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RESEARCH ARTICLE

Microhabitat partitioning correlates with opsin gene expression in coral reef cardinalfishes (Apogonidae)

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Abstract

1. Fish are the most diverse vertebrate group, and they have evolved equally diverse visual systems, varying in terms of eye morphology, number and distribution of spectrally distinct photoreceptor types, visual opsin genes and opsin gene expression levels.
2. This variation is mainly due to adaptations driven by two factors: differences in the light environments and behavioural tasks. However, while the effects of large-scale habitat differences are well described, it is less clear whether visual systems also adapt to differences in environmental light at the microhabitat level.
3. To address this, we assessed the relationship between microhabitat use and visual system features in fishes inhabiting coral reefs, where habitat partitioning is particularly common.
4. We suggest that differences in microhabitat use by cardinalfishes (Apogonidae) drive morphological and molecular adaptations in their visual systems. To test this, we investigated diurnal microhabitat use in 17 cardinalfish species and assessed whether this correlated with differences in visual opsin gene expression and eye morphology.
5. We found that cardinalfishes display six types of microhabitat partitioning behaviours during the day, ranging from specialists found exclusively in the water column to species that are always hidden inside the reef matrix.
6. Species predominantly found in exposed microhabitats had higher expression of the short-wavelength-sensitive violet opsin (*SWS2B*) and lower expression of the dim-light active rod opsin (*RH1*). Species of intermediate exposure, on the other hand, expressed opsins that are mostly sensitive to the blue-green central part of the light spectrum (*SWS2A*s and *RH2*s), while fishes entirely hidden in the reef substrate had a higher expression of the long-wavelength-sensitive red opsin.
7. We also found that eye size relative to body size differed between cardinalfish species, and relative eye size decreased with an increase in habitat exposure.
8. Retinal topography did not show co-adaptation with microhabitat use, but data suggested co-adaptation with feeding mode.
9. We suggest that, although most cardinalfishes are nocturnal foragers, their visual systems—and possibly those of other (reef) fishes—have also adapted to the light intensity and the light spectrum of their preferred diurnal microhabitats.

KEYWORDS
eye size, fish, *LWS*, microhabitat partitioning, opsin gene expression, retinal topography, *SWS2*, vertebrate visual system evolution

1 | INTRODUCTION

Animal visual systems are functionally diverse, with differences at the morphological and the molecular level. In fish, this diversity is mainly driven by differences in the availability of light (Hauser & Chang, 2017; Land & Nilsson, 2002), but can also be due to differences in habitat complexity (Collin & Shand, 2003; Hughes, 1977) or specific behavioural tasks, for example, foraging or sexual selection (reviewed in Hauser & Chang, 2017; Price, 2017). In aquatic environments, differences in light mainly arise from wavelength selective light absorption and scattering due to depth, the size of suspended particles, and the reflectance of such particles or the substrate (Lythgoe, 1979). For example, the deep-sea has a blue-shifted light environment and consequently, deep-sea species generally possess photoreceptors that are maximally sensitive to blue light (~480 nm; Partridge, Archer, & Vanoostrum, 1992).

Morphologically, visual systems may differ in eye size, shape, and at the retinal level in functional type, number and/or distribution of neural cells including photoreceptors. Morphological changes to boost sensitivity in low-light conditions, for example, may include rod-dominated retinas, increased relative eye size or a higher photoreceptor-to-ganglion cell summation ratio (de Busserolles & Marshall, 2017; Kelber & Roth, 2006; Warrant, 2004).

At the molecular level, the part of the electromagnetic spectrum to which a photoreceptor is maximally sensitive (λ_{max}) may vary, primarily due to changes in its photopigments (Hunt & Collin, 2014). Photopigments are molecules comprised of opsins—membrane-bound proteins with receptor function—to which a vitamin A-derived chromophore is covalently bound. While in vertebrates only two chromophore types (vitamin A1 or A2) occur, opsins are more variable. They are classified according to their λ_{max} values, their phylogeny and their specificity to morphologically distinct photoreceptor types (Hunt & Collin, 2014). Opsins may alter photoreceptor λ_{max} via (a) variations in their amino acid sequences or (b) via differential expression of the different opsin genes (reviewed in Carleton, Dalton, Escobar-Camacho, & Nandamuri, 2016). In vertebrates, five opsin classes are found: rod-specific rhodopsin (*RH1*) used for scotopic vision, and four cone-specific classes used for photopic and colour vision: short-wavelength-sensitive 1 (*SWS1*), ultraviolet; short-wavelength-sensitive 2 (*SWS2*), violet/blue; rhodopsin-like 2 (*RH2*), blue-green/green and long-wavelength-sensitive (*LWS*), yellow/red; Hunt & Collin, 2014). In percomorph fishes, gene duplication has resulted in a more diversified repertoire consisting of rod-specific *RH1*, single cone-specific *SWS1*, *SWS2B* (violet), *SWS2A*α and *SWS2A*β (blue), and double cone-specific *RH2B* (blue-green), *RH2A* (green) and *LWS* (Cortesi et al., 2015; Hunt & Collin, 2014).

In fish, visual systems adapt to large-scale lighting differences due to habitat depth, type (e.g. reef vs. open ocean) or season (Lythgoe, 1979; Lythgoe, Muntz, Partridge, Shand, & Williams, 1994; Muntz, 1982). However, fish vision may also be tuned to light differences between habitats on smaller scales for species sharing the same general habitat at similar depths. For example, in some African cichlids, opsin gene expression differs depending on the associated substrate (e.g. rock vs. sand; Sabbah et al., 2011). The photoreceptor spectral sensitivities of surfperch living among California's kelp forests are tuned to light in structurally distinct parts of that general habitat (e.g. canopied vs. not-canopied; Cummings & Partridge, 2001). However, although suggested (Lythgoe, 1979; Marshall, Jennings, McFarland, Loew, & Losey, 2003), it remains to be tested whether this phenomenon may be acting on an even smaller—microhabitat—scale, and thus contributing to the remarkable diversification of colour vision among fishes living on coral reefs, one of the most diverse ecosystems on earth, where species-based habitat partitioning, at times within a single coral head, is particularly common (reviewed in Williams, 1991).

