Light modulated cnidocyte discharge predates the origins of eyes in Cnidaria

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1. Abstract

Complex biological traits often originate by integrating previously separate parts, but the organismal functions of these precursors are challenging to infer. If we can understand the ancestral functions of these precursors, it could help explain how they persisted and how they facilitated the origins of complex traits. Animal eyes are some of the best studied complex traits, and they include many parts, such as opsin-based photoreceptor cells, pigment cells, and lens cells. Eye evolution is understood through conceptual models that argue these parts gradually came together to support increasingly sophisticated visual functions. Despite the well accepted logic of these conceptual models, explicit comparative studies to identify organismal functions of eye-precursors are lacking. Here, we investigate how precursors functioned before they became part of eyes in Cnidaria, a group formed by sea anemones, corals and jellyfish. Specifically, we test whether ancestral photoreceptor cells regulated the discharge of cnidocytes, the expensive single-use cells with various uses including prey capture, locomotion, and protection. Similar to a previous study of *Hydra*, we show an additional four distantly related cnidarian groups discharge significantly more cnidocytes when exposed to dim blue light compared to bright blue light. Our comparative analyses support the hypothesis that the cnidarian ancestor was capable of modulating cnidocyte discharge with light. Although eye-precursors might have had other functions like regulating timing of spawning, our findings are consistent with the hypothesis that photoreceptor cells which mediate cnidocyte discharge predated eyes, perhaps facilitating the prolific origination of eyes in Cnidaria.

Keywords: light sensing, nematocysts, ocelli, photoreception, photosensitivity

2. Introduction

Complex biological traits often evolve by combining previously separate parts, which we herein term "precursors" , that originally served other organismal functions. Understanding ancestral functions of precursors will help us understand whether and how they were conserved over time, ultimately informing how complex traits originate. An attractive system for exploring the ancestral functions of precursors is animal eyes, which are complex organs composed of modules with known functions, including photoreceptors, pigments, and often lens cells (Oakley and Speiser, 2015). These modules also function outside of eyes, yet only when combined do they facilitate the complex visual tasks that eyes can do. According to a functional model, modules gradually accrued during eye evolution, sequentially adding photoreceptors, pigments, and lenses to support the acquisition of increasingly advanced visual tasks (Nilsson, 2013). The modules did not evolve de novo within eyes, but probably were recruited from elsewhere, while also serving functions outside of eyes (Swafford and Oakley, 2019). As such, understanding the functions of precursor modules destined to later join forces and become eyes is particularly important for understanding eye origins.

Photoreceptor cells are a logical starting point for understanding eye origins because they are the keystone module of animal eyes. These cells, sometimes called dispersed or extraocular photoreceptors, function outside of eyes, lacking a visual function, and simply sense the ambient intensity of light with no ability to determine the direction the light is coming from (Ramirez *et al.*, 2011). Still, they provide non-directional information on light levels that is useful to organisms for many sensory tasks, including shadow responses, circadian and seasonal entrainment, depth gauges, and other organismal functions (Nilsson, 2009). From the perspective of the functional model of eye evolution, extraocular photoreceptors predated their incorporation into eyes by functioning as simple light gauges for non-directional photoreception (Nilsson, 2013). Although generally associated with non-directional photoreception, the organismal-level functions of eye-precursors often go untested.

We propose cnidarians (sea anemones, corals and jellyfish) are a particularly interesting system for examining possible early functions of eye precursors. Cnidarians convergently evolved eyes of many types in the jellyfish stage, including lensed-eyes with crystallins in box jellyfish (Picciani *et al.*, 2018). At the same time, ancestral cnidarians lacked eyes altogether but possessed opsin genes probably capable of sensing light (Picciani *et al.*, 2018). Therefore, any functions relying on non-directional light sensing in the cnidarian ancestor may represent an early role of eye precursors. Non-directional light sensing in Cnidaria is associated with various sensory tasks, including larval settlement and synchronized mass spawning in corals (Boch *et al.*, 2011; Mason *et al.*, 2012), vertical migration and spawning in jellyfish (Miller, 1979; Schuyler and Sullivan, 1997; Quiroga Artigas *et al.*, 2018), tentacle expansion and retraction in corals and sea anemones (Sawyer, Dowse and Shick, 1994; Gorbunov and Falkowski, 2002), and cnidocyte discharge in *Hydra* polyps (Plachetzki, Fong and Oakley, 2012). Among these light responses, so far we know that at least two of them are opsin-based: light-induced spawning in the hydrozoan jellyfish *Clytia* (Quiroga Artigas *et al.*, 2018) and light modulation of cnidocyte discharge in *Hydra* (Plachetzki, Fong and

