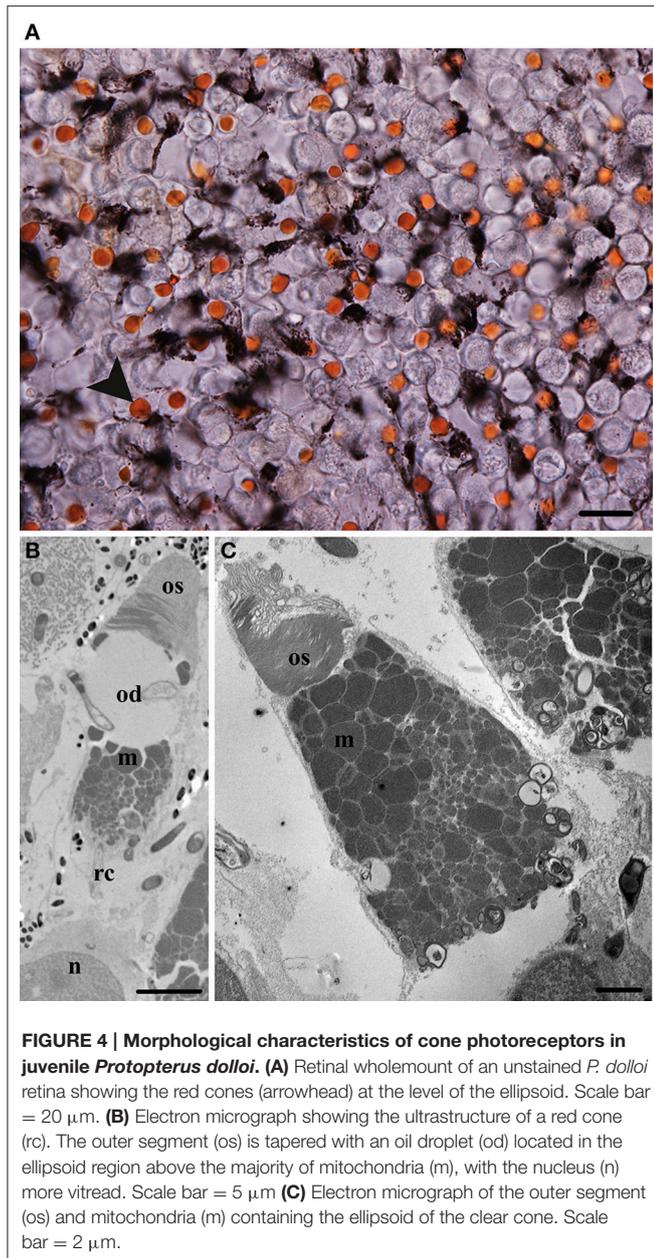
FIGURE 3 | Transmission electron micrographs detailing the ultrastructure of a juvenile *Protopterus dolloi* rod photoreceptor. (A) The rod (rd) is large with one large oil droplet (od) situated within the mitochondria (m) of the ellipsoid, with smaller oil droplets (*) in the mitochondria, closer to 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.
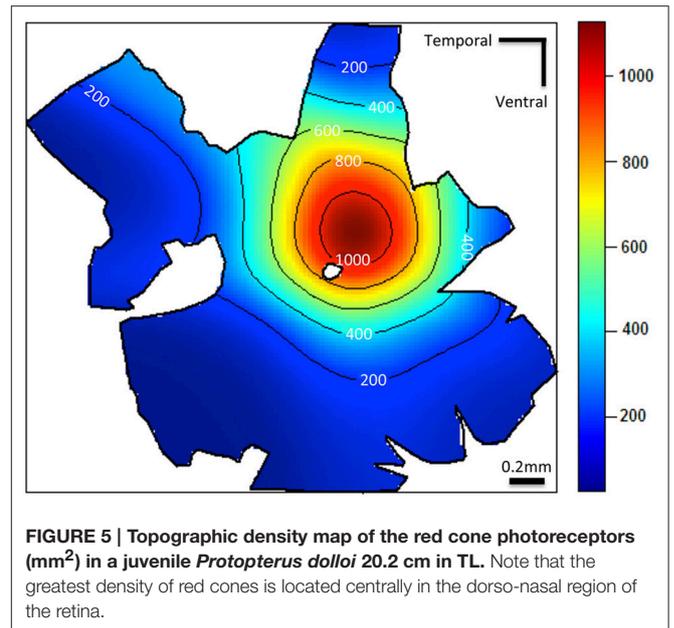
(1968). However, our investigation did not reveal double cones. Pfeiffer (1968) described the retina of *P. dolloi* in paraffin wax sections, and reported single cones with oil droplets, and double cones with only one member containing an oil droplet. Our investigation of *P. dolloi* photoreceptors did 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.

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

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).

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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