Here, to control for potentially confounding factors like phylogenetic constraint, we focused on a group of closely related reef fishes with remarkable visual system diversity, the cardinalfishes (Apogonidae; Fishelson, Ayalon, Zverdling, & Holzman, 2004; Luehrmann, Carleton, Cortesi, Cheney, & Marshall, 2019). These fishes are common on shallow tropical coral reefs, are one of the most abundant reef fish families and are predominantly nocturnal foragers (Marnane & Bellwood, 2002). However, during the day, they aggregate in large multi-species groups in and around the reef matrix, in particular caves and branching corals (e.g. *Porites cylindrica*; Gardiner & Jones, 2005; Greenfield & Johnson, 1990), where they carry out social behaviours, such as pair formation and mating (reviewed in Vagelli, 2011). A previous survey of seven species found that in these multi-species aggregations fish display fine-scale microhabitat partitioning among the same diurnal refuge sites, with some species found predominantly outside, and others within or below reef structures (Gardiner, 2010).

To test whether their visual system is related to microhabitat use, we compared the microhabitat partitioning behaviour of 17 cardinalfish species to morphological and molecular differences in their visual systems. First, we conducted an ecological assessment of habitat partitioning in these focal species. Second, we tested whether their opsin gene expression, and/or relative eye size—as a proxy for light sensitivity (Land, 1990) — correlated with diurnal microhabitat use. We used our previous opsin expression data which showed that cardinalfishes express multiple visual opsins, and that based on differences in opsin gene expression and spectral sensitivities, species could be placed into five, possibly functionally distinct, groups (Luehrmann et al., 2019). Third, the retinal photoreceptor and/or ganglion cell topographies of

five cardinalfish species from different microhabitats were determined to gain additional insight into these fishes' adaptations to their environment. Finally, visual system diversity was also tested for correlation with cardinalfish feeding ecology and activity period.

2 | MATERIALS AND METHODS

2.1 | Microhabitat use assessment

Underwater visual surveys were conducted on SCUBA to determine microhabitat use of 23 cardinalfish species (Table 1) on reefs surrounding Lizard Island (14°40′S, 145°28′E), Great Barrier Reef, Australia (Figure 1). Fish counts were conducted between 6.30 a.m. and 4 p.m., from 3 to 14 March 2017. Data for *Apogonichthyoides melas* and *Pterapogon cf. mirifica* were taken from counts between 10 February and 20 April 2015, as these species were not found during counts in 2017. Counts were performed as spot counts at 111 sites distributed over eight different locations at depths between 1 and 6 m, with a site defined as a separate coral head, outcrop or boulder located >5 m apart (Figure 1b). When a site was encountered, we approached slowly and waited for several minutes to ensure fish behaviour was not disturbed. Then, we recorded individual animal numbers to 20 and estimated larger groups to the nearest 50. We avoided double counting of sites by navigating around the locations systematically. We counted fish in four distinct microhabitat partitions defined as per Figure 1c. For microhabitat partitioning analysis, we only used species for which at least 10 individuals were counted at >3 different sites, and then calculated the frequency of occurrence at each microhabitat partition as a proportion of total individuals counted per species (Figure S1; Table 1). To identify patterns of microhabitat use, we then used hierarchical cluster analysis (Ward.D2, bootstrap = 100, R-package: PVCLUST, R Core Team, 2014; Suzuki & Shimodaira, 2006; Figure 2).

TABLE 1 Summary of cardinalfish species sampled in microhabitat assessment

Species	Individuals (*n*)	Sites (*N*)
Apogon crassiceps[a]	3	3
Apogonichthyoides melas[a]	2	2
Cheilodipterus artus	227	19
C. macrodon	18	7
C. quinquelineatus	1,800	57
Fibramia thermalis	65	3
Nectamia fusca	35	3
N. savayensis	50	6
N. viria[a]	10	1
Ostorhinchus compressus	104	14
O. cookii	44	11
O. cyanosoma	2,247	50
O. doederleini	235	24
O. nigrofasciatus	52	29
O. novemfasciatus[a]	3	2
Pristiapogon exostigma	44	11
P. kallopterus[a]	9	3
Pterapogon cf. mirifica[a]	2	2
Rhabdamia gracilis	1,410	6
Taeniamia fucata	2,811	13
T. zosterophora	75	3
Zoramia viridiventer	4,835	15
Z. leptacantha	510	6

Note: n = total individuals counted across all sampling sites and locations. *N* = number of sampling sites at which each species was found.

[a]Omitted from microhabitat partitioning analysis.

2.2 | Relative eye size

Cardinalfish species used for anatomical studies (*n* = 24, Table S1) were either collected on the reefs surrounding Lizard Island between February 2015 and April 2017, or obtained through an aquarium supplier (Cairns Marine Pty Ltd). Fishes from Lizard Island were collected on SCUBA or snorkel using clove oil, hand nets and barrier nets, under the following permits: Great Barrier Reef Marine Park Authority (GBRMPA) Permit G12/35005.1, GBRMPA Limited Impact Permit UQ006/2014 and Queensland General Fisheries Permit 140763. After collection, animals were returned to the laboratory and anaesthetized using a clove oil solution (10% clove oil, 40% ethanol, 50% sea water) before being euthanized by decapitation.

The standard length (SL) of each individual was measured, eyes were removed from the socket and the horizontal eye diameter was measured to the nearest 0.1 mm using callipers. After removal of the cornea, the lens was extracted and its diameter measured (Table S1). Species were identified based on morphology and colouration and, where possible, subsequently confirmed via RNA sequencing and by cross-referencing cytochrome c oxidase subunit 1 gene (*COI*) sequences to public databases (boldsystems.org; Luehrmann et al., 2019).

Analyses were performed using base R (R Core Team, 2014) and the CAPER package (Orme, 2013). Relative eye size, lens diameter, eye diameter and SL were $\log_{10}$ transformed. As the ratio between lens diameter and eye diameter was highly proportionate (phylogenetic least squares regression [PGLS], $F_{1,21} = 367.9$, $r^2 = .94$, $p < .001$; Figure 3b), further comparative analyses were performed on eye diameters only. Relative eye size was calculated as the ratio of $\log_{10}$ eye diameter to $\log_{10}$ SL. As data were non-normally distributed (Shapiro–Wilk, $w = 0.981$, $p = .003$), analysis of variance for the entire dataset was performed using a Kruskal–Wallis test. Differences between cardinalfish relative eye sizes on the genus level were identified through post-hoc pairwise comparisons (Dunn test; Dunn, 1961). To account for multiple comparisons, *p* values were adjusted using Benjamini-Hochberg corrections (Table S2). Genera for which three or fewer individuals were measured were omitted from the analysis (Table S1).