Oakley, 2012). In the jellyfish *Clytia*, a gonad-specific opsin (*opsin9*) controls secretion of a neuropeptide that causes oocyte maturation (Quiroga Artigas *et al.*, 2018). Blue/cyan light induces the highest levels of oocyte maturation followed by gamete release, both of which fail to occur in genetically modified gonads that lack *opsin9*. In turn, an opsin (*HmOps2*) expressed in photosensory cells in the tentacles of *Hydra* polyps may modulate the discharge of neighboring stinging cells, the cnidocytes, in response to different intensities of blue light (Plachetzki, Fong and Oakley, 2012). Here, the evidence for opsin is indirect, relying on a pharmacological agent that targeted a co-expressed ion channel known to be involved in opsin-based phototransduction.

Because cnidocytes were clearly present in ancestral cnidarians and benefit from strong sensory regulation, we hypothesize modulation of cnidocyte discharge by light was an ancestral function in cnidarians. A cnidocyte is a powerful weapon that produces a ballistic organelle, the cnidocyst, which is discharged upon proper cues (Figure 1; Kass-Simon *et al.*, 2002). The cnidocyst itself is a capsule containing toxins with a harpoon-like tubule that releases its contents after the explosive firing. Cnidocytes are strongly regulated because they are single-use and energetically costly to replace (Anderson and Bouchard, 2009).

Therefore, multiple sensory modalities, including chemosensation, mechanosensation, and photosensation regulate cnidocyte discharge, with cnidocytes in the tentacles being highly regulated for efficient prey capture (Anderson and Bouchard, 2009). Assuming sensory regulation was always important for cnidocytes, then both function (regulation) and structure (cnidocyte) may date to the origin of cnidarians. In this study, we investigate whether this non-visual light response occurs in distantly related groups of Cnidaria other than *Hydra*. Using well-established cnidocyte capture assays and phylogenetics, we test if the intensity of blue light also affects the discharge of cnidocytes in other four eyeless species and whether this light response dates to the cnidarian ancestor. Our study brings into focus the early functional history of light responses in Cnidaria and how ancient sensory tasks may have facilitated eye origins by sustaining simple roles for extraocular photoreceptor cells.

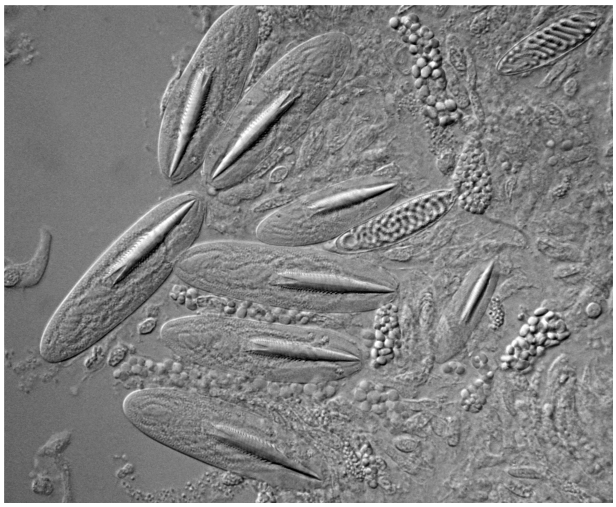

Figure 1: Undischarged cnidocysts from an anthozoan polyp.

3. Materials and Methods

Taxon sampling

We tested how light conditions affect cnidocyte capture in four distantly related species, which represent four orders (Corallimorpharia, Actiniaria, Pennatulacea, Semaeostomeae),

three subclasses (Hexacorallia, Octocorallia, Discomedusae), and two classes (Anthozoa, Scyphozoa). Most of these species occur in the coast of California, and can be cultured over long periods of time, facilitating cnidocyte capture assays.

