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Octopus skin 'sight' and the evolution of dispersed, dermal light sensing in Mollusca

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by

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Abstract

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We now know that co-option, or reuse of ancestral components, plays a prominent role in the evolution of emergent systems, like the reuse of gene regulatory networks in the evolution of developmental programs for morphology. Do the evolutionary origins of animal behaviors show evidence of modular reuse that we find at other levels of biological organization? I found that the skin of *Octopus bimaculoides* is intrinsically light sensitive, and that bright light causes colored chromatophore organs in octopus skin to expand, even without input from the central brain or eyes. Because this Light-Activated Chromatophore Expansion (or LACE) behavior relies on evolutionary novel chromatophore organs, LACE is also an evolutionary novelty. As such, I can pinpoint its origin in evolutionary time and ask whether the ability of mollusc skin to sense light existed prior to the evolution of cephalopod chromatophores and LACE. I found expression of the same r-opsin based phototransduction genes in both *O. bimaculoides* eyes and skin, and the spectral sensitivity of LACE closely matches that of the r-opsin in octopus eyes, consistent with the hypothesis that r-opsin phototransduction underlies LACE. The r-opsin phototransduction cascade can be traced back to at least the last common ancestor of bilaterians, so did the reuse of the cascade in octopus skin arise before, in time with, or after the evolution of cephalopod chromatophores? After surveying 28 mollusc mantle transcriptomes for opsins, I found that r-opsin cascade genes are expressed across the molluscs, from multiple species in each of the major mollusc classes, and an ancestral state reconstruction suggests that the last common ancestor of molluscs expressed r-opsin in its mantle. Taken together, these results

suggest that the evolution of LACE required co-option of an ancient phototransduction module, and that like the evolution of development and other emergent systems, reuse may play a fundamental role in the macroevolution of animal behaviors.

Contents

Chapter 1

Introduction

Animal behaviors are the ephemeral, emergent outputs of biology across multiple levels of organization. From an evolutionary standpoint, animal behaviors are a key interface between genes and the environment, providing a conduit through which natural selection can act on lower biological levels. Niko Tinbergen, one of the founding fathers of the field of ethology (animal behavior) proposed his now famous 'four questions' to explain the 'how' and 'why' of an animal behavior. We need to not only study a behavior's current use and adaptive value, but also understand the contributions of the development of behavior over an animal's lifetime, the mechanisms that produce the behavior and the evolutionary history of the lineage of animals producing that behavior (Tinbergen 1963). While Tinbergen's questions isolated the role of history into just one of his questions, we also know that behaviors themselves evolve, and that the evolution of their development and their underlying mechanisms create a vital foundation for evolutionary changes in behavior. While we have spent many decades understanding the current utility and selective pressures that shape animal behaviors, as a field we have neglected to consider Tinbergen's other questions. For the most part, we are just starting to explain 1) the specific mechanisms (e.g. genetic, neurobiological) that underlie most behaviors, and 2) how the evolution of those mechanisms

have impacted behaviors across species and through macroevolutionary timescales.

Because there are currently so many unknowns, it is unclear how, and indeed, whether, the evolution of behavior differs from evolution at other levels of biological organization. For example, behaviors, as emergent properties, may evolve in a manner similar to other emergent biological processes, like morphological development. The field of evolutionary developmental biology (evo-devo) has made great strides in understanding how evolution of developmental programs drives the evolution of morphology. Through studies in this field, we now know that the genes that regulate development of something like an eye can be shared across vast evolutionary time scales, even when eyes themselves have evolved many times in different animal lineages, a concept called deep homology (reviewed in Shubin et al. 2009). Deep homologies arise through the re-use, or co-option, of components to build new traits, and this process of re-use is rampant in the evolution of development. Do these same processes drive the evolution of other kinds of emergent systems, like animal behaviors? If so, we might expect to find patterns of gene, cell, neural circuit or morphology reuse and sharing in the evolution of new behaviors over vast evolutionary timescales, as we see in the deep homology and co-option of gene regulatory networks that drive development. This background leads to the overarching question of my dissertation: Can we find deep homology and reuse in the evolution of a novel light-sensing behavior in octopus skin?

I begin in chapter 2 by setting the stage for what we know about the a particular form of extraocular light-sensing often referred to as the "dermal light sense," a review that assembles molecular, physiological and behavioral data about dermal light sensing in 4 invertebrate phyla. From this work, it is clear that dispersed light sensing is pervasive in these 4 phyla based on decades of behavioral data, but the molecular underpinnings are essentially unknown.

In chapter 3 I show that octopus skin is intrinsically light sensitive, and in response to

bright light, the pigmented organs in the skin, chromatophores, expand. Chromatophore organs of this type are found only in the coeloid cephalopods (octopus, squid, and cuttlefish). The same phototransduction genes expressed in octopus eyes are also expressed in their skin. Together, these data suggest that ancient r-opsin phototransduction genes have been used for multiple light sensing purposes in multiple parts of the octopus body. This reuse suggests that novel behaviors, like Light-Activated Chromatophore Expansion (LACE), may arise through new combinations of evolutionarily old components (opsin phototransduction pathway) and new components (the chromatophores themselves, which are evolutionary novelties).

In chapter 4, I retrace the evolutionary history of light sensitive opsin proteins to ask when the kernels of extant opsin diversity in bilaterians arose. I found that contrary to expectations about the "simple" nature of early animals, I was able to infer that the ancestor of all animals possessed 4 opsins, and that the last common ancestor of almost all bilaterians must have possessed at least 9 different opsin types. Despite the very early origins of this diversity, no extant bilaterian today has orthologs of all 9 original bilaterian duplicates. Instead, the evolutionary history of opsins in animals is dynamic, with many gains and also many losses.

Finally, in chapter 5, I wrap up by asking when r-opsin phototransduction cascade expression in mantle skin arose relative to the origins of the other major component of octopus LACE, the chromatophores. I surveyed 28 molluscan transcriptomes for phototransduction genes, and used ancestral state reconstruction to infer when r-opsins came to be expressed in mollusc mantle skin. I was able to infer that r-opsin based light sensing is likely at least as old as the last common molluscan ancestor. This result suggests that the multiple independent origins of eyes in bivalves and chitons, plus the LACE response in octopus skin (Chapter 3) arose from genes that may have been present in the mantle of their most recent common ancestor.

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1. Chapter 2 was first published in "Ramirez, M. D., Speiser, D. I., Pankey, M. S., & Oakley, T. H. (2011). Understanding the dermal light sense in the context of integrative photoreceptor cell biology. Visual neuroscience, 28(4), 265-279." Under copyright of Cambridge University Press, 2011 and reprinted with permission.

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Chapter 2

Understanding the dermal light sense in the context of integrative photoreceptor cell biology

2.1 Abstract

While the concept of a dermal light sense has existed for over a century, little progress has been made in our understanding of the mechanisms underlying dispersed photoreception and the evolutionary histories of dispersed photoreceptor cells. These cells historically have been difficult to locate and positively identify, but modern molecular techniques, integrated with existing behavioral, morphological, and physiological data, will make cell identification easier and allow us to address questions of mechanism and evolution. With this in mind, we propose a new classification scheme for all photoreceptor cell types based on two axes, cell distribution (aggregated vs. dispersed) and position within neural networks (first order vs. high order). All photoreceptor cells fall within one of four quadrants created by these axes: aggregated/high order, dispersed/high order, aggregated/first order, or dispersed/first order. This new method of organization will help researchers make objective comparisons between different photoreceptor cell types. Using integrative data from four major phyla (Mollusca, Cnidaria, Echinodermata, and Arthropoda), we also provide evidence for three hypotheses for dispersed photoreceptor cell function and evolution. First, aside from echinoderms, we find that animals often use dispersed photoreceptor cells for tasks that do not require spatial vision. Second, although there are both echinoderm and arthropod exceptions, we find that dispersed photoreceptor cells generally lack morphological specializations that either enhance light gathering or aid in the collection of directional information about light. Third, we find that dispersed photoreceptor cells have evolved a number of times in Metazoa and that most dispersed photoreceptor cells have likely evolved through the co-option of existing phototransduction cascades. Our new classification scheme, combined with modern investigative techniques, will help us address these hypotheses in great detail and generate new hypothesis regarding the function and evolution of dispersed photoreceptor cells.

Keywords Extraocular photoreceptors, Non-visual photoreception, Evolution, Phototransduction, Invertebrates

2.2 Introduction

Light infiltrates almost every environment on Earth and strongly impacts most animals lives. Animals detect light using sensors known as photoreceptor cells. Photoreceptor cells are best known from the retinas of animal eyes, but they are also found outside the eyes, where they are often called extraocular photoreceptor cells (EOPCs). EOPCs are found in both eyed and eyeless animals, and in some cases, they confer a particular form of photoreception known as the 'dermal light sense'. Millott (1968) defined the dermal light sense as a widespread photic sense that is not mediated by eyes or eyespots and in which light does not act directly on an effector. How is the dermal light sense employed by animals? Do the same biochemical and physiological mechanisms underlie the dermal light sense in all animals? How are the photoreceptor cells that confer the dermal light sense related to the photoreceptor cells found in animal eyes? Answering these and other questions requires knowledge about the structure and function of the dermal light sense in a wide variety of animals.

Unfortunately, our understanding of the dermal light sense has not progressed much since Millottś (1968) work; it is clear that we still know relatively little about this form of light perception. Although we have identified many behaviors that may be mediated by a dermal light sense, in most cases, we have not identified the primary sensory cells and/or biochemical pathways involved in these behaviors. Additionally, we know that many animals possess eyes, extraocular photo-organs, and perhaps also dermal photoreceptor cells, but we often do not understand how the separate contributions of these specific systems or photoreceptor cell types relate to particular behaviors. In fact, the term dermal light sense

may itself be misleading, as it is unclear if the receptors that confer this sense are located at or directly below the skin surface. Finally, we rarely know how many receptors are involved in the dermal light sense, how these receptors are distributed in space, or whether these receptors are primary sensory cells (like retinal photoreceptor cells) or fine processes extending from higher-order neurons (Kennedy, 1960; Wiederhold et al., 1973).

Despite a wealth of unanswered basic questions regarding dermal photoreceptor cell identity and function, recent advances in our understanding of the molecular basis of phototransduction offer new ways to study and understand the dermal light sense. Thus, we have three goals for this paper. First, we will clarify the meaning of the dermal light sense by providing a new classification scheme for all photoreceptor cells. Next, we will present data related to the following three hypotheses about dispersed photoreceptor cells: a) that they are involved in behavioral tasks that do not require true spatial vision (i.e., the ability to form images); b) that they do not express morphological features that enable retinal photoreceptor cells to maximize light gathering power or restrict the direction from which light is collected, such as expanded membrane surface areas or pigmentation, respectively; and c) that dermal light senses have evolved a number of times in animals, and, in some instances, may have originated through the co-option of existing phototransduction pathways. Finally, we will discuss the implications our hypotheses hold for the evolution of dermal light photoreceptor cells and illustrate how detailed comparisons between objectively categorized photoreceptor cells deepen our understanding of the evolution of photosensory systems in general.

2.3 (Re)defining the dermal light sense

A first goal for this review is to outline a new classification scheme for photoreceptor cells that relies on both a receptors' anatomical distribution within an animal (e.g., dis-

persed or aggregated) and its position within a neural network (e.g., primary/first order or higher order). A new and objective way to group photoreceptor cells is necessary to make more meaningful comparisons between different cells. We have purposely excluded other methods of receptor classification from our scheme because they often do not apply to the cells conferring the dermal light sense and may rarely apply to EOPCs in general. One such method of classification divides receptors by the types of information that they gather about light, for example, temporal (changes in light intensity over time), directional (differences in intensity in a gradient), or spatial (true image formation). Furthermore, these types of information are also often thought to be linked to particular structures or photoorgans; for example, it is thought that spatial vision is generally restricted to eyes however, see Hypothesis I in the section titled Hypotheses and data for dispersed photoreception. Photoreceptor cells have also been traditionally categorized as either ciliary or rhabdomeric (*sensu* Eakin, 1972), but dermal photoreceptor cells often do not possess either of these types of morphological modification however, see Hypothesis II in the section titled Hypotheses and data for dispersed photoreception. We believe that the characters we have chosen, distribution and neural identity, can be used to describe a wider set of light sensitive neurons than these prior classification schemes. By explicitly defining the photoreceptor cells that confer the dermal light sense, we will be better able to explore functional and evolutionary differences and similarities between receptor cell types and propose specific hypotheses regarding the origin and evolution of dermal light sense photoreceptor cells.

We propose that all photoreceptor cells can be classified using two axes (see Fig. 1), provided that we rely on characters of the photoreceptor cells themselves and not on characters of the organs to which these cells might belong. The first axis in our classification scheme is continuously varying and describes the extent to which photoreceptor cells are dispersed or aggregated on the surface of an animal's body. While elaborating specific details is beyond the scope of the current contribution, we propose that this dispersed versus aggregated

axis should be fully quantifiable using spatial point pattern statistics (e.g., Diggle, 2003). For example, a null model in spatial statistics is complete spatial randomness (CSR), and departures from CSR can occur by aggregation or dispersion (repulsion). We imagine that photoreceptor cell distribution patterns can thus be quantified on a continuous axis, ranging from highly aggregated to highly dispersed. In addition, whether photoreceptor cells are aggregated or dispersed could be the subject of discrete statistical tests. Photoreceptor cells that are relatively close together, like those in eyes, are strongly aggregated. In contrast, other photoreceptor cells may be dispersed across the surface of an animal's body and rarely occur next to each other. Some photoreceptor cells, like the paired pigmented cells used for directional photoreception in animals such as acoel worms or receptors associated with the eyespots of some spiralians, will likely be statistically indistinguishable from CSR and will be neither statistically aggregated nor dispersed. Classifying photoreceptor cells by the extent of their dispersion also offers a more quantitative definition of what constitutes an eye. For example, eyes can be described as collections of aggregated photoreceptor cells that provide spatial vision (Land & Nilsson, 2002).

The second axis in our classification scheme has two discrete states that identify receptors as either first- or higher-order neurons. First-order neurons are primary sensory cells that transduce external stimuli into electrical signals, then pass these signals onto other neurons via synapses. Classic retinal photoreceptor cells, like rods and cones in the vertebrate eye, fit this description. While the best-characterized photoreceptor cells are first-order neurons, higher-order neurons can also be light sensitive. These higher- order neurons (or 'interneurons') have many synaptic connections with other neurons and can both send and receive electrical signals. Many higher-order neurons do not directly receive sensory stimuli from outside the animal. Nevertheless, photoreceptive interneurons have been described in a wide range of animals: examples include ipRGCs in vertebrates (Provencio et al., 2000), certain neural tissues in mammals (Tarttelin et al., 2003), abdominal ganglia in

the gastropod molluscs *Aplysia* (Arvanitaki & Chalazonitis, 1961) and *Onchidium* (Hisano et al., 1972a), and abdominal ganglion cells in arthropods such as crayfish (Kennedy, 1960) and lobsters (Wilkens & Larimer, 1972).

Taken together, these two axes for receptor classification produce four separate quadrants (Fig. 1). As described in the preceding paragraphs, photoreceptor cells can fall into one of two distinct categories of neural identity on the y-axis, either first order or higher order, and can vary continuously in their amount of dispersion on the x-axis. Quadrant I contains aggregated high-order neurons, such as those found clustered in ganglia from the marine gastropod *Onchidium* (Hisano et al., 1972a) and the crayfish *Procambarus clarkii* (Kennedy, 1963). These receptors tend to be morphologically similar to the other neurons in the ganglia where they occur. Quadrant II houses dispersed high-order neurons. Curiously, we have yet to identify any examples of photoreceptor cells that are both dispersed and higher order. Quadrant III houses aggregated first-order neurons typified by the retinal photoreceptor cells used for image formation in many animal eyes. This quadrant also contains the photoreceptor cells found in well-characterized extraocular photoorgans like frontal organs/parietal eyes in non- mammalian vertebrates and parolfactory glands/epistellar bodies in cephalopods. These cells are aggregated first-order neurons that often bear classic retinal photoreceptor cell morphologies (reviewed in Nishioka et al., 1962, 1966; Adler, 1976). Finally, Quadrant IV contains cells that are dispersed first-order neurons. We argue that the receptors that confer the dermal light sense belong in this fourth quadrant. As we are primarily interested here in defining and studying the dermal light sense, the remainder of this review will focus solely on these dispersed primary sensory cells. Henceforth, we will refer to these cells as 'dispersed photoreceptor cells,' part of a 'dispersed' photoreception system and avoid the less specific terms 'dermal' and 'dermal light sense.' Dispersed first-order neurons in Quadrant IV are typified by the sensory neurons that tile the body wall of *Drosophila melanogaster* larvae (Xiang et al., 2010). More

examples of dispersed photoreceptor cells may be found in Table 1.

Finally, we present three notes concerning the scope of this review. a) Although many larval photo-organs and photoreceptor cells can be included in our classification scheme, for space considerations, we are restricting our review to photoreceptor cells found in adult metazoans. b) For this review, we define photoreceptor cells as the neurons that convert light into an electrical signal via a signal transduction cascade (per Richter et al., 2010). This definition restricts our discussion to neural cells; however, we must note that there are other types of cells that also transduce light. Instead of relaying an electrical signal, this type of cell, called an effector cell, most often converts light directly into mechanical energy. For example, the alga *Chlamydomonas* uses light-sensitive ion channels (channelrhodopsin-1 and -2) to directly drive the flagellar beating responsible for positive and negative phototaxis (reviewed in Hegemann, 2008). It is worthwhile to consider the functions and evolution of effector cells, but for the purpose of this review, we will not consider them further. c) Finally, we will not discuss other photopigments besides opsins, like cryptochromes. Cryptochromes are well known to mediate circadian rhythms in many organisms and can be found in numerous cell types (reviewed in Cashmore et al., 1999). However, while cryptochromes are clearly associated with EOPCs, these cells are usually aggregated higher-order cells, and not dispersed photoreceptor cells, and are thus beyond the scope of this review.

2.4 The molecular basis of photoreceptor function

Although our understanding of dispersed photoreception may have changed little since 1968, enormous progress has been made toward understanding the biochemical and molecular basis of light sensitivity in animals. This molecular synthesis has facilitated deeper insights into photoreceptor cell morphology, physiology, and evolution (Arendt, 2003). While

the molecular synthesis was forged primarily from data on retinal photoreceptor cells, we assume here that all photoreceptor cells, including those underlying dispersed photoreception, may be considered within the same framework. This perspective generates testable hypotheses about the genetics and physiology of dispersed photoreception and provides the potential for a more unified understanding of the evolution of all photoreceptor cells in animals (see Table 1). Photoreceptor cells can be categorized by the degree of similarity between the molecular components that make up their phototransduction cascades. Phototransduction begins with a photon of light being absorbed by a visual pigment that consists of a chromophore (often the vitamin A derivative retinal) bound to a seven transmembrane domain G-protein coupled receptor known as an 'opsin.' Metazoan opsins appear to be monophyletic and to have originated before the common ancestor of Cnidaria-Bilateria (Plachetzki et al., 2007; Suga et al., 2008). Based on recent reconstructions of opsin phylogeny, opsins can be categorized into four separate clades defined by the G-protein with which they interact. The resulting categories include Gt-opsins, Gq-opsins, Gs-opsins, and Go-opsins. The well-characterized Gt- and Gq-opsins are generally found in cells with ciliary or rhabdomeric morphology, respectively, and are thus often referred to as 'c-opsins' and 'r-opsins' (Arendt & Wittbrodt, 2001). A third clade, the Gs-opsins (or 'cnidops') is known only from cnidarians. The fourth clade includes Go-opsins, which were first discovered in scallop ciliary photoreceptor cells (Kojima et al., 1997). Although relatively poorly known, other Go-opsins have been found in lizard parietal eyes (Su et al., 2006), amphioxus ocelli (Koyanagi et al., 2002), and mammalian neural tissue (Tarttelin et al., 2003). Furthermore, genomic surveys indicate that Go-opsins may be found across Metazoa (unpublished observation).

The four opsin clades are each associated with distinct sets of downstream secondary messengers and ion channels. For example, Gt-opsins activate transducin, which signals through a cyclic nucleotide second messenger that closes cyclic nucleotide gated (CNG)

ion channels (Fu & Yau, 2007). In contrast, Gq-opsins involve the secondary messenger inositol triphosphate that leads to intracellular calcium release and the opening of transient receptor potential cation (TRPC) channels (Hardie, 2001). In a box jelly, the Gs-opsin cascade involves adenylate cyclase (AC) (Koyanagi et al., 2008), and in the hydrozoan *Hydra*, a closely related opsin colocalizes with CNG (Plachetzki et al., 2010). Finally, although the majority of known photoreceptor cells use opsin-based phototransduction cascades, other methods of light detection exist. For example, lite-1 and its homologue, Gr28b, are light-sensitive gustatory receptors in *Caenorhabditis elegans* and *D. melanogaster*, respectively (Edwards et al., 2008; Xiang et al., 2010). Interestingly, lite-1 and Gr28b use a mix of secondary messengers and ion channels; for example, lite-1 interacts with Gi/o-proteins, guanylate cyclase, and cGMP to open CNG ion channels (Liu et al., 2010), while Gr28b uses TRPA1 ion channels (Xiang et al., 2010). As these unusual results clearly suggest, discovering previously unknown photoreceptor cells may reveal unique molecular solutions for detecting light. Elucidating distinct phototransduction cascades contributes to a mechanistic understanding of variation in photoreceptor cell physiology and vice versa, as the state change of the ion channel following phototransduction changes the membrane potential of the cell. The direction of the voltage change depends on the type of phototransduction pathway involved. Using this link between biochemistry and physiology, we can generate testable hypotheses about which phototransduction cascade a cell utilizes through electrophysiological investigations of photocurrents using intracellular or patch- clamp recordings (e.g., Nasi & Gomez, 2009). For example, hyperpolarization (an increase in membrane potential) is seen in cells employing the Gt-opsin cascade, which involves CNG ion channels and phosphodiesterase (PDE). Depolarization (decrease in membrane potential) is seen in cells using the Gq-opsin cascade, and more specifically, the TRPC ion channel. However, membrane depolarization is also associated the Gs-opsin pathway, which uses CNG instead of TRPC, but differs from the Gt-opsin pathway by using AC instead of PDE. Using either

PDE or AC alters whether the second messenger decreases (PDE) or increases (AC). How the second messenger acts on CNG ion channels depends on the direction of change in second messenger concentration and can cause hyperpolarization (for Gt-PDE cells) or depolarization (in Gs-AC cells) (see Su et al., 2006). Thus, while physiological data by itself can inform hypotheses about the underlying molecular machinery for phototransduction, integrating other types of data allows us to better understand photoreceptor cell functions and compare functions across cells types to address evolutionary questions.