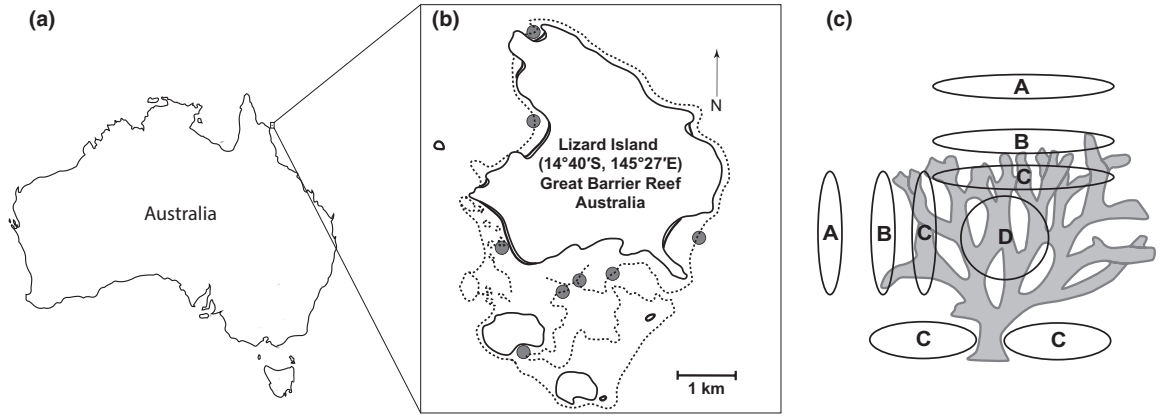

FIGURE 1 Sampling location and microhabitat assessment. (a) Location of the field site relative to the Australian mainland. (b) Overview of the sampling locations (grey circles) around Lizard Island, Queensland, Australia. (c) Schematic of microhabitat classification used for partitioning assessments. Microhabitat A: fully exposed, 1–2 m above or next to structure (coral head, coral outcrop, boulder); microhabitat B: fully exposed, directly above or adjacent to structure; microhabitat C: semi-hidden (visible from outside), between coral branches, in crevices, under overhangs or ledges; microhabitat D: entirely hidden (not visible from outside), inside coral outcrops, deep inside crevice/cave structures

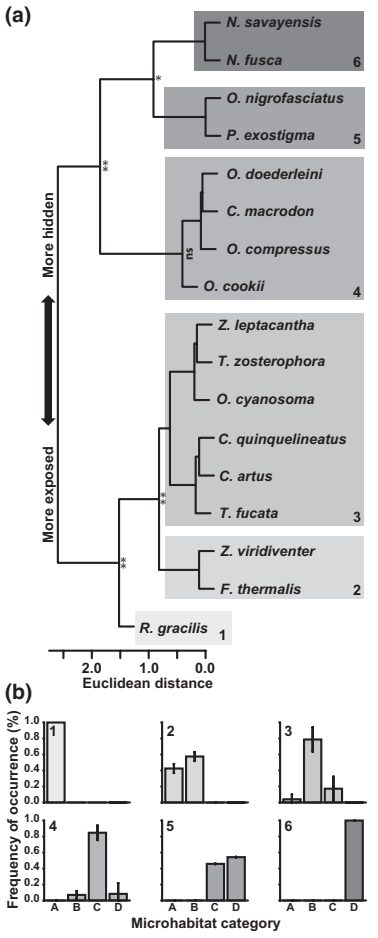

FIGURE 2 (a) Clustering (1–6) of microhabitat counts across the four different microhabitat categories (A–D) using a Ward. D2 cluster analysis. Different microhabitat partition clusters are indicated by numbers 1–6. Significance levels of bootstrap analysis are designated by: $^{**}p < .01$, $^{*}p < .05$. (b) Mean (±*SD*) microhabitat (A–D: A, fully exposed away from structure; B, fully exposed adjacent to structure; C, semi-hidden; D, entirely hidden) distribution of species comprised in each identified cluster (1–6)

2.3 | Retinal cell topography

In five cardinalfish species (see Figure S3), enucleated eyecups were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline at room temperature for at least 24 hr, then stored at 4°C. Retinal whole mounts were prepared following the methods outlined in Coimbra, Marceliano, Andrade-da-Costa, and Yamada (2006) and Ullmann, Moore, Temple, Fernández-Juricic, and Collin (2012). Few individuals were investigated in this study due to the challenge in processing such small eyes using this method. However, previous studies (de Busserolles, Fitzpatrick, Marshall, & Collin, 2014; de Busserolles, Marshall, & Collin, 2014) have shown a low intraspecific variability in retinal topography in fishes, and the analysis of two individuals of *Ostorhinchus doederleini* (Figure S2) suggests low intraspecific variability in cardinalfishes also. Using the stereological software StereoInvestigator (Microbrightfield), topographic distribution of photoreceptors and ganglion cells was assessed using the optical fractionator technique designed by West, Slomianka, and Gundersen (1991) and modified by Coimbra, Nolan, Collin, and Hart (2012). The counting frame and grid sizes were carefully chosen to maintain the highest level of sampling and achieve an acceptable Schaeffer's coefficient of error (CE < 0.1; Glaser & Wilson, 1998; Slomianka & West, 2005) following the sampling protocols described in de Busserolles, Marshall, et al. (2014) and de Busserolles, Fitzpatrick, et al. (2014; see Table S3 for a summary of counting parameters). For ganglion cell analysis, displaced amacrine cells were included in the counts as they were difficult to distinguish from ganglion cells based on morphological criteria alone. The inclusion of amacrine cells in the analysis has previously been shown not to influence the overall topography of fish retinae (e.g. Collin & Pettigrew, 1988c). Topographic maps were constructed using R v3.1.2 (R Core Team, 2014) with the results exported from StereoInvestigator according to Garza-Gisholt, Hemmi, Hart, and Collin (2014). The upper limit spatial resolving power (SRP), expressed in cycles per degree (cpd), was estimated for each

