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Convergent evolution of eyes with divergent gene expression in jellyfish

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requirements for the degree
Doctor of Philosophy
in Ecology, Evolution and Marine Biology

by

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December 2020

The dissertation of Natasha Picciani de Souza is approved.

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November 2020

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Without my friends and distant yet virtually connected family, I certainly would have had a harder time adjusting to life in Santa Barbara. I am grateful for all my family and lifelong friends that even far still make themselves present in my daily life, always bringing me memories of joyful days and multiplying the happiness I ended up finding in a foreign land. I am deeply thankful for each of my life decisions that brought my partner Alex Pulido into my days, a butter heart who is always there for me, loving me unconditionally, buying thirty raffle tickets (and actually getting the prize!) to support my Science Pub talk, and sharing the passion and admiration for a culture and country that I will always carry with me. Together with Alex came our dog Freya, who became my emotional support through graduate school and especially during my last year, who I can pet and brush whenever a code does not run just so I can calm down. I would like to also especially thank Fernanda Ribeiro, who got a free ticket to annoy me even more after I became her next door neighbor, but who was always there to celebrate accomplishments with me, make first lonely Christmas holidays feel warm, or simply listen whenever victories just looked like a far away thing. Additionally, I could never forget to thank my friend Carol Camargo, my secret spy in the MCDB

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Natasha Picciani de Souza

EDUCATION

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MSc	Zoology Department, National Museum Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil PI: Drs. Carla Zilberberg and Clovis B. Castro	2011-2013
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PEER-REVIEWED PUBLICATIONS

Picciani N, Kerlin J, Hensley N, Jindrich K, Gold DA, Oakley TH (2020) Light modulated cnidocyte discharge predates the origin of eyes in Cnidaria. (accepted for publication in the journal Ecology and Evolution)

Picciani N, Kerlin J, Sierra NC, Swafford AJ, Ramirez MD, Roberts NG, Cannon JT, Daly M, Oakley TH (2018) Prolific origination of eyes in Cnidaria with co-option of non-visual opsins. Current Biology, 28: 2413-2419 (***featured in the journal cover**).
Press release: Nature Highlights (www.nature.com/articles/d41586-018-05755-0)
The Current (www.news.ucsb.edu/2018/019128/without-batting-eye)

Picciani N, Seiblitz I, Castro CB, Zilberberg C (2016) Geographic patterns of *Symbiodinium* diversity associated with the coral *Mussismilia hispida* (Cnidaria, Scleractinia) correlate with major reef regions in the Southwestern Atlantic Ocean. Marine Biology, 163: 236.

Picciani N, Pires DO, Silva HR (2011) Cnidocysts of Caryophylliidae and Dendrophylliidae (Cnidaria: Scleractinia): taxonomic distribution and phylogenetic implications. Zootaxa, 3135: 35-54.

NON PEER-REVIEWED PUBLICATIONS

Picciani N, Garrido AG, Castro CB, Zilberberg C (2016) Do equador aos trópicos: as zooxantelas na dimensão do espaço. (Book Chapter, Conhecendo os Recifes Brasileiros, Coral Vivo)

Garrido AG, **Picciani N,** Zilberberg C (2016) Simbiose coral-zooxantelas em um mundo em transformação. (Book Chapter, Conhecendo os Recifes Brasileiros, Coral Vivo)

GRANTS AND AWARDS (*upcoming)

Gaylord Donnelley Postdoctoral Fellowship, Yale University (~$120,000)	*2021-22
Worster Award (~5,000)	2020
Wake Award for Best Student Poster Presentation, SICB Annual Meeting	2020
Graduate Division Dissertation Fellowship, UC Santa Barbara (~ $10,000)	2019
Summer Fellowship, Santa Barbara Museum of Natural History (~ $8,000)	2019
Conference Travel Award, Cnidofest ($500)	2018
Conference Travel Award, UC Santa Barbara ($200)	2018
Travelling Fellowship, The Company of Biologists ($1,700)	2018
Single Cell Analysis Course, Cold Spring Harbor Labs ($800)	2018
Research Block Grant, EEMB Department ($1,600)	2018
Student Research Fellowship, Friday Harbor Laboratories ($1,500, declined)	2018

Travel Grant, Society for the Study of Evolution ($500, declined)	2018
Travel Award, European Molecular Biology Laboratory ($500)	2017
Charlotte Mangum Student Support Award, SICB ($100)	2016,2017,2018
Broadening Participation Travel Award, SICB ($500)	2016,2017
Pre-doctoral Fellowship, CAPES ($182,000)	2014-18
Honorable mention in Dissertation Seminars	2012
Field Trip Grant, The Coral Vivo Project	2011
Graduate Fellowship, CNPQ ($10,400)	2011-13
Undergraduate thesis approved with honors	2010
Undergraduate fellowship, Petrobras Research Center	2010
Undergraduate teaching assistant fellowship	2009-10

CONFERENCE PRESENTATIONS

Society for Integrative and Comparative Biology Annual Meeting	2016,2017,2018,2020
Society for Molecular Biology and Evolution Annual Conference	2019
Cnidofest	2016,2018
Annual EEMB Graduate Student Symposium	2018
Cnidofest (Bodega Bay, CA)	2016
Marine Biology Laboratory (Woods Hole, MA)	2016
Annual EEMB Graduate Student Symposium (UCSB, Santa Barbara, CA)	2015
Annual Meeting of the Willi Hennig Society	2011

TEACHING ASSISTANT EXPERIENCE

Introductory Biology (EEMB3L, EEMB2L; UC Santa Barbara)	W'19, S'17, S'16
Invertebrate Zoology (EEMB 112; UC Santa Barbara)	F'19, F'18
Macroevolution (EEMB 102; UC Santa Barbara)	Summer 2018, S'18, S'19, S'20
Guest lecturer in Zoology I (Federal University of Rio de Janeiro)	April 2013
Federal Rural University of Rio de Janeiro	April 2009-January 2010

ADDITIONAL COURSEWORK

Single Cell Analysis (100h, Cold Spring Harbor Labs, NY)	2018
Marine Animal Models in Evolution and Development (100h, EMBL, Fiskebackskil, Sweden)	2017
Workshop on Molecular Evolution (100h, Woods Hole, MA)	2016
Phylogenetics in the –Omics era (100h, EMBO, Iquitos, Peru)	2016
Topics in Marine Biology (50h, University of São Paulo, São Sebastião, Brazil)	2010
Advanced Methods for Phylogenetic Analysis (62h, EMBO, Rio de Janeiro, Brazil)	2009
Introduction to Phylogenetic Analysis (56h, State University of São Paulo, Rio Claro, Brazil)	2008
Internship Program in the Project "Coral Vivo" (200h, Arraial D'Ajuda, Brazil)	2008

ORGANIZING COMMITTEE MEMBER

Cnidofest (Davis, CA) **Invited Student Representative**	2020
Darwin: 150 years (Seropédica, RJ, Brazil)	2009
Environmental June (Seropédica, RJ, Brazil)	2009
XXVIII Biology Week (Seropédica, RJ, Brazil)	2009

RELATED PROFESSIONAL EXPERIENCE

University of California Santa Barbara 2016- 2018

Reader in the core courses of Introductory Biology

Assist the academic team with preparation, application and grading of the lecture and lab exams from Introductory Biology (up to ~ 5,000 exams per quarter)

Instituto Coral Vivo (Rio de Janeiro, RJ, Brazil) 2013

Co-Founder

The "Instituto Coral Vivo" is a Brazilian non-profit organization that promotes the conservation and sustainable use of marine and coastal environments as well as the production and dissemination of related technical and scientific knowledge

BioConsult Ambiental Ltda. (Rio de Janeiro, RJ, Brazil) 2013-2014

Technical consultant, freelancer

Provided support on the identification of anthozoans, especially deep-sea corals, in a deep-sea biodiversity project sponsored by Petrobras. Duties included examining the external morphology of the samples and carrying out histological analyses to determine taxonomic identification

OUTREACH

Volunteer at the Santa Barbara Museum of Natural History	2017-2019
Science Pub Night, Santa Barbara Museum of Natural History	2019
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Scientist Day with first graders, McKinley Elementary School	2016,2020
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SOCIETY MEMBERSHIPS

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Abstract

Convergent evolution of eyes with divergent gene expression in jellyfish

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Convergent evolution of complex phenotypes like eyes offers us an important opportunity to investigate the redeployment and divergence of genetic components used to build those complex phenotypes. While eyes evolved convergently among animals with sophisticated neural machinery to process visual information, they surprisingly also evolved in animals with simpler nervous systems and highly capable of eye regeneration, such as jellyfish. Because eyes are formed by discrete parts, each with known genetic pathways in model systems, they are an ideal system for understanding the evolutionary trajectories underlying convergent evolution of complex phenotypes. Did eyes in jellyfish evolve convergently? And if so, to what extent do they employ similar genes and where did their parts come from? I explored these questions by integrating evidence at several levels of biological organization. First, I inferred the largest cnidarian species phylogeny to date, which allowed me to test how many times jellyfish eyes evolved. I found that eyes originated convergently at least eight times among the swimming jellyfish stage. Next, I focused on three species with convergent eyes to investigate the extent to which vision genes differentially expressed in their eye-bearing tissues were similar. I found that most genes involved in eye development and phototransduction pathways in convergent eyes are upregulated in a lineage-specific way. Comparing these findings with previous knowledge from a few other jellyfish species suggested

that eyes belonging to the same evolutionary origin might employ a very predictable set of vision genes. Finally, I asked what the ancestral organismal function of photoreceptor cells was before they became part of jellyfish eyes. Based on experiments in four species of cnidarians, I found support for the hypothesis that ancestral photoreceptor cells modulated the discharge of stinging cells in the cnidarian ancestor. Such an ancient photosensory function could have long sustained a role for photoreceptor cells and perhaps facilitated multiple eye origins in the group. My dissertation work advanced a new emergent system for comparative research on eye evolution, development and function, underscored the potential for convergent eyes to have mostly unique evolutionary trajectories, and uncovered ancient sensory tasks that predated eye origins.

Contents

Chapter 1

Introduction

Convergent evolution occurs when distinct evolutionary lineages of organisms independently evolve similar phenotypic traits. Many processes can drive phenotypic convergence, including similar responses to selective pressures, stochasticity and developmental constraints (Losos et al. 2011, Lau & Oakley 2020). Ultimately, these processes will generate patterns that are traceable at the macroevolutionary scale with phylogenetic comparative methods. Convergence allows us to leverage naturally replicated experiments in order to seek explanations for patterns of increased similarity (Stayton 2015). That is particularly interesting for understanding the origins of complex phenotypic traits, such as eyes and flowers, which are modular and composed by several discrete parts (see McShea 1996 for a discussion on biological complexity). How similar were the evolutionary trajectories used to repeatedly build those complex novel traits? When a complex trait originates during evolution, multiple parts, old and new, can be combined to form that complex trait (Muller & Wagner 1991, Brigandt 2002, Oakley & Speiser 2015). As such, if a complex trait repeatedly originates via convergent evolution, it gives us the chance to investigate its multiple origins coupled with the evolutionary histories of its underlying components.

A classic example of a complex trait that evolved convergently among animals and yet is made of parts that are recognizable for their function and genetic basis are animal eyes. Animal eyes are morphologically diverse, variably complex, and serve as an ideal system for the study of convergence. Despite their morphological diversity, eyes have basic building blocks - photoreceptor cells, pigment cells and lens proteins – that are each capable of functioning outside eyes but can operate as an integrated unit to allow for directional photoreception and vision (Nilsson 2009, Nilsson 2013). Photoreceptor cells, for instance, can occur in non-visual tissues, including the skin and brain of several animals, and sense ambient light to mediate behavior (Arendt et al. 2004, Halford et al. 2009, Ramirez et al 2011, Fernandes et al. 2012). Likewise, pigments such as melanin can be responsible for UV protection and scavenging reactive oxygen species (Nosanchuk & Casadevall 2006, Kvam & Dahle 2003, Tada et al. 2010, Ostrovksy & Dontsov 2019) and play a role in immune system response against pathogens (Marmaras et al. 1996, Tapia et al. 2014). In turn, lens proteins are typically derived from stress related proteins and only acquire a visual refractive role when expressed in high amounts by lens cells (Piatigorski 1993, 2003). These building blocks are recognizable across vast evolutionary distances, leading to hypotheses that the parts accrued gradually to evolve complex eyes (Salvini-Plawen & Mayr 1977, Nilsson & Pelger 1994, Nilsson 2013).

At a lower level of biological organization, photoreceptor cells, pigment cells and lens cells are themselves characterized by distinct molecular and genetic pathways. Photoreceptor cells in animal eyes can sense light and transmit signals via phototransduction cascades that start with a visual pigment composed by an opsin protein bound to a light sensitive chromophore molecule (reviewed in Shichida & Matsuyama 2009). Phototransduction cascades are modular, with opsins, G-protein alpha subunits, intermediary enzymes, and ion channels varying and defining divergent cascades among animals (Shichida & Matsuyama 2009; Plachetzki et al.

2010). In turn, pigment cells can synthesize pigments (e.g. melanin or ommochromes) that serve as a light shield and inform photoreceptor cells of light direction (Nilsson 2013). We know of several biosynthetic pathways to generate eye pigments, including their genes, enzymes, and final products (Sugumaran & Barek 2016, Figon & Casas 2019). Similarly, the molecular and genetic bases to produce eye lenses are also well studied in model systems (Cvekl & Ashery-Padan 2014). This extensive knowledge on eye components together with studies on the basis of eye morphogenesis (reviewed in Koenig & Gross 2020) allows us to understand to what extent animals redeployed similar or divergent pathways to convergently build eyes. As pointed out by Koenig & Gross (2020), while traditional genetic models deepened much of our understanding of animal eye evolution, current technological advances support novel studies in many non-model species, so that we can gain a better understanding of how animal eye diversity originated.

Eye evolution is primarily informed by studies of bilaterian animals (arthropods, molluscs, and vertebrates), which almost invariably evolved with sophisticated neural machinery to process visual information. Increases in neural tissue mass is often coupled with elaboration of sensory systems, which incurs in increased energetic costs due to physiological processes for visual processing, such as signal transduction and transmission (Nivel & Laughlin 2008, Moran et al 2015). Yet eyes, including true image-forming lensed eyes, also exist in Cnidaria (jellyfishes, corals, and sea anemones), which are ancient non-bilaterians with simple nervous systems made of dispersed and condensed neurons for locally processing information. With approximately 11,000 known species, cnidarians are distributed in two main groups: Anthozoa (~7,500 spp.) and Medusozoa (~3,500 spp.). These sister lineages differ drastically in their life history: while anthozoans are exclusively marine benthic polypoid forms, medusozoans may alternate polyp and medusa stages and occupy pelagic and benthic habitats (Daly et al. 2007). Complex life histories and physiological adaptations allowed cnidarians to occupy rocky shores, soft sediments, tide pools, freshwater and marine habitats from shallow waters to the deep sea in worldwide open ocean and coastal areas. Both pelagic and benthic jellyfish can have eyes of many types, ranging from simple eyespots (photoreceptor cells side by side with pigment cells) to very complex eyes similar to those of bilaterians with a cornea, crystallin lens and a retina (Salvini-Plawen & Mayr 1977, Martin 2002, Martin 2004). Investigating how eyes evolved in this animal system will help us understand evolutionary processes and trajectories that led to the emergence of complex sensory organs in a relatively unusual animal group. An integrated synthesis of eye diversity and gene expression, with an emphasis on vision genes, will allow us to evaluate which aspects of cnidarian eye evolution could be more prone to constraints as well as the extent of variability within and among eye origins.

My dissertation aimed at understanding the convergent evolution of a complex novel trait with comparative methods that targeted several levels of biological organization, from the complex trait itself to its underlying components and genes. Using jellyfish eyes as a biological system, I addressed the following questions:

1. *How often did eyes of varying complexity, including true image-forming eyes, arise in animals with simple neural circuitry such as cnidarians?*

2. *Do convergent cnidarian eyes differentially express a similar set of vision genes?*

3. *What was the ancestral organismal function of photoreceptor cells that later became part of cnidarian eyes?*

To answer each of these questions, I made extensive use of phylogenetic comparative methods together with experimental approaches and literature synthesis. In Chapter 2, I generated a novel pipeline that allowed me to use all publicly available cnidarian nucleotide sequences to date so I could infer what became the largest cnidarian species tree ever published and fine map their eye diversity. With such a large species tree, I placed knowledge of eye diversity in Cnidarian into a phylogenetic context, which revealed many instances of eye convergence. In Chapter 3, I was able to use an unbiased approach to pinpoint several genes upregulated in convergent eyes that play a role in vision in other animal systems. Due to limitations of candidate gene approaches, many of these vision genes were yet unreported in cnidarian eye structures, so that my analyses revealed an extensive new set of vision genes possibly playing a role in phototransduction and eye development/maintenance. It furthermore brought up the possibility that vastly distinct vision gene families were recruited to form eyes at each eye origin. Lastly, I use experimental approaches in Chapter 4 to test whether the ancestral function of photoreceptor cells prior to them acquiring a visual role was to modulate cnidocyte discharge. I found evidence that supports this hypothesis and imply that such a sensory task could have long sustained a role for phototransduction pathways and facilitated eye origins in the group by enabling those pathways to be exploited in vision.