Animal cultures

We cultured polyps of the sea anemone *Diadumene lineata* (Verrill, 1869) [=*Haliplanella luciae*] (Actiniaria, Hexacorallia) and the scyphozoan *Aurelia aurita* (Linnaeus, 1758) ("species 1" strain, Semaeostomeae, Discomedusae) in natural seawater at room temperature (22^{o}C ± 1^{o}C) under a 12:12 h photoperiod. We also cultured specimens of the corallimorph *Corynactis californica* Carlgren, 1936 (Corallimorpharia, Hexacorallia), collected from oil platforms off Santa Barbara, California (USA) on February 18th 2015 and colonies of *Renilla koellikeri* Pfeffer, 1886 (Pennatulacea, Octocorallia), collected in the Santa Barbara Channel on June 10th 2015, in a seawater open system (16^{o}C ± 2^{o}C) with a 12:12 h photoperiod. Animals were fed 2-days-old Selcon®-enriched *Artemia* nauplii (San Francisco Strain Brine Shrimp Eggs) on a daily basis. We performed all experiments with animals starved for 24 hours.

Cnidocyte assays

Because the polyp is widely accepted to be the ancestral stage among cnidarians, while the pelagic jellyfish evolved later in Medusozoa, we reasoned that comparing the cnidocyte response to light among jellyfish might not be informative for the ancestral state in Cnidaria, and therefore we focused our experiments on polyp stages only. Additionally, there are three types of cnidocytes (spirocytes, ptychocytes and nematocytes) among anthozoans, but only the nematocytes are widely distributed across cnidarians. As such, when we refer to cnidocytes throughout the text, we are specifically referring to nematocytes.

Cnidocyte capture assays followed the method described in Watson and Hessinger (1989). After double-coating fishing line with 20% (w/v) gelatin pre-heated to ∼ 70^{o}C, 2 cm-long monofilament fishing line probes (Essentials South Bend®) were left to dry for ∼ 20 min and then used for contacting one tentacle of each individual. We exposed healthy individuals to one of two different light intensities (dim light, 0.1 W/cm^{2}; bright light, 2.8 W/cm^{2}) from a blue LED (SuperBright LEDs) light source with a spectral peak at 470 nm for, approximately, two (*A. aurita*, N=33), three (*C. californica*, N=30; *R. koellikeri*, bright light, N=39; dim light, N=27; maintained at ∼ 16^{o}C in a cold chamber during experimentation) or four hours (*D. lineata*, dim light, N=40; bright light, N=33). Because polyps took different amounts of time to relax after being moved into the experimental set-up, they were exposed for varying amounts of time. Light intensity was measured using a Jaz spectrometer (Ocean Optics). Gelatin-coated probes were mounted in 100% glycerol, and discharged nematocysts were counted at 400X or 600X magnification of an Olympus BX61 microscope. We counted nematocysts by searching the whole length and width of the probe (one probe per individual) with proper focal adjustments. Probes were discarded whenever counting could not be done by the lack of a focal point or agglomeration of nematocysts.

Phylogenetic analysis

We used a maximum likelihood approach to infer the ancestral states (light modulated cnidocyte discharge, present or absent) on the time calibrated phylogeny from Picciani *et al.* (2018). We used R 3.4.1 and the function rayDISC from the R package corHMM v1.22 (Beaulieu *et al.* 2013) to estimate the marginal likelihoods of internal nodes with symmetrical rates model since the asymmetrical one was not significantly better and could lead to overparameterization (likelihood ratio test; chi-square test; df=1; p=0.1). Additionally, because outgroups lack cnidocytes altogether, we used a root prior to fix the root state as absent.

Statistical analysis

We analyzed counts of nematocysts captured in the gelatin probes using R 3.6.1. For every species, data were non-normal (Shapiro-Wilk test, $p<0.001$; except for *D. lineata,* which had data from treatment with dim light following a normal distribution) and frequency distributions were highly skewed though they had roughly the same shape. Given that, we used the Wilcoxon Rank-Sum test to compare sample means of each light treatment, assuming a significance level (α) of 0.05.