2.5 Hypotheses and data for dispersed photoreception

Armed with our current understanding of the molecular basis of photoreception, we can incorporate molecular techniques, such as antibody staining and *in situ* hybridization, with existing data on behavior, morphology, and electrophysiology to locate and identify different photoreceptor cell types. By integrating data from different experimental approaches and taxa, we will gain a more comprehensive understanding of dispersed photoreception that we can use to form specific hypotheses about its mechanisms and evolution. Although there are data for dispersed photoreceptor cells from many taxa, we have chosen to focus on four phyla that we believe currently provide the least incomplete data sets. In this section, we will present data from the literature that address three hypotheses regarding dispersed photoreceptor cells. Before embarking, however, it is worth defining spatial vision, a key idea for two of our hypotheses. An organ that provides spatial vision must be able to form at least a crude image and so must possess two or more photoreceptor cells (Land & Nilsson, 2002). This strict definition of spatial vision excludes cases where a single photoreceptor cell gathers directional information about light and allows an animal to move up or down a light gradient.

2.5.1 Behaviors mediated by dispersed photoreceptor cells

Hypothesis I

Dispersed photoreceptor cells are used for many different tasks, but rarely any that require true spatial vision.

Mollusca Behaviors mediated by dispersed photoreceptor cells are relatively well documented within the mollusks. These behaviors include phototaxis, which is the directional movement of an animal towards or away from light (Jekely, 2009), and the 'shadow response,' which describes an animal's defensive response to a sudden decrease in illumination. Neither of these tasks require an image-forming eye, only the ability to detect broad spatial or temporal differences in light intensity. Eyeless bivalves display both phototaxis and a shadow response. *Lasaea rubra*, an eyeless lamellibranch, is negatively phototactic; photosensitivity is located at the animals' foot, not the relatively small and immobile siphon (Morton, 1960). However, in a number of other eyeless lamellibranchs, like *Mya* (Hecht, 1919; Light, 1930) and *Spisula* (Kennedy, 1960), siphon retraction in response to sudden increases or decreases in illumination is well documented. In these bivalves, the siphon tip is the most sensitive to light, although reduced responses can be elicited from other parts of the siphon (Light, 1930). Gastropods also use dispersed photoreceptor cells for phototaxis and a shadow response. For example, the pond snail *Lymnaea stagnalis* orients positively to light and withdraws its head and foot under its shell when shaded (Willem, 1892; Liche, 1934, as cited in Duivenboden, 1982; Pieron, 1911; Dawson, 1911, as cited in Cook, 1975). These responses are observed even when an animal has been blinded or had its eyes and tentacles denervated (Cook, 1975, but see also Stoll, 1972, 1976; Duivenboden, 1982). *Nassarius reticulatus* also retracts its siphon and lowers its shell in response to shadows; again, both responses persist after eye removal (Crisp, 1972). Similarly, in *Onchidium ver-*

ruculatum, the shadow response persists following removal of stalk and dorsal eyes, but not after the removal of the labial palps and peripheral region of the mantle (Hisano et al., 1972b). Photosensitive central nervous system neurons in *Onchidium* do not respond to sudden changes in light and thus cannot contribute to the shadow response (Hisano et al., 1972b). Finally, siphons isolated from *Aplysia californica* habituate to both electrical stimuli as well as light, suggestive of dispersed photoreception (Lukowiak & Jacklet, 1972). In polyplacophoran mollusks (or 'chitons'), dispersed photoreceptor cells again appear to govern both phototaxis and a shadow response. In the eyeless *Chiton tuberculatus*, younger and older animals are negatively and positively phototactic, respectively; photosensitivity is likely conferred by dispersed receptor cells in the girdle and aesthetes, which are a set of projections from the peripheral nervous system that fill narrow channels in the dorsal shell plates (Arey & Crozier, 1919). Negative phototaxis has also been observed in a number of other eyeless chitons, including *Acanthochiton spiculosus* (Grancher, 1920), *Ischnochiton purpurascens* (Grancher, 1920), and *Ischnochiton maorianus* (Boyle, 1972). Nearly all chitons, including those without eyes, also display a defensive shadow response (Arey & Crozier, 1919; Boyle, 1972; Speiser et al., 2011), at times to very small changes in illumination, such as that caused by a fly passing overhead (Hyman, 1967). Dispersed photoreception may also be present in cephalopods. Chromatophores, the pigmented neuromuscular organs responsible for dermal color patterning in these animals, may directly respond to light; however, descriptions of this phenomenon are minimal (Steinach, 1901 as cited in Steven, 1963). Chromatophores in denervated or low motor tone skin respond to brief flashes of light, after a 1 s delay, and in whole animals, populations of chromatophores in illuminated skin respond by expanding, whereas shaded skin pales (Packard & Brancato, 1993).

As demonstrated by the above examples, dispersed photoreception may be prevalent within mollusks. Furthermore, dispersed photoreceptor cells in this phyla mediate be-

haviors that are clearly important for an individual's survival, such as phototaxis and the shadow response Because these behaviors can be evoked in animals that naturally lack eyes, or even in experimentally blinded animals, it is evident that they do not rely on photoreceptor cells that confer spatial vision, a finding consistent with our hypothesis that dispersed photoreceptor cells generally mediate behaviors that do not require true spatial vision.

Cnidaria Among the eyeless Cnidaria, behavioral responses to light vary (reviewed in Martin, 2002). In the anthozoan sea anemone *Metridium senile*, isolated mesenteries contract under light, even after anesthetization with magnesium chloride, which suggests that the parietal muscle may be directly photosensitive (Bohn, 1906 as cited in North, 1957; North & Pantin, 1958; Marks, 1976). Another sea anemone, *Calamactis praelongus*, has concentrations of nerves associated with regions of translucent skin in its oral disk and tentacles (Marks, 1976). Like *Metridium*, *Calamectis* also shows light sensitivity by some muscle cells, which leads to column bending. They can also detect light with sensory cells located near other muscles that are not themselves light sensitive (Marks, 1976). Yet another anemone, *Anthopleura elegantissima*, exhibits phototactic behavior correlated with the presence of symbiotic zooxanthellae (Pearse, 1974). This species (*A. elegantissima*) may also tune the photosynthetic behavior of its symbiotes in response to longterm changes in light conditions (Shick & Dykens, 1984). A related anthozoan, *Anthopleura xanthogrammica*, displays a range of wavelength-dependent behaviors: different wavelengths of UV and visible light are associated with specific behaviors such as tentacle flexion, tentacle retraction, and oral disk flexion (Clark & Kimeldorf, 1971).

So-called dispersed responses to light in animals without pigmentation or eyes are also known from polyps of each of the four Cnidarian classes (reviewed in Martin, 2002). For example, even though the hydrozoan *Hydra magnipapillata* lacks eyes or ocelli, dark-adapted animals display a series of predictable and repeatable postures that culminate in a tight

retraction of the animal into its most condensed state upon presentation with bright light (Passano & McCullough, 1962; Tardent & Frei, 1969). Overall, behaviors mediated by dispersed photoreceptor cells in Cnidaria are consistent with our behavioral hypothesis, as they mostly consist of phototactic movements and responses to changes in illumination. Furthermore, since many Cnidaria lack eyes entirely in one or more life stage, at least some of these behaviors cannot rely on photoreceptor cells that confer spatial vision.

Echinodermata Light-influenced behaviors are well documented in echinoderms. These responses include phototaxis and a shadow response, as observed earlier in mollusks, as well as changes in pigmentation, podia extension and withdrawal, spine movement, covering, conspecific aggregation, and dark shelter seeking (see Millott, 1975). Many echinoderms have a classic shadow response, but brittle stars are also negatively phototactic and react strongly to direct illumination by moving towards darker areas (Cowles, 1910; Hendler, 1984). Several sea urchin species also use spatial information to detect and crawl towards (or away) from dark targets of certain sizes (Blevins & Johnsen, 2004; Yerramilli & Johnsen, 2010). Interestingly, the two urchin species in the above studies had different numbers and densities of spines, and the species with the more densely packed spines was able to detect smaller targets. Thus, spatial resolution in sea urchins may correlate with spine spacing, meaning that dispersed photoreceptor cells in urchins may act like the individual ommatidia of a compound eye spread across an entire animal's body (Woodley, 1982; Yerramilli & Johnsen, 2010). Brittle star chromatophores may serve a function similar to sea urchin spines, at least when it comes to light perception: the chromatophores are positioned at the skin surface and it is possible that they screen underlying photoreceptor cells in a manner that facilitates spatial vision (Aizenberg et al., 2001).

Unlike the other animals discussed thus far, sea urchins contradict our behavioral hypothesis by demonstrating that dispersed photoreceptor cells can provide spatial vision.

Although further verification is necessary, brittle stars may provide a second counterexample to our hypothesis. Nevertheless, the most common light responses in echinoderms, phototaxis and shadow responses, do not necessarily require cells or organs specialized for spatial vision.

Arthropoda Finally, although behaviors mediated by EOPCs have been reported in arthropods, behaviors specifically attributed to dispersed photoreception are rare. Some butterflies use a small set of EOPCs located at the end of their abdomens to control copulation in males and oviposition in females (Arikawa et al., 1997; Arikawa & Takagi, 2001). Recently, Xiang et al. (2010) determined that some light avoidance behaviors in *D. melanogaster* larvae are controlled by neurons that tile the body wall. In particular, these dispersed neurons mediate negative phototaxis in response to high-intensity short- wavelength light (blue-UV). Although data within Arthropoda are limited, the examples above demonstrate behaviors that are mediated by light intensity and wavelength, not spatial information, and are thus consistent with our behavioral hypothesis.

2.5.2 Morphology and neurophysiology of dispersed photoreceptor cells

Hypothesis II:

Dispersed photoreceptor cells are rarely used for true spatial vision and so should not have the morphological features that allow other photoreceptor cells to maximize light gathering power or restrict the direction from which light is collected, such as expanded membrane surface areas or pigmentation, respectively.

Mollusca Only a handful of dispersed photoreceptor cells have been identified in mollusks, so the morphology of these cells is not well established. Within bivalves, potential photoreceptor cells were identified by morphology in siphons from the clam Mya, but their presence has not been confirmed by other means (Light, 1930). These cells are similar in structure to phaosomes, photoreceptor cells best known from annelids that have a central intracellular cavity filled with large microvillous membranes (Uhlich et al., 1970). Pallial and peripheral siphonal neurons showed both excitatory and inhibitory response to light in the surf clam *Spisula* (Kennedy, 1960). However, the author could not determine if the recorded neurons were primary sensory cells responding directly to light or were higher-order cells responding to input from other photoreceptor cells.

Within the gastropods, sensory-type cells in N. reticulatus were identified in the siphon, but it is not clear if these cells function as photoreceptor cells (Crisp, 1972). Potential photoreceptor cells have also been identified in the tentacles, lips, and foot of *Lymnaea* (Zylstra, 1971). These cells possess a few (1-3) cilia, lie below the epidermal surface, and project dendrites to the surface between epidermal cells (Zylstra, 1971). The firing of inferior pedal nerves in *Lymnaea* are inhibited by light, although it is again not clear whether the recorded nerves are themselves primary sensors (Chono et al., 2002). Based on these limited data, the morphologies of putative photoreceptor cells in mollusks are consistent with our morphological hypothesis for dispersed photoreceptor cells. For example, the putative photoreceptor cells described above lack pigmentation and are not associated with pigment cells. Many of these cells do possess cilia or elaborated microvilli, however, which are morphological modifications associated with enhanced receptor sensitivity.

Cnidaria Currently, there are no morphological studies of putative dispersed photoreceptor cells in Cnidaria, but neurophysiological experiments have confirmed and localized neural responses to light in this phylum. Marks (1976) recorded consistent pulses from the

nerve net of *Calamectis* when light was directed at the upper portion of this anemone. He subsequently focused the light on 1-mm diameter spots, which occasionally evoked a similar neurophysiological pulse, although only when light was shone on the outer margin of the oral disc, and then only with longer exposure times than when the whole upper portion was illuminated. The specific sensory cells involved in this light response were not identified. An experiment on *A. elegantissima* (Sawyer et al., 1994) suggested that this animal's light response is conferred by endodermal cells, which runs counter to the observation that photoreceptor cells are generally confined to the ectoderm. Due to the sparseness of the data available in Cnidaria, it is difficult to draw any conclusions about dispersed photoreceptor cell morphology in these animals. Many cnidarians are unpigmented and lack the discrete pigment cells that are often associated with other types of photoreceptor cells. In this way at least, dispersed receptors in cnidarians are consistent with our morphological hypothesis.

Echinodermata The morphological basis of dispersed photoreception in echinoderms is perhaps best understood in brittle stars (Ophiuroidea). The calcite skeleton of the brittle star *Ophiocoma wendtii* includes plates that cover the arms and form a three-dimensional mesh with relatively regular small openings called stereom. Within the dorsal arm plates in *O. wendtii* and other photo-responsive brittle stars, some stereom contain transparent lens-shaped objects. It is hypothesized that these 'microlenses' focus light onto bundles of neurons; it is also thought that they are actively shaded in bright environments by nearby chromatophores (Hendler & Byrne, 1987; Aizenberg et al., 2001). Extracellular recordings from the *O. wendtii* radial nerve cord confirm that photoreception occurs within this animal's arms (Cobb & Hendler, 1990). However, it is unknown whether the neurons that lie underneath the stereom are actually photosensitive. Ciliated cells at the tips of the arms of the brittle star *Ophiura ophiura* have also been identified as putative photoreceptor cells

(Cobb & Moore, 1986). No recordings have been taken from these cells in either *O. ophiura* or *O. wendtii* due to the technical difficulty of accessing them under the skeleton (Cobb & Hendler, 1990). Like the brittle star stereom, sea urchin spines may allow dispersed photoreceptor cells to gather spatial information (Yerramilli & Johnsen, 2010). As in most echinoderms, relatively little is known about the location of sea urchin photoreceptor cells; however, recent evidence suggests that these cells may be found in sea urchin tube feet (Lesser et al., 2011).

Our morphological hypothesis predicts that dispersed photoreceptor cells will lack elaborated membranes and/or associations with pigmented cells because they are not used for spatial vision. As we have outlined earlier, behavioral studies indicate that dispersed photoreceptor cells in echinoderms may gather information that facilitates spatial vision. Although there have been no detailed morphological studies of dispersed photoreceptor cells in this phylum, these cells are often associated with pigmented cells. Thus, the morphology of dispersed photoreceptor cells in echinoderms is inconsistent with what we see in other phyla, but it is possible that echinoderms have evolved a unique method for gathering spatial information that relies on dispersed photoreceptors cells.

Arthropoda There is limited evidence for dispersed photoreception among arthropods, but the examples we do know about provide us with our most detailed understanding of dispersed photoreceptor cell morphology. In the first example, dispersed photoreceptor cells in the butterfly *Papilio xuthus* have been described in microscopy studies. Here, there are four photoreceptor cells located near the *Papilio* genital region, two cells per side, associated with specific male or female anatomic structures (Arikawa et al., 1980). These cells are similar in structure to phaosomes, which are annelid photoreceptor cells with large intracellular microvillous membranes (Miyako et al., 1993; Arikawa & Miyako-Shimazaki, 1996). From extracellular recordings, we know that *Papilio's* dispersed photoreceptor cells

respond to flashes of light with a pattern of rapid firing, which decreases in frequency as light intensity decreases (Arikawa & Aoki, 1982). Ablation of these photoreceptor cells dramatically affects both male copulation and female oviposition behaviors (Arikawa et al., 1997; Arikawa & Takagi, 2001).

In a second example, green fluorescent protein expression in *D. melanogaster* larvae showed a set of photoreceptor cells (called class IV dendritic arborization neurons), which are tiled across the surface of their body wall; the dendritic arbors of these neurons fill much of the space between cell bodies (Grueber et al., 2002; Xiang et al., 2010). Short-wavelength light directed at these cells generated increased signals of the calcium indicator GCaMP3, which indicated that these cells directly respond to light. Genetic ablation of the class IV dendritic arborization neurons also decreased the light avoidance response of *Drosophila* larvae, while expression of channelrhodopsin-2 and stimulation with green light was sufficient to increase light avoidance in these animals, even when their larval eyes (Bolwig organs) were ablated (Xiang et al., 2010). Taken together, these results clearly indicate that the class IV dendritic arborization neurons are required for D. melanogaster larvae to avoid short wavelength light.

We hypothesize that dispersed photoreceptor cells lack the morphological modifications commonly seen in photoreceptor cells that provide spatial information. Consistent with our hypothesis, the dispersed photoreceptor cells in the two arthropods described above lack pigmentation. However, these cells do possess elaborated membranes. Expanded membrane surface area increases the number of visual pigment molecules potentially expressed by a cell, which in turn increases the proportion of available photons that a photoreceptor can gather. If a higher proportion of photons are collected by a photoreceptor, the photoreceptor is considered to have a higher sensitivity. It is evident then that dispersed photoreceptor cells in arthropods are modified so that their sensitivity is improved, but, because they lack any association with pigmented cells, it is unlikely that they gather spatial

information. Thus, dispersed photoreceptor cell morphology in arthropods is consistent with our hypothesis that these cells are not used for spatial vision.

2.5.3 Molecular basis of dispersed photoreception

Hypothesis III:

Dispersed photoreception systems originated a number of times during evolution and may have co-opted existing phototransduction pathways.

Mollusca With the exception of the pond snail *Lymnaea* and the cuttlefish *Sepia*, the phototransduction pathway genes involved in dispersed photoreception have not been identified in mollusks. In the gastropod L. stagnalis, the shadow responses of both sighted and blinded snails are not affected by a TRPC channel inhibitor, suggesting that the r-opsin (Gq-opsin) phototransduction pathway does not contribute to dispersed photoreception in this species (Pankey et al., 2010). However, the shadow response in this species is significantly hindered by exposure to the CNG channel inhibitor L-cis-diltiazem, which suggests that dispersed photoreception in *Lymnaea* is provided by CNG-dependent photoreceptor cells (Pankey et al., 2010). Based on consistent, observed associations between TRPC channels and light-induced cell membrane depolarization and between CNG channels and light-induced membrane hyperpolarization, these results indicate that a c-opsin (Gt- or Go- opsin) phototransduction cascade is used by *Lymnaea* dispersed photoreceptor cells. In contrast, the opsin messenger RNAs (mRNAs) expressed in the skin of the cuttlefish *Sepia officinalis* are similar to known cephalopod r-opsins (Mathger et al., 2010).

Previous investigations into the molecular basis of phototransduction in the retinal cells of *Lymnaea* (Chrachri & Nelson, 2005; Sakakibara et al., 2005) and *Sepia* (Brown & Brown, 1958; Bellingham et al., 1998) indicate pathways initiated by r-opsin (Gq- opsin). The ob-

servation that *Sepia* dispersed photoreceptor cells, like the retinal photoreceptor cells, rely on r-opsin suggests that retinal and dispersed photoreceptor cells in this animal share a common photoreceptor ancestor. In contrast, the use of CNG ion channels, instead of TRP channels, by dispersed photoreceptor cells in *Lymnaea* suggests independent origins for dispersed and retinal photoreceptor cells in this snail. Finally, although there is insufficient information at this point about dispersed phototransduction cascades in other mollusks to generalize more broadly, *Lymnaea* and *Sepia* appear to use different phototransduction pathway genes for dispersed photoreception. This, combined with the differences between the phototransduction cascades employed by *Lymnaea* dispersed and retinal photoreceptor cells, suggests different evolutionary origins of dispersed photoreceptor cells in these groups.

Cnidaria The molecular components of dispersed photoreceptor cells have recently been determined in *H. magnipapillata* (Plachetzki et al., 2007; Plachetzki et al., 2010). These components in *Hydra* are similar to those involved with retinal cell phototransduction in the cubozoan (or 'box jelly') eye (Koyanagi et al., 2008; Kozmik et al., 2008). The *Hydra* genome contains multiple opsin genes that, together with opsins from other cnidarians, form a distinct clade called cnidops (Plachetzki et al., 2007; but see Suga et al., 2008; Plachetzki et al., 2010). *In situ* hybridization indicates that these opsins are expressed in neurons throughout *Hydra* polyps, particularly those surrounding the hypostome (the apical region near the *Hydra* mouth). This pattern of opsin expression is consistent with the involvement of dispersed opsin-expressing photoreceptor cells in the light-induced contraction response observed in these animals. In addition, other phototransduction genes in *Hydra*, including CNG (Plachetzki et al., 2010), are co-expressed in the same cells as opsin. Behavioral assays further support a role for CNG in cnidarian phototransduction: the CNG channel inhibitor l-cis-diltiazem ablates the light-induced contraction response (Plachetzki

et al., 2010). Finally, retinal photoreceptor cells in cubozoan eyes employ a previously unknown phototransduction pathway wherein cnidops initiates a Gs-AC cascade that leads to an increase of cAMP (Koyanagi et al., 2008). The full degree of similarity between this cubozoan phototransduction cascade and the cnidops-CNG pathway from *Hydra* dispersed photoreceptor cells remains unknown. If the cnidops-based cascade in cubozoans is also employed by *Hydra*, it will suggest that cubozoan retinal photoreceptor cells and hydrozoan dispersed photoreceptor cells may share an evolutionary history.