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Chapter 2

Prolific origination of eyes in Cnidaria with co-option of non-visual opsins

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2.0 Abstract

Animal eyes vary considerably in morphology and complexity and are thus ideal for understanding the evolution of complex biological traits. While eyes evolved many times in bilaterian animals with elaborate nervous systems, image-forming and simpler eyes also exist in cnidarians, which are ancient non-bilaterians with neural nets and regions with condensed neurons to process information. How often eyes of varying complexity, including image-forming eyes, arose in animals with such simple neural circuitry remains obscure. Here, we produced large-scale phylogenies of Cnidaria and their photosensitive proteins and coupled them with an extensive literature search on eyes and light-sensing behavior to show that cnidarian eyes originated at least eight times, with complex, lensed-eyes having a history separate from other eye types. Compiled data show widespread light-sensing behavior in eyeless cnidarians and comparative analyses support ancestors without eyes that already sensed light with dispersed photoreceptor cells. The history of expression of photoreceptive opsin proteins supports the inference of distinct eye origins via separate co-option of different non-visual opsin paralogs into eyes. Overall, our results show eyes evolved repeatedly from ancestral photoreceptor cells in non-bilaterian animals with simple nervous systems, co-opting existing precursors, similar to what occurred in Bilateria. Our study underscores the potential for multiple, evolutionarily distinct visual systems even in animals with simple nervous systems.

Keywords: light sensing, photoreception, convergence, visual systems, non-bilaterians

2.1 Results and Discussion

Traits like eyes have long challenged biologists to explain steps leading to the evolution of complexity (Oakley & Speiser 2015). Animal eyes are made of smaller building blocks, minimally including photoreceptor cells and pigment cells, sometimes having lenses or mirrors for improved spatial resolution. Components of eyes are recognizable across vast evolutionary distances, leading to hypotheses that the parts accrued gradually to evolve complex eyes. Eye evolution is primarily informed by studies of bilaterian animals (arthropods, molluscs, and vertebrates), which almost invariably evolved with sophisticated neural machinery to process visual information. Yet eyes also exist in Cnidaria (jellyfishes, corals, and sea anemones), which are ancient non-bilaterians with nervous systems of dispersed and condensed neurons for locally processing information and no typical bilaterian central nervous system (but see Garm et al. 2006).

The number of times eyes originated in this ancient animal group with simple nervous systems remains unresolved. Cnidarian eyes express transcription factors homologous to those expressed in bilaterian eyes, leading to claims of a single origin of all eyes, including those of cnidarians (Suga et al. 2010). However, cnidarians also show differences in structural details of photoreceptors, leading to suggestions of four or five origins of eyes within Cnidaria (Salvini-Plawen & Mayr 1977). Finally, a morphological phylogeny of major cnidarian groups using eyes as one of many traits suggests eyes appeared at least twice in Cnidaria (Marques & Collins 2004). Overall, cnidarian eye evolution remains controversial because previous studies were either non-phylogenetic or lacked the extensive taxon sampling necessary to address origins of eyes in a group as diverse and variable as Cnidaria.

2.1.1 Phylogenetic support for multiple origins of eyes among adult pelagic medusa

To address questions of eye evolution in an ancient group with simpler nervous systems, we produced a large-scale molecular phylogeny of Cnidaria (1102 species) and performed ancestral state estimation with parsimony, maximum likelihood (ML), and Bayesian approaches, using extensive data on presence of eyes gathered from published literature for adult medusae (Table S1). An eye is minimally defined as a region made of photoreceptor cells adjacent to pigment cells (Arendt 2001). Some researchers restrict the term eyes to image-forming organs and use ocelli for those that do not form images (Nilsson 2004). But morphological variation among ocelli and eyes typically forms a continuous gradation, making it difficult to distinguish clear boundaries among these organs (Salvini-Plawen & Mayr 1997). For this reason, and because we lack ultrastructural and functional data for photoreceptive organs of many cnidarian species, we refer to them all as eyes. Our ancestral state reconstruction strongly suggests that the last common cnidarian ancestor (Figure 1A), as well as key ancestors in major medusozoan classes (Staurozoa, Scyphozoa, Cubozoa and Hydrozoa) lacked eyes (Figure 1B). Thus, eyes probably originated repeatedly, at least eight times among distantly related medusoid cnidarians, and up to sixteen times with less conservative counting of state transitions (Figure 1). First, reconstructions under parsimony indicate ten to sixteen origins of eyes as equally most parsimonious, depending on the transformation criterion (accelerated or delayed) (see supplemental results, Data availability in STAR Methods). Second, using ML, we estimated rates of character transitions assuming an asymmetric, two-state Markov model, generating marginal likelihoods for both states (presence or absence of eyes) at every internal node on our species phylogeny. Eyes originated thirteen times in cnidarians under ML, when counting character transitions where one state has a significantly higher proportion of marginal likelihood (Figure 1; see also Figure S1). More conservative counting leads to fewer inferences of eye gain. We conservatively infer one gain instead of more in each of two hydrozoan clades (gains 4 and 7 on Figure 1; see also Figure S1) where the likelihood of eye presence in some internal nodes was substantially, but not significantly, higher than absence. Furthermore, we conservatively infer one scyphozoan origin instead of two due to a lack of observations on eye for some species. In sum, even conservative counting of ML ancestral states infers eight origins, one in the lineage leading to box jellyfishes (Cubozoa), one in scyphomedusa (Scyphozoa), and six in hydromedusa (Hydrozoa). Finally, Bayesian character state analysis (Bayes Factor Test) supports the estimate of multiple eye origins, in agreement with ML and parsimony results. The Bayes factor test of independent origins assumes known rates of gains and losses and a continuous-time Markov model of evolution. We compared the probability of observing our data under the hypothesis that eyes originated in Cnidaria less than eight times to the alternative hypothesis that eyes originated at least eight times. This led to a Bayes factor test consistent with ML and parsimony results, strongly favoring at least eight origins in Cnidaria across several priors on rates of eye gains and losses. Furthermore, the Bayes Factor test still strongly and consistently favors hypotheses of more than a single origin, two origins, and four to five origins, as suggested by previous studies on cnidarian eye evolution, also across several priors on gains and losses rates (see Table S2, Data availability in STAR Methods).

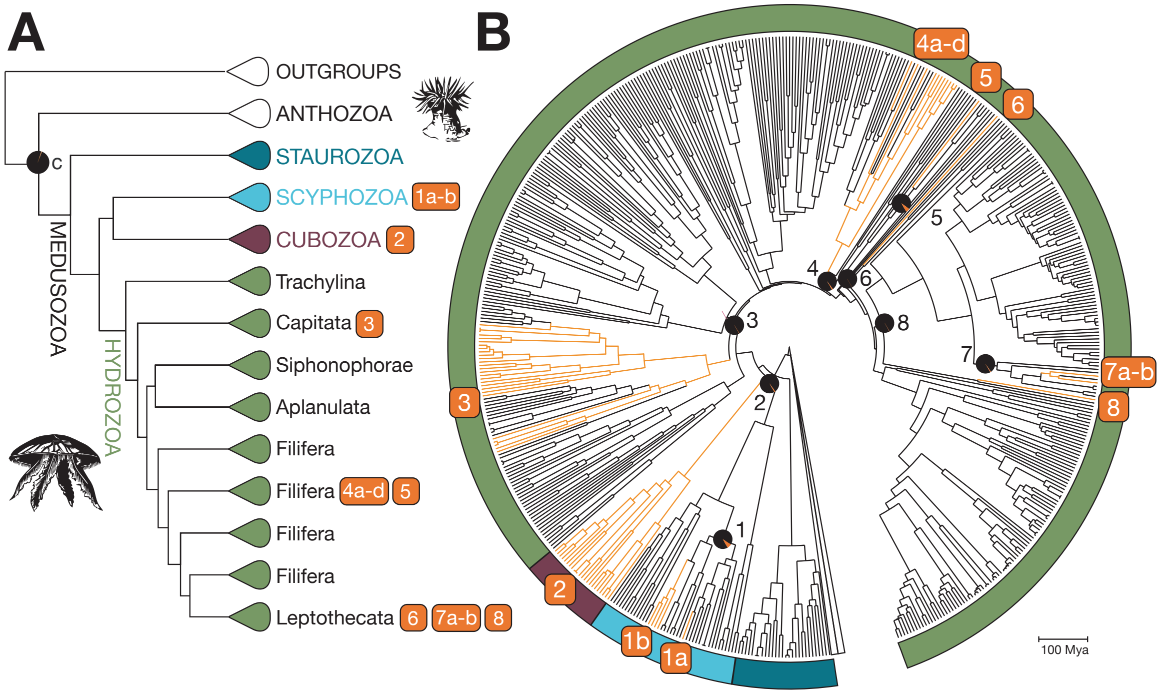

Figure 1.1. Eyes Originated between 8 and 13 Times in Cnidaria. (A) Summary of the cnidarian tree topology used for ancestral state reconstructions illustrated with major clades collapsed. The eyeless cnidarian ancestor is indicated as ''c.'' (B) Time-calibrated cnidarian tree with medusozoan clades expanded to show eye origins. Eyes are only found in medusoid cnidarians, which have corresponding colors in (A) and (B). Pie charts at internal nodes represent selected ancestral states with marginal likelihoods for absence (black) and presence (orange) of eyes reconstructed using a two-state Markov model (1–7) ML estimate of transition rates was 0.08 (gains:losses). Numbers 1–8 in orange squares denote eight most conservative, separate origins of eyes mapped on the ML tree inferred from a concat- enated dataset of five genes (18S, 28S, COI, 16S and 12S; 6,629 nucleotides) from 1,106 taxa (Anthozoa: 548, Medusozoa: 554, outgroups: 4) under a GTR+R10 model. We used TreePL to transform branch lengths to be proportional to time. Origins 1, 4, and 7 correspond to two or four eye origins as represented by ''a,'' ''b,'' ''c,'' and ''d'' (see text for discussion). The Bayes factor test of independent origins strongly favors the alternative hypothesis (H_1) of at least eight origins as opposed to fewer than eight (H_0) (Bayes factor $H_0/H_1 = 9.3 \times 10^{-25}$). Orange branches represent transitions from eye absence to eye presence inferred with parsimony ancestral state reconstruction using the accelerated transformation criteria. All node support values and ancestral state reconstructions are available in Figures S1 and S2. See also Table S1 and Data S1.

We did not find our inferences of eye history to be sensitive to reasonable variations in the species tree. Most importantly, the Bayes factor test of independent origins incorporates uncertainty in species tree topology using a distribution of trees from bootstrapped pseudoreplicates of our molecular data using ML. Second, we considered multiple phylogenetic hypotheses from different analytical strategies, including data partitioning and varying outgroups (see STAR Methods for details). Most living cnidarians belong to two historically well supported groups (but see Kayal et al. 2013): Anthozoa (~7,500 species) and Medusozoa (~3,500 species). We excluded a third group recently confirmed to be cnidarians (Chang et al. 2015), the Myxozoa (~2,200 species), from our analyses because they are challenging to place phylogenetically without very large datasets, are extremely divergent from other lineages, and as endoparasites lack eyes. Despite consistent support for these three major groups in the literature, relationships within each are still contentious, and differences within Medusozoa in particular, could impact our ancestral state estimations. Our analyses consistently place Staurozoa as sister to other medusozoan groups as suggested previously by rDNA analyses (Collins et al. 2006), but in disagreement with recent phylogenomic studies (Zapata et al. 2015, Kayal et al. 2017). However, differences in the phylogenetic relationships within medusozoan groups (especially within Hydrozoa) did not affect our conservative inference of at least eight origins of eyes in Cnidaria (see supplemental results, Data availability in STAR Methods).

Our model of evolution for ancestral state reconstruction relied on scoring cnidarian species as having eyes simply ‘present’ or ‘absent’. Differences in morphology and development of eyes in different cnidarian lineages offer additional data to address the hypothesis of 8 eye origins and provide insight into the evolutionary processes that shaped eye evolution in cnidarians. Therefore, we compiled descriptions of fine structure and development of eyes. We find morphological details are often different among eyes we infer to be of separate origin, as expected in the absence of strong convergent evolution. For example, Origin 1 includes scyphozoans *Aurelia aurita* (Linnaeus 1758) (Origin 1a) and *Cassiopea xamachana* Bigelow 1892 (Origin 1b), whose eyes differ from those of other medusozoans in having pigmented photosensory cells (Bouillon & Nielsen 1974, Yamasu & Yoshida 1973). Origin 2 includes the unique and sophisticated lensed eye of box jellyfishes (Figure 2D), which have a three-layered retina, unique crystalline lens, and cornea (Yamasu & Yoshida 1976, O'Connor et al. 2010). Although we count cubozoan eyes as having a single origin separate from other cnidarians, cubozoans themselves have multiple eye types, including pit and slit eyes and planular eyes in single individuals (Nordstrom et al. 2003), all of which are absent in close relatives. Therefore, each of these eye types could have a separate origin, if they are not derived from each other. Origins 3-8 encompass the eyes of hydrozoans, which comprise two cell types: pigment cells and photosensory ciliary cells. Origin 3 includes the everted pigment cup eyes of *Cladonema radiatum* Dujardin 1843 (Figure 2C), which have compact lenses formed of subunits from distal cytoplasmic portions of pigment cells that synthesize lens proteins (Bouillon & Nielsen 1974, Weber 1981). Origin 4 is represented by *Bougainvillia principis* (Steenstrup 1850), which also have everted pigment cup eyes (Figure 2B), that differ from those of Origin 3 in having a lens-like body (but without *Cladonema*-like subunits) formed from agglomerations of lateral projections of pigment cells, which are spatially separated from multiciliated photosensory cells (Singla 1974). Origin 5 includes *Leuckartiara octona* (Fleming, 1823) (Figure 2A), in which the two cell types are interspersed in a single flat layer to form an eyespot (Singla 1974). Here, the photosensory cells are different from other cnidarian eyes in possessing an exceptional supranuclear vacuole and cilia without striated rootlets (Singla 1974). Origins 6 and 8 have never been the subject of ultrastructural work. Finally, Origin 7 is represented by inverted pigment cup eyes of *Tiaropsis multicirrata* (Sars, 1835) which have ectodermal photosensory cells and endodermal pigment cells (Singla 1974).

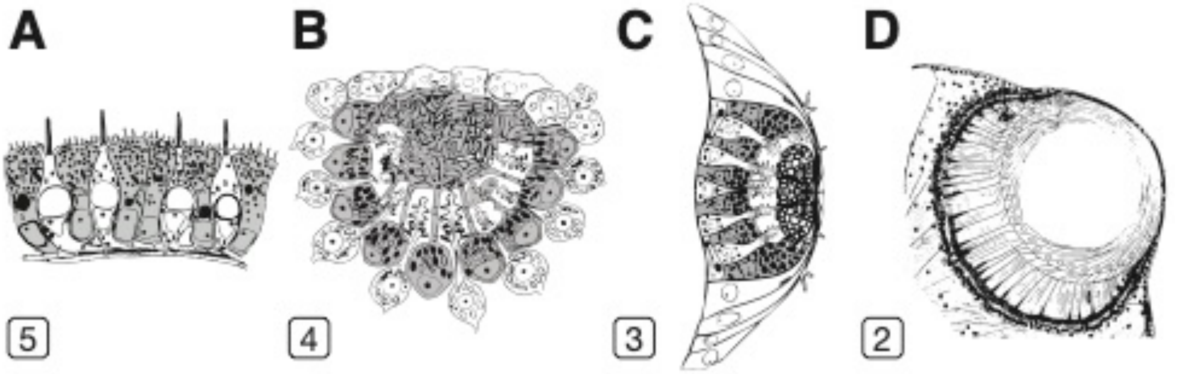

Figure 1.2. Eyes of Separate Origin Differ in Ultrastructural Details. (A) Eyespot from the hydrozoan *Leuckartiara oc- tona* (Fleming 1823), Origin 5). Pigment and spin- dle-shaped photosensory ciliary cells alternate in a single layer, with ciliary cells having an exceptional supranuclear vacuole. (B) Everted pigment cup from the hydrozoan *Bou- gainvillia principis* (Steenstrup 1850), Origin 4). Cuboidal pigment cells form a cup into which they project irregular tubular processes. Photosensory ciliary cells may bear one to three cilia, and their projections are spatially separated from those of the pigment cells. (C) Everted pigment cup with lens from the hydrozoan *Cladonema radiatum* Dujardin 1843, Origin 3. Basal parts of the photosensory ciliary cells are located between the pigment cells and their distal parts project microvilli that intermingle with those of adjacent cells. Cytoplasmic portions of the pigment cells extend to form a compact lens. (D) Lens eye of the cubozoan *Carybdea xaymacana* Conant 1897 (Origin 2). Cornea made of flattened ectodermal cells and spherical crystallin lens separated by a small space from a retina composed of columnar pigmented photosensory ciliary cells. Origin labels are in squares. Modified from Bouillon & Nielsen (1974), Weber (1981), Singla (1974), Berger (1900). See also Table S1.

Several of the separate origins we postulate are consistent with Salvini-Plawen and Mayr (1977), who lacked detailed phylogenetic hypotheses, but posited our Origins 1,2,3,5,7, based on morphology alone. Along with morphology, developmental genetic details differ between eyes we infer to have separate origins. *Pax* genes are typically regulators of animal eye development, including cnidarians (Suga et al. 2010). Whereas *Pax-B* is involved in the development of lensed-eyes of the box jellyfish *Tripedalia cystophora* (Origin 2), *Pax-A* regulates the development of eyes in the hydrozoan *Cladonema radiatum* (Origin 3) (Suga et al. 2010). Our phylogenetic analyses shed new light on eye morphology, indicating cnidarian eyes of separate origin evolved differently in morphological detail, perhaps using distinct developmental pathways.