3. Results

Light modulates cnidocyte discharge in distantly related cnidarians

Our analyses reveal a clear trend across distantly related cnidarians to use light for modulating the discharge of their cnidocytes (Figure 2), and indicate the cnidarian ancestor was also able to do so (Figure 3). Overall, the discharge of cnidocytes into probes was significantly higher for polyps exposed to a dim compared to bright blue LED light (Figure 2). Our statistical power was very high ($\sim$ 100%) for *D. lineata* and *A. aurita*, indicating that we can be very confident in the effect of light intensity on cnidocyte discharge in these two long-diverged species ($\sim$ 700 mya). Conversely, power was lower for the other two species (*R. koellikeri* and *C. californica*; 40.3% and 52.9% , respectively) so that despite significant effects ($p = 0.025$ in *C. californica*; p=0.022 in *R. koellikeri*), these should be considered with caution because low power may increase the chance of false positive results (Christley, 2010).

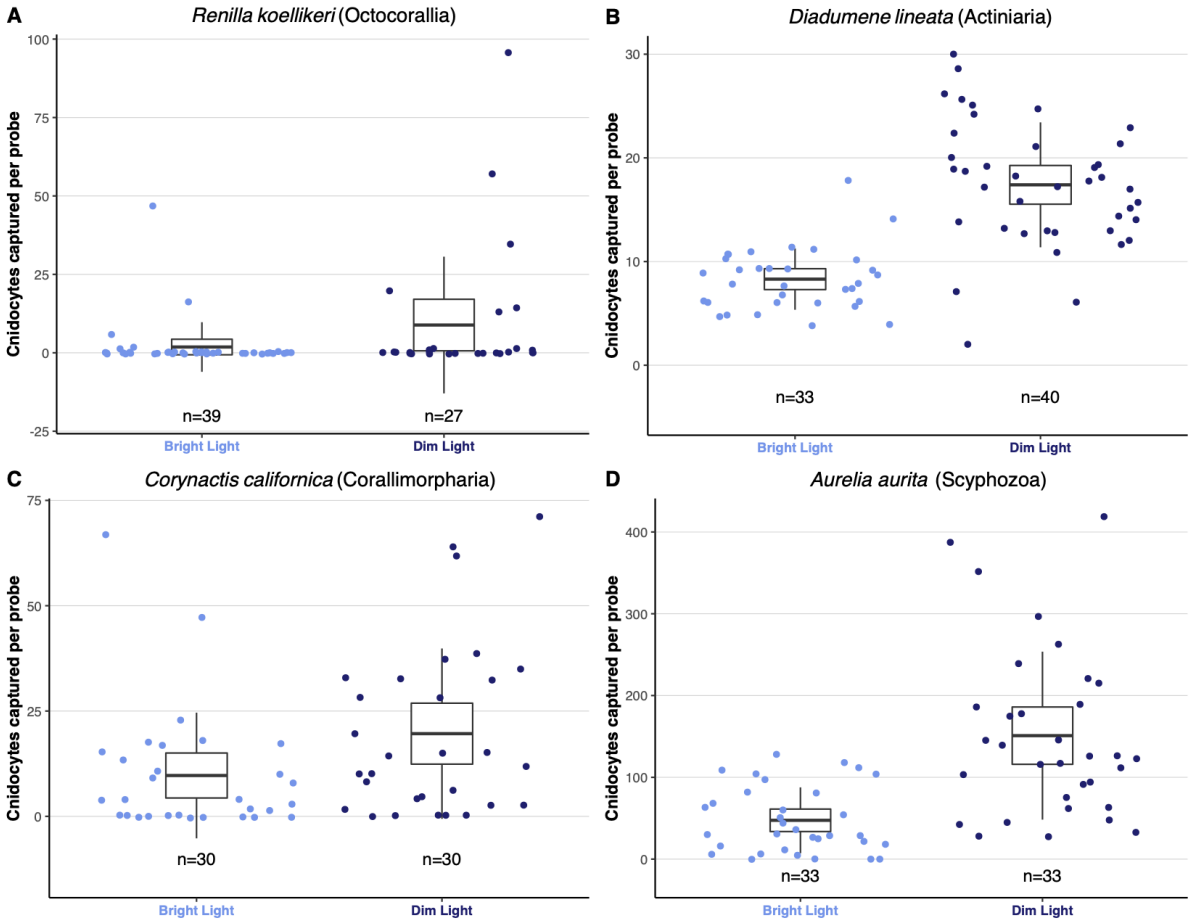

Figure 2. Cnidocyte discharge increases when polyps are exposed to dim blue light, a response conserved across long-diverged cnidarian species. Under dim blue light (470 nm; 0.1 W/cm^2), discharge of cnidocysts in the gelatin matrix was significantly higher than in bright blue light (470 nm; 2.8 W/cm^2) assays (Wilcoxon Rank-Sum Test, two-tailed; *A. aurita*: $p<0.0001$, *C. californica*: $p=0.025$, *D. lineata*: $p<0.0001$, *R. koellikeri*: $p=0.022$; see *Materials and Methods* for details). Center lines in box plots correspond to the sample mean, top and bottom extremes represent upper and lower 95% confidence interval points and whiskers are one standard deviation lines.

Species-specific variation in numbers of discharged cnidocytes

The octocoral *R. koellikeri* discharged substantially fewer cnidocytes on average (from each treatment) than all other species, while the scyphopolyp *A. aurita* discharged more cnidocytes than the octocoral, the sea anemone *D. lineata* and the corallimorph *C. californica*. That could be explained by either a comparable density of cnidocytes among species but differential use, variation on cnidocyte density in tentacles among species or a combination of both. For