Echinodermata The molecular components of phototransduction in echinoderms are known almost solely from genome sequence identity, rather than from functional studies. Six opsins were identified in an analysis of the sea urchin *Strongylocentrotus purpuratus* genome; all six of these opsins fall within the range of known metazoan opsins, but they are only distantly related to each other (Burke et al., 2006; Raible et al., 2006; Rubin et al., 2006). Furthermore, it was found that various opsins are expressed in the pedicellariae, tube feet, neural ring, and neural tube of adult animals (Raible et al., 2006). *In situ* expression patterns generated for several larval developmental stages and adult tube feet revealed that the urchin c-opsin homologue is expressed in widely dispersed cells (Ooka et al., 2010). Another study shows that urchin tube feet may express a second type of opsin as well (Lesser et al., 2011). Antibodies against bovine rhodopsin were also found to bind to optic cushions from sea star and brittle star arms (Johnsen, 1997). It is clear that several types of opsin are expressed in echinoderm dispersed photoreceptor cells, but, without functional data, it is difficult to properly categorize these photoreceptors or associate particular behaviors with their presence. Thus, it is difficult to evaluate how many times dispersed photoreceptor cells have evolved in echinoderms or whether these cells are closely related to any other described photoreceptors in Metazoa.

Arthropoda The only well-described phototransduction pathway for dispersed photoreception in arthropods is that found in *D. melanogaster* larvae. Light avoidance behaviors are maintained in D. melanogaster larvae that were mutants in rhodopsins and cryptochrome, suggesting that neither molecule was used by class IV dendritic arborization neurons to mediate light avoidance. Instead, the authors found that Gr28b, a Drosophila homologue of the C. elegans photopigment lite-1, was required for light responses using P-elements insertions and RNA interference (RNAi), although it is not yet not clear from these experiments whether Gr28b directly senses light (Xiang et al., 2010). Furthermore, dispersed photoreceptor cells in *D. melanogaster* larvae likely employ the thermosensor TrpA1, a homologue of the mammalian TrpA, for responding to light, as TrpA1 RNAi expression in class IV dendritic arborization neurons abolished light-induced changes in firing rates in these cells (Xiang et al., 2010). These phototransduction cascade genes, particularly Gr28b, do not fit into any canonical opsin-based pathway and represent unique molecular solutions to light detection in this species. It is not yet clear whether this new type of phototransduction cascade is used by any closely related insect species or whether it is widespread throughout the arthropods. Given its novelty, however, this cascade clearly represents an independent evolution of the phototransduction pathway for dispersed photoreceptor cells. We hypothesized that dispersed phototransduction cascades evolved from existing molecular components involved with phototransduction. The novel cascades found in *Drosophila* larvae contradict this hypothesis, as Gr28b is most closely related to gustatory receptors and TrpA1 is a member of an ion channel family typically associated with temperature detection.

2.6 Discussion

Dispersed photoreception, or the 'dermal light sense', has long presented a number of mechanistic and evolutionary conundrums for biologists. Some mechanistic questions include: What cells underlie dispersed photoreception? How is dispersed photoreception used by animals? Do the same physiological and molecular mechanisms underlie dispersed photoreception in all animals? We are also interested in evolutionary questions, such as how did dispersed photoreceptor cells originate in different groups and how are these receptors related to other photoreceptor cells? Our goal for this paper was to better understand the dermal light sense in the context of the integrative biology of photoreceptor cells. Specifically, we had three main goals for this review: a) to present a new objective classification scheme for photoreceptor cells that will help facilitate comparisons between different photoreceptor cell types, b) to provide key observations concerning what is known about distributed photoreceptor cells in different animals and to propose three hypotheses regarding dispersed photoreception, and 3) to discuss how the study of dispersed photoreceptor cells informs our general understanding of metazoan photoreceptor origin, evolution, function, and diversity.

2.6.1 Classification of photoreceptor cell types

Our proposed classification scheme allows us to place any photoreceptor cell within one of four quadrants. These quadrants are defined by two axes: the first indicates the spatial relationship between a given photoreceptor and the other photoreceptor cells in an animal, while the second describes the way a photoreceptor interacts with the rest of an animal's nervous system (see Fig. 1). Our classification scheme allows us to make a number of comparisons between photoreceptor cells that share a quadrant, much as we have done for some photoreceptor cells that are dispersed first-order neurons. It also lets us explore

hypotheses about the function and evolution of cells in a quadrant. For instance, the majority of photoreceptor cells used for spatial vision are aggregated first-order neurons that fall within Quadrant III. This grouping prompts a number of questions that we can now ask about these cells: Are there requirements for spatial vision that almost always necessitate that photoreceptor cells be aggregated? If there are, how do echinoderms like sea urchins, which seem to have spatial vision despite only having dispersed photoreceptor cells, overcome these requirements? Could their spherical body shape contribute? Similarly, we also classified photoreceptor cells by their neural network position as either sensory cells (first order) or other neural functions (higher order). Do first-order and higher-order cells mediate similar types of light-influenced behavior? Are certain types of cell better suited for particular tasks than others? Finally, we wonder why we have no good examples of dispersed higher-order photoreceptor cells. This might be due to discovery bias, as dispersed photoreceptor cells generally lack pigment and relatively few higher-order neurons have been investigated for light sensitivity.

2.6.2 Three hypotheses for dispersed photoreceptor cells

Dispersed photoreceptor cells are used for behaviors that do not require true spatial vision

Animals can use non-directional light information to set circadian cycles, gauge depth, monitor UV levels, detect a predator's shadow, or, in burrowing animals, find a substrate surface (reviewed by Nilsson, 2009). Additionally, behaviors like phototaxis require directional, but not necessarily spatial, information about light. In many cases, the photoreceptor cells mediating these tasks lie outside the eyes in so-called extraocular photoreceptor cells or EOPCs. Our classification scheme splits EOPCs into at least two groups: those that are aggregated high-order neurons (Quadrant I) and those that are dispersed first-order neurons (Quadrant IV). Given what we know about these two types of photoreceptor, it

appears that aggregated high-order neurons, like those found within ganglia in *Onchidium* or ipRGCs in mammalian eyes, are employed for a different set of non-visual tasks than dispersed first-order photoreceptor cells. For example, light-sensitive interneurons in the abdominal ganglia of *Onchidium* are thought to influence tactile and water pressure inputs associated with mantle-levitating or pneumostom-eclosing behaviors (reviewed in Gotow & Nishi, 2008). Melanopsin (r- opsin) expressing ipRGCs are important for circadian responses, such as pupil reflexes and photoentrainment, in mammals (Panda et al., 2002; Hattar et al., 2003). Thus, aggregated higher-order photoreceptor cells may preferentially be used for tasks associated with relatively long-term physiological responses like photoentrainment. In contrast, we have presented evidence that suggests that dispersed first-order photoreceptor cells are used for short-term movement- based behavioral responses such as phototaxis and shadow responses. Furthermore, based on these behaviors, dispersed photoreceptor cells are capable of collecting both directional and non-directional light information. While we have a lot to learn about these two very different classes of photoreceptor cells, it appears that each may be specialized for particular tasks related to either directional or non- directional light collection, but most often not true spatial vision.

Dispersed photoreceptor cells are morphologically unspecialized

Our hypothesis for the general lack of morphological specialization in dispersed photoreceptor cells is that they are rarely used to gather visual information and, thus, need neither pigmentation nor a close association with pigmented cells. Our hypothesis also implies that dispersed photoreceptor cells may not require the increased sensitivity afforded by elaborated membranes (reviewed by Nilsson, 2009). To the extent that dispersed photoreceptor cells have been positively confirmed in the four focal phyla, support for this hypothesis is ambiguous. Putative dispersed photoreceptor cells in bivalves and gastropods are not pigmented. Some appear to possess cilia, but the cilia themselves are not folded. However, those cells that resemble phaosomes, like those described in the clam *Mya*, do

have expanded microvilli. In the cnidarian *Hydra*, photoreceptor cells located near the group of battery cells associated with nematocysts lack both pigmentation and membrane folding. However, the morphologies of dispersed cells in echinoderms and arthropods do possess some specializations for directional light collection. In the echinoderms, putative photoreceptor cells beneath the brittle star stereom were identified; these cells possessed fine neural processes but lacked membrane elaboration or pigmentation. However, separate pigment cells in echinoderms, specifically chromatophores in brittle stars and spines in sea urchins, are thought to interact with these neurons in response to light. Finally, in the arthropods, both examples we present show evidence of membrane expansion but not pigmentation. The cells in butterfly genitalia resemble phaosomes, which possess an extracellular space that is filled with microvilli. In *Drosophila* larvae, the ligh-sensitive neurons bear large dendritic arborizations, which increase the surface area of each cell.

How are these photoreceptor cells used by animals in these four phyla? Shadow-response-like movement and contraction are particularly common in the mollusks and echinoderms, and hydra also retract into compact balls when illuminated. These types of behaviors do not necessarily require directional information about light. Conversely, some animals, such as many mollusks, appear to use dispersed photoreceptor cells for directional tasks like phototaxis. Other animals, such as sea urchins (and potentially brittle stars), are able to use dispersed photoreceptor cells for spatial vision. Thus, we can state that a close association between photoreceptor cells and pigment cells is normally required for spatial vision, but in some cases, the opaque body of an animal (or large portions of an animal) can provide the screening necessary for directional information to be gathered (Milne & Milne, 1956, as cited in Yoshida, 1979). In these cases, then, we might expect to see some membrane elaboration, which allows increased light collection, to compensate for photons lost through screening.

An alternative hypothesis for why some dispersed photoreceptor cells lack morpholog-

ical specializations is that these cells may be constrained, morphologically, by factors not directly related to photo- detection, namely the maintenance of multi- functionality. Empirical evidence for this hypothesis is scarce. However, multi- functionality can arise from dispersed receptors being either multimodal sensors or higher-order neurons that receive input from both other neurons and the external environment. For example, cells associated with nematocysts in *Hydra* express mRNAs that code for opsin and CNG (Plachetzki et al., 2010). From previous studies, we know that nematocyst firing is influenced by both mechano- and chemosensory stimuli (Watson & Hessinger, 1989, 1994), and it appears likely that these opsin-expressing cells in *Hydra* may also contribute to the nematocyst firing response. Finally, although they do not fall under the definition of dispersed photoreceptor cells, multimodal sensory neurons have been identified in C. elegans. These ciliated cells respond to both light and electrical stimulation (Gabel et al., 2007; Ward et al., 2008).

Some dispersed photoreceptor cells may also be higher-order neurons (in that they receive input from other cells). Although they are not dispersed photoreceptor cells, retinal ganglion cells (RGCs) in the vertebrate eye are known to be photosensitive third-order neurons; functionally similar interneurons could very well be common. The abdominal ganglion photoreceptor cells found in the marine gastropod *Onchidium* are another well-documented example of photosensitive higher-order neurons. These cells function as both interneurons and photoreceptors but maintain a fairly typical neural morphology that allows them to interact with many other neurons via synapses (reviewed in Gotow & Nishi, 2008). Multi-functionality could thus constrain the morphology of these higher-order photoreceptor cells by not allowing them to evolve the elaborated membranes that help bolster the sensitivity of retinal photoreceptor cells.

Dispersed photoreceptor cells use a variety of phototransduction pathways

We have noted that dispersed photoreceptor cells use a variety of phototransduction

pathways. Thus, we conclude that dispersed EOPCs have evolved a number of times within the Metazoa, possibly by co-opting existing phototransduction cascades. This conclusion appears to hold for our four focal phyla. We have evidence that dispersed photoreceptor cells may have evolved more than once within the mollusk, as the cuttlefish *Sepia* uses r-opsin for both retinal and dispersed photoreceptor cells, whereas dispersed phototransduction in the pond snail *Lymnaea* relies on CNG and, potentially, c-opsin. The cnidarians appear to use a unique form of the opsin protein, cnidops, as well as CNG for their ion channels. Echinoderms have at least six different opsins, and we do not yet have a clear consensus about the specific type of phototransduction cascade employed by echinoderm dispersed photoreceptor cells, which makes it difficult to conjecture about the evolutionary origins of these cells. Finally, the presence of non-opsin based light sensitivity in cells tiling *Drosophila* larvae clearly indicate a system evolutionarily unrelated to opsin-based systems. Overall, molecular evidence relating to phototransduction cascades suggests that at least some dispersed photoreceptor cells have evolved independently in mollusk, cnidarians, and arthropods and that these cells may have even evolved more than once with each phylum.

2.6.3 Dispersed photoreceptor cells in the context of photoreceptor cell evolution

Given that dispersed photoreceptor cells have likely evolved multiple times during Metazoan history, how did these cells originate? Also, what is the evolutionary relationship between dispersed photoreceptor cells and other receptors, including other photoreceptor cells within the same animal? Here our classification scheme provides characters that, combined with a greater understanding of the phototransduction cascades employed by different photoreceptor cells, may help unravel the relationship between different photoreceptor cell types. For instance, we suggest that when different dispersed photoreceptor

cells use different phototransduction cascade genes, they likely evolved separately. We can apply this same logic to photoreceptor cells types within an individual animal: differences in phototransduction cascades within the same animal suggest possible independent evolution of photoreceptor cell types. This may be the case in the pond snail *Lymnaea*, as this snail's dispersed photoreceptor cells seem to use CNG, while their retinal photoreceptor cells depolarize with light stimulation, implicating TRP (or possibly CNG coupled with AC) as the ion channel responsible for retinal phototransduction.

The scenario described above may not be unusual in animals; for instance, the melanopsin expressing intrinsically light-sensitive RGCs in vertebrate eyes have only been identified relatively recently. Thus, vertebrate eyes possess two types of photoreceptor cells that likely use distinct phototransduction pathway genes: the canonical c-opsin pathway found in rods and cones, as well as a pathway initiated by melanopsin, which is closely related to the r-opsin found in invertebrate eyes (Hattar, 2002; Tarttelin et al., 2003). These two photoreceptor types also fall into different quadrants within our classification scheme, rods and cones into the aggregated/first-order quadrant, and RGCs into the aggregated/higher-order quadrant. Given the morphological and molecular differences between these two types of vertebrate photoreceptor cells, we could propose hypotheses to account for the differences we see. For instance, we could ask whether RCGs, which are relatively morphologically unspecialized, are constrained by their function as interneurons or whether they are sufficiently sensitive to light without extensive membrane elaboration.

Finally, we may be able to ask broader evolutionary questions regarding photoreception systems. For instance, what the ancestral Metazoan photoreceptor may have looked like, how phototransduction cascade genes evolved and diversified and what the evolutionary relationship may be between phototransduction and other signal transduction pathways and other sensory modalities. By understanding different types of photoreceptor cells and photoreception systems, we may be able to better understand the evolution of eyes, a ques-

tion that has challenged many evolutionary biologists, including Darwin.

2.6.4 Acknowledgments

We thank Tom Cronin and our reviewers for their helpful suggestions. This work was supported by grant IOS-1045257 from the National Science Foundation to T.H.O.

After this review was accepted for publication, Ullrich-Luter et al. (2011) reported that Sp-op4 (a r-opsin) and Sp-pax6 are expressed by photoreceptor cells in the tube feet of adult sea urchins. These cells possess surface areas expanded via microvilli, but lack any pigmentation; they also appear to confer true spatial vision to sea urchins. The morphology of these photoreceptors, alongside r-opsin expression, suggests that they are rhabdomeric type cells. Overall, these new results are consistent with two of our hypotheses for dispersed photoreceptor cells, as they show that sea urchin photoreceptors are first order cells that lack pigmentation and use r- opsin for true spatial vision (no other deuterostome is known to use an r- opsin for this purpose). Finally, this paper highlights how uncovering the mechanisms that underlie dispersed photoreceptor cells is important to understanding the evolution of photo-sensory systems generally.

Table 1. *Types of photoreceptor cells found in different metazoan phyla*

Taxon	Photoreceptive tissue	Photoreceptor quadrant	Photoreceptor ultrastructure *sensu* Eakin	Photoreceptor neurophysiology	Phototransduction components	References
Cnidaria						
Medusazoa	Rhopalia/ocelli	III	Ciliary	—	Cnidopsin, Gs, CNG	Yamasu and Yoshida (1973); Suga et al. (2008); Kozmik et al. (2008); Koyanagi et al. (2008)
	Planula	III	Rhabdomeric	—	—	Nordstrom et al. (2003)
	Epithelial cells	IV	Neither	—	Cnidops, Arrestin, CNG	Haug (1933); Eakin and Westfall (1962); Rushforth et al. (1963); Yamasu and Yoshida (1973); Singla (1974); Plachetzki et al. (2007, 2009); Suga et al. (2008)
Echinodermata	Armtip ocelli, Tentacular eyespots	III	Rhabdomeric (*Asterias*) both (*Henricia*)	—	—	Cobb and Moore (1986); Cobb and Hendler (1990); Johnsen (1997)
	Dispersed photoreceptor cells	IV	—	—	c-opsin, r-opsin?, Go-opsin?	Woodley (1982); Aizenberg et al. (2001); Burke et al. (2006); Raible et al. (2006); Rubin et al. (2006); Yerramilli and Johnsen (2010); Ooka et al. (2010)
Cephalochordata	Dorsal pigmented ocelli (organs of Hesse), Frontal eye-Lamellar bodies	III	Rhabomeric (ocelli) Ciliary (lamellar bodies, frontal eye)	Depolarizing (*ocelli*)	Peropsin, r-opsin, c-opsin	Eakin and Westfall (1962); Ruiz and Anadon (1991); Koyanagi et al. (2002, 2005); Nasi & Gomez (2009)
	Joseph cells	IV	Rhabdomeric	Depolarizing	—	Lacalli (2004); Eakin and Westfall (1962); Del Pilar Gomez et al. (2009)
Urochordata	Larval ocellus, Siphon eyespots	III	Ciliary (larval) Rhabdomeric (adult)	Hyperpolarizing	c-opsin, Gi	Eakin (1972); Dilly and Wolken (1973); Kusakabe et al. (2001); Kusakabe and Tsuda (2007)
Vertebrata	Rods & cones, Pineal eyes, Parietal eyes	III	Ciliary	Hyperpolarizing	c-opsin, Gt, PDE, CNG	Eakin (1961); reviewed in Fu et al. (2007) reviewed in Mano and Fukada (2007)
	ipRGCs, Frontal organ	III	Neither	Depolarizing	r-opsin, Gq, PLC, TRP	Panda et al. (2002); Hattar et al. (2003)
	Chromatophores	IV	—	—	—	Bagnara and Obika (1967); reviewed in Oshima (2001)
Nematoda	ASJ, AWB, ASK and ASH neurons (*C. elegans*), Ganglionic photoreceptors	I	Neither	Hyperpolarizing	lite-1, Gi/o, CNG	Ward et al. (2008); Edwards et al. (2008); Liu et al. (2010)
	Ocelli, amphid photoreceptors	III	Rhabdomeric, ciliary	—	—	Hyman (1951); Siddiqui and Vigglierchio (1970)

Table 1. Continued.

Taxon	Photoreceptive tissue	Photoreceptor quadrant	Photoreceptor ultrastructure *sensu* Eakin	Photoreceptor neurophysiology	Phototransduction components	References
Arthropoda	Caudal photoreceptors	I	Neither	Depolarizing	—	Kennedy (1963); Wilkens and Larimer (1972)
	Larval ocelli/stemmata/bolwigs, Adult eyes, Genital photoreceptors	III	Rhabdomeric	Depolarizing	r-opsin, Gq, PLC, TRP	Bolwig (1946); Arikawa and Miyako-Shimazaki (1996); Eakin (1972); reviewed in Hardie (2001)
	Larval ddaC neurons	IV	Neither	—	Gr28b, TRPA1	Xiang et al. (2010)
Platyhelminthes	Epidermal cells	IV	Neither		—	Eakin (1972); Dörjes (1968)
	Ocelli	III	Rhabdomeric		—	Eakin (1968)
Annelida	Larval brain	I	—	—	c-opsin	Arendt et al. (2004)
	Cephalic eyes	III	Rhabdomeric	—	r-opsin	Arendt et al. (2004)
Mollusca	Phaosomes	IV	Neither	—	—	Rohlich et al. (1970)
Polyplacophora	Aesthete eyes	III	Rhabdomeric	—	—	Boyle (1969)
	Girdle	IV	—	—	—	Arey and Crozier (1919)
	Larval epithelium	IV	—	—	—	Heath (1904)
Cephalopoda	Eyes, Epistellar ganglia, Parolfactory vesicles, Photophore	III	Rhabdomeric	Depolarizing	r-opsin, Gq, PLC, TRP	Nishioka et al. (1966); Mauro (1977); Cobb et al. (1995); Cobb and Williamson (1998a,b) Hara and Hara (1980); Tong et al. (2009)
	Skin	IV	—	—	r-opsin	Mathger et al. (2010)
Bivalvia	Mantle eyes, Siphonal eyes	III	Rhabdomeric, ciliary	Hyperpolarizing, depolarizing	r-opsin, Go-opsin, Gq, Go, PLC, TRP, CNG	Barber and Wright (1969); Nilsson (1994); Kojima et al. (1997); Gomez & Nasi (2000)
	Phaosomes (siphon)	IV	Neither	—	—	Kennedy (1960)
Gastropoda	Oesophageal ganglia	I	Neither	Both	Gp, Gt, PDE, cGMP	Hisano et al. (1972b); Gotow and Nishi (2008)
	Dorsal ocelli	III	Ciliary	—	—	Yanase and Sakamoto (1965)
	Cephalic eyes	III	Rhabdomeric, ciilary (Heteropoda)	Depolarizing	r-opsin, Gq, PLC, TRP	Eakin and Brandenburger (1967); reviewed in Salvini-Plawen and Mayr (1977); Sakakibara et al. (2005); Chrachri and Nelson (2005)
	Dermis	IV	—	—	CNG	Zylstra (1971), Pankey et al. (2010)