2.1.2 Light sensitivity likely predates eye origins in Cnidaria

In addition to strong support for separate origins of eyes, we find light sensitivity, the first step toward evolving an eye (Nilsson 2013), to be present across all Cnidaria, not only those closely related to species with eyes. Light sensitivity is present in many eyeless organisms, which often perceive light through dispersed extraocular photoreceptor cells (Ramirez et al. 2011, Sumner-Rooney et al. 2018) using various molecular mechanisms (Porter 2016). Extraocular light sensitivity is found in Cnidaria, which respond to light and use it to tune essential activities like larval settlement, spawning, migration, feeding, or cnidocyte firing. We compiled reports of light-associated processes for eyeless species in our tree (Data S1) to show that light sensitivity is widespread across all cnidarian classes. We made no attempt to distinguish different mechanisms as these are usually unknown in cnidarians (but see Plachetzki et al. 2012, Levy et al. 2007). One genetic mechanism for light sensitivity is opsin-based phototransduction. Light sensitivity of opsin proteins has been tested directly in two cnidarian species (Koyanagi et al. 2008, Quiroga Artigas et al. 2018) and indirectly with electroretinograms in others (Weber 1982a, Weber 1982b, Coates et al. 2006, Ekstrom et al. 2008, Garm et al. 2007). Additionally, opsins were probably present in ancestral cnidarians (Feuda et a. 2012, Plachetzki et al. 2010, Feuda et al. 2014). Therefore, opsins serve as logical candidate genes for many extraocular light-sensing functions. Light-sensing functions across all cnidarian classes suggest that light sensitivity did not appear separately in groups with eyes, but was likely ancestral, long predating all origins of all cnidarian eyes. Consequently, if light sensitivity existed in the first cnidarians, we speculate light sensitivity acted through dispersed, extraocular photoreceptor cells expressing opsins for any of a variety of functions. Ancestral photoreception is consistent with a traditional idea that eyes evolve stepwise, building upon ancestral photoreceptor cells over evolutionary time (Oakley & Pankey 2008, Nilsson & Pelger 1994).

2.1.3 Separate co-option of distantly related extraocular opsins supports multiple eye origins

To test whether visual opsins are derived from extraocular genes in Cnidaria, as in bilaterian animals with central nervous systems (Porter et al. 2012), we inferred opsin history using new and published transcriptomes of 86 cnidarian species. Consistent with separate eye origins, we demonstrate that eyes from at least two different origins express distantly related opsin genes (Figure 1.3), and we show opsins now expressed in eyes evolved from extraocular opsins (Figure 1.3).

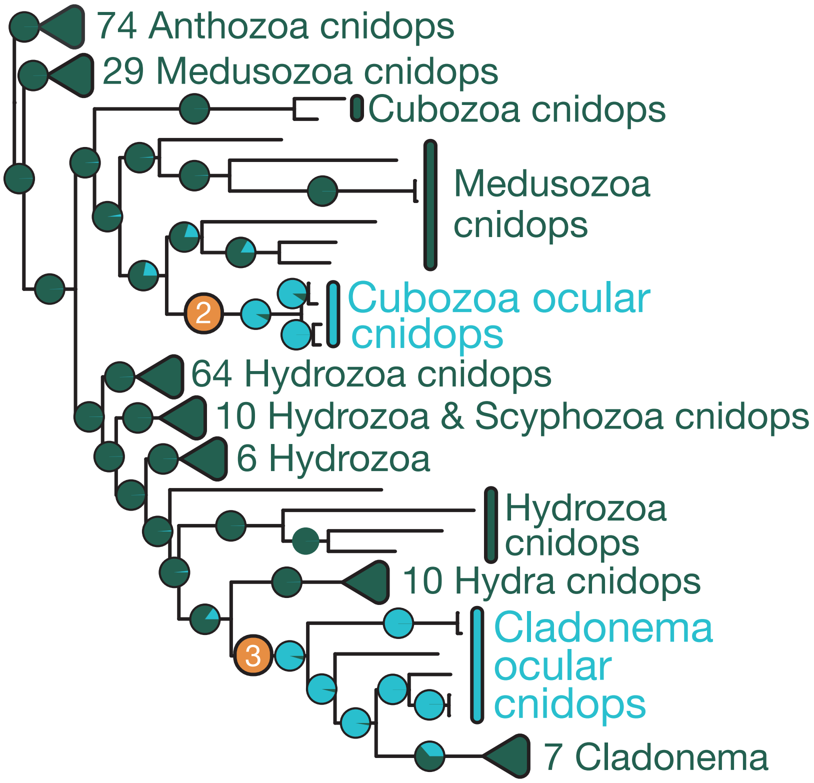

Figure 1.3. The Evolutionary History of Cnidarian Opsins Is Consistent with Multiple Eye Origins. We analyzed a large opsin dataset from all animals, including extensive new cnidarian sequences, with ML under a LG+F+R10 model in IQ-TREE. Illustrated here are cnidarian xenopsins (Ramirez et al. 2016) (cnidops, Plachetzki et al. 2007) and their pattern of ocular (light blue) and non-ocular (dark green) expression (see Figure S3 for full sequence names). Each pie chart displays the proportion of marginal likelihood for one state (ocular) versus the other (extraocular). Here, we also plot with orange squares separate origins of eyes from Cubozoa (box jellyfish; Origin 2) and *Cladonema* (Origin 3), species whose ocular opsins are known (Koyanagi et al. 2008, Suga et al. 2008, Liegertová et al. 2015, Bielecki et al. 2014). The ocular opsins of Origin 2 and Origin 3 are distantly related to each other, and each is descended from extraocular opsins. The alternative hypothesis of homology of eyes from origins 2 and 3 would predict their ocular opsins to be closely related, forming a monophyletic group. An alternative cnidops topology as recovered with a ML analysis under a GTR+G model in RAxML is also consistent with these conclusions (see Figures S1, S2, and S3).

To understand the history of ocular opsins, we tested two previous hypotheses of cnidarian opsin evolution using opsins from unprecedented taxonomic diversity, now including staurozoans, scyphozoans, siphonophores, and anthozoans. First, in an analysis including opsins from across all animals, we corroborate that all cnidarian opsins fall into three different subfamilies: chaopsins, anthozoa II opsins, and xenopsins (Feuda et al. 2012, Ramirez et al. 2016) (see supplemental results, Data availability in STAR Methods). We also corroborate cnidarian xenopsins (also called cnidops (Plachetzki et al. 2007)) are the only opsins known from medusozoans, the only cnidarians with eyes. We focused therefore on cnidops history to understand whether cnidarian eye origins were associated with separate co-option of extraocular genes. Following the cnidarian species tree, we find cnidops history to include an early divergence between anthozoan and medusozoan sequences, with opsins known to be expressed in cnidarian eyes belonging to the medusozoan opsin group (see full opsin tree 1 in supplemental results, Data availability in STAR Methods). With an alternative model of sequence evolution, GTR+G instead of LG+R10, cnidops topology is different, such that the medusozoan opsins no longer form a monophyletic group (see full opsin tree 2 in supplemental results, Data availability in STAR Methods).

By analyzing the expression history of cnidops in or out of eyes, we infer that at least two separate eye origins, Origins 2 and 3, were accompanied by shifts in opsin expression from extraocular to photoreceptor cells of newly evolved eyes (Figure 3). Using the cnidops topology obtained when using either GTR+G or LG+R10, our ancestral state estimates of cnidops expression shows that transitions from extraocular to ocular expression occurred separately when lensed eyes originated in *Cladonema radiatum* (Origin 3) and in cubozoans *Tripedalia cystophora* Conant, 1897 and *Carybdea rastonii* Haake, 1886 (Origin 2). Therefore, opsin history agrees with our inferences of multiple origins of eyes, with separate origins reflected in repeated events of extraocular opsins becoming ocular. Alternatively, if eyes had originated only once among cnidarians, followed by multiple losses, we would expect to find ocular opsins from eyed-species in a monophyletic group. That is, cnidops from the eyes of *Cladonema*, *Carybdea*, and *Tripedalia* would be closely related. Indeed, we find that *Carybdea+Tripedalia* ocular opsins form a monophyletic group as expected since their homologous eyes belong to Origin 2, which in turn are distantly related to *Cladonema* ocular opsins, present in eyes from Origin 3. Accordingly, our ML ancestor estimation infers separate transitions from extraocular expression to ocular in each of these groups.

2.2 Conclusions

Taken together, our results suggest cnidarian eyes evolved multiple times from ancestral photoreceptor cells, with opsins expressed in eyes of separate origin having evolved from divergent extraocular genes. These results make sense of previously published morphological and developmental details and make new predictions. Rather than representing stages of a single line of gradual evolution (16), cnidarian eyes originated prolifically in the absence of a central nervous system, often using different opsin paralogs, different morphological building blocks, and/or different developmental pathways. These perspectives provide rich opportunities to address fundamental evolutionary questions. To what extent are developmental, physiological, and genetic bases similar among cnidarian eyes within the same origin and different between origins? Do convergent eyes ever use the same developmental, physiological, or genetic basis? Do homologous cell types evolve differently in eyes versus outside of eyes, and if so, are cell types themselves convergent? The phylogenetic studies presented here provide a framework for such future studies.

2.3 Acknowledgements

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2.4 Author Contributions

Conceptualization, N.P. and T.H.O.; Methodology, N.P., M.D.R., and T.H.O.; Investigation, N.P., J.K., N.S., N.R., and J.C.; Software, A.S.; Resources, A.S, M.D.R., and M.D.; Data Curation, N.P.; Visualization, N.P., T.H.O.; Writing - Original Draft, N.P. and T.H.O.; Writing - Review & Editing, N.P., T.H.O., N.S., M.D., M.D.R., J.K.; Supervision, N.P., T.H.O.; Funding acquisition, N.P., T.H.O., and M.D.

2.5 Experimental Model and Subject Details

2.5.1 Animals

Polyps of *Haliplanella luciae* (=*Diadumente lineata*) used for transcriptome analysis were maintained in natural seawater at room temperature (22°C ± 1°C) under a 12:12 h photoperiod. Using a seawater open system (16°C ± 2°C; 12:12 h photoperiod), we cultivated colonies of *Renilla koellikeri* Pfeffer, 1886 (Anthozoa, Pennatulacea), collected in the Santa Barbara Channel on June 10th 2015. We fed the animals with 3-day-old Selcon® enriched *Artemia* nauplii (San Francisco Strain Brine Shrimp Eggs).

2.6 Method Details

2.6.1 Dataset assembly for species tree

We first retrieved DNA sequences (43,667) for all taxonomically non-redundant cnidarians available in the nucleotide database of NCBI on 10-26-2016. The database contains many taxonomically redundant sequences from the following: *Nematostella vectensis*, *Hydra oligactis, Hydra vulgaris, Corallium, Faviina, Porites, Stylophora, Acropora, Anthopleura elegantissima, Aiptasia, Aurelia aurita, Exaiptasia pallida* and *Hydractinia symbiolongicarpus.* In order to reduce computation time, we analyzed sequences from these taxa (120,554; also retrieved on 10-26-2016) separately in a second round of clustering analyses as explained below.

Because previous studies often sequenced non-overlapping portions of homologous genes, we inferred clusters of homologous gene regions from these bulk downloads as described in Yang & Smith (2014). We initially built a local blast database and performed all-against-all searches to recover pairwise similarity scores among sequences. After excluding BLAST results without a 50% minimum sequence overlap to avoid short sequences (with the python script 'blast-to-mcl'; Yang & Smith (2014)), we clustered remaining sequences using MCL 14-137 (Markov Clustering Algorithm; Van Dongen (2000)) with an inflation value of 2.0. We converted the MCL output to fasta files using the python script 'write-fasta-files-from-mcl' after shortening sequence names in the original fasta file (Yang & Smith 2014). Sequence names were matched back using a python script "seqmatcher" (available at https://bitbucket.org/swafford). By manually inspecting the resulting clusters, we selected those that encompassed a taxonomically diverse range of cnidarians. More specifically, we did not consider clusters with sequences from only one species, one genus or two genera for further analyses, unless clusters were not monospecific and contained species for which light sensing information is available. Most representative genes were partial regions of the 12S, 16S, cytochrome oxidase subunit 1 (COI) mtDNA in addition to 18S and 28S rDNA genes. After determining the suite of genes to be groomed in this first set of analyses, we repeated the clustering protocol with the molecular data set from the highly redundant taxa originally excluded. Sequence clusters with any of those genes were then merged with the selected raw clusters from the primary dataset for a third round of clustering analyses. In this third clustering analysis, we used a combination of 0.4 minimum sequence coverage for exclusion and 1.4 MCL inflation value for large but still alignable clusters.

We aligned genes with only one cluster each (18S and 12S) using MAFFT 7.305b (L-INS-i). Other genes (28S, 16S, COI) had taxa spread out in multiple clusters most likely due to sequencing of distinct regions of the same molecular marker. We merged clusters corresponding to the same gene and aligned the merged file using MAFFT 7.305b (E-INS-i), which accounts for multiple alignable and long unalignable regions among sequences Katoh & Standley (2013). We trimmed low quality regions of each gene alignment using trimAl 1.2 Capella-Gutiérrez et al. (2009) by removing positions with gaps in 40% or more sequences. Next, we removed spurious sequences by retaining only those with at least 65% nucleotides achieving a 60% overlap with those from other sequences. Merging 28S clusters resulted in a poor quality alignment with no sequences retained after using trimAl. Therefore, instead of merging 28S clusters, we selected the most taxonomically diverse 28S cluster to align, trim, and use for downstream analyses. We then discarded redundant sequences (keeping the longest sequence for a species, gaps ignored when counting nucleotide bases) with the tool "remove_phytab_dupes" (Oakley et al. 2014; available at Osiris on the Galaxy Bioinformatics Platform; http://galaxy-dev.cnsi.ucsb.edu/osiris). We concatenated processed alignments using Phylocatenator (also available at Osiris), retaining species with at least two genes. We manually included sequences from the following outgroup species: *Crassostrea gigas*, *Amphimedon queenslandica*, *Strongylocentrotus purpuratus* and *Trichoplax adhaerens*. With the concatenated data set (GenBank accession numbers in Table S3), we performed a preliminary maximum likelihood analysis and excluded unstable long-branch taxa (myxozoan species, *Juncella fragilis*, *Lepidisis olapa*, *Leptogorgia virgulata* and *Acropora* sp.).

2.6.2 Species tree reconstruction

With this final data set (1106 terminal taxa, 6629 aligned nucleotides), we performed model selection and maximum likelihood analyses using IQ-TREE multicore 1.4.2 (Nguyen et al. 2015) and explored the effects of partitioning to accommodate variation in substitution rates among sites and inclusion of distantly related outgroups on tree topology, branch lengths and node supports (see Kainer & Lanfear 2015, Bergsten 2005). We therefore analyzed our dataset with 4 strategies: (1) without partitioning, (2) with partitioning, (3) without partitioning and no outgroup, (4) with partitioning and no outgroup. When we partitioned the data set, we selected the best partitioning scheme and evolutionary models using PartitionFinder2 (Lanfear et al. 2017). We measured branch support in trees from all strategies with the ultrafast bootstrap algorithm, aBayes and SH-aLRT on 1,000 replicates using IQ-TREE. According to BIC (Bayesian Information Criterion), the best model for the unpartitioned dataset under strategies (1) and (3) was GTR+R10, and GTR+I+G for a partition of mitochondrial genes and SYM+I+G for that of nuclear genes under (2) and (4). Maximum likelihood analyses were run for a total of 10 replicates under each strategy. Trees without the outgroups were rooted with Anthozoa, as the split between Anthozoa and Medusozoa is well supported and we were mostly interested in the topology changes among medusozoan groups (see main text for discussion). We time-calibrated the main tree shown in Fig 1 before ancestral state reconstruction using penalized likelihood with six fossil calibrations in TreePL 1.0 (Smith & O'Meara 2012). Within Cnidaria, we used fossil calibration dates from Park et al. (2012), with the exception of family Rhizangiidae, for which we had no sequence data. We chose min and max dates for crown group Cnidaria from Van Iten et al. (2014), and for the root of the tree, we used 635 Ma as the lower bound for Metazoa based on biomarker evidence as per Cunningham et al (2017). Species names, genes and accession numbers of sequences concatenated for species tree are available at a github repository, as well as supplemental results from strategies 2-4 (see Data Availability section below).

We scored cnidarians in our phylogeny as possessing eyes or not based on an extensive literature survey (Table S1), with eyes defined minimally as a region with alternating pigment and photoreceptor cells (also called ocelli). One caveat of our scoring is that many species accounts are based on preserved material, which may lose pigmentation due to fixatives and hinder the recognition of eyes (André Morandini, pers. comm.). Nonetheless, high quality descriptions with fresh material show that many scyphozoans (e.g. *Chrysaora* spp.) truly lack eyes in their rhopalia (Morandini & Marques 2010) and taxonomic descriptions typically state explicitly when eyes are absent in the species. Considering the lack of mention of ocelli could be due to fixative issues, we assigned a missing state to species of scyphozoans and hydrozoans that had no mention of ocelli in descriptions, recognizing a possibility for absence or presence of eyes in those (see observations in Table S1). Pigment spots are often associated with light sensitivity, but their role in light perception needs to be validated with experimental or ultrastructural evidence. The broad scale of our analysis required us to rely on several observations made with light microscopy, so that in many cases we lack direct evidence for light perception. At present, there is therefore a fair amount of uncertainty on whether all cnidarian eyes are functional. Nonetheless, our phylogenetic results show that many eyes for which we do not have direct experimental evidence of light perception belong to close relatives of species that possess well-studied eyes (e.g. those shown in Figure 3).