instance, octocorals often lack cnidocytes altogether or possess only one small type indicating considerable lower density compared to other species. Conversely, scyphopolyps depend primarily on only one type of cnidocyte, the nematocyte, as opposed to anthozoans, which use nematocytes and spirocytes for lassoing prey. That the scyphopolyp relies solely on nematocytes only could explain its higher discharge compared to other species.

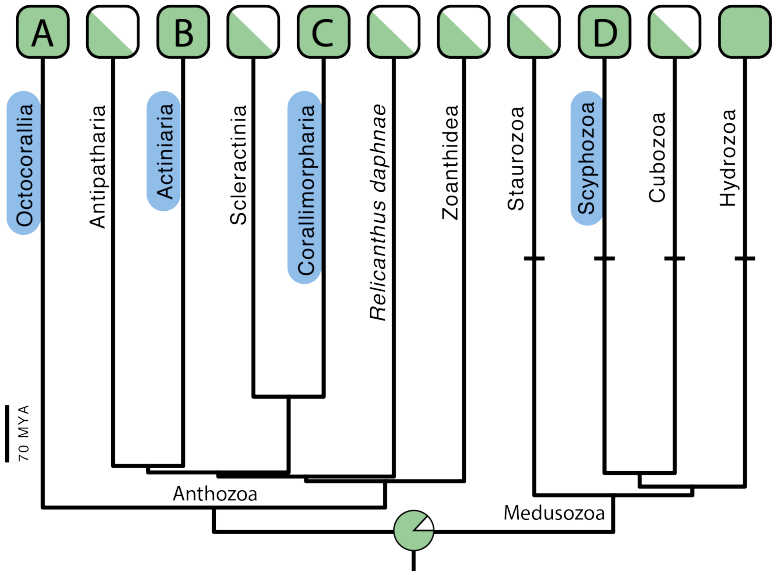

Figure 3. Maximum likelihood ancestral state reconstruction on the main phylogeny from Picciani et al. (2018). Marginal likelihoods of ancestral states (light modulated cnidocyte discharge present, green; absent, white) at the cnidarian ancestor node are shown in the pie chart, and inferred with a symmetric Markov two-state model (equal rates) of trait evolution. Letters and blue ovals shows where studied species are placed in the phylogeny (A: *R. koellikeri*, B: *D. lineata*, C: *C. californica*, D: *A. aurita*). Tip states of groups for which we lack information on light modulated cnidocyte discharge are scored as missing data and shown as rectangles half colored in green. Horizontal bars indicate lineages in which eyes convergently evolved. Scale bar denotes time in millions of years. See Figure S1 for the whole phylogeny with ancestral states.

4. Discussion

Our study presents empirical support for a sensory task that we suggest as a possible role for ancestral photoreceptors that predate cnidarian eyes. By testing whether the modulation of cnidocyte discharge by light occurs among long-diverged cnidarian lineages and reconstructing the state of the cnidarian ancestor, we find support for the hypothesis that this light response is a deeply conserved sensory task preserved over millions of years. Because we find a broad diversity of cnidarian polyps discharge significantly more cnidocytes during exposure to dim blue light compared to bright blue light, we suggest that ancestral photoreceptors in Cnidaria regulated the discharge of cnidocytes. Considering cnidocyte discharge is still the primary means of defense and prey capture of almost all cnidarians, such a long-standing photoreceptive function could have facilitated multiple convergent eye origins in the group.