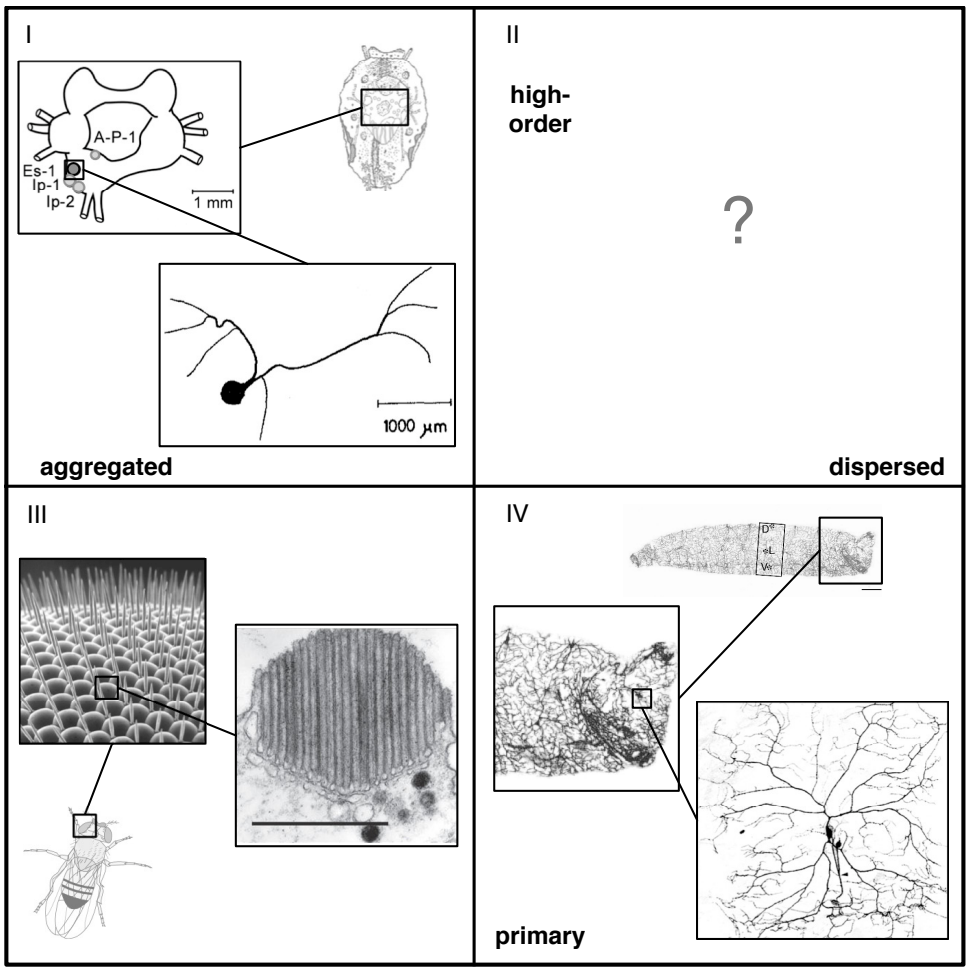

Figure 2.1: Photoreceptor cell distributions and neuron types. Photoreceptor cell distribution ranges from aggregated to dispersed on the x-axis. Photoreceptor cell type is either primary or higher-order neuron on the y-axis. (A) Drawing of *Onchidium verruculatum*, abdominal ganglion with photosensitive neurons Ip-1, Ip-2, Es-1 and A-P-1, and morphology of neuron Es-1 (adapted with permission from Springer Science & Business Media: Journal of Comparative Physiology A: Neuroethology, Gotow, 1975; Gotow & Nishi ©Rockefeller University Press, 2002. Originally published in Journal of General Physiology. 120:581-597. doi:10.1085/jgp.20028619). (B) No example of this type of photoreceptor cell. (C) Illustration of Drosophila melanogaster, micrograph of compound eye and micrograph of single rhabdomere within eye ommatidia (from Mrabet, 2008; Howard, 2008 and adapted by permission from MacMillan Publishers Ltd.: Nature, Hardie & Raghu, 2001). (D) Illustration of *D. melanogaster* larvae, class IV dendritic arborization neurons tiling the body wall, confocal image of a single class IV dendritic arborization neuron (adapted with permission from Development, Grueber et al., 2002; adapted with permission from MacMillan Publishers Ltd.: Nature, Xiang et al., 2010).

Chapter 3

Eye-independent, light-activated chromatophore expansion (LACE) and expression of phototransduction genes in the skin of *Octopus bimaculoides*

3.1 Abstract

Cephalopods are renowned for changing the color and pattern of their skin for both camouflage and communication. Yet, we do not fully understand how cephalopods control the pigmented chromatophore organs in their skin and change their body pattern. Although these changes primarily rely on eyesight, we found that light causes chromatophores to expand in excised pieces of *Octopus bimaculoides* skin. We call this behavior Light-Activated Chromatophore Expansion (or LACE). To uncover how octopus skin senses light, we used antibodies against r-opsin phototransduction proteins to identify sensory neurons that express r-opsin in the skin. We hypothesized that octopus LACE relies on the same r-opsin phototransduction cascade found in octopus eyes. By creating an action spectrum for the latency to LACE, we found that LACE occurred most quickly in response to blue light. We fit our action spectrum data to a standard opsin curve template and estimated the λ max of LACE to be 480 nm. Consistent with our hypothesis, the maximum sensitivity of the light sensors underlying LACE closely matches the known spectral sensitivity of opsin from octopus eyes. LACE in isolated preparations suggests that octopus skin is intrinsically light sensitive and that this dispersed light sense might contribute to their unique and novel patterning abilities. Finally, our data suggest that a common molecular mechanism for light detection in eyes may have been co-opted for light sensing in octopus skin and then used for LACE.

3.2 Introduction

Octopuses, like other coleoid cephalopods, create signals and camouflage themselves by altering the color, pattern and texture of their skin (Holmes, 1940; Hanlon and Messenger, 1988; Packard and Sanders, 1971). While light in the environment influences which

body patterns are produced, exactly how cephalopods gather and use environmental light to control their body patterning is still debated (Buresch et al., 2015). In general, body-patterning behaviors in cephalopods depend on three major components: the eyes, the central nervous system (CNS) and pigmented organs called chromatophores embedded in the skin (Messenger, 2001). Chromatophores are an evolutionary novelty because their morphology in coleoid cephalopods is distinct from those found in any other animal taxa, including other mollusks. Cephalopod chromatophores consist of an elastic sac filled with pigment granules and surrounded by radial muscles, which are innervated by nerves that extend directly from the brain (Cloney and Florey, 1968; Young, 1971, 1974). When chromatophore muscles contract, the pigment sac at the center is stretched out, showing the chromatophores' color. Cephalopods seem to use their well-developed, camera-type eyes to gather information about salient features of the light environment, such as brightness, contrast and edges, which strongly influence changes in the appearance of their skin (Messenger, 1979; Chiao and Hanlon, 2001; Zylinski et al., 2009). Chromatophores can be experimentally controlled with electrical stimulation of the eyes or various brain regions (e.g. optic, peduncle and chromatophore lobes), leading to an overall darkening of the skin tone and sometimes even distinct patterns, which also demonstrates the importance of the eyes and CNS in controlling the activity of chromatophores (Messenger, 1967; Boycott, 1961; Young, 1976; Dubas et al., 1986).

Despite the involvement of eyes for detecting light and the CNS for controlling chromatophore activity in cephalopods, several studies suggest that chromatophores might also be controlled locally by the peripheral nervous system. Both Florey (1966) and Packard and Brancato (1993) noted that squid and octopus chromatophores in dissociated or denervated skin seem to expand in response to light, but surprisingly, neither study investigated these observations further. These intriguing notes suggest that cephalopod skin may be intrinsically sensitive to light, and if so, raise the questions of how the skin senses light and to

what extent this ability contributes to rapid changes in the color and tone of cephalopod skin.

Recent work on the molecular basis for light sensing in the skin of myriad animals suggest that cephalopod skin could detect light using the same families of proteins that detect light in the eyes of animals, including a subfamily of G-protein-coupled receptor proteins (GPCRs) called opsins. There are at least three major groups of opsins: the r-opsins, c-opsins and Go/RGR (retinal G-protein-coupled receptor) opsins (Porter et al., 2012; Feuda et al., 2012). While c-opsins are typically thought to detect light in vertebrate eyes and r-opsins in invertebrate eyes, various opsins are expressed in the skin of many animals (Ramirez et al., 2011), and opsins have been localized to receptors dispersed across the body of animals from multiple phyla, including cnidarians, echinoderms, annelids and vertebrates (Plachetzki et al., 2012; Raible et al., 2006; Backfisch et al., 2013; Bellono et al., 2013; Fulgione et al., 2014). Because opsins are known to function as light receptors, the cells that express opsin may be dispersed light sensors that could underlie some light-mediated behaviors. While opsins have not been localized to particular cells in the skin of any cephalopods prior to this study, the same r-opsin used to detect light in the eyes of the cuttlefish *Sepia officinalis* is also expressed in its skin (Mäthger et al., 2010).

The preliminary observations that squid and octopus chromatophores respond directly to light in dissociated skin and the expression of opsin mRNAs in cuttlefish skin suggests: (1) that dispersed light sensitivity in the skin of cephalopods contributes to some chromatophore responses, perhaps separately from eye or CNS input; and (2) that cephalopods use the same r-opsin-based phototransduction genes to detect light with both their eyes and skin. We found that dispersed, dermal light sensitivity contributes to a direct response of *Octopus bimaculoides* chromatophores to light. We call this chromatophore response light-activated chromatophore expansion (LACE). LACE behavior in isolated octopus skin shows that the skin can sense and respond to light directly. Next, we found multiple r-

opsin cascade genes expressed in the skin of *O. bimaculoides* and localized r-opsin protein expression to ciliated sensory cells in the skin of hatchling octopuses. Finally, like the opsin found in the eyes of *Octopus vulgaris*, LACE in *O. bimaculoides* is maximally responsive to blue (470 nm) light. These results are consistent with the hypothesis that r-opsin-based phototransduction underlies LACE behavior in *O. bimaculoides*.

3.3 Results

3.3.1 *Octopus bimaculoides* exhibits LACE in dissociated skin preparations

Chromatophores in skin removed from the funnels of both hatchling and adult *Octopus bimaculoides* expand dramatically when illuminated by bright white light (absolute irradiance=2.60×10^{15} photon cm^{-2} s^{-1}; see Fig. 1). While we observed slow rhythmic contractions of the muscles beneath the skin under red light from an LED (absolute irradiance: 1.36×10^{14} photon cm^{-2} s^{-1}), the chromatophores themselves remained in their relaxed position and only expanded in response to either a gentle mechanical stimulus or bright white light. While the light remained on, the chromatophores remained expanded and appeared to pulse rhythmically, but would sometimes contract again after prolonged exposure to white light. When the white light was switched off and the chromatophores were illuminated with only red light, the chromatophores in fresh preparations contracted back to their original state. As preparations aged over the course of 1+ days, their responses to light became erratic: chromatophores would no longer respond to white light, or remain expanded, regardless of whether they were under white or red light. The direction of the response of the chromatophores to light (to increase in size) is consistent across samples (see Fig. 2; binomial sign test, N=10, P=0.002).

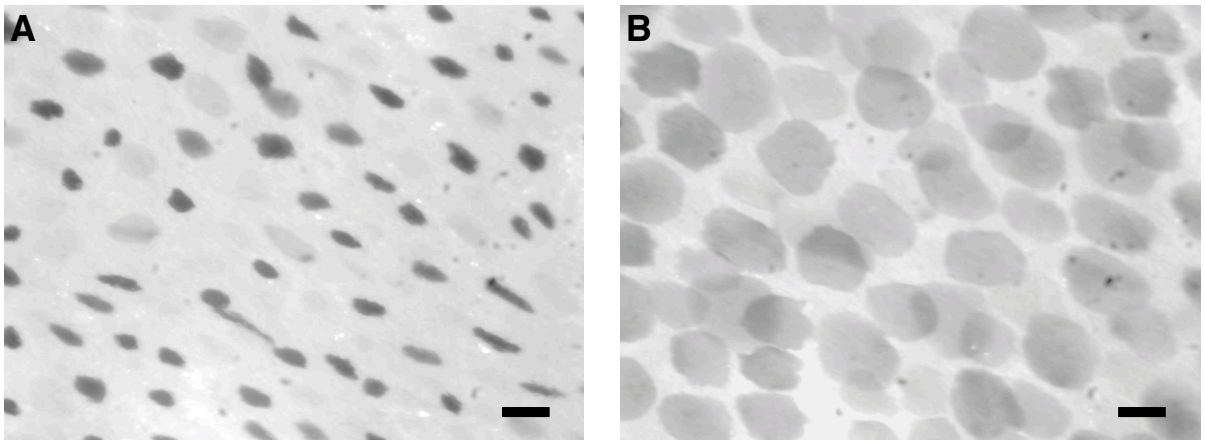

Figure 3.1: Chromatophores in isolated *Octopus bimaculoides* skin expand when illuminated. Stills from infrared video of isolated adult *O. bimaculoides* funnel skin showing LACE (light-activated chromatophore expansion). (A) Chromatophores remain in their contracted state after 3 s of exposure to bright white light. (B) Chromatophores have reached their maximum expansion after 6 s of exposure to bright white light. Scale bars: 100 μm.

LACE caused a statistically significant increase in the size of chromatophores in both adult and hatchling skin, a five-fold average increase for adults (one-sample t-test on log-ratio, t=8.9246, d.f.=8, P<0.0001; Fig. 3A) and a two-fold average increase for hatchlings (one- sample t-test on log-ratio, t=11.915, d.f.=200, P<0.0001; Fig. 3B). Although both adult and hatchling chromatophores expanded significantly after LACE, the log-ratio of the increase differed significantly between adults and hatchlings (two-sample t-test, t=5.4578, d.f.=9.245, P<0.001). The mean latency of LACE from the beginning of the white light stimulus to the time when maximally expanded was significantly different between adult (6.54±2.42 s) and hatchling (15.37±12.74 s) samples (two sample t-test, t=-5.19, d.f.= 64.06, P<0.001; Fig. 3C). Once they began expanding, chromatophores took an average of 4.97±5.1 s to expand fully (Fig. 3). There was no significant difference in the duration of chromatophore expansion between adults and hatchlings (two sample t-test, t=-1.48, d.f.=83.68, P=0.14; Fig. 3D).

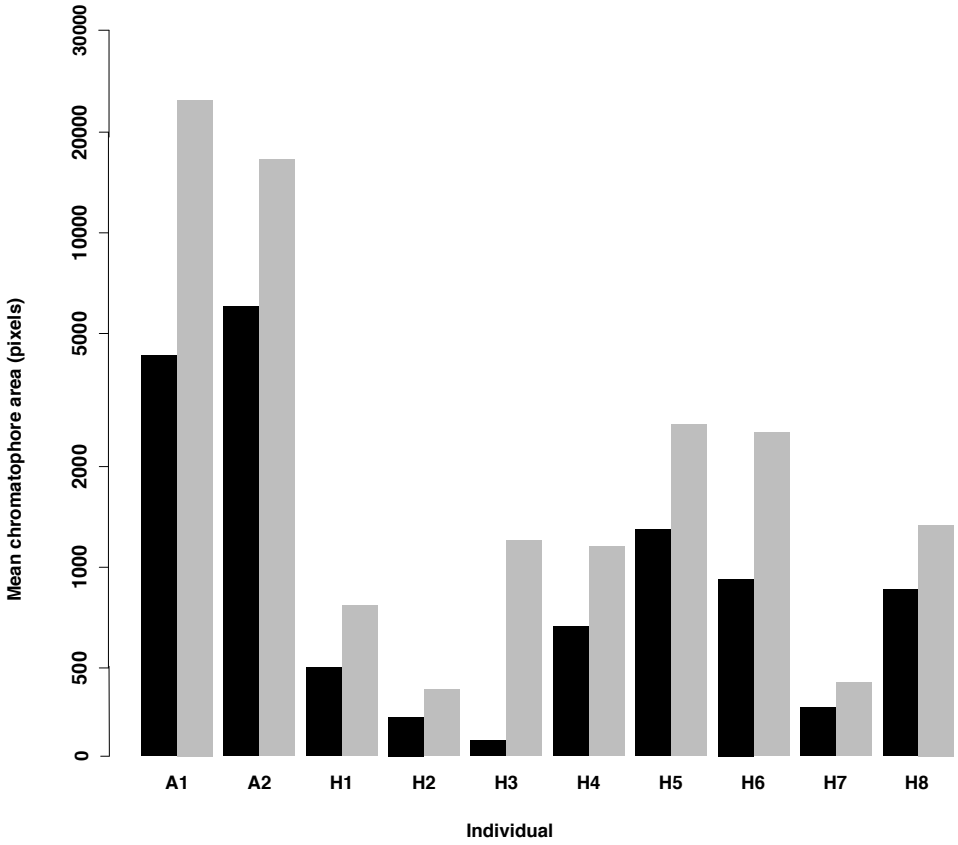

Figure 3.2: Chromatophores expand dramatically under bright white light (binomial sign test, N=10, P=0.002). Paired bar plots of mean chromatophore areas (in pixels) before and after LACE. Each bar is the average size of a single chromatophore measured from at least three trials per animal. A1 and A2 are adult samples and H1-H8 are hatchling samples.

3.3.2 R-opsin phototransduction cascade genes are expressed in *Octopus bimaculoides* skin

We searched for the molecular components of r-opsin phototransduction using degenerate PCR. Based on PCR amplification, we found opsin expressed in adult skin samples (N=5) from the dorsal mantle. These sequences are essentially identical to the r-opsin expressed in *O. bimaculoides* eyes, with only one confirmed nucleotide difference in skin sample 3, indicating that the opsin expressed in the skin is also an r-opsin (GenBank accession no. KR140162).

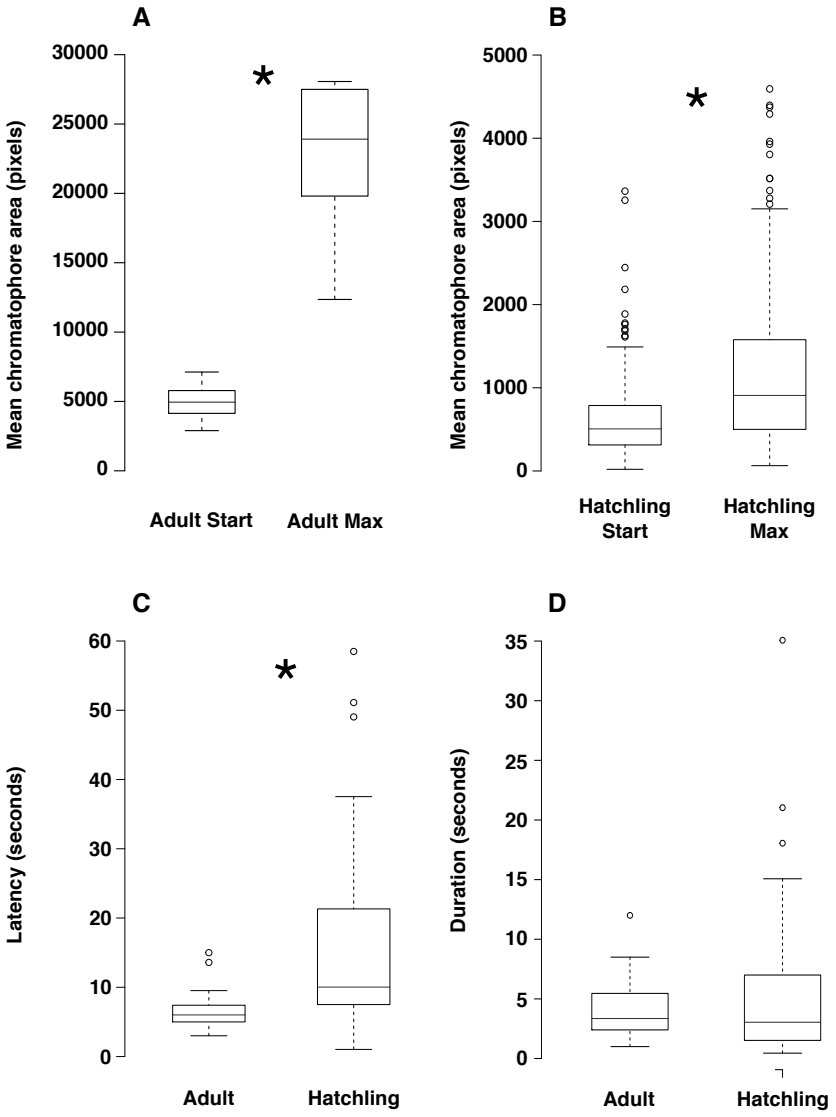

Figure 3.3: Box plots of mean adult and hatchling chromatophore size before and after LACE, latency to LACE and duration to maximum chromatophore expansion. (A) The mean size of adult chromatophores at the beginning of LACE and at their maximum expansion. (B) The mean size of hatchling chromatophores at the beginning of LACE and at their maximim. (C) The mean latency to expansion of the chromatophores from the start of the white light stimulus to the beginning of LACE responses (Adult, N=2; hatchling, N=8). (D) The mean length of time from the beginning of LACE to maximum expansion of the chromatophores (Adult, N=8, hatchling, N=8). Asterisks indicate statistically significant differences ($P<0.05$).