Besides the adult medusae, other life stages can also be very active, such as the free-swimming planula larvae and the young scyphozoan medusae or ephyra. Detailed studies describe single-cell pigment cups in the planula of cubozoans despite their lack of a nervous system (Nordstrom et al. 2013). Among scyphozoans, if the adult is eyeless, their ephyra tends to be eyeless as well (see 69). For instance, the ephyra of the scyphozoan *Pelagia noctiluca* (Forsskål, 1775) bears no eyes and remains eyeless as an adult medusa (Hertwig 1878, after Russell 1970), but the opposite occurs in *Aurelia aurita* (Linnaeus, 1758) and *Nausithoe aurea* Silveira & Morandini 1997, whose eyes start developing in the young ephyra and reach full size in the adult medusa (Yamasu & Yoshida 1973). Although eyes in other stages will substantially add to our knowledge on cnidarian eye evolution, we restrict this study to eyes in the well-studied adult medusae in order to facilitate a broad scale analysis.

2.6.3 RNA-Seq library preparation and sequencing

For 9 anemone species (*Actinia equina*, *Aiptasia pallida*, *Anthopleura elegantissima*, *Bunodosoma cavernata*, *Calliactis parasitica*, *Metridium senile*, *Sargatia elegans*, *Stomphia coccinea, Triactis producta*), tissue excised from the polyp was flash frozen in liquid nitrogen and stored at -80° C. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), following manufacturer-supplied protocols. Small (1.5-2.0 mm), sterile ceramic beads were added to each sample along with Buffer RLT and then each tissue sample was homogenized using a Mini-Beadbeater-8 (BioSpec Products).

RNA extractions were quantified on the Qubit 2.0 (Life Technologies) and RIN values determined on the BioAnalyzer (Agilent Technologies). First strand synthesis, library construction, and paired-end 100 base sequencing were conducted at The Genomics Shared Resource Center of the The Ohio State University James Comprehensive Cancer Center (Columbus, OH, USA). For *Haliplanella luciae* (=*Diadumene lineata*) and *Renilla koellikeri*, total RNA from polyp tentacles was extracted using the Nucleospin RNA II kit (Macherey-Nagel) or the Qiagen RNEasy Mini kit. First and second strand cDNA synthesis was made with the SMARTer cDNA Kit and Advantage 2 PCR kits (Clontech). Libraries were constructed using the Illumina TruSeq kit and sequencing was performed on Illumina HiSeq 2000 and 3000 platforms.

2.6.4 Dataset assembly for opsin tree

To identify candidate opsin genes, we screened 109 transcriptomes of 86 cnidarian species, including 98 deposited in the NCBI SRA (Sequence Read Archive; NCBI) and TSA (Transcriptome Shotgun Assembly; NCBI) databases and 11 newly generated data sets (species and accession numbers listed in Table S4). Paired or single-end raw reads from the SRA and newly generated reads were trimmed for quality (minimum Phred score of 33) and adaptors (automatically detected) using either Trim Galore 0.4.2 [a wrapper for Cutadapt 1.12 (Martin 2011) and FastQC 0.11.5] or Trimmomatic 0.32 (Bolger et al. 2014) followed by assembly using Trinity 2.2.0 (Grabherr et al. 2011). We detected open reading frames with at least 30 amino acids among transcripts in each transcriptome using TransDecoder r2012-08-15 (Haas et al. 2013). We used PIA (Speiser et al. 2014) to identify candidate opsin genes. We modified PIA to include a reduced opsin bait set with two or three sequences (one deuterostome, one protostome and one cnidarian representative if applicable) of each opsin group described in Ramirez et al. (2016). For initially estimating the placement of candidate sequences we used the opsin data set of (Feuda et al. 2012), which comprises cnidarian opsin sequences and non-opsin GPCR outgroups. We performed blastp searches against the transcriptomes using the opsin bait set with an e-value of 1e-10 and added all candidate opsin sequences onto Feuda's master opsin alignment with MAFFT-profile. With the evolutionary placement algorithm implemented in RAxML 8.2.9 (Stamatakis 2014), we placed new candidate opsin sequences on Feuda's tree using a maximum likelihood criterion. We used the python script "supercuts" (available at https://bitbucket.org/swafford/supercuts) to retain cnidarian query sequences closely related to opsins, placopsins and melatonin receptors for further phylogenetic analysis. We used tblastn to blast all the selected candidate opsin protein sequences against the 2014 NCBI nucleotide database on Galaxy Platform and removed potential contaminants (observed to match queries with unexpected highly significant e-values and identity; available at https://github.com/npicciani/picciani_et_al_2018/contaminants.fasta). We also removed redundant duplicates and partial sequences for each species in our cnidarian data set using CD-HIT 4.6 (Fu et al. 2012) with an identity threshold of 100% (final set of candidate opsin sequences in Table S5).

2.6.5 Opsin tree reconstruction

We added all sequences from Ramirez et al. (2016) and representatives of melatonin receptors, placopsins and chytropsins (Ahrendt et al. 2017) as outgroups to our cnidarian data set as these are some of the closest groups to canonical type-II opsins (Feuda et al. 2012). We rooted our opsin trees with the chytropsins. We removed duplicates from the cnidarian species that were already in the Ramirez data set using AliView 1.18 (Larsson 2014) and aligned the final opsin data set using MAFFT 7.304b (E-INS-i; 47). We used trimAl 1.2 (Capella-Gutiérrez et al. 2009) to remove positions with gaps in 90% or more sequences and spurious sequences that did not have 65% of their residues achieving an overlap of 50% with those of other sequences. Our final alignment consisted in 1591 protein sequences and 457 amino acid residues. We carried out model selection and tree reconstruction under a maximum likelihood (ML) criteria using IQ-Tree multicore 1.4.2 (Nguyen et al. 2015). We performed 35 ML searches under the LG+F+R10 model. We measured branch support using the Ultrafast Bootstrap (UFBoot) algorithm with 1000 replicates, the SH-aLRT and abayes (approximate transformation Bayes test). The ultrafast bootstrap support values did not converge in the best ML search, therefore we re-ran an extra search with the best tree as a starting tree and ensured the UFBoot values converged (i.e. achieving a bootstrap correlation coefficient of split occurrence frequencies > 0.99). In addition to the LG+F+R10 model, we reconstructed the opsin tree using the GTR+G model in RaxML 7.4.3 (Stamatakis 2014) and generated bootstrap values based on 100 replicates.

After generating the opsin phylogeny, we restricted the ancestral state reconstruction to the cnidops subfamily. As such, we used the cnidops topology to reconstruct the location of expression for ancestral cnidops sequences, whether it was ocular or non-ocular. Our scoring considered not only the sample from which the transcriptome was generated but also in-situ hybridization data on opsin expression from previous studies. We considered ocular opsins to be those expressed in photoreceptor cells of eyes, and non-ocular those expressed outside photoreceptor cells of eyes (Table S6). When an opsin sequence is expressed ubiquitously across body regions including photoreceptor cells (which only is known from *Cladonema*), we scored it as polymorphic or ambiguous as it is both non-ocular and ocular. Note also that many cnidarians are eyeless, and in instances where we find opsins in those species, we called them non-ocular without the need for data on localization. While common to conduct ancestral state reconstruction on time-calibrated trees, as we did for Cnidarian species, we did not scale branch lengths to time for the opsin gene tree because constraining nodes with fossils requires confident identification of orthologs, which is challenging for opsins (Ramirez et al. 2016). Instead of attempting a rough chronogram to map the history of opsin expression, we scaled branch lengths by amino acid substitutions in opsins. This assumes shifts in location of gene expression location are reflected by substitution rates, reasonable because expression divergence is often correlated with sequence divergence (Li et al. 2005). We estimated the ancestral states (ocular vs non ocular expression) across the opsin phylogeny as described below (see Quantification and Statistical Analysis). We used an asymmetric Markov two-state model (transition rates different) given its best fit compared to a symmetric Markov two-state model according to a likelihood ratio analysis (chi-square test; df=1; $p<0.05$).

2.6.6 Quantification and Statistical Analysis

To statistically infer ancestral states, we used the function RayDISC in the R package "corHMM" to compute the marginal state likelihoods at internal nodes in rooted species trees (Beaulieu et al. 2013). When the state is missing or the species is polymorphic, RayDISC assigns equal likelihoods to both states (presence or absence of eyes). We compared the fit of two models of character evolution, an asymmetric Markov two-state model (transition rates different) and a symmetric Markov two-state model (equal transition rates), to our data. A Markov transition rate model of evolution assumes the probability of change of a character does not depend on previous states, that character transition along a branch is independent of transitions in other parts of the tree, and that rates of transition do not change along the branches of a tree (Mooers & Schluter 1999). Our likelihood ratio test indicates a significantly better fit of an asymmetric Markov two-state model to our data under the topologies of all species tree reconstruction strategies (chi-square test; df=1; $p<0.05$ for all topologies). For each ancestral node, we considered the marginal likelihood of eye presence (P) to be significantly better than the marginal likelihood of eye absence (A) when $|\ln(P) - \ln(A)| > 2$ (Pagel 1999). Because we were only able to include very distantly related outgroups, we did not score them for eyes and used a root prior that assumes equal likelihoods for both states (presence and absence of eyes). Additionally to these likelihood reconstructions, we accounted for phylogenetic uncertainty in cnidarian relationships and used the R package "indorigin" to perform a Bayes Factor Test of Independent Origins comparing the probability of the observed data under the null and alternative hypothesis of less or at least a specified number of origins, with a set of 1,000 bootstrap replicates produced under strategy 1 (unpartitioned analysis, described above) with estimated transition rates from corHMM (see more details about the Bayes Factor Test in Pankey et al. (2014). Parsimony ancestral state reconstruction was made with Mesquite 1.0 (Maddison & Maddison 2018). Finally, we gathered reports of light-associated processes among cnidarians in our phylogenetic data set from the literature (Data S1).

2.7 Data and software availability

The accession numbers for opsin sequences and transcriptomes generated in this study are MH586782-MH586815, SRA: SRP152591 in BioProject PRJNA464357. Additional method details, sequences and analytical results (including supplemental results referenced throughout the main text) are available at https://github.com/npicciani/picciani_et_al_2018.

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Chapter 3

Gene expression in jellyfish convergent eyes reveals some conservation yet extensive divergence in expression of vision genes

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3.0 Abstract

Convergent evolution is central to our understanding of the pathways to phenotypic similarity. If we can understand the genetic basis of phenotypic convergence, we can pinpoint instances of conservation and divergence in each convergent trajectory. Animal eyes are one of the most famous examples of convergence, evolving in many distantly related lineages. While the eyes of bilaterian animals are relatively well studied, eye convergence in simpler animals such as jellyfish remains poorly understood. Jellyfish eyes evolved independently at least nine times, and yet we know of only a few cnidarian genes related to eye development and function in very few species. Here, we investigate gene expression in convergent eyes and other non-visual tissues from three species of jellyfish. We demonstrate that tissues with eyes invariably express a very distinctive set of genes that differentiates them from other non-visual tissues. Overall gene expression indicates that non-visual and homologous tissues such as the manubrium display a profile that is very similar across species, while tissues with eyes seem to be more divergent from one species to another. Moreover, a few vision related gene families upregulated in tissues with eyes are conserved across all species, with most of them being upregulated in a lineage-specific way. Our study highlights the possibility that distinct gene families were recruited at each eye origin, with lineages using a few conserved building blocks and lineage-specific ones possibly associated with details of eye morphology and phototransduction.

Keywords: independent evolution, photoreception, light sensing, development

3.1 Introduction

Convergent evolution can lead to phenotypic or functional similarity across distantly related lineages. It provides us with replicated natural experiments that are key to infer pathways underpinning the origin of similar traits. Understanding the basis of convergent traits can help us dissect conserved and divergent genetic routes that lineages separately used to build those traits. Eyes are convergent traits that appeared several times in evolution and consist of building blocks with discrete functions, such as photoreceptors, pigments and lenses. Because animal eyes are complex and modular, they provide us with a unique opportunity to understand the sets of genes repeatedly used to produce their building blocks. While convergence of eyes in vertebrates and cephalopods is relatively well studied (Ogura, Ikeo, and Gojobori 2004), other groups of animals, such as cnidarians (e.g jellyfish), have received very little attention. Only recently did we realize the extent of convergent evolution that occurred among species of jellyfish, with eyes of many types originating at least nine times (Picciani et al. 2018; Miranda and Collins, 2019). Leveraging these many origins across jellyfish can help us gauge the amount of shared (conserved) or lineage-specific (divergent) genes expressed in convergent eyes. Ultimately, that will allow us to pinpoint the multiple routes that animals can use to repeatedly build convergent traits.

Even though eyes occur in several jellyfish lineages, we know of a few genes involved in vision and eye development in only two groups, mostly based on studies with a priori selection of genes. One group, Cubozoa or box jellyfish, possess true image forming and other simpler eye types. Box jellyfish eyes are located on sensory organs called rhopalia, which also contain a large part of their nervous system. Genes encoding components of their visual phototransduction (opsins, G-proteins and intermediary enzymes such as adenylate cyclase) and regulating eye development (*pax*B) are known for box jellyfish eyes (Kozmik et al. 2003; Bielecki et al. 2014; Koyanagi et al. 2008). A second group, the Hydrozoa, comprises small jellyfish, or hydromedusae, that lack rhopalia but possess eyes sometimes with lens-like structures on their so-called tentacle bulbs. One species of hydromedusae, *Cladonema radiatum*, uses *pax*A in the development of its lensed-eyes, as well as members of the *Six* gene family (Suga et al. 2010; Stierwald et al. 2004). Knowledge of vision related genes in a third major group of jellyfish, the Scyphozoa, is relatively limited [but see (Gold et al. 2019)], even though several genes related to nervous system development are known to be expressed in the scyphozoan rhopalia (Nakanishi et al. 2010, 2015; Nakanishi, Hartenstein, and Jacobs 2009). While these studies have advanced our knowledge of genes involved in cnidarian vision and eye evolution, unbiased approaches that do not rely on a priori candidate genes can provide us with a more global perspective on vision-related genes and allow for an objective comparison of jellyfish convergent eyes.

Here, we present for the first time a cross species comparison of genes differentially expressed in convergent jellyfish eyes and other non-visual tissues. We show that tissues with eyes (tentacle bulbs and rhopalia) from three groups of jellyfish (Scyphozoa, Cubozoa, Hydrozoa) with convergent eyes invariably display a gene expression signature very distinctive from that of other tissue types. By comparing their complement of vision related genes, we show that convergent eyes express a few gene families across all three species, whereas they express many more gene families in a lineage-specific way. Our results corroborate previous studies on gene expression in jellyfish eyes, and, by comparing our newly generated data with previously published data, it further suggests that gene expression in eyes that are homologous are extraordinarily similar. Our study provides an outstanding starting point for other comparative studies aiming at investigating other aspects of jellyfish eye convergence.

3.2 Methods

3.2.1 Animal culturing

We cultured polyp colonies of the hydromedusae *Sarsia tubulosa* (M. Sars, 1835) in a closed system with sea water at 18°C and 37‰ salinity as well as a 12:12 photoperiod. We obtained polyps of the scyphozoan *Aurelia aurita* sp.1 (Linnaeus, 1758) from the Santa Barbara Sea Center and kept them in an open water system with temperatures varying according to those on the coast of Santa Barbara, California. We fed polyps three-day old artemia nauplii enriched with Selco (Self-Emulsifying Lipid Concentrate; Brine Shrimp Direct) every three days. *Aurelia* jellyfish were kept in a goldfish bowl with oxygenation from an air tube creating a circular flow.

3.2.2 RNA extraction for tissue-specific library construction and sequencing

We starved jellyfish for at least 24h prior to dissections in order to avoid brine shrimp contamination. We dissected tentacles/marginal lappets, tentacle bulbs/rhopalia and mouth (Fig. 1A) using UV-sterilized tools further treated with

RNase AWAY™ Surface Decontaminant (Thermo Fisher Scientific). We transferred each dissected tissue directly into either the RNeasy Mini Kit lysis buffer or chilled TRIzol® Reagent (Invitrogen) (see Table S1 for details on tissue samples and their RNA extraction methods). We proceeded according to the kit manufacturer's protocol for samples extracted with the RNeasy Mini Kit. For those kept in TRIzol, we proceeded with a liquid nitrogen freezing step followed by maceration with a mini pestle and RNA extraction using chloroform. We additionally performed RNA extraction with TRIzol of whole body jellyfish for a reference assembly. We sent RNA samples to Novogene Corporation (Sacramento, CA) for quality control tests (quantitation and RNA integrity checks using Nanodrop, Agarose Gel electrophoresis, Agilent 2100), library preparation and paired-end Illumina Hiseq PE150 sequencing at 150 bp read lengths. We sequenced a total of 15 paired-end RNA-seq libraries from four tissue types from *Sarsia* and *Aurelia* with, at least, two biological replicates per tissue per species (*Sarsia*, tentacles: 2, tentacle bulbs: 2, manubrium: 2; *Aurelia*, rhopalia: 3 and manubrium: 6). Additionally, we used tissue-specific paired-end RNA-seq publicly available at the NCBI SRA Database from tentacles (SRR8101526), rhopalia (SRR8101523) and manubrium (SRR8101518) of the box jellyfish *Tripedalia cystophora* Conant, 1897.