Organization of cnidocytes and their sensory apparatus vary extensively between cnidarian classes (Anderson and Bouchard, 2009), yet a similar innervation pattern (Anderson, Thompson and Moneypenny, 2004) suggests photoreceptor cells could still have persisted in the circuitry controlling cnidocyte discharge. Spatial positioning of cnidocytes in tentacles varies considerably - from patchy in hydrozoans and scyphozoans to uniform in sea anemones and corals (Anderson and Bouchard, 2009). Additionally, receptor complexes associated with

cnidocytes can be produced solely by the cnidocytes themselves or receive projections from nearby ciliary cells (Watson and Mire-Thibodeaux, 1994). Given such seemingly divergent organization, an alternative to homology of light modulation of cnidocyte discharge would be convergence of such light responsiveness via repeated co-option of photoreceptor cells into cnidocyte circuitry. If convergent, the ancestral cnidocyte circuitry would have lacked photoreceptor cells, which would have been later independently assimilated into cnidocyte circuitry. But cnidarian photoreceptor cells are strongly peptidergic (Martin, 2002, 2004; Plickert and Schneider, 2004) and cnidocytes are innervated by networks of peptidergic neurons in all cnidarian classes regardless of their cnidocyte organization (Anderson, Thompson and Moneypenny, 2004; Westfall, 2004). These observations on peptidergic neurons, coupled with our inference that light modulation of cnidocyte discharge was ancestral, is consistent with a hypothesis that the cnidarian ancestor possessed photoreceptor cells that could send modulatory signals to cnidocytes, and that these cells likely persisted in cnidocyte circuitry over evolutionary time.

Of the various light-sensing genes in cnidarians, only xenopsins (called cnidops in cnidarians) occur in both Medusozoa and eyeless Anthozoa, suggesting that xenopsins could be used to sense light for cnidocyte discharge. For instance, different light sensing molecules, either non-opsin proteins or opsin types other than xenopsin, could be used for light-detection in species of anthozoans. Anthozoans can sense light with cryptochromes and two opsin types besides xenopsin, all of which seem to be completely absent in medusozoans like *Hydra* (Gornik *et al.*, 2020; Reitzel, Tarrant and Levy, 2013; Ramirez *et al.*, 2016; Picciani *et al.*, 2018). Only the xenopsin is used by both groups, and, interestingly, it is both the light sensitive molecule in photoreceptor cells of eyes and photosensory neurons that modulate the discharge of cnidocytes in *Hydra* (Plachetzki, Fong and Oakley, 2012). It is likely that the broadly distributed xenopsin would underlie an ancestral light response, yet demonstrating that anthozoans use xenopsin to modulate cnidocyte discharge would reinforce that those photoreceptors belong to the lineage of eye precursors.

Other roles besides modulation of cnidocyte discharge are also possible for photoreceptors in the cnidarian ancestor, thought to be a solitary polyp lacking symbionts (Kayal *et al.*, 2018). First, several functions, including larval settlement and phototaxis, could be ancestral - but we do not yet know if they use opsins. If not opsin-based, it seems unlikely such photoreceptors became assimilated into eyes that invariably use opsin. A topic for future research would be to test whether other light-dependent functions are opsin-based, and if so, whether the functions are ancestral in Cnidaria. Second, opsin-expressing ectodermal cells in the gonads of *Clytia* control oocyte maturation (Quiroga Artigas *et al.*, 2018), so that spawning is another candidate for an ancestral photoreceptive function in cnidarians. Testing whether light-influenced spawning is ancestral would require a survey of other species besides *Clytia*. A broad survey could be facilitated by the many available reports of light-influenced spawning in Cnidaria (see Item S1 in Picciani *et al.* (2018)). Understanding the phototransduction pathways underlying spawning across species using genetic and experimental approaches would also be important to uncover the identity of photoreceptor cells

and their relationship to eye precursors.