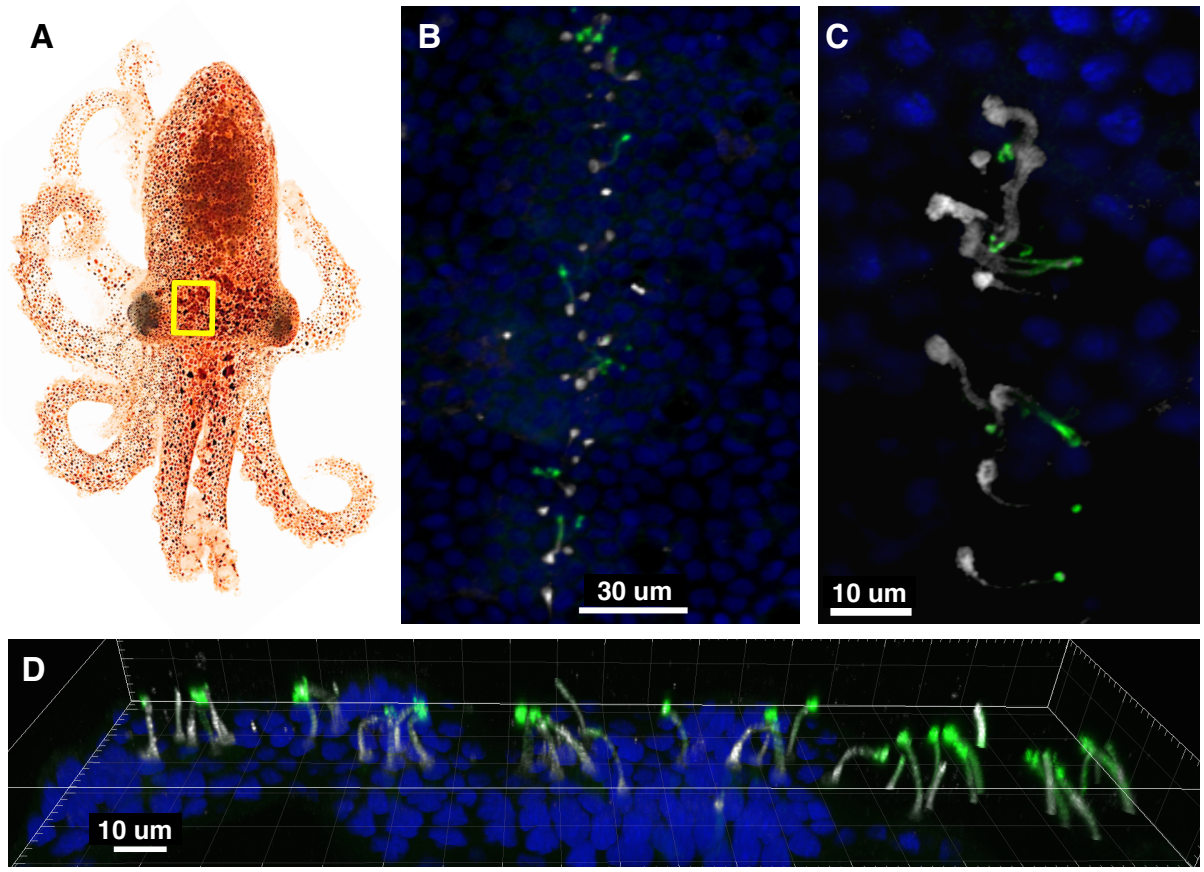

Figure 3.4: Peripheral sensory neurons in the head and siphon skin of hatchling *Octopus bimaculoides* express r-opsin proteins. (A) A hatchling *O. bimaculoides*; the yellow rectangle indicates the region enlarged in B. (B) Fluorescent confocal z-stack of one of four lines of peripheral sensory neurons on the head of a hatchling octopus. (C) Fluorescent confocal z-stack projection of peripheral sensory neurons that comprise the lines found on the head and funnel skin of hatchling octopuses. (D) 3D z-stack projection of r-opsin-expressing peripheral sensory neurons in the head and siphon skin of hatchlings. The cilia bundles attached to sensory neurons embedded in the skin of octopus hatchlings project out onto the skin surface. R-opsin proteins are expressed along the lengths of the cilia bundles and the tops of the cell bodies. Blue, cell nuclei stained with DAPI; green, α- and β-tubulin antibody labeling; white, r-opsin antibody labeling. Hatchling photo credit: Markos Alexandrou.

Peripheral sensory neurons express r-opsin proteins in hatchling *Octopus bimaculoides* skin We found that α- and β-tubulin antibodies bind to many multi- ciliated peripheral sensory neurons spread over the entire epidermal surface of the mantle, head and arms. Typically, the cilia of these cells were packaged into bundles, although sometimes the indi-

vidual cilia were visible. A set of these peripheral sensory neurons form four lines on the head and one on the funnel of the *O. bimaculoides* hatchlings (Fig. 4).

The octopus r-opsin antibody specifically binds to the cilia of many of the primary sensory neurons on the mantle epidermal surface. When the opsin stain is co-localized with tubulin in these cells (Fig. 4), the length of the cilia binds the opsin antibody, but the tip of each cilium appears to only bind tubulin, not opsin. In some cases, the opsin antibody also bound to the topmost portion of the cell body.

3.3.3 LACE action spectrum

We found that LACE responses occurred more quickly (shorter latency) under blue light (470-480 nm) than other wavelengths of the visible spectrum (Fig. 5A). We estimated the λ max of the LACE response to be 480 nm when fitting to the Govardovskii opsin template (Fig. 5B). The Govardovskii calculated spectral sensitivity of opsin from O. vulgaris eyes using data mined from Brown and Brown (1958) matches what the authors reported for octopus opsin, with a λ max of 474 nm (Fig. 5B).

3.4 Discussion

Here, we show definitive evidence of dispersed light sensing in octopus skin and document the expression of a candidate light sensor in skin of the same species, *Octopus bimaculoides*. Two previous studies have speculated that cephalopod skin may be intrinsically sensitive to light, noting that chromatophores in both squid and octopus skin seem to expand when the skin is illuminated, but neither study provided more than preliminary observations (Florey, 1966; Packard and Brancato, 1993). We found that chromatophores in the skin of *O. bimaculoides* expand significantly and repeatedly when exposed to bright

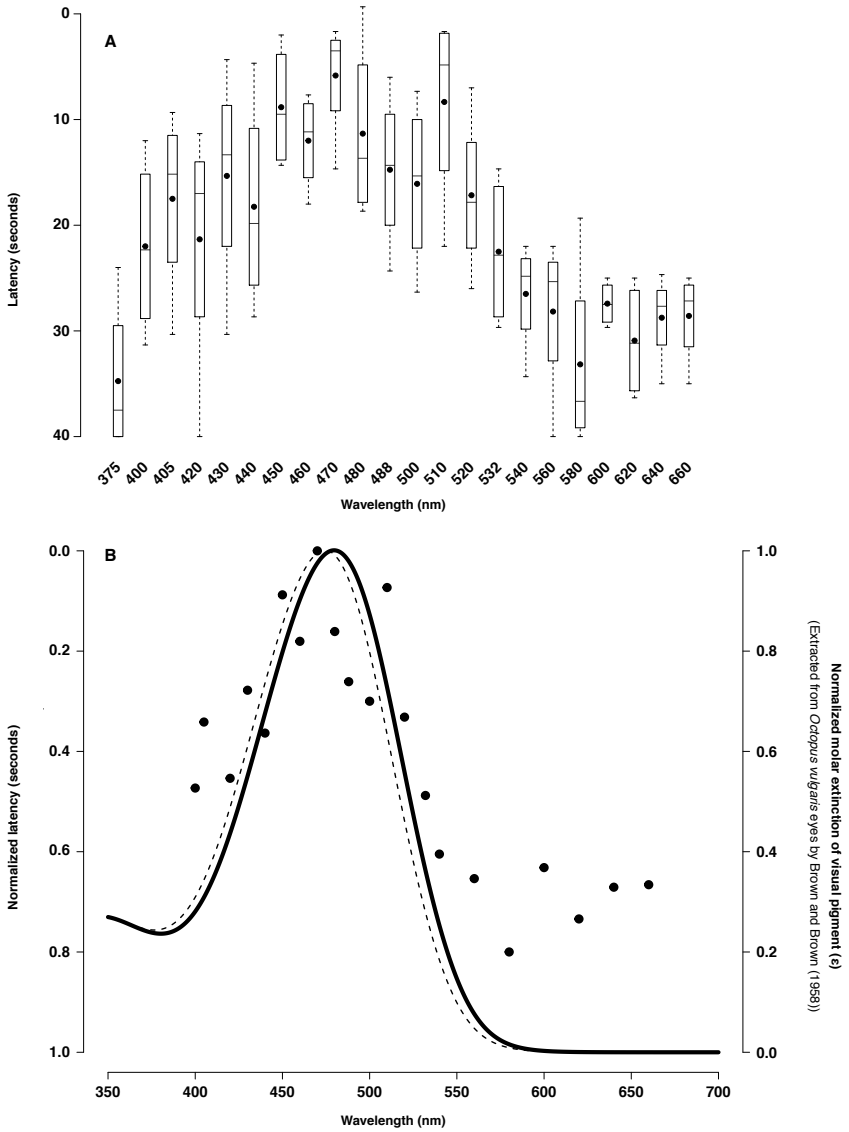

Figure 3.5: LACE behavior is maximally sensitive to blue light. (A) The action spectrum for *Octopus bimaculoides* LACE shows that the latency for LACE is shortest between 470 and 480 nm. Each box represents four data points for each wavelength, corresponding to the mean latencies across three trials of one skin sample from four adult animals. Black dots are the means at each wavelength average across four animals. (B) Govardovskii opsin models for octopus LACE latency (solid line) and data on the spectral sensitivity of *Octopus vulgaris* eye opsin mined from Brown and Brown (1958) (dotted line). The predicted λmax for octopus LACE is 480 nm. The Govardovskii predicted λmax for octopus eye opsin is 474 nm, the same λmax reported by Brown and Brown (1958).

white light, a behavior we call light-activated chromatophore expansion, or LACE. We attribute LACE to light, as we minimized heat reaching the samples by using fiber optics to

illuminate the skin, which itself was submerged underwater. LACE responses clearly show that *O. bimaculoides* skin can detect light by itself, independent of eyes.

While octopus LACE is a robust behavior, we found that some of the parameters of LACE differ from those noted by Packard and Brancato (1993). For instance, they report that chromatophores in denervated *Octopus vulgaris* skin expand 1 s after a flash of bright white light, which differs from the average 6 s (adults) or 15 s (hatchling) latency for LACE we found in *O. bimaculoides*. This incongruence in latency may be attributable to differences between the species and/or the preparation itself, as it seems that Packard and Brancato did not isolate skin samples, but denervated portions of skin still attached to the whole animal. We observed a high degree of variation in both the latency of LACE and the time to full expansion of the chromatophores in our preparations and attribute at least some of this variation to differences in the time between dissecting the tissue and running LACE experiments. We also observed differences in LACE between the hatchlings and adults, where adult skin responded more consistently and robustly than the skin from younger animals. We speculate that this could be caused by the presence of more light sensors in adult versus hatchling skin. However, despite these differences from preliminary reports, our data are the clearest demonstration to date that *Octopus bimaculoides* skin is intrinsically light sensitive, and that light detected by the skin causes the chromatophores to expand.

We hypothesized that r-opsin, a key light sensing protein in the eyes of octopuses and other animals, may also detect light in octopus skin and underlie LACE. To support this hypothesis, we looked for evidence of opsin expression in the skin and determined the action spectrum for LACE. Consistent with our hypothesis, we found that r-opsin is expressed in the skin of *O. bimaculoides*. This result is similar to Mäthger et al. (2010), who detected r-opsin mRNA from one PCR trial of skin from another cephalopod, the cuttlefish *Sepia officinalis*, although it is not yet known whether cuttlefish have LACE. Additionally, opsin

expression by itself is weak evidence for the ability of skin to detect light. Other essential r-opsin cascade genes, including G-protein α (q) and phospholipase C, are also expressed in the skin of *O. bimaculoides*, suggesting that the necessary genes for functional opsin-based phototransduction are expressed in octopus skin (Speiser et al., 2014). Finally, the LACE action spectrum is also consistent with our hypothesis. Spectral sensitivity analysis of the opsin from the eyes of another octopus O. vulgaris shows a λmax of 474 nm (Brown and Brown, 1958). If the same opsin found in octopus eyes underlies octopus LACE, then LACE activity should peak close to the known spectral sensitivity of octopus opsin. Indeed, we found that the latency to LACE is shortest in blue light, and fitting the Govordovskii curve to the action spectrum data gives a λmax of 480 nm. Taken together, these data strongly support our hypothesis that opsin phototransduction underlies LACE. Future work should continue to test this hypothesis by manipulating the function of opsin phototransduction proteins and observing how they affect LACE.

Because r-opsin is known to function in light sensing, cells in octopus skin that express opsin are excellent candidates for dispersed light sensors that could underlie LACE. We identified ciliated peripheral sensory neurons in the skin of hatchling *O. bimaculoides* using α- and β-tubulin antibodies. These cells were similar in morphology and position (Sundermann-Meister, 1978; Sundermann, 1983; Mackie, 2008; Buresi et al., 2014) to cells described as mechanoreceptors in both squid and cuttlefish (Budelmann and Bleckmann, 1988; Bleckmann et al., 1991). It is not yet known whether these peripheral sensory neurons act as mechanoreceptors in the skin of *O. bimaculoides*. Intriguingly, we localized r-opsin expression to these same peripheral sensory neurons in hatchling skin, raising the possibility that aside from a mechanoreceptive function, these sensory cells may also be dispersed light receptors in octopus and other cephalopods. Unfortunately, the precise connections between candidate dispersed light sensors in octopus skin, the chromatophores and the CNS remain unclear, as does their relationships with LACE and merits further in-

vestigation to test the hypothesis that the r-opsin-expressing neurons detect light.

Our finding of opsin expressed in known mechanoreceptors raises the question of whether opsin has a role in mechanoreception, in addition to its well-established role in light detection. While our work is the first description of this opsin expression pattern in mollusks, opsin-expressing mechanoreceptors have been recently described in the annelid *Platynereis*, zebrafish and *Drosophila* (Backfisch et al., 2013; Senthilan et al., 2012). From work on mechanoreception in *Drosophila* antennae, we now know that opsin is required for anntenal mechanoreceptors to detect vibrations, highlighting a previously unknown role for opsin in senses besides light detection (Senthilan et al., 2012). We do not yet know whether the opsin-expressing cells we found in hatchling *O. bimaculoides* skin function as mechanoreceptors, light sensors or both, or the extent to which opsin is required for detecting either of these stimuli. Still, our results compel future research into the role of opsins in senses other than photoreception. We believe that the phylogenetic spread of opsin expression in mechanoreceptors among vertebrates, annelids, arthropods and now mollusks, suggests that such mechano-sensory roles for opsin could be ancient in animals.

Finally, uncovering dispersed light sensitivity in octopus skin raises the question of how it evolved to underlie LACE in octopuses. Our study is the best evidence so far for light-sensitive skin in cephalopods and we hypothesize that LACE may play a role in modulating body patterning for camouflage, alongside the canonical control exerted by the CNS. However, while cephalopods are unique among mollusks for their body-patterning abilities, we know that most other mollusks, especially bivalves, gastropods and chitons, are able to sense light with their skin. There is rich literature describing behaviors like phototaxis or shadow responses and physiology linked to light sensing in the skin of other mollusks (Ramirez et al., 2011). We do not yet know if or how cephalopods use their light-sensing skin for these other more typical molluscan behaviors. However, the widespread distribution of dispersed light sensing and associated behaviors throughout the phylum suggests

that dispersed light sensitivity could be an ancestral molluscan trait that has been co-opted in the cephalopod lineage to mediate novel body-patterning behaviors in response to light. Understanding the underlying molecular mechanisms for dispersed light sensing across the mollusk classes would help clarify the evolutionary history of dispersed light sensing and associated behaviors. Our study provides a framework for future comparative work that can integrate already known behavioral data with molecular data for light-detecting components in various mollusks. This work could address the question of whether diverse mollusk behaviors that rely on dispersed light sensing share a common molecular mechanism for light detection, and thus whether dispersed light sensing was present in ancestral mollusks.

3.5 Materials and Methods

3.5.1 Sample collection

We obtained 11 adult *Octopus bimaculoides* Pickford and McConnaughey, 1949 from marine collectors at the University of California, Santa Barbara from 2010-014. We housed the animals in flow-through tanks supplied with filtered seawater. Our hatchling octopuses came from a clutch laid by a captive female in the winter of 2013, and the animals hatched during the following summer. *Octopus bimaculoides* hatch as fully developed octopuses that are immediately able to hunt and change body patterning. The hatchlings we used for these experiments were between 0 and 4 months old. To kill animals, we first anesthetized them in a seawater solution containing 5% ethanol and 7.5% isotonic magnesium chloride until the chromatophores no longer responded to gentle poking and ventilation slowed, followed by quick decerebration (Moltschaniwskyj et al., 2007; Andrews et al., 2013).

3.5.2 LACE behavior under white light

We used insect pins to mount dissected funnels from adult and hatchling octopus (N=10) to Sylgard-lined Petri dishes filled with fresh seawater. To record the activity of the chromatophores on these isolated funnels, we used an infrared CCD camera (LCL902HS, Watec, Newburgh, NY, USA) mounted on a dissecting microscope. We measured the absolute irradiance for our light sources using a spectrophotometer (Jaz, OceanOptics, Dunedin, FL, USA) placed at an equivalent distance from the light source as experienced by the skin samples. We recorded under red LEDs (max intensity: 636 nm, full width at half maximum: 16 nm, absolute irradiance: 1.36×10^{14} photon cm^{-2} s^{-1}), which did not stimulate LACE behavior. We allowed skin samples to dark adapt under red light for at least 2 min between trials. The white light stimulus was provided by a fiber optic light source set to maximum brightness (peak intensity: 681 nm, full width at half maximum: 150 nm, absolute irradiance: 2.60×10^{15} photon cm^{-2} s^{-1}). The light stimulus lasted until the chromatophores reached maximum expansion or 2 min, whichever was shortest.

We measured multiple aspects of LACE, including the latency of the beginning of LACE from the onset of the stimulus and the time to maximum chromatophore expansion from the video recordings. We also captured individual still images from the video at the beginning of LACE and at the time of maximum chromatophore expansion to measure the change in chromatophore size. These images were processed by thresholding and analyzing particles in FIJI (Schindelin et al., 2012), which allowed us to count the number of pixels of the chromatophore before and after LACE. We performed multiple light trials on each sample, but because the chromatophores do not behave independently, we only measured one randomly selected chromatophore per trial, and averaged the chromatophore area pixel count within each of the 10 samples to get the mean chromatophore size before and after LACE. For all statistical tests, we assumed that similar mechanisms underlie LACE in both adult

and hatchling octopuses, but because we found significant differences in the specific values for latency and chromatophore change after LACE, we analyzed the adults and hatchlings separately. To test the hypothesis that exposure to light causes an increase in the size of the chromatophores, we used a binomial sign test (N=10, P=0.5). Because we wanted to compare LACE-dependent changes in chromatophore size between individual animals which also varied in size, we report the log-ratio of the mean area pixel count before and after LACE for each sample. To test whether the change in mean chromatophore size after exposure to light is significant, we used a one-sample t-test of the log-ratio change in chromatophore size after LACE.

3.5.3 Identifying opsin phototransduction cascade gene expression

For long-term storage of dissected skin and eyes prior to RNA extraction, we placed samples in RNAlater (Life Technologies, Carlsbad, CA, USA) and stored them at -20°C. We extracted mRNA from adult eyes and dorsal mantle skin samples stored in RNAlater using the Nucleospin RNA XS kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. To make a single- stranded cDNA library for each sample, we used the Superscript II RT reaction kit following the manufacturer's protocol. We stored all cDNA libraries at -20°C, and diluted them (1:200) before using them as PCR templates.

We created species-specific PCR primers using the coding sequence for *O. bimaculoides* eye opsin found in Genbank (accession no. AY545172.1). The forward primer sequence was: GCGGCATCAAGAAAATGTCC; and the reverse primer sequence was: TGCAAGAAGAGCGATGATGG. These primers amplify an approximate 340 bp region of the opsin cDNA. The PCR thermocycler program was as follows, repeated for 40 cycles: denaturation, 94°C for 15 s; annealing, 55°C for 30 s; extension, 72°C for 120 s. After 40 cycles, there was a 7 min hold at 72°C for final elongation. To sequence the PCR products, we cloned them

into TOP 10 cells (Invitrogen, Carlsbad, CA, USA), extracted the product and sent to UC Berkeley for Sanger sequencing. We used MUSCLE (Edgar and Sjolander, 2004) in Seaview (Gouy et al., 2010) to align the sequences. We amplified and sequenced opsin products from the eyes (n=2) and dorsal mantle skin (n=5) of adult animals.

3.5.4 Antibody staining

We fixed samples for antibody staining in 4% formaldehyde in phosphate buffered saline (PBS) overnight at room temperature. We washed them with PBS, dehydrated them stepwise into 100% methanol and stored them at -20°C. We rehydrated formaldehyde-fixed samples into 100% PBS, and dissected as necessary. The samples were then blocked (4% donkey serum, 10% bovine serum albumin in PBS/0.1% Tween-20) for at least 1 h. Next, we incubated the samples in primary antibody solution (1:2000 Îijl antibody in blocking solution) using anti-octopus rhodopsin (LSL- LB-5509, Cosmo Bio USA, Carlsbad, CA, USA) and α- and β-tubulin as neural markers (α- tubulin: T7451, Sigma-Aldrich, St Louis, MO, USA; β-tubulin: E7, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) for 4 h at room temperature or 24 h at 4°C. We then washed in PBS three times for 5 min, and transferred them to the secondary antibody solution (1:250 goat anti-mouse Cy3: A10521; goat anti-rabbit Cy5: A10523, Life Technologies, Carlsbad, CA, USA) to stain for 2 h at room temperature. Samples were then washed in 100% PBS twice for 5 min each, then transferred to PBS containing DAPI (0.5:1000 Îijl) for at least 10 min before two more washes in 100% PBS. Samples were mounted in glycerol and visualized using a confocal microscope (Fluoview 1000 Spectral Confocal, Olympus America Inc., Center Valley, PA, USA).

3.5.5 Creating the LACE latency action spectrum

To generate an action spectrum, we collected 6-mm-diameter skin punches from the distal surface of adult *O. bimaculoides* funnels (N=4). We used the same white light source, red LED light, video camera, digital converter and computer software as described for the initial LACE trials. Briefly, each skin sample was visualized using a dissecting scope and infrared CCD camera. We lit the samples with narrow-bandwidth light through a 1 mm fiber optic cable that we positioned just above the surface of the water with a micromanipulator, such that it illuminated the area of skin captured by the video camera. The fiber optic cable was not moved from this position during a trial, although the exact position differed slightly between trials and samples. On the other side of an opaque partition, we used a white scope light (LG-PS2, Olympus America Inc., Center Valley, PA, USA) as the initial light source and lenses (Qioptic, Fairport, NY, USA) directed white light through one of two color filter wheels (Thorlabs, Newton, New Jersey, USA) before the filtered light was directed into the fiber optic cable. Each filter wheel contained 11 colored filters. Together the filters spanned the visible spectrum from 375 to 660 nm (375, 400, 405, 420, 430, 440, 450, 460, 470, 480, 488, 500, 510, 520, 532, 540, 560, 580, 600, 620, 640 and 660 nm).