3.2.3 Assembly of a low-redundancy reference transcriptome for *Sarsia*

Due to the lack of a genome for *Sarsia*, we assembled a reference transcriptome using ~70M paired-end raw reads from whole body jellyfish. We evaluated the quality of raw reads using FastQC v0.11.9, and used Trinity v2.8.5 for trimming raw reads with Trimmomatic v0.36 and assembling the clean reads. According to quality assessment via BUSCO v3.1 (Seppey, Manni, and Zdobnov 2019), this original assembly possessed 96.6% Metazoa Complete BUSCOs, with a high proportion of duplicated BUSCOs (44.9%) among complete BUSCOs. These high levels of duplicated genes in the reference assembly led to many multimapped reads in preliminary downstream analyses, sometimes comprising almost half of the total number of mapped reads. Multimapped reads can lead to overestimation of sequenced molecules, and, as such, are often discarded prior to differential expression analyses at the gene level [see (Deschamps-Francoeur, Simoneau, and Scott 2020)]. We thus tested several ways to reduce the number of duplicates (or redundant sequences) in the assembly, while maintaining BUSCO completeness and obtaining high percentages of uniquely mapped reads. After testing several approaches, we lowered transcriptome redundancy by keeping only the longest transcript per 'gene' ('gene' as defined by Trinity gene ids) following (Liang et al. 2019). We used the same strategy to build a reference assembly for *Tripedalia*, which also lacks a genome, based on its original assembly available at the NCBI TSA database [GHAQ00000000.1; (Khalturin et al. 2019)]. By doing that, we lowered the number of sequences in our initial *Sarsia* and *Tripedalia* reference assemblies from 176,963 and 154,192 sequences, respectively, to 27,090 and 20,979. This step increased the downstream amount of uniquely mapped reads (mapping details below) from ~40-45% to ~75-85%.

3.2.4 Read mapping and counting

We used STAR v2.7.5b (Dobin et al. 2013) to build an index and perform read mappings of tissue-specific libraries (see Table S2 for details on library sizes and quality statistics). We counted reads mapped to genes using featureCounts v2.0.1 (Liao, Smyth, and Shi 2014) with an annotation GTF file containing only exon entries generated with a custom python script. For *Aurelia* we used its published genome (Gold et al. 2019) as a reference for read mapping (29,964 gene models; 15,884 annotated genes).

3.2.5 Differential gene expression analysis

We used estimated fragment counts from featureCounts to perform pairwise contrasts to identify genes differentially expressed among tissues using the R package DESeq2 (Love, Huber, and Anders 2014). We normalized raw counts using the DESeq2 method, which generates pseudocounts by taking into account sequencing depth. Outlier libraries were removed according to Cook's distance. Additionally, we used the independent filtering approach from DESeq2 to remove low expressed genes prior to each tissue contrast. For each pairwise contrast, the independent filtering optimizes removal of low expressed genes in order to maximize the number of rejections (Benjamini Hochberg adjusted p-values lower than $\alpha=0.05$) over the mean of normalized counts. Each pairwise contrast was performed using the Wald statistics, as implemented in DESeq2. We also transformed the data with a variance stabilizing transformation to facilitate data visualization and clustering. We produced venn diagrams of overlapping differentially expressed genes among tissues using the R package VennDiagram v1.6.20.

3.2.6 GO enrichment analysis

We performed functional annotation of each transcriptome using Trinotate (Bryant et al. 2017), which carries out homology searches using BLAST+SWISSProt/UniProt. We used gene annotations (15,884) for the *Aurelia* genome available from Gold et al. (2018). We retrieved annotations for 14,935 genes from *Sarsia* and 11,981 from *Tripedalia*. We tested whether Gene Ontology (GO) terms related to biological processes were significantly enriched among upregulated genes with the Fisher's exact test and the GO processing algorithm "weight01", with a GO processing algorithm ("weight01") that takes into account the hierarchical nature of gene ontology terms (Alexa, Rahnenführer, and Lengauer 2006) which accounts for the hierarchical structure of gene ontology terms, using the R package topGO (Alexa, Rahnenführer, and Lengauer 2006).

3.2.7 Phylogenetic analysis of opsin sequences

We retrieved opsins from *Sarsia* and *Tripedalia* transcriptomes using a customized python version of PIA [Phylogenetically Informed Annotation; (Speiser et al. 2014)] with the opsin tree from (Picciani et al. 2018). Candidate opsin proteins from *Aurelia* were selected based on its genome annotation. We then combined the original matrix of opsin proteins from (Picciani et al. 2018) with those we pulled from each reference for estimating their phylogenetic relationships. After aligning the sequences using MAFFT v7.407 (Katoh and Standley 2013), we trimmed them using trimAl (Capella-Gutiérrez, Silla-Martínez, and Gabaldón 2009). We used that final trimmed alignment as an input to IQ-TREE v2.0.3 (Minh et al. 2020) for estimating both the best fit model of sequence evolution (LG+F+R10, according to the Bayesian Information Criteria) and their evolutionary tree using a maximum likelihood approach (with ten replicate runs).

3.3 Results

3.3.1 Eye tissues possess a gene expression signature enriched in vision-related genes and very distinct from other body tissues

We first investigated whether tissues with eyes have their own gene expression signature. One tentacle sample from *Aurelia* corresponded to an outlier sample and was excluded from downstream analysis. To do that, we inferred differentially expressed genes (DEGs) between tissues with eyes or other tissue types (tentacles and manubrium) within species (Wald test, $p_{adj} < 0.05$). In each species, variation among samples is mostly explained by tissue types (Figure 3.1A-C). Gene expression in tissues with eyes strongly distinguishes them from other tissues, even adjacent tentacles (Figure 3.1A-C). Across all species, tissues with eyes (rhopalia and tentacle bulbs) and tentacles share the lowest number of DEGs, while tissues with eyes and manubrium show the most pronounced difference (Table 1). Our analysis reveals a few eye tissue-specific genes (Figure 3.1D-F), with many of them not functionally annotated and possibly taxonomically restricted. To understand the biological meaning of upregulated DEGs, we assessed which processes were enriched across eye tissues. Across all species, upregulated DEGs are significantly enriched (Fisher's exact test, p<0.05) in several GO categories, typically including neuropeptide signaling (GO:0007218), synaptic transmission (GO:0007270), phototransduction (GO:0007602), visual perception (GO:0007601) and absorption of visible light (GO:0016038). Interestingly, the rhopalia from *Aurelia* and *Tripedalia* display overrepresented processes related to visual learning (GO:0008542) and memory (GO:0007613), suggesting these tissues might be key for sophisticated information processing.

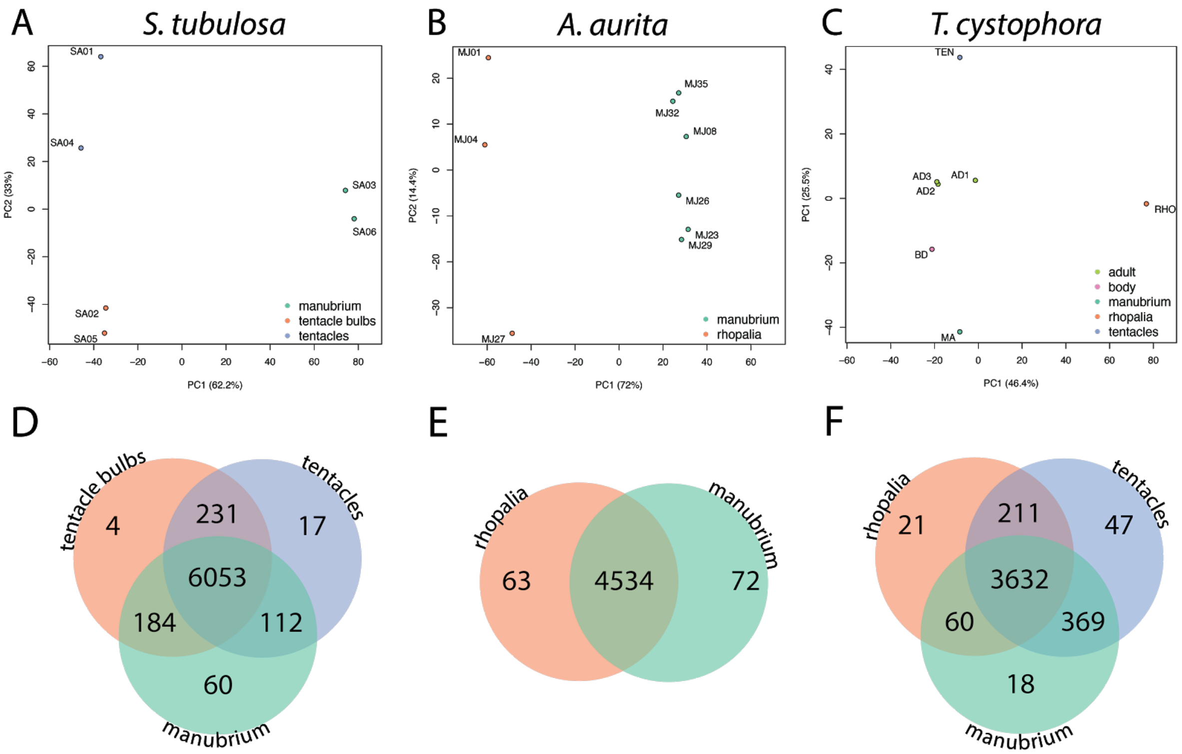

Figure 3.1. Gene expression signature among tissue types from *Sarsia* (A, D), *Aurelia* (B, E) and *Tripedalia* (C, F). A-C: Principal component analysis of tissue samples included in this study. The first two principal components, PC1 and PC2, account for 95.2% (A), 86.4% (B) and 71.9% (C) of variation in gene expression across tissue samples from each species. D-F: Overlapping differentially expressed (DE) genes among tentacle bulbs, rhopalia, manubrium and tentacles from *Sarsia* (D), *Aurelia* (E) and *Tripedalia* (F). Non-overlapping DE genes (or unique) are those that are differentially expressed among tissues yet are only detected in only one type of tissue. DE genes correspond to ~24% of genes

in *Sarsia* (6,661 out of 27,090), ~13% of genes in *Aurelia* (4,669 out of 35,273), and 20% in *Tripedalia* (4,358 out of 20,979). List of DE genes from each pairwise comparison between tissue types per species is available in Table S3.

3.3.2 Gene expression across species is more divergent between eye tissues than manubrium

We then sought to estimate overall gene expression dissimilarity in tissues with convergent eyes (*Aurelia* rhopalia, *Tripedalia* rhopalia and *Sarsia* tentacle bulbs) compared to other tissue types (tentacles, manubrium) across species. We first inferred the orthologous gene groups across our reference assemblies using Orthofinder v2.4. We detected a total of 14,213 groups of orthologous genes (so-called orthogroups; in which ~77.4% of genes from all references were placed), among which 3,488 corresponded to single copyorthogroups (strict orthologs). We merged gene counts of these 3,488 single copy orthogroups, from tissue samples across species, regressed out batch effects to remove species signals, and compared their gene expression with distance metrics. By estimating the euclidean distances among samples and using a hierarchical clustering algorithm, we found that gene expression in the manubrium was highly similar across species and formed one cluster. Conversely, tissues with eyes split into two main groups: one composed of rhopalia from *Aurelia* + *Tripedalia*, and the second with *Sarsia* tentacle bulbs. Additionally, tentacle samples were nested within eye tissue groups, without following any species-specific patterns (Figure 3.2). Using Pearson correlation coefficients instead of euclidean distances for a clustering analysis produces identical results (not shown).

Table 1. Number of up- and downregulated DEGs (p_{adj} <0.05) in pairwise contrasts between tissue types* in each jellyfish.

	Sarsia		*Aurelia*		*Tripedalia*	
	up	**down**	**up**	**down**	**up**	**down**
Rhop/TBulbs vs Tent	1,430	1,604	na	na	984	1,677
Rhop/TBulbs vs Manu	1,902	1,143	2,734	1,935	1,342	1,701
Manu vs Tent	2,293	2,868	na	na	651	1,131
Total DEGs	6,661		4,669		4,358	
%DEGs out of total genes	~24%		~13%		~20%	

*Rhop: rhopalia, Tbulbs: tentacle bulbs, Tent: tentacles, Manu: manubrium.

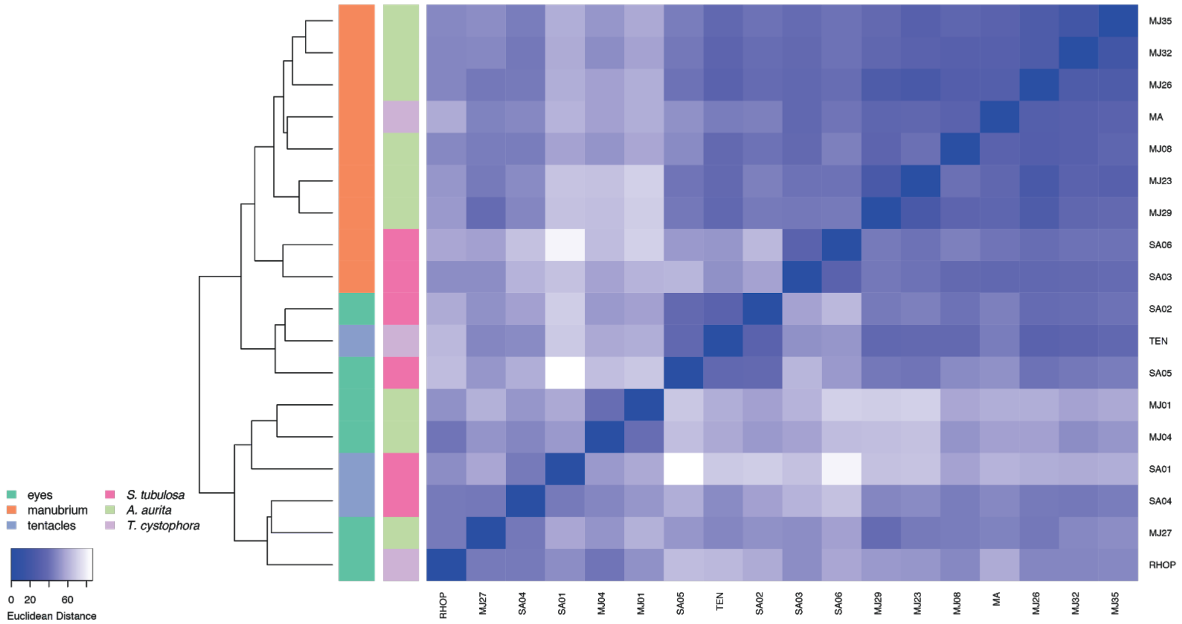

Figure 3.2. Euclidean distances (dissimilarity) among tissue samples from *Sarsia*, *Aurelia* and *Tripedalia*. Original counts from 3,488 single copy orthogroups were transformed to the log2 scale and corrected for batch effects (species signal) prior to visualization. Cluster dendrogram on the left displays hierarchical cluster solution of analysis of tissue samples using the complete linkage method.

3.3.3 Gene families associated with phototransduction processes, visual system specification and the retinal determination gene network are upregulated in tissues with eyes

Next, we inspected the identity of genes associated with visual processes that were substantially upregulated (log2 fold change > 2) in tissues with eyes. Candidate homologs from several well-known gene families related to phototransduction, visual system specification, melanin synthesis, and the retinal determination gene network are upregulated in tissues with eyes. Moreover, these 'vision-related' genes seem to be conserved across species at different degrees, with only eight gene families shared among all three species, while others either shared by two species only, or many more specific to one species (see Table S4). Below we describe genes found to be broadly conserved and those that are lineage-specific in expression.

Conserved genes across species. Cyclic nucleotide gated (CNG) ion channels mediate visual/non-visual phototransduction in several animals, including cnidarians, and are thought to belong to their ancestral phototransduction cascade (Plachetzki, Fong, and Oakley 2010). Likewise, pigment cells are fundamental eye structures and synthesize screening pigments for directional photoreception. Accordingly, both CNG ion channel genes and *tyrosinase*-like genes are upregulated in eye tissues from all species, suggesting they might be involved in visual phototransduction and pigment synthesis. While these two genes are related to structural components implicated in vision, we find *six3/6* (sine oculis) homeobox gene, a regulator of eye development in flies, also upregulated across all species.

Other conserved vision related genes yet, to our knowledge, unreported in cnidarian eyes include *neuroglobin*, *notch*, the homeobox *lhx1*, the nuclear receptor *tlx* and *wnt-4*-like genes.

Lineage-specific genes. Another *six* member, *six1/2*, is upregulated only in *Sarsia* tissues with eyes together with one *pax*A homolog. Even though *Sarsia* expresses an *eyes absent* (*eya*)-like gene, we find it is not differentially expressed among the tissues we sampled, though with a slightly higher expression in the manubrium. These expression patterns are consistent with those described in *C. radiatum* (Stierwald et al. 2004; Graziussi et al. 2012), a closely related hydromedusae with eyes homologous to *Sarsia*. While *Aurelia* and *Tripedalia* express *Pax* genes, none of them are upregulated in their tissues with eyes - they are either not differentially expressed or upregulated in other tissue types.

Genes encoding lens proteins are upregulated only in *Sarsia* and *Tripedalia*, which have each a different type of lens. The lens in *Tripedalia* eyes are made of crystallins similar to those in other animal eyes, so that J1A and J1B-*crystallin* genes are upregulated only in its rhopalia. One upregulated *alpha(B)-crystallin*-like gene is a heat-shock protein, while other types of J-*crystallin* genes (J2 and J3) previously reported from *Tripedalia* are not upregulated in its rhopalia. Crystallins are so far not known from other cnidarians, but *Sarsia* is nonetheless able to produce a lens-like electron dense material (Singla and Weber 1982). Rather than crystallin genes, we retrieved two *lens fiber membrane intrinsic protein*-like (*Lim2*-like) genes upregulated in *Sarsia* eye tissues. *Lim2* genes in mouse and bovine encode structural constituents of their eye lenses, pointing out to a possibly similar functional role.