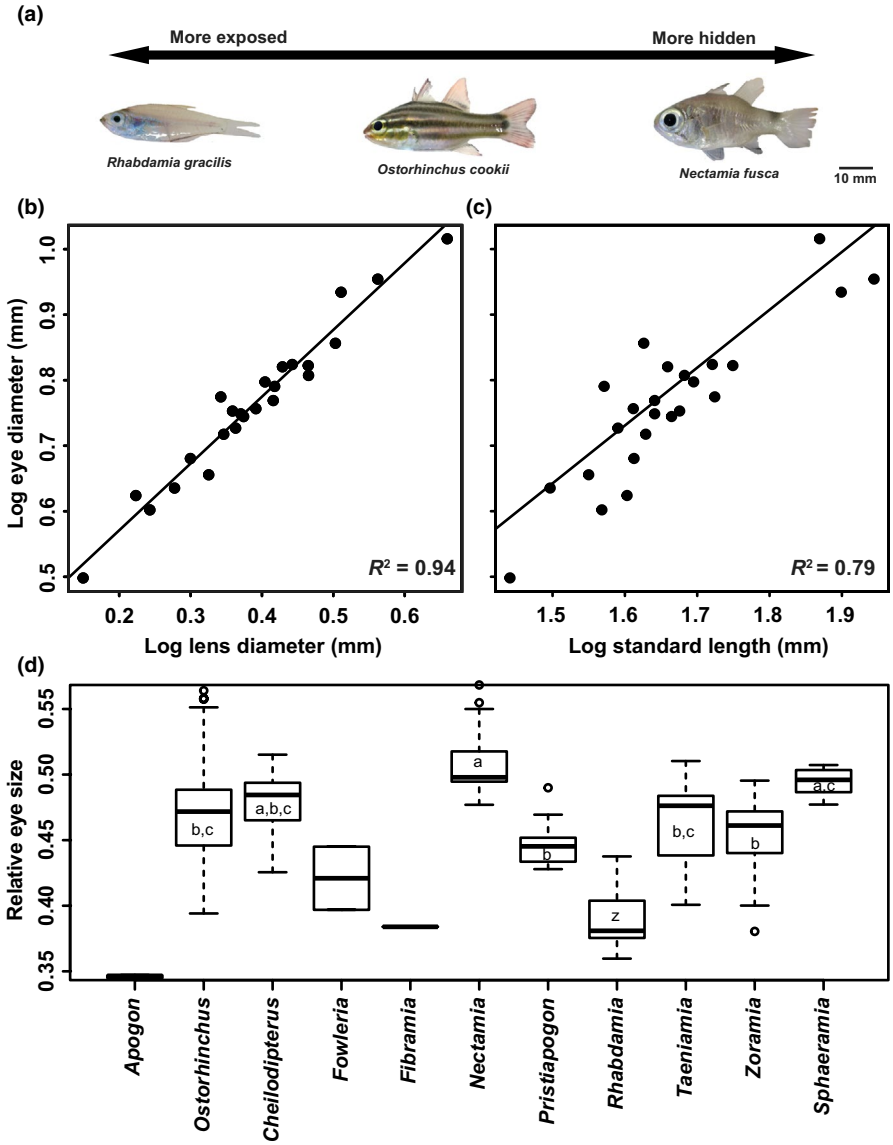

FIGURE 3 Differences in eye size relative to body length in cardinalfishes. (a) Different relative eye size in three cardinalfish species, from left to right: Luminous cardinalfish (*Rhabdamia gracilis*); Cook's cardinalfish (*Ostorhinchus cookii*); Sava Cardinalfish (*Nectamia savayensis*). Relationships of (b) horizontal eye diameter and lens diameter, and (c) eye diameter and standard length. Fitted lines represent the phylogenetically corrected linear regressions using phylogenetic comparative analysis. (d) Comparison of relative eye size by genus as per Mabuchi, Fraser, Song, Azuma, and Nishida (2014). Different letters indicate significant differences based on Dunn's post-hoc tests. Genera without letters were excluded from the analysis (see Table S1)

individual using the ganglion cell peak density as described by Collin and Pettigrew (1989). Note that, since amacrine cells were included in the ganglion cell counts, SRPs will be slightly overestimated.

2.4 | Opsin gene expression, activity patterns and foraging mode

We used proportional opsin gene expression data from our previous work on 26 cardinalfish species collected from the same locations (Luehrmann et al., 2019; Table S5). These included all of the species used for microhabitat partitioning analysis (Table 1) and those for which relative eye sizes and retinal topography maps were obtained (Tables S1 and S3). We also characterized each species as being nocturnally or diurnally active, and their foraging mode as exclusively benthivorous, benthivorous and planktivorous, or exclusively planktivorous based on previously published research (see Table S5 for references).

2.5 | Phylogenetic comparative analyses

We tested whether the ecological parameters (microhabitat use, foraging mode, activity period) correlated with visual system composition (relative eye size, proportional opsin gene expression) of cardinalfishes using PGLS. For comparative analyses, we used the cardinalfish phylogeny from Luehrmann et al. (2019). Each predictor was independently tested against each dependent variable and no correlations between predictors were assessed due to different sample sizes. To account for multiple testing, *p* values were adjusted using Bonferroni corrections: for eight tests each in the cases of proportional opsin gene expression versus microhabitat and foraging mode, respectively; and for seven tests each in the cases of proportional opsin gene expression versus activity period and versus relative eye size, respectively (Figure 5; Tables S5 and S6). Analyses were performed in R version 3.1.2 (R Core Team, 2014) using the CAPER package (Orme, 2013).

3 | RESULTS

3.1 | Microhabitat distribution

We found marked variability in abundance and microhabitat distribution among different cardinalfish species (Table 1; Figure S1). Several species displayed microhabitat specializations (e.g. *Rhabdamia gracilis*, *Nectamia savayensis*, *N. fusca*, *Taeniamia zosterophora*, *Zoramia leptacantha*, *Ostorhinchus compressus*), while others showed more generalist microhabitat preferences (e.g. most *Cheilodipterus* and *Ostorhinchus* species; Figure S1).