To ensure that each skin sample within a trial was exposed to similar photon counts for each wavelength of light, we measured the photon counts for each filter at maximum intensity of the white light source. Within the range of the spectrum we expected to find the peak sensitivity of the LACE behavior (between 440 and 660 nm), we adjusted the power of the light source such that photon counts for each filter were equivalent to the max photon count at the wavelength with the least power in the spectrum. For our particular white light source, we calibrated the photon counts for filters between 440 and 660 nm to the photon count at 510 nm. To do this, we used a spectrophotometer (Jaz, Ocean Optics, Dunedin, FL, USA) to measure the absolute irradiance (in photons cm^{-2} s^{-1} in the position of the sample,

and another spectrophotometer (SM700, Milwaukee Instruments Inc., Rocky Mount, NC, USA) to monitor the overall output of the white light coming from the back of the scope light. For each colored filter, we adjusted the power of the light source until the photon counts matched those measured at 510 nm, and recorded the output of the monitor white light.

During the experimental trials, we used these lux values to adjust the power of the white light to standardize photon counts that the skin received. Our white light source was less powerful for wavelengths under 440 nm, and so photon counts for 375-430 nm consistently fell below the 510 nm count standard. For these wavelengths, we used the maximum power allowed by the scope light to maximize the photon counts for the filters. Receiving fewer photons potentially increased the latency of the LACE response at these shorter wavelengths, but in doing so we maximized the likelihood of LACE occurring at the wavelengths surrounding our predicted λmax, based on previous reports of λmax of opsin from the eyes of *Octopus vulgaris* (Brown and Brown, 1958).

To induce LACE, we illuminated the samples with light through each filter for 45 s, then allowed them to sit under red light for 90 s as a dark adapt period before trying another wavelength. We recorded the activity of the chromatophores continuously within a trial, and performed three trials for all but sample 3, which only had two trials for technical reasons. Each trial consisted of 22 light stimuli, presented to the skin in randomized order. From the video we recorded the start time of the light stimulus and the time when a LACE event began (marked by first noticeable expansion of the chromatophores) after the start of the stimulus. This duration is the latency of LACE for that particular wavelength. We excluded LACE data at 375 nm from our analyses because we were unable to measure the photon count at 375 nm.

3.5.6 Quantifying the spectral sensitivity of the light-sensing protein

We estimated the λ max for the octopus LACE action spectrum using the Govardovskii model to fit an opsin specific template (Govardovskii et al., 2000) to the data by minimizing the sums of squares using the optimize function in R (R Core Team, 2013). We extracted data on the spectral sensitivity of O. vulgaris eye opsin using Data Thief (Tummers, 2006) from Brown and Brown (1958).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.D.R. and T.H.O. designed the study and wrote the paper. M.D.R. performed the experiments, imaging and statistical analysis. T.H.O. created the R code for the Govardovskii opsin curve.

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Chapter 4

The last common ancestor of most bilaterian animals possessed at least nine opsins

4.1 Abstract

The opsin gene family encodes key proteins animals use to sense light and has expanded dramatically since it originated early in animal evolution. Understanding the origins of opsin diversity can offer clues to how separate lineages of animals have repurposed different opsin paralogs for different light-detecting functions. However, the more we look for opsins outside of eyes and from additional animal phyla, the more opsins we uncover, suggesting we still do not know the true extent of opsin diversity, nor the ancestry of opsin diversity in animals. To estimate the number of opsin paralogs present in both the last common ancestor of all bilaterians, and the ancestor of Cnidaria + Bilateria, we reconstructed a reconciled opsin phylogeny using sequences from 15 animal phyla, including the traditionally poorly-sampled echinoderms and molluscs. Our analysis strongly supports a repertoire of nine opsin paralogs in the bilaterian ancestor and four opsin paralogs in the last common ancestor of Cnidaria + Bilateria. Thus, the kernels of extant opsin diversity arose much earlier in animal history than previously known. Further, opsins likely duplicated and were lost many times, with different lineages of animals maintaining different repertoires of opsin paralogs. This phylogenetic information can inform hypotheses about the functions of different opsin paralogs and be used to understand how and when opsins were incorporated into complex traits like eyes and extraocular sensors. Key words: reconciled tree, eye evolution, extraocular photoreceptors, phototransduction, vision

4.2 Introduction

As the protein component of visual pigments, opsins are used in the majority of light-detecting cells found in animals (Nilsson 2013). Opsins are G-protein coupled receptors which bind a light-sensitive chromophore via a Schiff base linkage at a conserved lysine

residue (Terakita 2005). When the chromophore absorbs a photon, conformational changes in the chromophore and opsin protein result in the activation of a G-protein based signal transduction cascade (Terakita 2005). Despite their widespread importance in animal photosensitivity, most work on the function and evolution of opsins focused initially on those expressed in the eyes of vertebrates and arthropods (Nathans and Hogness 1983; O'Tousa et al. 1985). Only recently has work on opsins included those expressed outside eyes or from other animal phyla (Velarde et al. 2005; Radu et al. 2008; Hering et al. 2012; D'Aniello et al. 2015; Hering and Mayer 2014). We now know the evolutionary history of opsins is one of many gains and losses of genes across time and among species (Colbourne et al. 2011; Henze and Oakley 2015; Davies et al. 2015; Liegertova et al. 2015; Feuda et al. 2016). This kind of high gene turnover requires broad taxonomic sampling of opsins to fully reconstruct their evolutionary origins, simply because we know that ancient losses may result in the complete absence of some opsin paralogs, even in major groups of animals. Previous large-scale opsin phylogenies have also found many sequences that fall outside of the well-known opsin groups, typically identified in phyla for which we have sparse data, e.g. arthropsins in Daphnia or Echinopsins B in echinoderms (e.g. Colbourne et al. 2011; D'Aniello et al. 2015). Most analyses do not address the nature of these orphaned sequences. While they may be recently-diverged, lineage-specific duplications, another possibility is that they represent entire opsin paralogs that are not found within the phyla that have been most heavily sampled, and have thus not been recognized. Without an accurate picture of how opsin paralogs are distributed among animals, it is challenging to address how diverse opsins really are, when that diversity arose, and how different opsins integrated into different kinds of light-detecting structures through evolution.

Opsins evolved very early in animals (Plachetzki et al. 2007; Feuda et al. 2012; Oakley and Speiser 2015), likely first expressed in light-sensitive cells and later in more complex structures like eyes (Arendt and Wittbrodt 2001; Nilsson 2013). Historically, opsin diver-

sity has been partitioned among three clades which we will refer to as 'canonical c-opsins', 'canonical r-opsins', and 'tetraopsins', formerly 'ciliary', 'rhabdomeric', and 'Group 4 opsins' *sensu* (Porter et al. 2012; Liegertova et al. 2015), respectively. A possible fourth clade of opsins, cnidops, are currently known only from cnidarians (Plachetzki et al. 2007; Feuda et al. 2012). To understand how many opsin paralogs were present in the last common eumetazoan and bilaterian ancestors, we need to understand when these major opsin clades arose and how they are related to each other. Because cnidarians are one of the earliest branching animal lineages with opsins, the opsin repertoire of cnidarians likely represents opsin paralogs present in the last common ancestor of eumetazoans. However, relating cnidarian opsins to the major animal opsin paralogs has proved difficult, and hypotheses on how cnidarian and bilaterian opsins relate vary widely between analyses. For example, the recent prevailing view suggests most recent ancestor of eumetazoans had three opsin paralogs: c-opsins, r-opsins and tetraopsins (Suga et al. 2008; Feuda et al. 2012; Feuda et al. 2014). But cnidarian genomes have been hypothesized to encode either the cnidarian-specific cnidops alone (Porter et al. 2012; Liegertova et al. 2015), both cnidops and c-opsins (Plachetzki et al. 2007; Vopalensky and Kozmik 2009) or c-opsins, r-opsins and tetraopsins (Group 4) in common with bilaterians (Suga et al. 2008; Feuda et al. 2012; Feuda et al. 2014). Based on in-vitro assays, an opsin from the coral Acropora palmata couples with the same G-protein q alpha subunit used by r-opsins (Lee et al. 1994; Mason et al. 2012). Together with the hypothesized phylogenetic position of this opsin, the functional test suggests that some cnidarians may possess canonical r-opsins (Mason et al. 2012). Still, the exact placement of cnidarian opsins is highly sensitive to the specific substitution models and gene sampling regime used in each analysis. At the same time, a solid understanding of their placement is important for understanding the origins of bilaterian opsin diversity.

The reconstruction of opsin evolution in the bilaterians poses yet more challenges. Early

estimates of opsin diversity in the last common bilaterian ancestor identified two (Nilsson 2005) or three (Plachetzki et al. 2007; Porter et al. 2012; Feuda et al. 2012; Feuda et al. 2014) paralogs, corresponding to the canonical c-opsins and canonical r-opsins, or canonical c-. r- and tetraopsins respectively. No bilaterians seemed to have direct orthologs of cnidops, the most commonly identified opsin paralog in cnidarians. Recent sampling efforts to survey new taxa and extraocular tissues have expanded our current view of opsin diversity, and we now recognize that multiple clades of opsins found in extant animals were present in the last common ancestor of bilaterians, based on their presence in both deuterostome (e.g. vertebrates and echinoderms) and protostome (e.g. arthropods and molluscs) genomes. This raises the minimum opsin paralog count in the last common ancestor of bilaterians to five (Terakita 2005; Vopalensky and Kozmik 2009; Suga et al. 2008) or six (Hering and Mayer 2014; Liegertova et al. 2015; Feuda et al. 2014), distributed between the bilaterian c-, r- and tetraopsins. With these additions, a pattern emerges – as we catalog opsins in diverse phyla and from different types of light receptors, we uncover a greater diversity of opsin paralogs.

Thus, the goal of our analysis is to reconstruct a more taxonomically comprehensive evolutionary history of animal opsins to understand the origins of bilaterian opsin diversity. We achieve this in two ways. First, we include newly published opsin sequences from multiple studies that have yet to be synthesized in a large scale phylogenetic analysis. Second, we identify additional new opsins from both publicly available transcriptomes and nine unpublished mollusc transcriptomes, as molluscs are the second most speciose phylum but lag far behind other large taxa in terms of representation in opsin phylogenies to date. With this more comprehensive data set, we produced the first large-scale reconciled opsin phylogeny to better estimate the number of opsins present in the last common bilaterian ancestor. This approach allows us to infer nine opsin paralogs were likely present in the last common bilaterian ancestor. Further, we find that all cnidarian opsins are sister

to three opsin paralogs found in other animals, rather than forming cnidarian-specific paralogs. From this distributions of cnidarian opsins, we infer that the last common ancestor of eumetazoans had at least four opsin paralogs. These results suggest a rapid radiation in opsin diversity prior to the origin of bilaterians, followed by unique patterns of duplications and losses specific to different animal lineages. Finally, these results urge a renewed focus on surveying opsins in understudied phyla (prime candidates include Annelida, and non-bilaterians like Cnidaria and acoels), on including sufficiently diverse sequences when resolving opsin relationships, and on performing functional experiments to determine both the roles of non-visual opsins and the extent to which orthologous opsins in divergent phyla perform similar functions.

4.3 Methods

4.3.1 Data collection:

We searched both NCBI and UniProt using BLAST (Gish and States 1993) with a bait set of 5 opsin sequences (accession numbers: BAG80696.1; NP_001014890.1; CAA49906.1; O15974.1; P23820.1, see Suppl. Table 2 for more info) and an e-value cutoff of 1e-5. Our goal was to maximize the identification of potential opsins from understudied taxa, so we excluded vertebrates and arthropods from our BLAST search on NCBI and downloaded the top 250 hits per opsin bait. We searched Uniref90 with the same bait sequences and cutoff value, then downloaded only lophotrochozoan (NCBI taxonomic ID: 1206795) sequences/clusters. We combined all the sequences we recovered from NCBI and Uniref90 with sequences from other publications, which include tardigrades, arthropods, ambulcraria, cubozoan cnidarians and vertebrates (Hering and Mayer 2014; Henze and Oakley 2015; D'Aniello et al. 2015; Liegertova et al. 2015; Davies et al. 2015). To this initial

database of published sequences, we added mollusc opsins that we gathered by running Phylogenetically Informed Annotation, PIA, (Speiser et al. 2014) on transcriptomes from 7 cephalopods, 5 chitons, 1 gastropod, and 1 bivalve (see Suppl. Table 1 for species and sequence Genbank accession numbers).

4.3.2 Data grooming:

Because our initial data collection was permissive, our raw dataset (over 1,600 sequences) contained both duplicates as well as a number of non-opsin GPCRs. We used CD-HIT (Li and Godzik 2006; Fu et al. 2012) to cluster together sequences that were more than 90% similar to each other to remove duplicates and short sequences that were identical to longer sequences already in the dataset. This also allowed us to reduce the sample size in the alignment by cutting highly similar sequences, while maintaining overall diversity of sequences in the dataset. To remove non-opsin GPCRs, we first ran the dataset through SATé-II (Liu et al. 2012) using the automatic settings. SATé-II runs FastTree 2 (Price et al. 2010) on an initial MAFFT (Katoh and Standley 2013) alignment, then subdivides the alignment into subproblems (maximum size is 200 for the auto setting), which are each realigned with MAFFT. The realigned subproblems are then merged using MUSCLE (Edgar 2004), and a new tree produced by FastTree, and the maximum likelihood (ML) score is calculated. This process is iterated until a pre-defined stopping point. For multiple sequence alignments, SATé-II performs best overall compared to other alignment programs like MAFFT or MUSCLE (Pervez et al. 2014). We used FigTree (Rambaut 2007) to visualize the tree from our SATé run, rooted with melatonin receptors (Feuda et al. 2014). We then trimmed this tree to exclude non-opsins using a custom python script called Supercuts (Swafford 2016) and retained the ingroup clade for subsequent analyses. Next, we removed any sequences from the alignment that lacked the conserved lysine residue ho-

mologous to K296 of bovine rhodopsin. We also manually trimmed the beginning and end of the alignment to the first and last aligned blocks using Aliview (Larsson 2014). Finally, although they lack the conserved lysine, we added the Trichoplax adherens placopsins back to our dataset as a close outgroup to root our tree, as Feuda et al. (2012) showed that placopsins are sister to all other animal opsins. In total, our groomed dataset had 789 opsins with the conserved K296 (plus three placozoan opsins without the lysine) from 368 species across 15 phyla.

4.3.3 Tree estimation and support values:

To create the final alignment for our dataset, we ran SATé on our dataset using the following configuration: a subproblem fraction of 0.025, stopping iterations after 5 unimproved ML scores and FastTree under the GTR gamma model. We used the MPI version of IQ-TREE 1.4.0 (Nguyen et al. 2014), to select a substitution model based on our SATé alignment, infer a maximum likelihood tree, and compute support values. IQ-TREE incorporates an approach for calculating ultrafast bootstraps (UFBoot), which have fewer biases compared to other bootstrapping methods (Minh et al. 2013). We were also able to perform the SH-like approximate likelihood ratio test (SH-aLRT) and the approximate Bayes test as implemented in IQ-TREE to assess support for single branches to complement our UFBoot analysis (Guindon et al. 2010; Anisimova et al. 2011). SH-aLRT branch supports are often more consistent and conservative than bootstrapping methods (Simmons and Randle 2014; Simmons and Norton 2014). The IQ-TREE substitution model test selected the LG+F+R8 model for our alignment based on BIC. Because we had a large number of relatively short sequences, we performed 50 ML tree searches varying the perturbation value (0.1-0.5). We also extended the number of trees IQ-TREE searched once it found a tree with a better ML score to 500. This helped ensure that the algorithm was exploring

the tree parameter space and not getting stuck at a local maximum. Two trees had virtually identical high log-likelihood scores, and so we ran IQ-TREE again, setting each tree as the starting tree, to break the tie and to get UFBoot, SH-aLRT and aBayes values for the final, highest log-likelihood tree. The code used for this analysis, our dataset and the resultant tree are available on BitBucket (UCSB Phylogenetics).

4.3.4 Tree reconciliation and rearrangement:

We used NOTUNG 2.8 (Chen et al. 2000) to reconcile the gene tree with a metazoan species tree based on NCBI Taxonomy. This animal phylogeny places sponges as sister to all other animals, and unresolved relationships between ctenophores, cnidarians and bilaterians. While the order of branching in early metazoans is contentious, our results are unaffected by this uncertainty. To perform both a reconciliation and rearrangement of weakly supported branches, NOTUNG requires a fully resolved species tree. We used the ape package (Paradis et al. 2004) in R (R Core Team 2016) to randomly resolve polytomies present in the species tree. Because our analysis focuses on major splits in the animal phylogeny that are well supported, e.g. protostomes vs deuterostomes, the random resolution of more shallow nodes did not impact our results. We set the penalty for duplications to 1.5, losses to 1.0 and the edge weight threshold for rearrangement to 95.0. After reconciling the tree, we used NOTUNG to rearrange nodes with UFBoot supports that fell below the 95.0 threshold to create our final reconciled tree.

4.3.5 Tree visualization:

We used FigTree 1.4.2 (Rambaut 2007) and TreeGraph2 (Sötver and Müller 2010) to collapse opsin clades by hand according to major taxonomic group (chordates, echinoderms, lophotrochozoans or ecdysozoans), and Evolview (Zhang et al. 2012) to format

the tree color, branch length, etc. for Figure 1. We used iTOL (Letunic and Bork 2011) to combine the tallies of opsins per phylum or molluscan class with animal and mollusc phylogenies (Figures 4 and 5). We made final adjustments to the outputs of these programs using OmniGraffle Pro (v. 6.5, Omni Group, Seattle, WA).

4.4 Results

From our reconciled tree containing 789 unique sequences, we infer nine bilaterian opsin paralogs spread across four eumetazoan paralogs (Figures 1 & 2, complete gene and reconciled gene trees in Suppl. Figures S1 & S2). We recover the six bilaterian opsin paralog groups described in previous publications: canonical c-opsin, canonical r-opsin, Go-opsin, RGR/retinochrome/peropsin, neuropsin and arthropsin. Our broader taxonomic sampling also allows us to infer three previously undescribed bilaterian paralogs, which we have named 'xenopsins', 'bathyopsins' and 'chaopsins'. Because adding so many new bilaterian opsins changes the relationships between paralogs, we establish new, named hypotheses for these relationships, as often done in species-level phylogenetic analyses (see Table 1). In addition to new clade names, we also use Roman numerals for eumetazoan paralogs and Arabic numerals for bilaterian paralogs to help clarify which opsin clades are inferred as eumetazoan versus bilaterian paralogs at a glance in the text and figures.

4.4.1 The last common eumetazoan ancestor had at least 4 opsin paralogs

All cnidarian opsins included in our analysis fell within three opsin paralogs also shared by bilaterians: the canonical visual opsins (I), chaopsins (II), and xenopsins (III) (see Figure 1 and Suppl. Figs S1 & S2). In our gene tree, Anthozoa II (Hering and Mayer 2014) is

Last common eumetazoan ancestor opsin paralogs	Last common bilaterian ancestor opsin paralogs	Previously named clades within each group	UFBoot support
I- Canonical visual opsins	1. Bathyopsins	echinoderm and inarticulate brachiopod bathyopsins	99
	2 .Canonical c-opsins	chordate TMT, chordate encephalopsins, echinoderm encephalopsin-like, arthropod pteropsins, *Platynereis* c-opsin, vertebrate visual c-opsins	96
	3. Non-canonical r-opsins	lophotrochozoa, ambulacrarian and cephalochordate 'arthropsins'	**
	4. Canonical r-opsins	lophotrochozoan visual r-opsins, platyhelminthes r-opsin, chordate melanopsins, arthropod visual r-opsins, arthropod arthropsins	99
II- Chaopsins	5. Chaopsins	echinoderm Echinopsins B and Anthozoa I	100
III- Xenopsins	6. Xenopsins	cnidarian cnidops, lophotrochozoan xenopsins	99
	7. RGR/Peropsins/ Retinochromes	chordate Rrh/RGR/peropsin, echinoderm RGR-like, mollusc retinochrome/peropsin-like/ arthropod peropsin-like	98
IV- Tetraopsins	8. Go-opsins	echinoderm, cephalochordate and lophotrochozoan Go-opsins	100
	9. Neuropsins	chordate, non-mammalian vertebrate, ambulacrarian, lophotrochozoan and arthropod neuropsins/opn5	100

Table 4.1: Summary of opsins present before bilaterians and present in the last common bilaterian ancestor. We considered UFBoot values above 95 as strong support for the monophyly of that group, but allowed branch rearrangements below that threshold. Asterisks (**) indicate support for the node based on reconciliation with the species tree.