Lastly, G protein α subunits are important phototransduction molecules that define animal cascades. We find genes encoding distinct types of G protein α subunits, namely Gα-i and Gα-s, upregulated in eye tissues of *Aurelia* and *Tripedalia*, respectively. The use of Gα-i in the visual phototransduction cascade of *Aurelia* is yet to be tested. In turn, Gα-s has been shown to mediate phototransduction in the box jellyfish *Carybdea rastonii* together with adenylate cyclase (Koyanagi et al. 2008), both of which are encoded by genes also upregulated in *Tripedalia* rhopalia. Even though *Sarsia* has genes that encode Gα-o, Gα-i, Gα-s and Gα-q-like subunits, all these types are expressed in similar amounts in its tissues.

3.3.4 Opsins with highest expression in tissues with eyes are closely related to known visual opsins

Because opsins are the light sensor in animal eyes, we next investigated the identity of the visual opsin gene in each species based on expression levels and their relationship to other known visual opsins. To estimate the diversity of opsin genes expressed by each jellyfish species, we used a python version of PIA (phylogenetic informed annotation; Speiser et al. 2014) to pull candidate opsin protein sequences from species transcriptomes. While we detected many candidate opsin protein sequences from both *Sarsia* and *Tripedalia* (37 and 19, respectively), a lot of them are short and end before the lysine residue (K296) to which the photosensitive chromophore gets attached, possibly representing technical artifacts (6 short sequences both in *Sarsia* and *Tripedalia*). We retrieved 37 protein sequences functionally annotated as opsins from the protein models available for the genome of *Aurelia*. After compiling these candidate opsin sequences, we performed a thorough phylogenetic analysis using these candidate opsins together with the set of opsin protein sequences from Picciani et al. (2018). We found only 3 out of 37 protein sequences (encoded by 3 genes) annotated as opsins in the genome from *Aurelia* belong to the canonical animal opsin group (type 2). In turn, we found 37 and 13 opsin proteins sequences (encoded by 23 and 11 genes) from *Sarsia* and *Tripedalia* respectively. Consistent with previous studies, we found that opsin genes from each species all belong to the cnidops clade (Figure 3.3A).

We first aimed at describing how expression of the already known visual opsin from *Tripedalia* compared to its other opsins. Compared with other opsin genes, the visual opsin gene from *Tripedalia* [comp72978_c0; referred to as lens-eye opsin in Bielecki et al. (2014)] is the one with highest expression in the rhopalia.

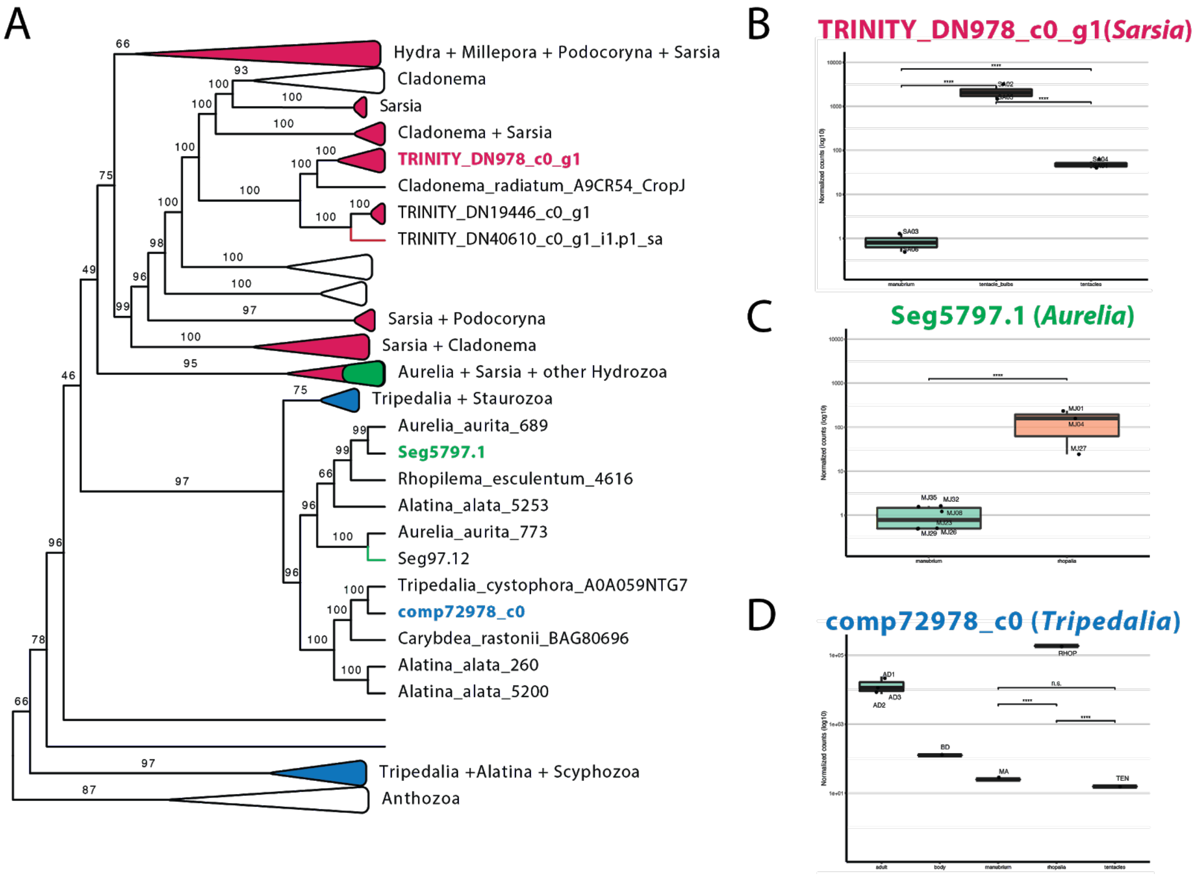

Figure 3.3. Cnidops gene tree (A) and expression of candidate visual opsin genes (B-D). Complete maximum likelihood tree (Figure S1) inferred from a sequence alignment with animal opsins and non-opsin G-protein coupled receptor sequences (1,879) with 296 aminoacid residues under a LG+F+R10 model in IQ-TREE. Ultrafast bootstrap support values shown above branches and calculated from 1,000 replicates. Clades are collapsed for visualization, with groups encompassing opsins from *Tripedalia*, *Sarsia* and *Aurelia* colored, respectively, in blue, pink and green.

Strikingly, it is upregulated in the rhopalia by three to five orders of magnitude compared with other tissues (Figure 3.3D). Other *Tripedalia* opsin genes (except for one gene) are similarly upregulated in its rhopalia (two of them unique to rhopalia), with an almost negligible expression in the manubrium and tentacles. Using a similar basis for comparison, we identified the opsin gene both upregulated and most highly expressed in tissues with eyes from other species. With a complete opsin tree, we also sought to visualize the relationship between these candidate visual opsins to those known from *Tripedalia* and *C. radiatum*. A total of 12 opsin genes are upregulated in *Sarsia* tentacle bulbs, with one being unique to it (TRINITY_DN10602_c0_g1). The *Sarsia* opsin gene upregulated in its eye tissues with the highest expression among others is TRINITY_DN978_c0_g1, which is encodes a protein that is closest sequence to CropJ, an eye-specific protein in *C. radiatum* (Figure 3.3B). The three opsin genes from *Aurelia* are upregulated in the rhopalium, yet none of them is unique to it. Among these three genes, the one with the highest expression (Seg5797.1; Fig. 3C) is closely related to other scyphozoan opsins in a group that is sister to rhopalia-specific opsins from cubozoans, which includes the previously mentioned visual opsin from *Tripedalia*.

3.4 Discussion

Our results demonstrate that separate eye origins in the moon jellyfish (*Aurelia*), box jellyfish (*Tripedalia*) and hydromedusae (*Sarsia*) are extensively associated with expression of familiar animal eye gene families. Yet, our study suggests that upregulation of only few of these gene families is conserved across species while their majority are species-specific. Furthermore, gene expression is substantially more dissimilar among tissues with eyes across species than the manubrium, which is thought to be a homologous tissue. Together, these results suggest some level of conservation but rampant divergence in vision genes upregulated in tissues with eyes across species, consistent with their independent evolutionary origin.

Tissues with eyes are transcriptionally very distinct from other tissue types, and consistently more dissimilar to the manubrium than to tentacles. Likely because jellyfish eyes are placed in specialized structures near the tentacles themselves (e.g. tentacle bulbs) or in sensory organs ontogenetically derived from tentacles (e.g. rhopalia), we find tissues with eyes to be far less distinctive from tentacles than manubrium in their gene expression. This is reflected both in the larger number of upregulated genes in eye tissues when contrasted with the manubrium as opposed to tentacles, and also the clustering patterns of euclidean distances among tissue samples across species, with tentacles often nested within groups of eye tissues. Tentacles display very disparate morphology among cnidarians, and some studies suggest they might not be homologous across species (Gold et al. 2015). Lack of homology could explain the split of tentacle samples into separate clusters, with tentacles nested within groups of eye tissue samples. It could also be possible that their gene expression is very disparate due to species specializing to feed on distinct prey types. Conversely, the manubrium of jellyfish species share many hallmarks of transcription factors also expressed in oral regions of anemones and bilaterians, suggesting they are homologous tissues with conserved patterns of gene expression (Kraus et al. 2015; Hejnol and Martindale 2008). This agrees with our results showing gene expression in manubrium samples is the least divergent among tissue types. Notably, eye tissues seem more divergent in gene expression profile and separate in two clusters: one with tentacle bulbs from *Sarsia* and a second with rhopalia from both *Aurelia* and *Tripedalia*. Because the rhopalia are a novelty shared by box jellyfish and moon jellyfish lineages (Marques and Collins 2005), their gene expression similarity could also be due to their homology, potentially being driven by genes related to their sensory structures other than eyes, such as gravity-measuring statocysts and sensory ciliated epithelia.

Upregulation of some vision related gene families occurs across species, but a lot more of them are expressed in a lineage-specific way. Such modular expression of vision related genes suggests that jellyfish could possibly rely on a basic framework to build eyes yet recruit disparate genes for perhaps elaborating or specializing their eye function and morphology. Shared across all species are genes controlling eye development, such as a *six6-like* gene, and encoding basic components of phototransduction and pigment cells, as CNG ion channels and tyrosinases. Other shared genes include transcription factors yet not studied in cnidarian eyes that possibly regulate jellyfish eye development. Consistent with the idea that genes can then be independently recruited to elaborate on a basic eye toolkit, lens genes used in convergent eyes with lenses are disparate. For example, *Tripedalia* is the only jellyfish with crystallin lenses, and, accordingly, it is the only jellyfish with crystallin genes upregulated in eye tissues. Conversely, *Sarsia* lacks crystallin lenses but is able to produce a lens-like dense material around swollen tips of its eye photoreceptor cells (Singla and Weber 1982) possibly using lens fiber (*Lim2*-like) genes. Likely because *Aurelia* eyes lack lenses, it also does not upregulate any kind of lens-related genes. Interestingly, *Aurelia* has the simplest eyes among the three jellyfish we studied and does not upregulate many eye genes in a lineage-specific fashion, whereas *Tripedalia* and *Sarsia* do so. Lineage-specific genes upregulated in *Sarsia* eye tissues, such as *pax*2 and *six*1/2, were demonstrated to be involved in eye development and regeneration in *C. radiatum*. This corroborates that eyes that share an evolutionary origin also share a very similar genetic basis, encompassing lineage-specific eye genes. Following a similar reasoning, convergent eyes in *Aurelia* and *Tripedalia* might use visual phototransduction cascades with distinct G-protein α subunits: one with Gα-i and the other with Gα-s, respectively. The use of Gα-s was demonstrated in *Carybdea rastonii*, another box jellyfish, together with activity of adenylate cyclase (Koyanagi et al. 2008). We find genes encoding Gα-s and adenylate cyclase upregulated in *Tripedalia* rhopalia. While an earlier study suggested that the visual phototransduction cascade in *Tripedalia* was Gα-i/o-based with phosphodiesterase (PDE6) as its intermediary enzyme (Kozmik et al. 2008), we do not find these genes to be upregulated in *Tripedalia* rhopalia. Our data suggests that the visual phototransduction cascade in *Tripedalia* might be similar to *C. rastonii*, with Gα-s and adenylate cyclase, and if so, would be contrary to the idea that box jellyfish visual opsins are "promiscuous" [see (Vopalensky and Kozmik 2009)].

Retinoid signaling controls vertebrate lens crystallin gene expression (Gopal-Srivastava, Cvekl, and Piatigorsky 1998; Cvekl and Wang 2009) but while crystallin genes are upregulated in *Tripedalia* eye tissues from *Tripedalia*, retinoid signaling genes are not. The *RXR* (retinoic acid receptor) nuclear receptor gene in *Tripedalia* is thought to possibly control the expression of its crystallin genes (Kostrouch et al. 1998); Galliot 2009). *Tripedalia* expresses one copy of this gene (comp72990_c6), but it is not upregulated in eye tissues or among DEGs, though it is relatively highly expressed across all tissues. Because RXR expression seems to be temporally bound to earlier stages of eye development (Kostrouch et al. 1998), we likely missed it by investigating the rhopalia of young adults. Yet, instead of RXR, another type of nuclear receptor is upregulated across all species: a nuclear receptor subfamily 2 group E (TLX)-like gene. Nuclear receptors are essential for vision in other animal groups (Forrest and Swaroop 2012; Kitambi and Hauptmann 2007; Yu et al. 2000), and several members of the subfamily 2 occur in cnidarians (Grasso et al. 2001). It is possible that this tlx-like gene plays an important role in functional maintenance of jellyfish eyes and is involved in the recruitment of lens genes.

While eye tissues are enriched in vision-related genes, the rhopalia express other genes possibly involved in memory and visual learning. The rhopalia serve as centers for neural processing that possibly exchange information, acting as subsystems in the jellyfish neuro-sensory system (Garm et al. 2006). These sense organs express orthologs of bilaterian genes, such as genes of *Otx* and POU groups, that regulate nervous system development, suggesting that they share some degree of conservation of neural system components with other animals (Nakanishi et al. 2010). Our analysis unraveled several genes associated with memory and visual learning, such as a FoxB1-like and HRH2-like histamine receptor genes, supporting that these sense organs are fundamental for advanced visual behavior. Alternatively, these genes could be involved in setting the circadian clock, which would also agree with dopamine and GABA receptor genes being upregulated in these tissues.

Candidate visual opsins further support a scenario of lineage-specific recruitment of opsin genes for a visual function. We first used *Tripedalia* in order to understand the expression pattern of its previously characterized visual protein. Its visual opsin is both upregulated and also the most highly expressed opsin in eye tissues. Searching for opsins with the same expression pattern, we inferred the candidate visual opsins in *Sarsia* and *Aurelia*. The candidate visual opsin in *Sarsia* is nested within a group formed by eye-specific opsins in *C. radiatum* (Cr-opsins). Other *Sarsia* opsins belong to other clades less closely related to Cr-opsins, with some being closely related to fire coral opsins and *Podocoryna* (another hydromedusae) opsins. Cr-opsins have their spatial expression localized to several parts of the retina, and as such, the closest relative of this putative visual *Sarsia* opsin is one Cr-opsin (CropJ) localized near the retina margin (Suga, Schmid, and Gehring 2008). The candidate visual opsin from *Aurelia* that follows this pattern is closely related to an opsin sequence from an eyeless scyphozoan, *Rhopilema esculentum*, forming a group sister to the rhopalia-specific opsins from box jellyfish. Functionally testing these candidate visual opsin genes and also sampling opsins from other cubozoans and scyphozoans will help us understand eye evolution in these systems.

One caveat of our study is that because of our approach to use the longest transcript per gene we are unable to perform the differential gene expression analyses at the isoform level, which could provide an even finer resolution to our comparative analyses. For that type of analysis though, longer reads rather than short ones could be more ideal. Isoforms can have conserved and and variable regions, so that short reads could be multimapped to conserved regions, potentially leading to loss of information. Additionally, expression patterns that we detected are restricted to the developmental stage of young adult jellyfish, and we could be missing key eye genes that show more dynamic temporal expression. Despite potential limitations, our results agree with many of the few reports that are available for eye development, vision and phototransduction in Cnidaria. This reinforces that our approach is valid and provides a powerful starting point for further comparative studies on cnidarian eyes.

3.5 Conclusions

Our results show that many genes upregulated in eye tissues are potential homologs of bilaterian gene families involved in vision, which corroborates earlier studies suggesting a common basis between cnidarian and bilaterian eyes. However, our gene expression analysis highlights the possibility that distinct gene families were recruited at each eye origin, with lineages using conserved building blocks and lineage-specific ones possibly associated with details of eye morphology and phototransduction. Our approach allowed us to identify several key genes possibly involved in vision and eye evolution in Cnidaria. It points to many exciting new questions about underlying constraints due to shared evolutionary history or development. For instance, are these lineage-specific genes recruited for building eyes in certain species because of any sort of phylogenetic constraint? What are the roles that orthologs of these genes play in other species? Could the polyp stage and the way it produces jellyfish have facilitated the recruitment of certain genes for vision and eye development? Analyzing eyeless species will also help us understand if genes coopted for vision were ancestrally present in tentacle bulbs or rhopalia with a distinct role.