Hierarchical cluster analysis revealed that cardinalfishes can be broadly classified into six habitat specialization clusters (Figure 2a), based on the microhabitat(s) they were most frequently found in Figure 2b. Cluster 1 contained only *R. gracilis*, which was exclusively found away from, but within 1–2 m of, the reef structure in midwater (microhabitat A; bootstrap, $p < .01$). Cluster 2 contained species (*Fibramia thermalis*, *Z. viridiventer*) predominantly found in exposed locations either away from structure (microhabitat A) or close to structure (microhabitat B), for example, hovering above the tips of branching corals ($p < .01$). Cluster 3 consisted of species of the genera *Taeniamia*, *Ostorhinchus*, *Cheilodipterus* and *Zoramia* that were predominantly found in exposed locations close to structures (microhabitat B), but that were rarely also found exposed and away from structure (microhabitat A) or in cover (microhabitat C; $p < .01$). Cluster 4 consisted of species (*O. cookii*, *O. compressus* and *O. doederleini*) that were predominantly found in cover, at the bottom of corals underneath branches, beneath rock ledges or between the tips of branching corals (microhabitat C; $p < .01$). They were, however, easily spotted from outside. Cluster 5 comprised species (*Pristiapogon exostigma*, *O. nigrofasciatus*) that were always hidden, for example, under ledges, between coral branches (microhabitat C) or inside caves (microhabitat D), where they were sometimes hard to spot ($p < .05$). Finally, cluster 6 comprised species (*N. savayensis*, *N. fusca*) found exclusively hidden inside the reef matrix, mostly deep inside branching corals (microhabitat D; $p < .05$).

3.2 | Relative eye size

Eye diameter was proportional to body size (PGLS, $F_{1,21} = 85.11$, $r^2 = .79$, $p < .001$), but showed considerable variation between species (Kruskal–Wallis, $\chi^2 = 116.434$, $df = 10$, $p < .001$; Figure 3, see Table S1 for an overview of morphometric measurements). Post-hoc pairwise comparisons of relative eye size at the genus level furthermore revealed three distinct size categories in this family (Figure 3d; Table S2). Members of the genus *Nectamia* had the largest eyes relative to their body sizes. Species of the genera *Ostorhinchus*, *Cheilodipterus*, *Pristiapogon*, *Taeniamia* and *Zoramia*, on the other hand, had intermediate sized eyes, while showing greater variability. *Ostorhinchus* species, in particular, showed a wide range of eye-diameter-to-standard-length ratios. *Sphaeramia nematoptera* had consistently large eyes, but not statistically larger than *Ostorhinchus* (Dunn, $z = -2.121$, $p = .036$), *Cheilodipterus* ($z = -1.04$, $p = .2$) and *Taeniamia* genera ($z = 2.103$, $p = .035$) due to the broad range of eye sizes in these groups. *R. gracilis* had the smallest eyes overall, even when compared to the *Zoramia* ($z = -4.373$, $p < .001$) and *Taeniamia* ($z = -4.628$, $p < .001$) species, which had the second smallest relative eye sizes. *Apogon crassiceps* appeared to have even smaller eyes, however, as only three specimens were sampled, this species was omitted from the analysis. In summary, species that have intermediate sized eyes showed greater variability than species with consistently large or small eyes (Figure 3; Tables S1 and S5).

Microhabitat partitioning correlated with relative eye size (PGLS, $F_{5,11} = 7.66$, $p = .02$; Figure 5a; Table S6). Relative eye size showed a positive correlation with decreased microhabitat exposure, with *R. gracilis* (microhabitat A) having the smallest, and *N. savayensis* and *N. fusca* (microhabitat D) having the largest eyes. Interestingly, species occurring predominantly in both completely hidden (microhabitat D) and partially hidden (microhabitat C) microhabitats (cluster 5) had surprisingly small eyes (*P. exostigma*, *O. nigrofasciatus*; Figure 5a). Relative eye size showed no significant correlation with either activity period or foraging mode (Table S6).

3.3 | Retinal neural cell topography

Topographic maps of ganglion cell densities revealed two specialization types, one characterized by increased cell density in the central and temporo-ventral part of the retina (*R. gracilis*, *T. fucata*), and one characterized by increased cell density in the central part of the retina (area centralis), which extends into a weak horizontal streak (*O. cyanosoma*; Figure 4).

Photoreceptors were mostly arranged in a square mosaic pattern composed of one single cone surrounded by four double cones. However, in some species, this pattern was not consistent over the entire retina with some areas showing more irregular single cone patterns (Figure S4). Photoreceptor cell topographies differed between genera, and in one case between species of the same genus (*O. notatus* differed from *O. cyanosoma* and *O. doederleini*; Figure 4). Topographic maps of total cone and double cone densities were nearly identical and revealed three specialization types (Figure 4; Figure S3): an area centralis (*T. fucata*); an increase in cell density in a large area of the central and temporo-ventral part of the retina and extending into a weak horizontal streak (*O. notatus*); and a pronounced horizontal streak with two areas centralis (*O. cyanosoma* and *O. doederleini*). For those species for which both ganglion cells and photoreceptors were investigated (*T. fucata*, *O. cyanosoma*), total photoreceptor cell distributions were similar to the ganglion cell distributions. Single cone topography was noticeably different from double cone topography in two species (*T. fucata*, *O. notatus*). These two species showed the highest proportion of single cones of all species investigated and *T. fucata* had irregular single cone patterns (Figure S4). In *T. fucata*, single cone distribution showed two large areas of increased cell density in the nasal and temporal part of the retina, while double cone distribution showed a single area centralis around the optic nerve (Figure 4). In *O. notatus*, single