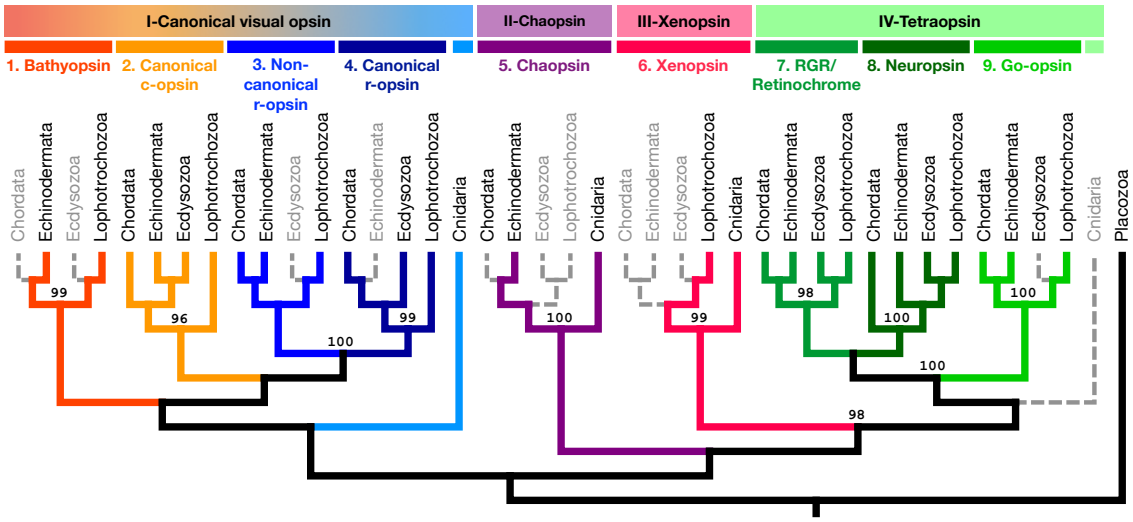

Figure 4.1: There are nine bilaterian opsin paralogs spread among four major eumetazoan opsin paralogs. The four major eumetazoan opsin paralogs are indicated at the top with roman numerals. The nine bilaterian opsin paralogs are indicated with arabic numerals and are color coded to match the corresponding branches. Each opsin clade has been reconciled and collapsed into five major taxonomic groups: chordates, echinoderms, ecdysozoans, lophotrochozoans, and cnidarians. Colored branches indicate the presence of an opsin in at least one species within the major taxonomic group. Light gray dashed branches indicate absence of an opsin paralog from the taxa indicated at the tips. These absences likely represent true losses of opsin paralogs. Ultrafast bootstrap (UFBoot) supports from IQ-TREE are given at the nodes they support. All unlabeled nodes had UFBoot supports <95% and were rearranged during tree reconciliation.

sister to the canonical c-opsins, but with mixed support from UFBoot (67) and single branch tests (aLRT = 91.2; aBayes = 0.998). Our reconciliation analysis minimizes duplications and losses using only bootstrap support values, and so places Anthozoa II sister to both the bilaterian paralogs containing both the canonical c- and canonical r-opsins as the most parsimonious arrangement (as seen in Figure 1). However, given the difference in support for the placement of Anthozoa II based on bootstraps vs. single branch tests vs. parsimony, we cannot confidently place these cnidarian opsins. On the other hand, the cnidarian Anthozoa I (Hering and Mayer 2014) are well supported as sister to echinoderm chaopsins (UFBoot = 100; aLRT = 98.6; aBayes = 1.0). Similarly, the cnidarian-wide cnidops (Plachetzki et al. 2007) are also strongly supported (UFBoot = 99; aLRT = 95.4; aBayes =

1.0) as sister to lophotrochozoan sequences, together comprising the xenopsins. Because all cnidarian opsin paralogs fall sister to bilaterian sequences, we infer that these three opsin paralogs arose prior to the split of cnidarians + bilaterians, and were thus present in the last common ancestor of eumetazoans. Although we did not find extant cnidarian tetraopsins, our reconciled tree infers that the last common eumetazoan ancestor did have a tetraopsin, raising our estimate of eumetazoan opsin paralogs to four.

4.4.2 The bilaterian ancestor at least 9 different opsin paralogs

I-Canonical visual opsins

This grouping consists of multiple clades of both previously and newly described opsins, encompassing the canonical visual opsins in both vertebrates and invertebrates. Because the relationships between these clades generally received low UFBoot support, their current placement together comes primarily by our reconciliation analysis.

1. New opsin group: Bathyopsins

The opsin paralog we have named bathyopsins is a small but well supported, monophyletic, bilaterian clade (see Figure 3, UFBoot=99). Sequences from the echinoderms, Echinopsins A (D'Aniello et al. 2015), represent deuterostomes, and sequences from the genome of the brachiopod Lingula represent prostostomes.

2. Canonical c-opsins

We have renamed as 'canonical c-opsins' the monophyletic clade of bilaterian opsins such as vertebrate visual and brain c-opsins, arthropod pteropsins (Velarde et al. 2005), and Platynereis c-opsin (Arendt et al. 2004). We recovered the canonical c-opsins with high support (UFBoot=96, see Figure 3). Despite mining numerous mollusk transcriptomes for

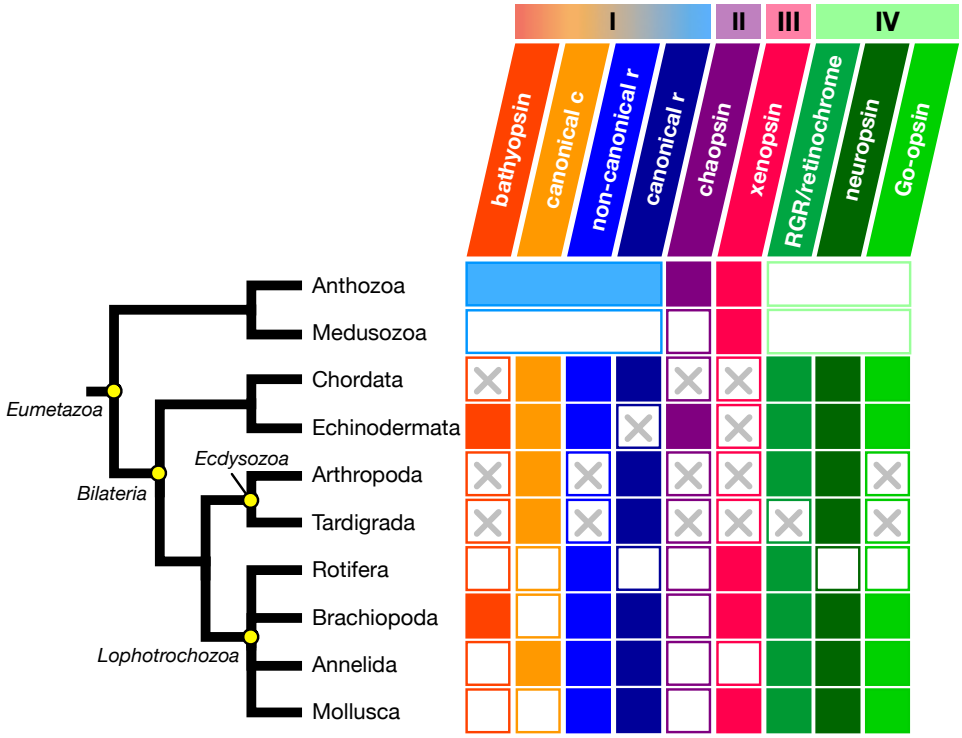

Figure 4.2: The history of opsins is marked by ancient diversity and subsequent losses of paralogs along different animal lineages. Summary of known opsin complements in major animal phyla. Major subdivisions of metazoans are indicated on the phylogeny as yellow dots with italic labels. Phyla are represented at the tips, except for cnidarians, which are broken down into the two major cnidarian splits. Colored bars with roman numerals indicate opsin paralogs present in the most recent ancestor of eumetazoans. The nine bilaterian opsin paralogs are indicated by slanted colored bars and full opsin names. Filled squares represent presence, empty squares absence of at least one sequence from the opsin paralog group for each phylum listed. Gray Xs mark losses of opsins that are strongly supported, based on absence of that opsin paralog in any genomes from the phylum. Note that no extant phylum included in our analysis seems to have the full complement of bilaterian opsins. The maximum is seven opsin paralogs in both echinoderms and brachiopods. The anthozoan I- canonical visual opsin paralog falls sister to bilaterian orthologs, and is indicated by the light blue bar.

opsin sequences (n=14), we did not recover any additional lophotrochozoan or protostome c-opsins that clustered with the canonical c-opsins besides the single c-opsin reported from the annelid *Platynereis dumerilii*.

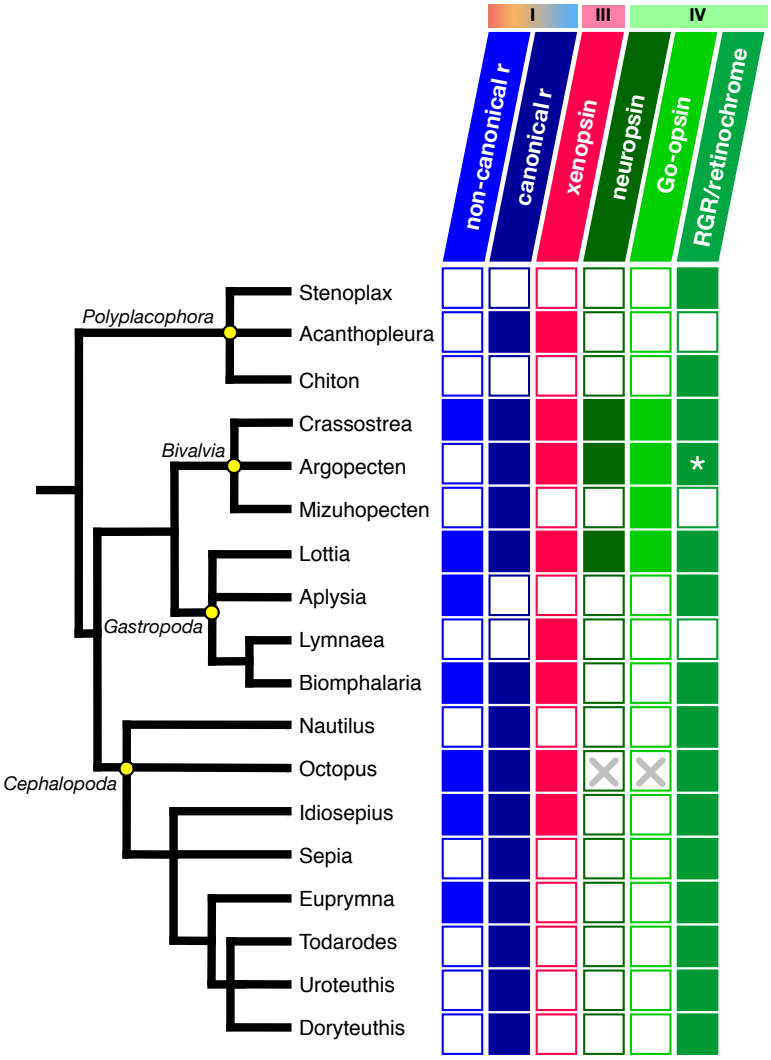

Figure 4.3: The ancestral mollusc likely had seven opsins from six of the bilaterian paralog groups. Summary of known opsin complements within the molluscs. Colored bars with roman numerals indicate opsin paralogs present in the most recent ancestor of eumetazoans. The nine bilaterian opsin paralogs are indicated by slanted colored bars and full opsin names. Filled squares represent presence, empty squares absence of at least one sequence from the opsin paralog for each genus listed. Gray Xs mark losses of opsins that are strongly supported, based on absence of that opsin paralog in the Octopus bimaculoides genome. The major classes of molluscs are noted with yellow dots and italic labels. Argopecten irradians retinochrome was not included our original analysis, but is present, noted by an asterisk.

3. Non-canonical r-opsins

The non-canonical r-opsins are sister to the canonical r-opsins with high support (UFBoot=100, see Figure 1), though we do not have strong support for the monophyly of the

non-canonical r-opsins, even after reconciliation (see Figures 1 & 3; Suppl. Figure 1). The non-canonical r-opsins contain sequences from deuterostome lineages like echinoderms, hemichordates and cephalochordates, and previously unannotated sequences from protostomes groups that include annelids, brachiopods and molluscs.

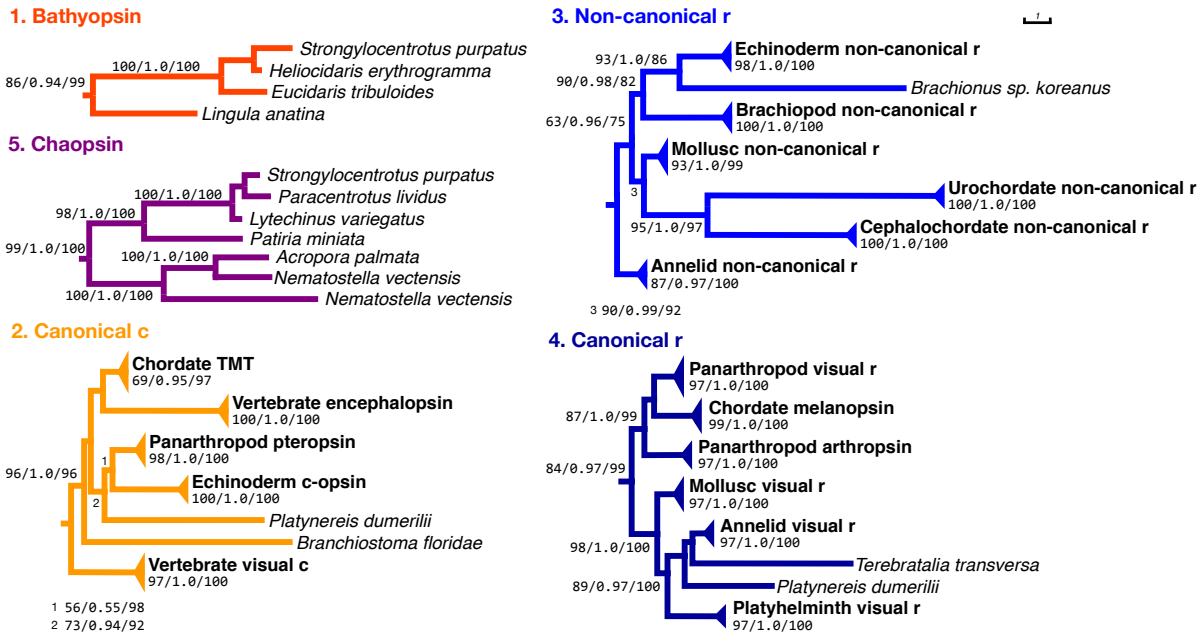

Figure 4.4: Opsin paralog trees for the Gq-opsins and C-opsins, representing the relationships between opsin orthologs by phylum. Each tree shows opsin sequences collapsed by clade. Values below the clade name represent SH-aLRT/aBayes/UFBoots. Only clades with bootstrap supports >75% are shown.

4. Canonical visual r-opsins

This opsin group is well supported by our tree (UFBoot=99, see Figure 3) and includes the following four clades: the canonical visual opsins of arthropods; the chordate melanopsins and arthropod arthroposins; the canonical visual opsins of mollusks; and the (presumably) visual r-opsins from annelids, brachiopods and platyhelminths.

II-5. New opsin group: chaopsins

The opsin group we have named chaopsins consists of two previously described clades of opsins, a group of cnidarian opsins called Anthozoa I (Hering and Mayer 2014) and the echinoderm Echinopsins B (D'Aniello et al. 2015). The grouping of these anthozoan and echinoderm sequences as monophyletic chaopsins is well supported (see Figure 3, UFBoot=100; aLRT=98.6; aBayes=1.0).

III-6. New opsin group: xenopsins

The opsin group we call xenopsins consists of sequences from a variety of lophotrochozoan protostomes (molluscs, rotifers and brachiopods) and cnidarian cnidops. This clade is well supported in our tree (see Figure 2, UFBoot=99; aLRT=95.4; aBayes=1.0). We did not find support for any other protostome (e.g. ecdysozoan) or deuterostome xenopsins. We recovered both the lophotrochozoan xenopsins and cnidarian cnidops with strong support (see Suppl Figure 1). The xenopsins are well supported as sister to the tetraopsins (UFBoot=98; aLRT=90.8; aBayes=1.0, see Figure 1 and Suppl. Figure 1).

Many xenopsins were initially described as c-opsin-like in previous analyses, including sequences from the genomes of the mollusks *Crassostrea gigas* and *Lottia gigantea* and the rotifer *Branchionus* sp., and those from gene expression data generated from the larval eyes of the articulate brachiopod *Terebratalia transversa*, the optic lobes of *Octopus bimaculoides* and the adult eyes of *Idiosepius paradoxus* (Passamaneck et al. 2011; Albertin et al. 2015; Yoshida et al. 2015). However, we believe that the limited taxonomic scope of previous analyses lead to the incorrect classification of these sequences as c-opsin-like. Our tree is the first to include all of these sequences into a single analysis, and our results clearly support them as a monophyletic clade. Finally, in addition to xenopsins that were previously described, we found 7 new mollusk xenopsins from combing through transcriptomes (see

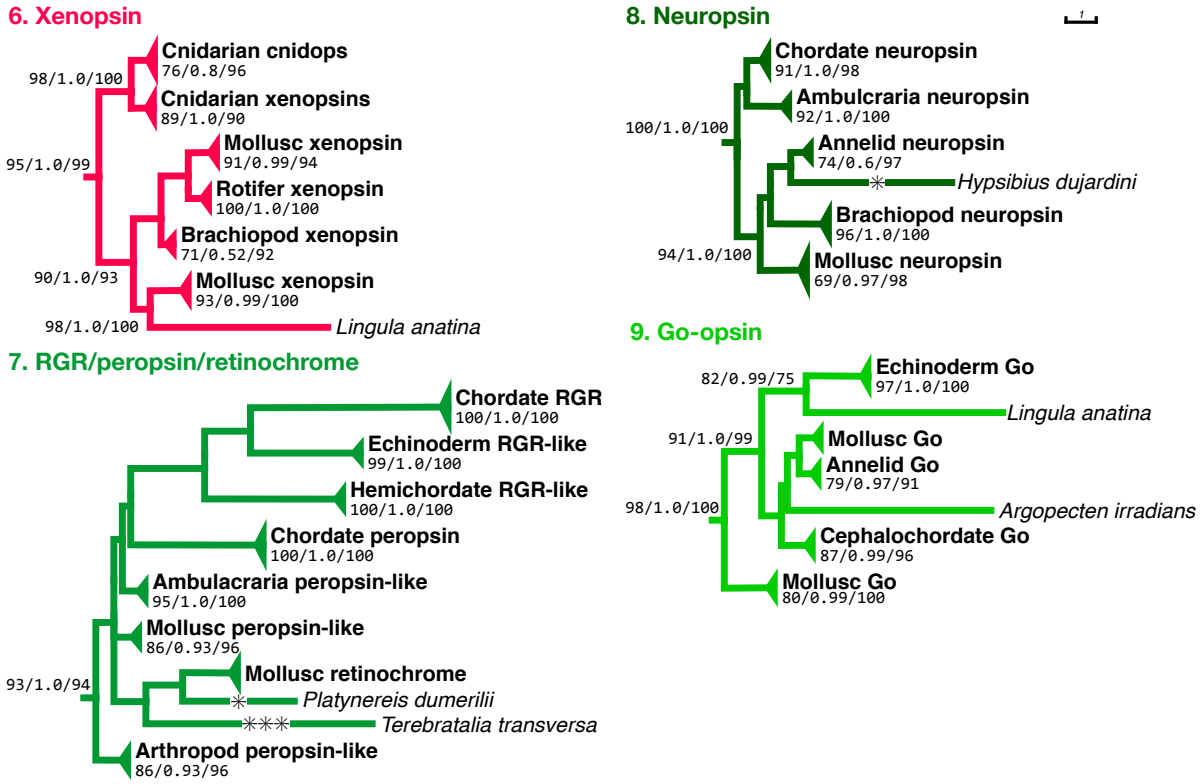

Figure 4.5: Opsin paralog trees for the tetraopsins and xenopsins, representing the relationships between opsins by phylum. Each tree shows opsin sequences collapsed by clade. Values below the clade name representSH-aLRT/aBayes/UFBoots. Only clades with bootstrap supports >75% are shown. Each asterisk '*' on a branch represents a shortening by five branch length units.

Suppl. Table 1).

IV- Tetraopsins

Similar to previous analyses (Porter et al. 2012; Hering and Mayer 2014; Feuda et al. 2014), we recover the tetraopsins (IV), formerly 'RGR/Go' or 'Group 4' opsins, as a monophyletic group with strong support (UFBoot = 100; aLRT = 98.9; aBayes = 1.0). They consist of RGR/retinochromes/peropsins, Go-opsins, and neuropsins. Because our tree shows strong support for these opsins as most closely related to each other, we have renamed this clade of opsins tetraopsins. Further, we find that each of the previously rec-

ognized, major splits within tetraopsins has representatives from both protostomes and deuterostomes. (see Figure 1)

7. RGR/retinochromes/peropsins

The RGR/retinochrome/peropsin clade is well-supported by our tree (UFBoot=98, see Figure 2). Deuterostome RGRs include the original RGRs identified in vertebrates, as well as RGR-like sequences in cephalochordates, hemichordates, and echinoderms (Jiang et al. 1993; Holland et al. 2008; D'Aniello et al. 2015). Deuterostome peropsins include RRH from vertebrates as well as peropsin-like sequences from cephalochordates, hemichordates and echinoderms (Sun et al. 1997; Holland et al. 2008; D'Aniello et al. 2015). Protostome retinochromes include the originally described retinochromes from cephalopods, plus retinochrome-like sequences in bivalve and gastropod molluscs (Hara and Hara 1967; Katagiri et al. 2001). We recovered an additional 3 retinochrome-like sequences from mollusc transcriptomes, including 1 from the gastropod *Bithynia siamensis goniomphalos* and 2 from the chitons *Stenoplax conspicua* and *Chiton virgulatus*. In addition to the molluscs, we found retinochrome-like sequences in the brachiopod *Terebratalia transversa*, previously described as a Go-opsin (Passamaneck and Martindale 2013) and a sequence previously described as a peropsin in the annelid *Platynereis dumerilli* (Marlow et al. 2014). We also found a small clade of protostome sequences that fell outside of the protostome retinochromes, including 4 sequences from the genomes of the mollusks *Crassostrea gigas*, *Lottia gigantea* and *Octopus bimaculoides* (Albertin et al. 2015). Finally, non-insect arthropod peropsin-like sequences (Henze and Oakley 2015) also belonged in the clade of protostome retinochromes. It is unclear from our analysis whether RGR/retinochromes and peropsins are separate bilaterian paralogs. We did recover distinct groups, suggestive of of two bilaterian clades, but had low support values at these nodes, and so we collapsed the groups together (see Suppl. Figure 1).

8. Neuropsins

The split between the protostome and deuterostome neuropsins is well supported (UFBoot=100, see Figure 2). Deuterostome neuropsins/opn5 sequences include a large clade of vertebrate and cephalochordate neuropsins, a large clade of non-mammalian vertebrate neuropsins, plus neuropsin-like sequences from the Ambulacraria (including those from both hemichordates and echinoderms). Neuropsins from protostomes include sequences from annelids, both *Platynereis dumerilli* (Gühmann et al. 2015) and Capitella teleta (Simakov et al. 2012), bivalve and gastropod molluscs, and from the brachiopod *Lingula anatina* (previously annotated as a peropsin). We recovered an additional bivalve neuropsin from the transcriptome of the scallop *Argopecten irradians*. We also found two pan-arthropod neuropsin-like sequences from water flea *Daphnia pulex* (Hering and Mayer 2014; Brandon 2015) and the tardigrade *Hypsibius dujardini* (Hering and Mayer 2014).

9. Go-opsins

The deuterostome and protostome Go-opsins form a well supported clade of bilaterian opsins (UFBoot=100, see Figure 2). We recovered the same deuterostome Go-opsins from echinoderms and cephalochordates as identified from previous analyses (D'Aniello et al. 2015). From protostomes, we found previously described sequences of Go-opsins from both bivalve and gastropod mollusc, and also sequences from brachiopods and annelids. We also recovered a new Go-opsin from the transcriptome of the scallop *A. irradians*.