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Chapter 4

Light modulated cnidocyte discharge predates the origins of eyes in Cnidaria

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4.0 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

4.1 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 that would 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. When they are outside of eyes, photoreceptor cells are called extraocular, lack a visual function and simply sense the ambient intensity of light (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 lineages with the jellyfish stage, including lensed eyes with crystallins in box jellyfish (Picciani *et al*. 2018; Miranda and Collins, 2019). 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 mediated by opsins: 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 of the xenopsin type (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, another xenopsin (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 not via a knockout experiment, relying instead 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, to maximize efficient use, 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 four other eyeless species and whether this light response is likely to date 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.

4.2 Material and Methods

4.2.1 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.

4.2.2 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 (22ºC +_ 1ºC) 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 (16ºC ± 2ºC) 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.

4.2.3 Cnidocyte assays

Because the polyp is widely accepted to be the ancestral stage among cnidarians, while the pelagic jellyfish evolved later in Medusozoa (Kayal et al. 2018, Collins 2002, Collins et al. 2006), 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 70ºC, 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 (*Aurelia*, N=33), three (*Corynactis*, N=30; *Renilla*, bright light, N=39; dim light, N=27; maintained at 16ºC in a cold chamber during experimentation) or four hours (*Diadumene*, 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.

4.2.4 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.

4.2.5 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 *Diadumene*, 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.

4.3 Results

4.3.1 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 4.1), and indicate the cnidarian ancestor was also able to do so (Figure 4.2). Overall, the discharge of cnidocytes into probes was significantly higher for polyps exposed to a dim compared to bright blue LED light (Figure 4.1). Our statistical power was very high (~100%) for *Diadumene* and *Aurelia*, indicating that we can be very confident in the effect of light intensity on cnidocyte discharge in these two long-diverged species (~700 mya). Conversely, power was lower for the other two species (*Renilla* and *Corynactis*; 40.3% and 52.9%, respectively) so that despite significant effects (p=0.025 in *Corynactis*; p=0.022 in *Renilla*), these should be considered with caution because low power may increase the chance of false positive results (Christley, 2010).

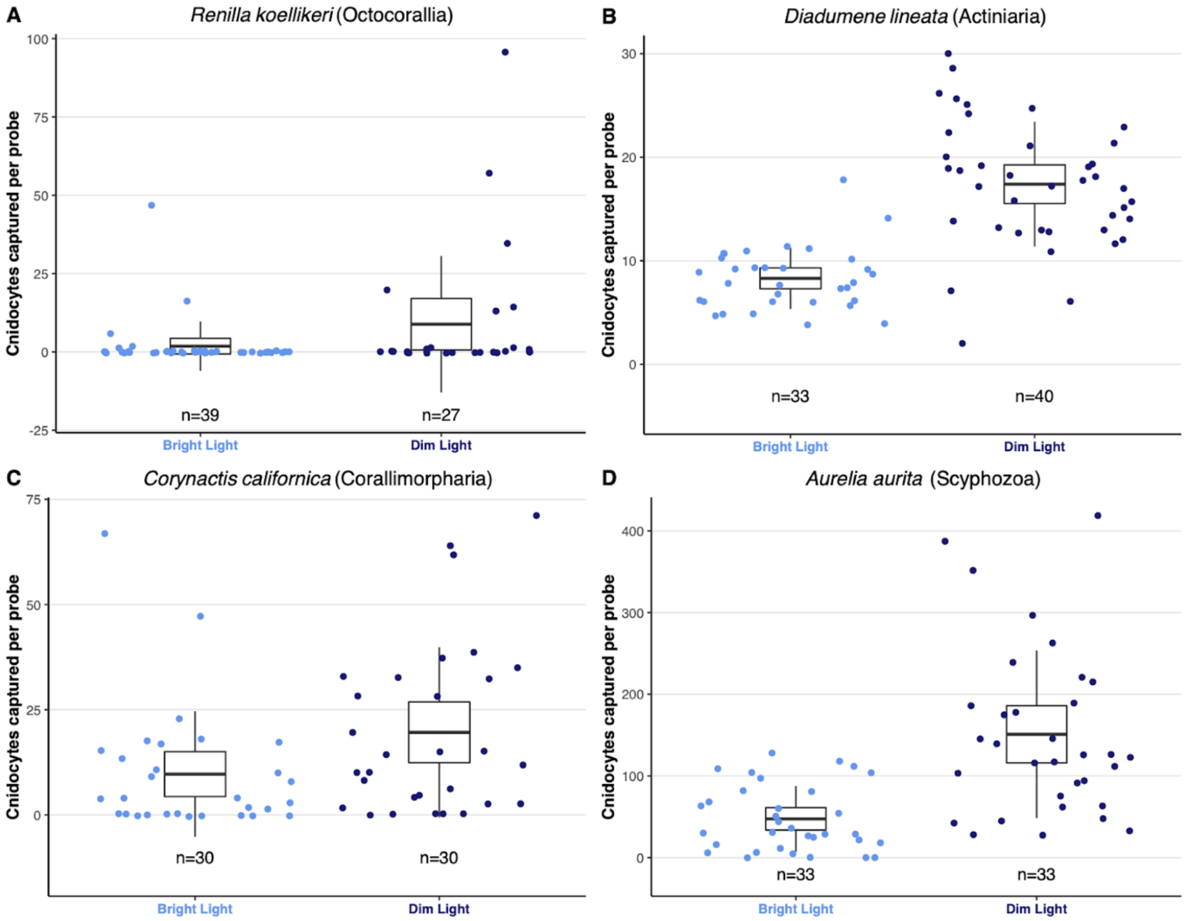

Figure 4.1. Cnidocyte discharge increases when polyps are exposed to dim blue light, a response conserved across long-diverged cnidarian species. Under dim blue light (470 nm; 0.1 W/cm²), discharge of cnidocysts in the gelatin matrix was significantly higher than in bright blue light (470 nm; 2.8 W/cm²) assays (Wilcoxon Rank-Sum Test, two-tailed; *Aurelia* : p<0.0001, *Corynactis*: p=0.025, *Diadumene*: p<0.0001, *Renilla*: 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.

4.3.2 Species-specific variation in numbers of discharged cnidocytes

The octocoral *Renilla* discharged substantially fewer cnidocytes on average (from each treatment) than all other species, while the scyphopolyp *Aurelia* discharged more cnidocytes than the octocoral, the sea anemone *Diadumene* and the corallimorph *Corynactis*. 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 considerably 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 *Aurelia* relies solely on nematocytes only could explain its higher discharge compared to other species.

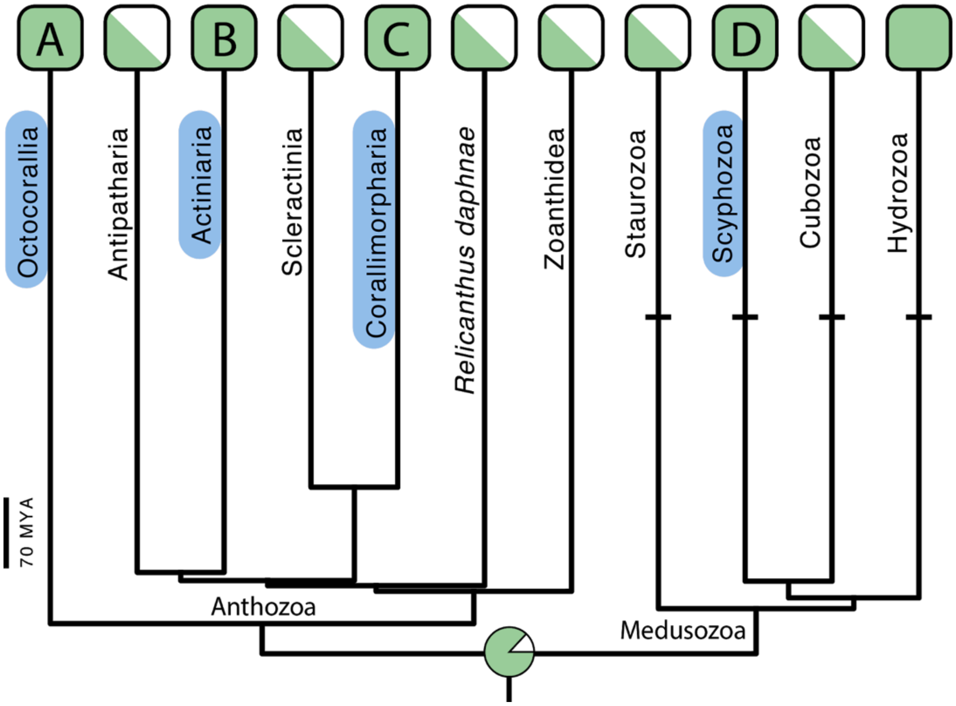

Figure 4.2. Maximum likelihood ancestral state reconstruction on the main phylogeny from Picciani *et al*. (2018). Marginal likelihoods of ancestral states (light modulated cnidocyte discharge present, green; absent, white) at the cnidarian ancestor node are shown in the pie chart, and inferred with a symmetric Markov two-state model (equal rates) of trait evolution. Letters and blue ovals show where studied species are placed in the phylogeny (A: *Renilla*, B: *Diadumene*, C: *Corynactis*, D: *Aurelia*). 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.

4.4 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 (Reitzel, Tarrant and Levy, 2013; Ramirez et al., 2016; Picciani et al., 2018), but only the xenopsin seems to be used by both groups. 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 a homologous light response would be mediated by a light sensing molecule shared among all cnidarians, such as the xenopsin. Demonstrating that the modulation of cnidocyte discharge in anthozoans is done with photoreceptors that use xenopsins 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-mediated, 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 mediated by opsins, 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 cnidarian 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

Anthozoa and Medusozoa (Esposito *et al.*, 2012; Dunlap *et al.*, 2013), which are the major cnidarian sister lineages, and therefore could also be ancestral. Melanin synthesis is involved in many biological processes outside of cnidarian eyes, including functioning 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 been 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.

4.5 Acknowledgements

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4.6 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).

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Chapter 5

Conclusions

In this dissertation, I investigated how eyes evolved convergently in an animal system with simpler neural circuitry compared with bilaterians. Below I summarize the conclusions from each overarching question that I addressed:

How often eyes of varying complexity, including true image-forming eyes, arose in animals with simple neural circuitry such as cnidarians? To infer the evolutionary history of cnidarian eyes, I first generated a novel pipeline for building what became the largest cnidarian species tree. I coupled this large-scale phylogeny of Cnidaria with a compilation of a vast literature on eyes and light sensing behavior to show that cnidarian eyes evolved convergently at least eight times from a photoreceptor precursor, with complex lensed-eyes of the box jellyfishes having a history separate from the others. By analyzing cnidarian photosensitive proteins, I also demonstrated that separate recruitment of non-visual photosensitive proteins for visual roles further supports multiple eye origins. With this first study, I showed that eyes evolved repeatedly from ancestral photoreceptor cells in non-bilaterian animals with simple nervous systems, recruiting existing precursors similarly to what occurred in Bilateria. These results made sense of published morphological and developmental details and showed that cnidarian eyes originated using different opsin paralogs and/or different developmental pathways. This study led me to the second overarching question in this dissertation: to what extent is gene expression similar among cnidarian eyes within the same origin and different between origins? Do convergent eyes ever use the same developmental, physiological, or genetic basis? The phylogenetic analyses presented in Chapter 2 provided a framework for my second overarching question.

Do convergent cnidarian eyes differentially express a similar set of vision genes? Gene expression levels underlying convergent complex organs can sometimes be predictable even in distantly related species. Considering cnidarian eyes evolved convergently so many times, I then asked whether convergent changes in phenotype lead to a similar gene expression profile. My results indicated that overall gene expression was very dissimilar among tissues with eyes, with a large dissimilarity between rhopalia samples and tentacle bulbs. It further showed that vision genes expressed by each species were shared (or conserved) at different degrees, with most gene families upregulated in eye tissues being specific to each species. This study helped us get a glimpse on the patterns underlying convergent evolution and highlighted that very different evolutionary trajectories may have led to eye origins that across jellyfish lineages.

What was the ancestral organismal function of photoreceptor cells that later became part of cnidarian eyes? Finally, after uncovering a lot of the evolutionary history behind eye diversity in cnidarians, I attempted to understand the ancient organismal function of photoreceptor cells before they acquired a role in eyes. To do that, I investigated the discharge of cnidocytes in response to light, a function known to be opsin-based, across species of cnidarians from groups that were key for inferring whether the cnidarian ancestor was able to do the same. I exposed polyps from several species to distinct light treatments and statistically analyzed the numbers of cnidocytes they discharged. After testing whether those species were able to use light to modulate the discharge of their cnidocytes, I used that information to perform an ancestral state reconstruction analysis and estimate the probability that this response dated to the cnidarian ancestor. I found support for that hypothesis, and as such, could pinpoint the modulation of cnidocyte discharge as an ancient function of opsin-based photoreceptor cells. This study advanced our knowledge about where the eye photoreceptor cells came from and provided important insights on sensory evolution in Cnidaria.

Appendix 1

Light-associated responses in Cnidaria

Observations marked with a star (*) are tentative indicators of light sensitivity. Taxonomy according to the World Register of Marine Species (as of March 03, 2018).

Phylum Cnidaria

Class Anthozoa

Subclass Hexacorallia

Order Actiniaria

Anthopleura elegantissima (Brandt, 1835)

Expansion and contraction of anemone *A. elegantissima* differs in individuals with or without endosymbiotic zooxanthellae.Pearse (1974) observes that *A. elegantissima* individuals that lack zooxanthellae do not show regular light response, while individuals that previously had or currently have zooxanthellae show light response through contraction and expansion of the tentacles.(Sawyer, Dowse, and Malcolm Shick 1994) determined photoreception in *A. elegantissima* likely occurs within the endoderm and hypothesized photoreception takes place either in sensory cells in a local nerve net or by the muscles themselves.

Eloactis producta Stimpson, 1856 [=*Haloclava producta* (Stimpson, 1856)]

Hargitt (1907) observed the anemone *E. producta* to expand its tentacles only at low light levels regardless of the time of day. Anemones placed in total dark at noontime were observed to expand fully, and when returned to light retracted fully into their burrows within five minutes.

Metridium senile Linnaeus, 1761

North and Pantin (1958) were able to measure light response of M. senile anemones by illuminating the organisms and measuring the bending and movement in response to the light. They observed local contraction of the longitudinal muscles in response to light, with maximum sensitivity at 490-520 nm.

Sagartia leucolena Verrill, 1866 [=*Diadumene leucolena* (Verrill, 1866)]

Hargitt (1907) observed the anemone *S. leucolena* to actively migrate to darker areas of the aquarium in which they were kept, creeping under rocks and other objects.

Urticina felina (Linnaeus, 1761)

The anemone U. felicina contracts within five minutes of exposure to bright light (Fleure and Walton (1907) after Martin (2002)).

Phylum Cnidaria

Class Anthozoa

Subclass Hexacorallia

Order Scleractinia

Acropora humilis (Dana, 1846)

To test the effects of light on broadcast spawning synchrony of the coral A. humilis, Boch et al. (2011) conducted tests with LED lights in three different treatments, and two different light/dark photoperiod cycles. Results indicated that light levels after sunset influence spawning synchrony, lunar photoperiod cues determine the critical threshold of spawning, and that the threshold may be wavelength dependent.

Diploria labyrinthiformis (Linnaeus, 1758)

Gorbunov and Falkowski (2002) measured the action spectrum of stony coral *D. labyrinthiformis* by illuminating polyps with varying wavelengths of actinic light and detecting contraction responses. When polyps that had extended tentacles in dark experiments were exposed to light illumination, the tentacles began contracting following a 2-3 minutes response time lag. When polyps with contracted tentacles in light experiments were placed into the dark, tentacles began extending and generally took over an hour to become fully extended. The action spectra obtained for *D. labyrinthiformis* was seen only in light with wavelengths <600 nm.

Eusmilia fastigiata (Pallas, 1766)

Gorbunov and Falkowski (2002) measured the action spectrum of stony coral *E. fastigiata* across a range of wavelengths. Polyps in a dark environment with open tentacles began contracting tentacles within 2-3 minutes following exposure to light.

Polyps in lit environments with contracted tentacles began to expand following change to a dark environment. Full expansion generally took over an hour. The action spectra obtained for *E. fastigiata* was similar to the spectra of invertebrate rhodopsins. By illuminating individual parts of the polyp with actinic light, they recorded photoresponse in parts that did not contain zooxanthellae (tentacles), but not in those that are zooxanthellae rich (mouth and base). *E. fastigiata* showed retraction responses in natural moonlight.

Favia favus (Forskål, 1775)

Levy (2003) compared the contractile responses of stony corals at different wavelengths to their concentration of zooxanthellae to determine if there was a relationship between diurnally expanded corals and the presence of photosynthetic zooxanthellae. *Favia favus*, a stony coral with a negligible number of zooxanthellae, was observed to contract tentacles in response to light (even at low irradiance levels) and expand only in the dark.

Manicina areolata (Linnaeus, 1758)

Gorbunov and Falkowski (2002) measured the spectral sensitivity of stony coral M. areolata at a range of irradiance levels. At low irradiance levels, a spectral response was maximal at 480nm (blue light) and was not observed at wavelengths overlapping the regions of absorption of photosynthetic pigments (above 600nm). This strongly suggests low-light reception in corals is not related to the photosynthetic activity of zooxanthellae but to photoreceptive systems of the host coral, and enables sensitivity to moonlight levels.