In addition to photoreceptor cells, other key precursor modules like pigments and crystallins probaby predated eye origins and served other organismal functions prior to visual function. For instance, one module - the biosynthesis machinery of melanin that includes tyrosinases - is present in species of both major sister lineages of Cnidaria (Esposito *et al.*, 2012; Dunlap *et al.*, 2013) and therefore could also be ancestral. Melanin synthesis is involved in biological processes outside of eyes, including as a trigger for scyphopolyps to strobilate and produce jellyfish (Van den Branden *et al.*, 1980; Van den Branden, Van den Sande and Decleir, 1980; Berking *et al.*, 2005). Moreover, melanin is also used by corals, sea fans, and anemones to create a physical barrier against pathogens, and melanin synthesis is correlated with disease resistance in corals (Petes *et al.*, 2003; Mydlarz *et al.*, 2008; Palmer, Mydlarz and Willis, 2008; Mydlarz and Palmer, 2011; Palmer, Bythell and Willis, 2012; Zaragoza *et al.*, 2014). Another precursor module, the crystallin proteins, form lenses in the eyes of box jellyfish, and may be derived from proteins with non-optical functions (Piatigorsky *et al.*, 1989, 2001; Piatigorsky, Horwitz and Norman, 1993). We know relatively little about the origins, both structural and functional, of box jellyfish lens crystallins, though they are thought to be closely related to vertebrate saposins (Piatigorsky *et al*. 2001). Crystallin homologs seem to occur in sea anemones (Nicosia *et al.*, 2014) and could perhaps be present in other lineages of eyeless cnidarians, or could have occurred ancestrally and lost in most eyeless species.

By testing a wide breadth of cnidarian diversity for a light influenced response known to involve a family of opsins used for vision, our results highlight that one possible early role for eye-precursors in Cnidaria was to modulate cnidocyte discharge. These results contribute to our understanding of eye evolution by using a phylogenetic context to propose an explanation for where the photoreceptor cells of eyes come from, and what functions they possibly had before becoming functionally integrated with other structures to mediate vision. It also raises interesting questions about how sensory tasks continued to evolve in lineages that acquired eyes. Which novel functions were cnidarians able to perform once they evolved directional photoreceptors and image-forming eyes? Did those new functions supersede ancestral functions? As proposed by Nilsson (2013), the evolution of increasingly complex visual tasks can be studied concomitantly with eye morphology so we can understand evolutionary trajectories accompanying both function and structure. By advancing a possible ancient role for cnidarian eye precursors, our study helps us to start dissecting the functional drivers that can elaborate morphological complexity.

5. Acknowledgements

We thank Professor Glen Watson for the donation of polyps of *Diadumene* (=*Haliplanella*) *lineata*, Jan Bielecki and Natalie Colburn for help with animal culture and data collection. We used computational facilities administered by the Center for Scientific Computing at the CNSI and MRL (NSF MRSEC; DMR-1720256) and purchased through NSF CNS-1725797, with incredible assistance from Fuzzy Rogers and Paul Weakliem. This study was sup-

ported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) through a doctoral fellowship to N.P. (process BEX-13130-13/7) in the program Science without Borders. T.H.O acknowledges funding from the National Science Foundation through grant IOS: 1754770.

6. Conflict of Interest

The authors declare no conflict of interest.

7. Author Contributions

Natasha Picciani: Conceptualization (equal); Data curation (lead); Methodology (equal); Investigation (equal); Formal Analysis (equal); Visualization (equal); Funding acquisition (equal); Writing - original draft preparation (lead); Writing - review and editing (equal). **Jamie R. Kerlin**: Investigation (equal); Writing - review and editing (equal). **Katia Jindrich**: Investigation (equal); Writing - review and editing (equal). **Nicholai M. Hensley**: Formal Analysis (equal); Visualization (equal); Writing - review and editing (equal). **David A. Gold**: Investigation (equal); Writing - review and editing (equal). **Todd H. Oakley**: Conceptualization (equal); Methodology (equal); Resources (lead); Funding acquisition (equal); Writing - original draft preparation (supporting); Writing - review and editing (equal).

8. Data Availability Statement

Raw datasets and analysis code are deposited in the Dryad repository doi:XX.XXXX/dryad.XX.

9. Ethical Approval

The authors followed all guidelines for ethical treatment of the animals

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