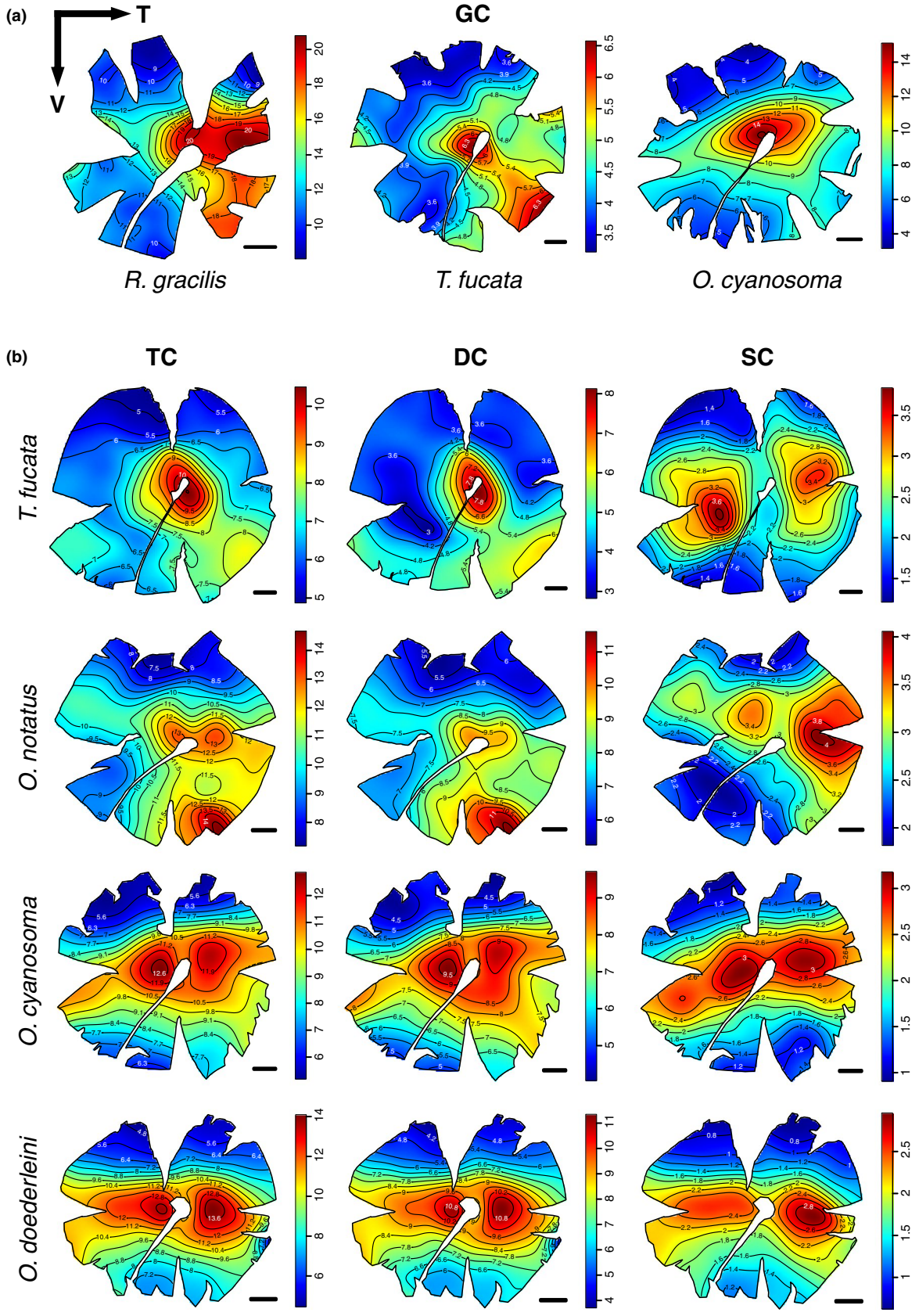

FIGURE 4 Topographic distribution of retinal neural cells in different cardinalfish species. (a) Ganglion cells (GC), (b) photoreceptors (total cone [TC], double cone [DC] and single cone [SC]). For two species, both ganglion and photoreceptor cells have been mapped. Black lines represent isodensity contours and values are expressed in densities × 10^3 cells/mm^2. Arrows indicate the orientation of the retinas: T, temporal; V, ventral. Scale bars = 1 mm