Montastraea cavernosa (Linnaeus, 1767)

Lasker (1979) observed M. cavernosa corals have two distinct morphs: a diurnal morph active both in day and night, and a nocturnal morph active only at night. In nocturnal specimens artificial shade caused marked increase in polyp expansion, and increases in light levels initiated polyp contraction. Night-time lighting resulted in full contraction of this morph. Artificial shade and night-time lighting had smaller effects on the more consistently active diurnal morphs, however small increases in expansion was observed in the dark condition, which reversed when colonies were returned to natural light. Addition of DMCU (a photosynthetic inhibitor) had no effect on expansion activity. Lasker (1979) concluded that expansion behaviour in *M. cavernosa* is controlled by direct light sensing and not by the sensing of a photosynthetic product. Gorbunov and Falkowski (2002) exposed polyps of *M. cavernosa* to different wavelengths of light, and determined the action spectrum by measuring the effect of each wavelength on the coral. The action spectra obtained at low light levels ranged between 400-600 nm, which does not overlap the absorption spectra of their photosynthetic symbionts. This suggests light sensitivity at low irradiance levels is not conferred by varying photoproducts but by photoreceptors of the host coral. The uniformity of the distribution of photoreceptor cells in tentacles was measured by illuminating individual parts of the polyp with light at a time. Retraction of tentacles from the light was seen in tentacles that did not contain zooxanthellae.

Montipora dilatata Studer, 1901

Hunter (1988) measured the effects of abiotic factors (including lunar cycles) on coral *M. dilatata* in 6 treatments: constant full moon, shifted-phase (14 days offset), constant new moon, lowered water temperature, shortened day length, and controls.

Spawning was synchronous between the control and constant full moon treatments. For the controls vs. shifted-phase treatment, the first spawning was synchronous, but the second spawning was offset 2-12 days. For the controls vs. constant new moon, the first spawning was synchronous, but the second spawning was offset by 8 days. In the shortened day length treatment, spawning happened about 1.5 hours after sunset in both experimental and control treatments.

Montastraea annularis (Ellis & Solander, 1786) [=*Orbicella annularis* (Ellis & Solander, 1786)]

Knowlton et al. (1997a) measured the effects of an artificial early sunset on spawning times of *M. annularis*, and found that columns of the coral exposed to an early sunset spawned earlier than the columns maintained in normal conditions.

Montastraea franksi (Gregory, 1895) [=*Orbicella franksi* (Gregory, 1895)]

Knowlton et al. (1997b) measured the effects of 3 different treatments of lighting conditions: control, 1 hour earlier than ambient sunset, or 2 hours earlier than ambient sunset on spawning of *M. franksi* corals. Fragments of *M. franksi* from the same colony were used in the treatments. The earlier sunset times caused earlier spawning, indicating that spawning timing is controlled by light.

Pocillopora damicornis (Linnaeus, 1758)

Jokiel, Ito, and Liu (1985) set up four different treatments to measure the effects of lunar irradiance on spawning synchrony of the coral *P. damicornis*: ambient lunar radiation, shifted-phase lunar radiation, constant full moon lunar radiation, and constant new moon lunar radiation. Spawning was shifted in shifted-phase lunar radiation treatments, and spawning was reduced in treatments with constant full or new moon lunar radiation. They conclude that spawning synchrony is influenced by night irradiance.

Phylum Cnidaria

Class Scyphozoa

Subclass Coronamedusae

Order Coronatae

**Atolla vanhoeffeni* Russell, 1957

Roe, James, and Thurston (1984) sampled from 4 different depths (ranging from 100-600 m) by trawling every hour for 48 hours to observe the migration pattern of organisms. During the daylight, deep-sea medusae *A. vanhoeffeni* were observed to have high abundances at 450-600 m, and moving toward 450 m just before sunset. At midnight, *A. vanhoeffeni* individuals had maximum abundance at 250 m.

**Atolla wyvillei* Haeckel, 1880

By sampling from 4 different depths of the water column between 100-600m every hour during a 48-hour period, Roe et al. (1984) observed the migration patterns of medusae *A. wyvillei*. It was observed that *A. wyvillei* have vertical migration patterns from over 600m in the day to 250m in the night, and juvenile individuals have shallower migration range.

Phylum Cnidaria

Class Scyphozoa

Subclass Discomedusae

Order Semaeostomeae

Chrysaora quinquecirrha (Desor, 1848)

Schuyler and Sullivan (1997) used mesocosm experiments to conduct a 27 day long term experiment, as well as 2 short term experiments, to observe the migration patterns of medusa *C. quinquecirrha*. Individuals were exposed to both natural light and dark, where the dark experiments took place during daylight hours, with mesocosms covered by a black sheet to determine if diel vertical migration of *C. quinquecirrha* is light-intensity dependent. Along with long light and dark periods, mesocosms were also exposed to random dark tests that lasted about 15 minutes each time. Measurements were taken by counting the number of individuals visible in the top 1 m of the mesocosm. Individuals were seen to have higher abundances in the surface meter during the dark experiments, and during the rapid cover tests, abundances in the surface water increased. When the cover was taken off of the mesocosm after the rapid cover test to allow light back in, individuals were observed to be either actively swimming away from the light, or to stop swimming upward to allow themselves to sink back to depth.

Phylum Cnidaria

Class Hydrozoa

Subclass Trachylinae

Order Narcomedusae

**Aegina citrea* Eschscholtz, 1829

A. citrea is a deep-sea species of medusa often found below 100 m and at daytime mainly below 300 m. Bleeker and van der Spoel (1988) suggests diurnal vertical migration evident as 42% of the population crosses the 300 m level daily.

Phylum Cnidaria

Class Hydrozoa

Subclass Trachylinae

Order Trachymedusae

Aglantha digitale (O. F. Müller, 1776)

Miller (1979) observed that if kept in constant illumination, spawning of medusae *A. digitale* was inhibited. Spawning was induced by placing the organism in the dark for several hours and then placing it under light. *A. digitale* spawned within 2 hours from being placed back into light. Lie et al. (1983) observed distinct vertical diel migrations in *A. digitale* from 10-40m at night to 45-80m depths during the day, with 20-40% of individuals found in the upper 20m during the day. Arai and Fulton (1973) sampled *A. digitale* individuals by trawling at 5m depth intervals between 0-25m, and at 100m. Samples were taken over a 24-hour period and were taken in the hours before, during, and after both sunrise and sunset. *A. digitale* individuals were seen only at 110m from the samples taken in the hours before sunset, the hour at sunset, as well as the hour at sunrise. The hour after sunset until the hour before sunrise, all individuals found were caught at 25m or above.

**Geryonia proboscidalis* (Forsskal, 1775)

Bleeker and van der Spoel (1988) observed diel migration of *G. proboscidalis* from night depths of 40-395m to daytime depths of 200-1090m.

**Liriope tetraphylla* (Chamisso & Eysenhardt, 1821)

Moreira (1973) monitored the vertical migration of *L. tetraphylla* medusae over 3 different 24-hour periods, and found this species migrates from the bottom to mid-water around 8 P.M. and 4 A.M., and surface water at midnight. The highest concentrations of organisms that were in bottom-water was at noon. Migration patterns were absent or less obvious with the presence of a thermocline.

Phylum Cnidaria

Class Hydrozoa

Subclass Hydroidolina

Order Leptothecata

Aequorea aequorea (Forsskal, 1775) [=*Aequorea forskalea* Peron & Lesueur, 1810)

Miller (1979) conducted light-induced spawning experiments on medusa A. aequorea. If kept in constant illumination, spawning was inhibited. Spawning was induced by placing the organism in the dark for several hours and then placing the organism in the light. A. aequorea spawned within less than 3 hours from being placed back into light.

Eutonina indicans (Romanes, 1876)

Miller (1979) observed that if kept in constant illumination, spawning by medusa *E. indicans* was inhibited. Spawning was induced by placing the organism in the dark for several hours and then placing the organism in the light. *E. indicans* spawned within less than 30 minutes from being placed back into light.

Orthopyxis caliculata (Hincks, 1853)

Miller (1979) conducted light-induced spawning experiments on hydroidolina *O. caliculata*. If kept in constant illumination, spawning was inhibited. By placing *O. caliculata* in the dark for several hours and then placing the organism in the light, spawning could be induced within less than 30 minutes after being placed into the light.

Phialidium hemisphaericum (Linnaeus, 1767) [=*Clytia hemisphaerica* (Linnaeus, 1767)]

P. hemisphaerica lack ocelli but are observed to spawn with light changes accompanying dawn or dusk (Arkett (1989) after Martin (2002)). Although individuals lack ocelli, a cilium-based photosensitive organelle is present (Bouillon and Nielsen 1974). This species uses light cues to modulate spawning through an opsin-based phototransduction cascade (Quiroga Artigas et al. 2018).

Tiaropsis multicirrata (Sars, 1835)

Miller (1979) conducted light-induced spawning experiments on medusa *T. multicirrata*. If kept in constant illumination, spawning was inhibited. Spawning was induced by placing the organism in the dark for several hours and then placing the organism in the light. *T. multicirrata* spawned within less than 30 minutes from being placed back into light.

Phylum Cnidaria

Class Hydrozoa

Subclass Hydroidolina

Order Anthoathecata

Suborder Aplanulata

**Ectopleura dumortierii* (Van Beneden, 1844)

(Moreira 1973) monitored the vertical migrations of *E. dumortierii* medusae over a 24-hour period, and found that individuals migrate to mid-water at sunset when the thermocline was present, and migrated down to depth before midnight.

Euphysa japonica (Maas, 1909)

Miller (1979) observed that if kept in constant illumination, spawning of *E. japonica* medusae was inhibited. Spawning was induced by placing the organism in the dark for several hours and then placing the organism in the light. *E. japonica* spawned within less than 30 minutes from being placed back into light.

Euphysa tentaculata Linko, 1905

Miller (1979) conducted light-induced spawning experiments on medusa *E. tentaculata*. If kept in constant illumination, spawning was inhibited. Spawning was induced by placing the organism in the dark for several hours and then placing the organism in the light. *E. tentaculata* spawned within less than 30 minutes from being placed back into light.

Hybocodon prolifer Agassiz, 1860

Miller (1979) observed that if kept in constant illumination, spawning by *H. prolifer* medusae was inhibited. Spawning was induced by placing the organism in the dark for several hours and then placing the organism in the light. *H. prolifer* spawned within an hour from being placed back into light.

Hydra magnipapillata Itô, 1947 [=*Hydra vulgaris* Pallas, 1766]

Plachetzki et al. (2012) exposed *Hydra magnipapillata* strain 105 to different blue light intensities and showed that more cnidocyte discharge occurred under dim light than under bright light. Co-expression of phototransduction genes on sensory neurons in addition to elimination of the cnidocyte response with pharmaceutical assays indicated a light-mediated response.

Hydra vulgaris Pallas, 1766

By exposing isolated *Hydra vulgaris* tentacles to different wavelengths of light, Guertin and Kass-Simon (2015) measured responses as the amplitude and frequency of the pulses of the tentacles. Significant changes in electrical activity of the tentacles were seen when these were exposed to 450-550 nm light.

Phylum Cnidaria

Class Hydrozoa

Subclass Hydroidolina

Order Siphonophorae

Suborder Cystonectae

Rhizophysa eysenhardtii Gegenbaur, 1859

Light and dark experiments by Purcell (1981) on the siphonophore *R. eysenhardtii* showed that extension of the tentacles and feeding depended on light; contraction of the tentacles occurred only in the dark. Miller (1979) conducted light-induced spawning experiments on medusa *S. tubulosa*. If kept in constant illumination, spawning was inhibited. Spawning was induced by placing the organism in the dark for several hours and then placing the organism in the light. *S. tubulosa* spawned within 2 hours of being placed back into light.

Phylum Cnidaria

Class Hydrozoa

Subclass Hydroidolina

Order Siphonophorae

Suborder Calycophorae

**Lensia conoidea* (Keferstein & Ehlers, 1860)

Pugh (1984) determined the average depth ranges of siphonophore *L. conoidea* at different times by taking sample hauls from different depths over a 48 hour period. *L. conoidea* was found to have an average depth range of 100-200 m during the day, and was found under 100 m at night. This shows a strong display of diel vertical migration of the species potentially induced by light.

Muggiaea atlantica Cunningham, 1892

Miller (1979) observed that if kept in constant illumination, spawning by siphonophore M. atlantica was inhibited. Spawning was induced by placing the organism in the dark for several hours and then placing the organism in the light. M. atlantica spawned within 8-10 hours from being placed back into light.

**Sphaeronectes gracilis* (Claus, 1873) [=*Sphaeronectes koellikeri* Huxley, 1859]

Pagés and Gili (1991) sampled the abundances of siphonophores in the water column to test for migratory behavior. By trawling at 5 different depths (ranging from 0m- 200m) over 48 hours, measurements of abundances of species at different depths and times were observed. *S. gracilis* was among the species to be found in both the Benguela waters and Angola current waters. At the boundary layer of these two water masses, *S. gracilis* was highly abundant in the surface waters in the night, and decreased in abundance in the daylight.

Phylum Cnidaria

Class Hydrozoa

Subclass Hydroidolina

Order Siphonophorae

Suborder Physonectae

**Agalma okeni* Eschscholtz, 1825

By trawling through 5 different midwater depths (ranging from 0m - 200m) over a 48 hour period, Pagés and Gili (1991) were able to sample abundances of a variety of cnidarian species at different depths and times. The siphonophore *A. okeni* was observed to have distinct migratory behavior, passing through the thermocline at night and increasing in abundance at surface waters. In the light, *A. okeni* was absent from surface waters at the boundary layer of the two water masses.

Phylum Cnidaria

Class Hydrozoa

Subclass Hydroidolina

Order Anthoathecata

Suborder Filifera

Hydractinia echinata (Fleming, 1828)

Ballard (1942) identifies light as the spawning induction mechanism for colonial hydroids *H. echinata*. Individuals were induced to spawn outside of normal windows using ectopic light signals. Results indicated that *H. echinata* spawned approximately an hour after being returned to light - consistent with their natural spawning times, which was observed approximately an hour after dawn. Spawning was found to be delayed indefinitely by leaving specimens in the dark.

Leuckartiara octona (Fleming, 1823)

Miller (1979) conducted light-induced spawning experiments on *L. octona* medusae. If kept in constant illumination, spawning was inhibited. Spawning was induced by placing the organism in the dark for several hours and then placing the organism in the light. *L. octona* spawned within 30 minutes of being placed into light.

Proboscidactyla flavicirrata Brandt, 1835

If kept in constant illumination, spawning of the medusa *P. flavicirrata* was inhibited indefinitely (Miller 1979). Spawning was induced by placing the organism in the dark for several hours and then placing the organism in the light. *P. flavicirrata* spawned within 30 minutes of being placed back into light.

**Proboscidactyla ornata* (McCrady, 1859)

Moreira (1973) monitored the vertical migration of *P. ornata* medusae over 3 different 24-hour periods, and found this species migrates from the bottom to mid-water around 8 P.M. and 4 A.M., and surface water at midnight. Migration patterns were absent or less obvious with the presence of a thermocline.

Rathkea octopunctata (M. Sars, 1835)

Spawning of the medusa *R. octopuncata* was inhibited when kept in constant illumination Miller (1979). Spawning was then induced by placing the organism in the dark for several hours and then placing the organism in the light. *R. octopuncata* spawned within an hour of being placed back into light.

**Turritopsis nutricula* McCrady, 1857

Moreira (1973) monitored the vertical migration of medusa *T. nutricula* over 3 different 24-hour periods, and found this species migrates from the bottom to mid-water at night in the presence of a thermocline.

Phylum Cnidaria

Class Hydrozoa

Subclass Hydroidolina

Order Anthoathecata

Suborder Capitata

Pennaria disticha Goldfuss, 1820 [=*Pennaria tiarella* (Ayres, 1854)]

Ballard (1942) identifies light as the spawning induction mechanism for the colonial hydroid *P. disticha*. Individuals were induced to spawn outside of normal diel windows using ectopic light signals. Results indicated that *P. disticha* spawned approximately 10-14 hours after being returned to light. Spawning was found to be delayed indefinitely by leaving specimens in the dark.

Polyorchis penicillatus (Eschscholtz, 1829)

If kept in constant illumination, spawning of medusa *P. penicillatus* was inhibited (Miller 1979). After 10-12 hours of light exposure, *P. penicillatus* spawned within an hour after being placed in total darkness. Action potentials in the neurons of *P. penicillatus* were recorded by Anderson and Mackie (1977), and a response was seen when light intensity was changed (reduced) over the ocelli of the organism. A swimming burst was observed in free swimming *P. penicillatus* immediately after a reduction in light intensity. Mills (1983) monitored the vertical migration pattern of *P. penicillatus* and found that there is no strong migration pattern toward the surface, but rather a general increase in activity in darkness. *P. penicillatus* is seen to have a strong swimming response to dark light, where individuals will temporarily begin swimming to the surface, but will return to depth. Increase in light intensity is also seen to induce swimming activity. Diel vertical migration is concluded to be light-dependent rather than dependent on an intrinsic clock. Swimming frequency of *P. penicillatus* was found to be proportional to rates of change in light intensity rather than a particular intensity. In slowly increasing light, swimming was inhibited, and "crumpling" behavior was observed, which may initiate dawn sinking in this crepuscularly migratory species. In decreasing light (sunset) as the rate of intensity change decreased, swimming frequency decreased and stopped when light intensity reached 0. The maximal response to shadows in monochromatic light occurred at 450-500nm (Arkett 1985).

**Porpita porpita* (Linnaeus, 1758)

Bleeker and van der Spoel (1988) - reported diel migration of the colonial hydroid *P. porpita* in the upper 50m at night and 460-870m depths during the day.

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