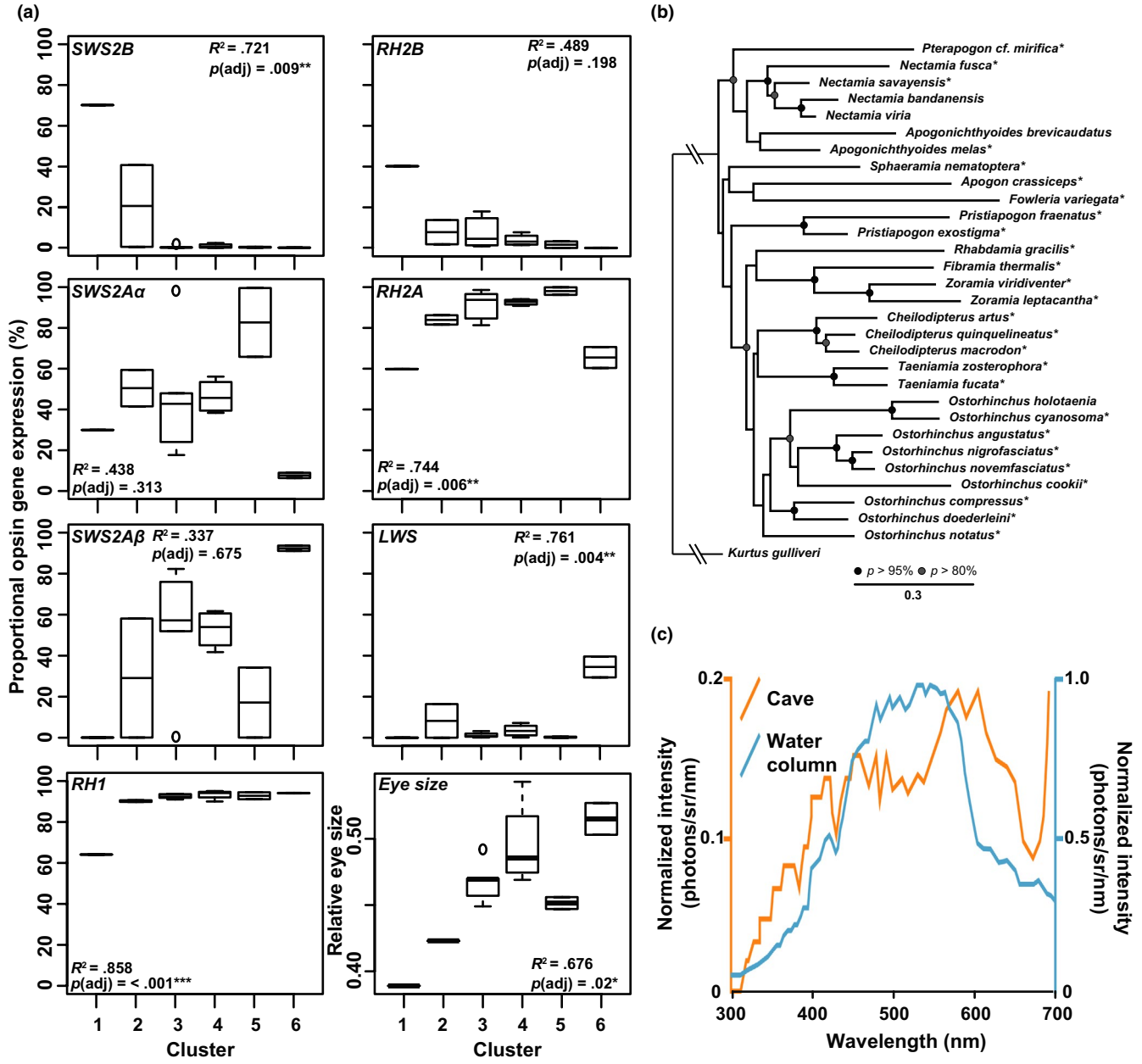

FIGURE 5 Phylogenetic comparative analysis (PGLS) of cardinalfish visual system characteristics in relation to ecological parameters and visual system specializations. (a) Proportional opsin gene expression and relative eye size in cardinalfishes categorized by microhabitat partitioning clusters. Bonferroni adjusted *p* values are shown where *<.05, **<.01, ***<.001. (b) Cardinalfish phylogeny used for PGLS analysis (see Luehrmann et al., 2019). Asterisks indicate species used and spheres indicate maximum likelihood support values. (c) Light environment in different microhabitats on coral reefs at Kaneohe Bay, Hawaii (after Marshall et al., 2003). Orange line, light inside a cave 1 m recessed. Blue line, light on the reef outside the cave. Measurements in relative photons/sr/nm

cone density was highest in the temporal region of the retina, extending into a horizontal streak, whereas double cone density was highest in the temporo-ventral part of the retina (Figure 4).

No clear relationship between retinal topography and microhabitat use and/or activity pattern could be identified. Species occupying the same microhabitat partition had very different topographies (e.g. *T. fucata* and *O. cyanosoma*; Figure 4; Table S5) and species mainly differing in activity pattern had similar topographies (e.g. *O. cyanosoma* and *O. doederleini*; Figure 4; Table S5). However, topography and foraging mode seemed to correlate, with pure planktivores having an area temporo-centralis (*R. gracilis*, *T. fucata*), while generalists (i.e. benthic and pelagic feeders) possessed streaks (*O. cyanosoma*, *O. doederleini*; Figure 4; Table S5). Moreover, the type of specialization appeared to follow the phylogeny, with closely related species having similar retinal topographies (Figure S3).

Total cell numbers and densities of both ganglion cells and photoreceptors varied between species, but were of similar order of magnitude (Table S4). Notably, peak ganglion cell density ranged from 8,289 cells/mm^2 in *T. fucata* to 23,051 cells/mm^2 in *R. gracilis*. Despite this, SRPs were similar in the three species assessed, with an average of 7.6 cpd.

3.4 | Opsin gene expression

We found that *SWS2B* (PGLS, $F_{5,11}$ = 9.283, p = .009), *RH2A* ($F_{5,11}$ = 10.31, p = .006) and *LWS* ($F_{5,11}$ = 11.17, p = .004) cone opsin expression, and the rod-opsin to cone-opsin ratio ($F_{5,11}$ = 20.37, p < .001) correlated with microhabitat partitioning (Figure 5a; Tables S5 and S6). *LWS* was highly expressed in *Nectamia* species, and, at lower levels, in *Fibramia*, but in virtually no other species. Therefore, *LWS* expression appeared to be high in species exclusively occupying hidden microhabitats (microhabitat D). *SWS2B*, in contrast, was highly expressed in species exclusively occupying exposed microhabitats (microhabitat A, *R. gracilis*; microhabitat B, *Z. viridiventer*). *RH2A*, while expressed in all species, showed the highest expression (>80% of double cone gene expression) in species occupying in-between microhabitats (clusters 2–5). *RH1* expression correlated negatively with microhabitat exposure, and thus was lowest in *R. gracilis*, followed by *F. thermalis* and *Z. viridiventer*. However, in all remaining species, *RH1* expression was nearly identical and accounted for >90% of total opsin expression (Figure 5a; Table S5). Activity period, foraging mode and relative eye size were not significantly related to any opsin expression profiles tested (Table S6).

4 | DISCUSSION

Our results show that microhabitat partitioning among different cardinalfish species correlates with adaptations in their visual systems. We found that a reduction in relative eye size, and therefore light sensitivity, was present in species from exposed microhabitats compared to species found hidden in the substrate. Opsin gene expression was also related to microhabitat use, with exposed species expressing a shorter-wavelength-shifted and hidden species expressing a longer-wavelength-shifted cone opsin palette, probably reflecting each microhabitat's light environment (Figure 5c). As to whether microhabitat partitioning could also explain differences in retinal neural cell topography, our results were inconclusive. Instead, a possible link between retinal topography and foraging mode was identified.

However, these trends were driven mainly by the few species showing extreme forms of adaptations to light conditions in microhabitats situated at the extreme ends of the light intensity and colour spectrum (microhabitats A and D). Most other species fall somewhere in between, with visual systems that seem suited to a broader colour and intensity range (see Figure 2). Since selection pressure is expected to be relaxed under those conditions, phylogenetic inertia may play a major role in shaping the visual systems in in-between cardinalfishes. This is also supported by the strong phylogenetic signal (Pagel's λ) when correlating relative eye size, *SWS2B* expression and *LWS* expression with microhabitat (Table S6).

Microhabitat partitioning behaviour occurs in many animals due to resource competition, such as for food, suitable mating sites or shelter from predators (e.g. Ross, 1986). With few exceptions, most cardinalfish species forage nocturnally and away from their diurnal refuge sites (Barnett, Bellwood, & Hoey, 2006; Marnane & Bellwood, 2002). For these species partitioning at their diurnal refuge sites is unlikely to be due to competition for food. An exception may be found in species with high expression of violet opsin (*SWS2B*) that also occur in exposed microhabitats and feed diurnally (*R. gracilis*). They may benefit from shorter-wavelength-shifted visual systems compared to other species (Luehrmann et al., 2019) in feeding contexts in midwater microhabitats since UV sensitivity can aid planktivory (e.g. Flamarique, 2016). Moreover, lower expression of rod opsin (*RH1*) in *R. gracilis* compared to other cardinalfish species (Luehrmann et al., 2019) may be a reflection of their diurnal lifestyle, though an in-depth analysis of total cone and rod photoreceptor numbers alongside the expression data would be needed to support this reasoning. Other cardinalfish species are well adapted to dim-light through higher *RH1* expression compared to diurnal reef fishes (Luehrmann et al., 2019; Musilova et al., 2019; Stieb, Carleton, Cortesi, Marshall, & Salzburger, 2016). However, even among those nocturnally foraging cardinalfishes the repertoire of cone opsins they use is on par with those of diurnal coral reef fishes (Luehrmann et al., 2019; Musilova et al., 2019). It remains to be tested whether cardinalfishes are capable of dim-light colour vision which could improve night time foraging efficiency, as reported from some gecko and anuran species (Kelber & Roth, 2006).

Cardinalfishes are heavily preyed on, making efficient defence mechanisms critical for their survival (e.g. Beukers-Stewart & Jones, 2004). Consequently, competition for shelter may drive microhabitat partitioning in this family, with those forced into the open needing to develop other means of protection. Indeed, several species generally found in microhabitats away from structure (microhabitat A and B), such as *R. gracilis*, *Z. viridiventer*, *T. zosterophora* or *Z. leptacantha*, are of silvery-translucent or pale appearance, providing excellent camouflage when viewed against a blue water background (Marshall, Cortesi, de Busserolles, Siebeck, & Cheney, 2018; Marshall & Johnsen, 2011). These species also form large schools, possibly further reducing predation risk (Pitcher, 1986). In contrast, species found in more sheltered microhabitats, for example, *O. doederleini*, *O. cookii*, *O. compressus*, or those always hidden inside the reef matrix, for example, *N. savayensis*, are darker in overall body colour, or like most *Ostorhinchus* species, have dark horizontal stripes. These species are also often solitary or live in smaller groups (Randall, Allen, & Steene, 1990). Light inside caves and crevices on tropical coral reefs is dim and red-shifted, the latter presumably caused by encrusting red-algae or other red-pigmented encrusting organisms, like sponges (Figure 5c; Marshall et al., 2003). Higher expression of *LWS* (red-sensitive) opsin, along with a complete absence of *SWS2B* expression, particularly in *Nectamia* species, may be an adaptation to the dim and/or red-shifted light conditions present in these microhabitats.

A longer-term effect of visual adaptation to different microhabitat light spectra may be an enhanced ability to recognize con- or heterospecifics under those lighting conditions, for example, for

predator avoidance or mate choice. For example, many cardinalfish species considered 'nocturnal' carry out courtship and mating behaviours at their resting sites during the day (reviewed in Vagelli, 2011). Mate colouration and visual co-adaptation may be important for sexual selection, as seen in cichlid speciation (Seehausen et al., 2008; Terai et al., 2006). Colour- or pattern-based heterospecific recognition, on the other hand, may be essential for the assortative aggregation behaviour of cardinalfishes at their diurnal refuge sites (Gardiner & Jones, 2005; Greenfield & Johnson, 1990), or predator recognition. Indeed, most cardinalfish species are colourful and many sport stripe patterns, as well as violet and/or UV markings, which are visible through their relatively UV transparent ocular media and SWS photopigments (Marshall, 2000; Siebeck & Marshall, 2001). However, unlike some reef fish (e.g. damselfishes) in which UV facial patterning can be slightly different between males and females and even between individuals (Siebeck, Parker, Sprenger, Mäthger, & Wallis, 2010), cardinalfishes do not exhibit sexual dimorphism. Males can only be distinguished from females by their enlarged jaws when incubating eggs during breeding season (Barnett & Bellwood, 2005).

The relationship of eye sizes to habitat exposure and brightness found here, with species occupying the most sheltered and dimmest microhabitats having the largest eyes and species occupying the least sheltered and brightest microhabitats having the smallest eyes, is consistent with previous studies in fishes (Schmitz & Wainwright, 2011), and confirms the importance of light availability as a driver of eye size evolution. However, some species (*P. exostigma*, *O. nigrofasciatus*) found in both completely hidden (microhabitat D) and partially hidden microhabitats (microhabitat C) had surprisingly small eyes, suggesting that other factors, such as phylogeny (Table S6; de Busserolles, Fitzpatrick, Paxton, Marshall, & Collin, 2013) or predation (Beston, Wostl, & Walsh, 2017), may have influenced their eye size evolution.

In fishes, the retinal topography usually reflects their habitat type, with visual streaks found in species living in open environments with an uninterrupted view of the horizon, and areas centralis found in species living in more enclosed environments (Terrain theory by Collin & Pettigrew, 1988a, 1988b; Hughes, 1977). Here, the lack of a relationship between microhabitat type and retinal topography in cardinalfishes could be explained by several factors: (a) we focused on the cone topography, but many of the species studied here are nocturnal and therefore may rely more on their rod photoreceptors; (b) habitat partitioning in this study was only assessed during the day and no data are available on habitat partitioning at night. Hence, it is possible that a correlation between microhabitat partitioning and retinal topography exists, but that it was missed due to different activity periods of species; (c) retinal topography in cardinalfishes may carry a strong phylogenetic signal (Figure S3). A phylogenetic constraint in retinal topography was first suggested in mammals (Stone, 1983) and has since been observed in a number of animals, including in deep-sea fishes (de Busserolles, Marshall, et al., 2014); (d) instead of diurnal microhabitats, retinal topography in cardinalfishes may be influenced by their feeding ecology. The streak in benthivorous/plantivorous species may allow them to scan a broad area of the sand–water interface, providing high acuity while minimizing eye movements (Collin & Pettigrew, 1988b). On the other hand, a higher density of cells in the temporo-ventral part of the retina in obligate planktivores may provide higher acuity to distinguish prey situated in front and above in the water column (de Busserolles, Marshall, et al., 2014; Collin & Pettigrew, 1988a).

5 | CONCLUSIONS

Our findings suggest that microhabitat partitioning is a factor contributing to visual system diversification in cardinalfishes, specifically those adapted to extreme microhabitats, whereas many show visual systems suited to broader microhabitat conditions. The colour vision of these nocturnal foragers is presumably linked to daytime activities in and around coral heads and the close-set nature of these social and other activities may in particular drive this site system co-adaptation. While there remains much to learn around the colours and their use in these engaging fish, our findings indicate that the availability of diverse microhabitats contributes to evolutionary sensory diversification in diverse ecosystems, such as tropical coral reefs, and perhaps in similar ways in terrestrial systems.

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AUTHORS' CONTRIBUTIONS

M.L., F.C., K.L.C., F.d.B. and N.J.M. conceived the ideas and designed the methodology; M.L. and F.C. collected the data; M.L. analysed the data; M.L. led the writing of the manuscript; all authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

All data are provided either in the main manuscript, the Supporting Information, or are available through publications cited accordingly, as well as through Dryad Digital Repository https://doi.org/10.5061/dryad.3xsj3txbr (Luehrmann, Cortesi, Cheney, de Busserolles, & Marshall, 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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