4.5 Discussion

Reconstructing the evolutionary history of opsins is vital for understanding how evolution produced light-detecting structures like eyes. Unfortunately, the problem of how and

when opsin diversity arose is made difficult by the large number of duplications and losses that have occurred within their evolutionary history. While most analyses of opsin diversity to date have focused on understanding opsin complements within a set of focal taxa (e.g. Plachetzki et al. 2007; Feuda et al. 2012; Hering and Mayer 2014; Feuda et al. 2014; D'Aniello et al. 2015), we included multiple poorly-sampled phyla to ensure the broadest phylogenetic scope to date, for a total of 324 species from 15 phyla. Our analysis reveals three previously unrecognized opsin paralogs in extant animals, and the surprising result that these three additional opsin paralogs likely arose early in the evolution of bilaterians, followed by losses and duplications within those opsins that remained.

Our first major finding is that the diversity of opsins in extant animals suggests the presence of at least seven separate opsin paralogs in the bilaterian ancestor, and we infer a total of at least 9 bilaterian opsin paralogs. In addition to the 6 previously identified bilaterian opsins (c-opsin, r-opsin, melanopsin, Go-opsin, peropsin/RGR/retinochrome and neuropsin), we propose three additional bilaterian opsins– xenopsins, bathyopsins and chaopsins. While we acknowledge the need for additional sequence and expression data to confirm the monophyly of these clades of opsin paralogs, our results are consistent with the hypothesis that these opsin paralogs were all present in the last common ancestor of bilaterians. Hints of the three new opsin groups we have identified can be seen in previous opsin phylogenies (Hering and Mayer 2014; D'Aniello et al. 2015), but hypotheses for how these orphaned sequences relate to other better-studied opsins remained obscure with less broad taxonomic coverage.

For example, cnidarian cnidops have historically been difficult to place consistently within opsin phylogenies. They are sister to the c-opsins in some analyses (Plachetzki et al. 2007; Porter et al. 2012; Hering and Mayer 2014; Liegertová et al. 2015), and the tetraopsins in others (Feuda et al. 2012; Feuda et al. 2014). The fact that cnidops changed positions between analyses suggests that the sequences in the clade are divergent com-

pared to others in the dataset. We found that cnidops fall sister to the lophotrochozoan xenopsins with high bootstrap and branch support, suggesting they are a monophyletic clade. Further, the hypothesis of xenopsins as the sister clade to the tetraopsins is also well supported both by UFBoot and single branch tests. If our reconciled gene tree is correct, the grouping of lophotrochozoan and cnidarian xenopsins suggests that xenopsins were present in both the bilaterian and eumetazoan ancestors. This differs significantly from previous opsin phylogenies (e.g. (Plachetzki et al. 2007; Feuda et al. 2012), since those did not include bilaterian xenopsin-like sequences, and so found that cnidops was its own eumetazoan opsin paralog that was lost from bilaterians entirely.

Lophotrochozoan xenopsins are a well-supported monophyletic clade, suggesting that xenopsins were present in the lophotrochozoan ancestor. Interestingly, xenopsins are absent from publicly available *Platynereis* opsins and the *Capitella* and *Helobdella* genomes. However, because our sampling from annelids is so sparse given the large number of species in the phylum, it seems likely that annelid xenopsins could be uncovered after broader sampling. Xenopsins are also absent from both the ecdysozoan and deuterostome taxa included in our analysis. Given that arthropods, chordates, and echinoderms are now well-sampled for opsin diversity, it seems unlikely, though possible, that xenopsins could be uncovered from unsampled species belonging to these phyla. Thus we hypothesize that the absence of xenopsins from these groups in our dataset reflects true losses of xenopsins from ecdysozoan and deuterostomes lineages. Given this hypothesis, we infer that xenopsins were lost at least three times in bilaterians: from ancestors of the annelids, Panarthropoda, and the deuterostomes.

Increased taxon sampling also allows us to hypothesize the bathyopsins and chaopsins as paralogs present in the last common ancestor of bilaterians. These opsins are are unusual because of their extreme phylogenetic sparseness, suggesting that if our gene tree inference is correct, these opsin paralogs were lost in the majority of bilaterians. How-

ever, we interpret this sparseness more as an indication that even our inclusive dataset may still be under-sampling true opsin diversity in animal phyla, rather than representing an accurate distribution of these opsins among animals. Bathyopsins are found in only two phyla so far, Echinodermata and Brachiopoda, and are well supported as a monophyletic clade in our tree. Given that bathyopsins are represented by one deuterostome and one protostome, we must infer that bathyopsins were present in the last common bilaterian ancestor. We have not yet found chordate or hemichordate representatives. In protostomes, we infer that the lophotrochozoan ancestor had bathyopsins, but since bathyopsins are unknown in ecdysozoa entirely, it is possible that they were lost in ecdysozoa after the lophotrochozoan/ecdysozoan split. Because opsins from chordates and arthropods are well sampled, it is unlikely, but possible, that these phyla possess bathyopsins. We have not uncovered annelid, mollusc or rotifer bathyopsins. However, because lophotrochozoans, and especially annelids, are underrepresented even in our analysis, it is possible that opsin surveys from lophotrochozoans will reveal additional members of the bathyopsins in these phyla.

We found chaopsins in only two phyla so far, echinoderms and cnidarians, and their monophyly is supported by both high UFboots and single branch tests. Given our data set and analyses, we hypothesize that chaopsins were lost up to three times in bilaterians: twice from deuterostomes (chordates and hemichordates) and once in the ancestor of all protostomes (including both ecdysozoans and lophotrochozoans). We also find that anthozoans are the only cnidarians that have chaopsins, which suggests another potential loss of chaopsins from the ancestor of hydrozoans and cubozoans. As with the other new opsin types we have described, we are more confident that chaopsins are truly lost from chordates and arthropods because of the extent to which those phyla have been sampled. We are much less confident regarding the loss of chaopsins from the lophotrochozoans, which are extremely poorly sampled, especially considering the amount of animal diversity found

in the group.

The second major finding is that the eumetazoan ancestor likely had at least four opsin paralogs, based on the distribution of cnidarian opsins in our analyses. This differs from previous reports which divide cnidarian opsins into their own lineage-specific clade (Suga et al. 2008; Porter et al. 2012), cnidarian specific and cnidarian c-opsin clades (Plachetzki et al. 2007) or as cnidarian members of the c-, r- and tetraopsins (Feuda et al. 2012; Feuda et al. 2014; Hering and Mayer 2014; Liegertová et al. 2015). We do find that extant cnidarian sequences fall sister to bilaterian opsins, but to different opsin paralogs than previously reported.

Our analysis places cnidops sister to the lophotrochozoan xenopsins with high support, whereas in the past, cnidops has typically formed its own clade or else fallen sister to the c-opsins (Plachetzki et al. 2007; Feuda et al. 2012; Feuda et al. 2014; Hering and Mayer 2014; Liegertová et al. 2015). Unlike the other cnidarian opsin paralog groups which contain only anthozoan sequences, cnidarian xenopsins have representatives from all the major classes of cnidarians. We did not recover strong support for Anthozoa II as sister the canonical c-opsins as found by other analyses (Plachetzki et al. 2007; Suga et al. 2008; Feuda et al. 2012; Feuda et al. 2014). Instead, we found ambiguous bootstrap vs. single branch support for this placement, and after reconciliation, Anthozoa II fell outside of the clade containing both canonical c- and r-opsins.

Unlike a number of recent analyses that placed chaopsins from Anthozoa I (Hering and Mayer 2014) with the canonical r-opsins (Feuda et al. 2012; Feuda et al. 2014; Hering and Mayer 2014), we recovered this relationship with moderate support in our gene tree only. After reconciling the gene tree chaopsins fell sister to the clade containing xenopsins + tetraopsins. Finally, from our phylogeny, we infer the loss of cnidarian opsins belonging to the tetraopsins. However, because cnidarians are not well sampled, it is possible that a cnidarian ortholog of the bilaterian tetraopsins may be uncovered. Overall, we success-

fully identified well-supported bilaterian orthologs of at least two cnidarian opsins, cnidops as xenopsins, and Anthozoa II opsins as chaopsins and infer that the last common ancestor of eumetazoans must have had at least 4 different opsins. Adding more opsins from non-bilaterians (Cnidaria and acoels) may help solidify deeper relationships between well documented opsin paralog like the canonical c- and r-opsins and the opsin paralogs we have identified in this analysis.

We used a traditional animal phylogeny to reconcile our gene tree, with ctenophores placed sister to cnidarians, and these two phyla together as sister to bilaterians. However, the traditional view of ctenophores as sister to cnidarian (Coelenterata hypothesis) is challenged by multiple studies that instead place ctenophores sister to all other animals (ctenophore-out hypothesis) (Dunn et al. 2008; Ryan et al. 2013; Moroz et al. 2014; Borowiec et al. 2015; Pisani et al. 2015; Halanych et al. 2016). There seem to be two opsin paralogs in ctenophores, but the relationship between those opsin paralogs and opsins from other animals is contentious, particularly the placement of Mnemiopsis 3 (Feuda et al. 2014; Schnitzler et al. 2012). Although Mnemiopsis 3 does have the conserved lysine that aligns at bovine rhodopsin position 296, it was excluded from (Hering and Mayer 2014) because there is an additional insertion that is absent from the other Mnemiopsis opsins. Its placement in the metazoan opsin phylogeny is also highly sensitive to outgroups as seen in (Schnitzler et al. 2012; Feuda et al. 2014). For these reasons, we did not include Mnemiopsis 3 in our analysis. Overall, the results of our analysis are not affected the current controversy about the relationship between ctenophores and other animals (Borowiec et al. 2015; Pisani et al. 2015; Halanych et al. 2016; Pisani et al. 2016). Our results also cannot support either hypothesis, as the ctenophore opsins we included were not placed in the animal opsin phylogeny with high support.

Our opsin dataset includes more sequences from more phyla than any previously published opsin phylogeny. These conditions meant that many nodes were difficult to resolve

with high statistical bootstrap support (see Supp. Figure 1) and so we used a unique combination of methods to produce our final tree, incorporating both maximum-likelihood (SATé and IQ-TREE) and gene tree-species tree reconciliation (NOTUNG). Because we were interested in knowing how many opsin paralogs were present in the common ancestor of bilaterians, we needed a better understanding of how different opsins were related to one another. However, because the history of duplications and losses of opsins can make a gene tree by itself difficult to interpret, we reconciled the opsin gene tree to a species tree using NOTUNG, which looked for the most parsimonious pattern of duplications and losses to account for known species relationships. This tells us how opsin paralogs might be related to each other, specifically whether any pair of opsins found between species arose from duplication events (paralogs) or speciation events (orthologs), and allowed us to determine which groups may have bilaterian or eumetazoan origins. It is worth noting that while reconciliation can bias counts of duplicates and losses (Hahn 2007), we estimate the same minimum number of opsin paralogs in the most recent ancestor of bilaterians with both our reconciled tree and the IQ-TREE gene tree (Suppl. Figures 1). Overall, where we have overlapping data, our final reconciled tree is generally consistent with other large-scale opsin phylogenies (Porter et al. 2012; Hering and Mayer 2014; Feuda et al. 2014).

Opsin evolution is surprisingly complex, and this complexity hints at just how much we have yet to learn about how animals use opsins, how these functions shaped the evolution of the gene family and the physiology and behaviors that require opsins. It is not yet clear to what extent the loss of an opsin paralog within an animal lineage suggests the concomitant loss of the organismal function, or whether other opsin paralogs can take over that function. At present, we have no functional data for the majority of the 700+ opsins included in this analysis. While we can make some inferences about the function of a particular opsin based on what we know about orthologous opsins, the data we do have suggests that different animal phyla use related opsins for different purposes, e.g. r-opsins likely mediate vision

in many protostome eyes, but the related orthologous melanopsins in vertebrate retinal ganglion cells only have roles in non-visual tasks.

The discovery of opsins restricted to traditionally understudied groups like echinoderms, molluscs, annelids, and brachiopods strongly underscores the need to look outside well-sampled phyla like vertebrates and arthropods for opsins. Additionally, many of these traditionally understudied animals are not well known for possessing eyes, and yet our analysis (among others) shows rich repertoires of opsins in these clades, urging us to look beyond animal eyes for opsin expression. By now it is exceedingly clear that opsins are expressed across the bodies of animals (Ramirez et al. 2011), and our results show that there may still be opsin paralogs to discover outside of animal eyes. Further, many analyses, including ours, use the presence of a conserved lysine residue (bovine rhodopsin K296) as diagnostic for opsins (Terakita 2005). However, we found that keeping this requirement eliminated 500 sequences recovered by BLAST. While many of these are likely closely related, but non-opsin GPCRs, some may be opsin duplicates that have lost the conserved lysine (Henze and Oakley 2015). Future analyses of these opsin-like sequences may reveal even more ancient diversity than what we have recovered so far.

Finally, our analysis raises an urgent and intriguing question– what was the last common ancestor of Bilateria are animals doing with 9 opsin paralogs. Cataloging opsin sequence diversity alone is insufficient to understand why animals have so many different opsins. We must also take what we learn from an analysis like our own to understand how changes to an opsin‘s sequence alter its function and how animals use opsins for different tasks. Besides spatial vision, opsins are used for myriad purposes, as depth-gauges, for circadian rhythms or enabling private communication between conspecifics (Bennett 1979; Lythgoe 1979; Bybee et al. 2012). Further, while opsins are canonical light detectors, two recent studies have shown roles for opsins in both heat sensing and detecting mechanical stimuli in *Drosophila* (Shen et al. 2011; Senthilan et al. 2012). These studies provide a tanta-

lizing glimpse into opsin functions in sensory modalities besides light detection. Without understanding the true extent of opsin diversity, we cannot understand opsin evolution, the evolution of eyes and other light sensors, or even how a complex trait like eyes can evolve.

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Chapter 5

Retracing the evolutionary origins of r-opsin photoreception in mollusc mantle

5.1 Abstract

At macroevolutionary timescales, do novel behaviors evolve through reuse and tinkering of underlying components? We asked whether deep homology of a dispersed light sense in mollusc mantle may have contributed to the evolution of Light-Activated Chromatophore Expansion (LACE), a novel behavior of the colored chromatophore organs in octopus skin that we have previously hypothesized uses the same phototransduction cascade to sense light as octopus eyes. We surveyed the literature and 45 publicly available mantle transcriptomes from five major molluscan lineages for the expression of opsin phototransduction genes, the primary molecular mechanism that animals sense light. After excluding transcriptomes that fell below our quality threshold, we kept 28 species in our analysis. A total of 20 mantle transcriptomes had at least one opsin expressed. R-opsin cascade genes are expressed in the mantles of 18 species from 5 molluscan classes. We combined these data with mantle expression data from a few species in the literature, bringing our total count of r-opsin expressing mantles to 21. The broad distribution of these data suggest that ancestral octopus skin could have been light sensitive prior to the evolution of their chromatophore organs. We performed an ancestral state reconstruction for r-opsin expression in the mantle of the last common ancestral mollusc, and found that this ancestor most likely expressed r-opsin in its mantle. These results suggest that an ancestral light sense existed before the origins of chromatophores in cephalopods and may have been co-opted during evolution and integrated with chromatophores. When this happened, it allowed octopus chromatophores to respond to changes in light in the absence of the central nervous system. The molecular basis for light sensing, r-opsin phototransduction, is already known as a deep homology, since r-opsin phototransduction arose, at latest, before the last common bilaterian ancestor. Our results are consistent with the hypothesis that a dispersed light sense in the mantle is also deeply homologous and pre-dates the origins of cephalopod

chromatophores and their LACE behavior in response to light.

5.2 Introduction

Chromatophores are small, pigmented organs embedded in the skin of coleoid cephalopods like octopuses, squid and cuttlefish. When chromatophores organs expand and contract, they change the tone and color of the animals' skin in an instant, creating visual displays for used for remarkably sophisticated camouflage and communication. No other molluscs have such a diverse and complex behavioral repertoire, and cephalopod chromatophores are a major reason why. This complexity is a lure for scientists interested in understanding behavior, but also raises the question of how such complex and novel behaviors evolve? Because behaviors are the output of underlying levels of biology, we can start to understand how behaviors evolve by understanding their component mechanisms and when those elements themselves evolved.

We consider cephalopod chromatophores as evolutionary novelties, or "novel traits or behaviors, or novel combinations of previously existing traits or behaviors, arising during the evolution of a lineage, and that perform a new function within the ecology of that lineage" (Pigliucci 2008). Chromatophores perhaps originated before the last common ancestor of the coleoid cephalopods (350-250 Ma), as they are not present in modern *Nautilus* spp., the sister taxon to the coleoids (Kroger et al. 2011; Warnke et al. 2011; Tanner et al. 2017). As organs, chromatophores differ in their structure and function compared to pigment cells found in other animals, changing shape via a unique mechanism. Instead of pigment granules dispersing within a fixed amoeboid cell membrane, the cell membrane containing pigment granules in cephalopod chromatophores is highly elaborated and elastic. When the radial muscles attached to the pigment cell contract, they stretch the cell membrane taut, allowing pigment granules to disperse within the now larger area of the

cell. The effect is a dramatic increase in the apparent size of the colored spot, and because the muscles are under direct neural, rather than physiological, control, the change in chromatophore size can happen almost instantly.

Chromatophores typically respond to environmental light indirectly, mediated through cephalopod eyes and brains. However chromatophores in the mantle skin of *Octopus bimaculoides* expand in response to light when completely isolated from the central nervous system and eyes, a behavior called Light Activated Chromatophore Expansion, or LACE (Ramirez and Oakley 2015). Since cephalopod chromatophores are evolutionary novelties and LACE is a behavior of chromatophores, we argue that LACE itself is an evolutionarily novel behavior. Though evolutionarily novel, LACE is also relatively simple, essentially requiring only the chromatophore organs and a local light sense in octopus skin. Though we know very little about the evolutionary or developmental origins of octopus chromatophores themselves, light sensing in animals is well studied at both the molecular and behavioral level in an evolutionary context, providing a solid foundation for understanding the evolutionary history of this component of LACE. We want to ask whether there is evidence of deep homology and evolutionary tinkering in light sense underlying LACE behavior, and in particular, whether the light sense itself might be a deeply homologous trait.

From previous work, we hypothesized that octopus skin likely senses light using opsin-based phototransduction, the same mechanism used by almost every animal eye, although different eyes use different opsin paralogs (Yau and Hardie 2009). Opsins are a large family of 7-transmembrane domain, G-protein coupled receptors found only in animals, though they are missing from the sponges (Feuda et al. 2012). Opsins seem to have originated early in the evolutionary history of animals, based on the shared distribution of opsin paralogs among distantly related phyla. As we estimated in Chapter 4, the last common ancestor of most bilaterians (excluding Xenacoelomorpha) may have had at least nine opsin paralogs,

setting the stage for large numbers of subsequent duplications, but also losses of distinct sets of paralogs in different bilaterian lineages (Ramirez et al. 2016).

The same r-opsin and associated phototransduction cascade genes that sense light in octopus eyes are expressed in octopus skin, and the spectral sensitivity of octopus eyes and LACE to blue light (474 and 480nm respectively) also supports the hypothesis that the same light receptive protein, r-opsin, is used in both octopus eyes and skin (Brown and Brown 1958; Ramirez and Oakley 2015). LACE has not been shown for chromatophores from other cephalopods (though see Florey 1966 and Packard and Brancato 1993), but r-opsin is also expressed in the mantles of the cuttlefish *Sepia officinalis* and several squid species, *Doryteuthis pealii, Euprymna scolopes* and *Uroteuthis edulis* (Mathger et al. 2010; Kingston et al. 2015; Kingston et al. 2015; Pankey et al. 2014). Outside of cephalopods, genes from the r-opsin phototransduction cascade are expressed in the mantles of bivalves *Argopecten irradians* and *Patinopecten yessosensis* and in the aesthetes of chitons (Porath-Krause et al. 2016; Wang et al. 2017; Ramirez et al. 2016). While we have these few specific examples, it is not clear how widespread expression of opsins in the mantle, including r-opsins, is across molluscs.

The likely use of r-opsin in LACE represents deep homology of the molecular mechanism for sensing light. The fact that r-opsin phototransduction genes are expressed in mantle tissue across multiple molluscan classes raises the question of whether this expression in the mantle represents independent evolutionary events in different molluscan lineages, or the inheritance of r-opsin expression in the mantle from an ancient molluscan ancestor. If the former, then the expression of r-opsin cascade genes in cephalopod mantle might have evolved at the same relative time as their chromatophores, and separately in the mantles of bivalves and aesthetes of chitons. If the latter, then not only would the molecular mechanism underlying LACE be deeply homologous, but also its tissue-specific expression pattern in the mantle, perhaps indicating the deep homology of the dispersed sensory cells

which express r-opsin and allow the mantle tissue to be sensitive to light.

We used a comparative transcriptomic approach as a first pass to try to pinpoint when in molluscan evolutionary history r-opsin cascade genes were expressed in mantle. We mined 28 mantle transcriptomes total from 9 cephalopods, 2 gastropods, 5 bivalves, 6 aplachophorans and 6 polyplachophorans (chitons) for genes used in opsin-based phototransduction cascades. While we were able to find almost all of r-opsin cascade components in the majority of the transcriptomes, the opsins themselves were more often not recovered. Although we did not recover an opsin from every transcriptome, the broad distribution of each opsin type in the mantle transcriptomes is consistent with the hypothesis that these opsins were expressed in the mantle of the last common molluscan ancestor. We used an ancestral state reconstruction framework to test this hypothesis more rigorously. The results of the ancestral state reconstruction suggest that the last common ancestor of all molluscs expressed r-opsin in its mantle, making the r-opsin expression in the mantle of modern molluscs deeply homologous. The results strongly support our hypothesis that LACE evolved by the incorporation of a phylogenetically old light sense in the mantle with the phylogenetically new chromatophore organs.

5.3 Methods

We gathered transcriptomes from mollusc mantles either from assembled transcriptomes provided by collaborators, or by assembling mantle transcriptomes from data available on NCBI in the SRA database.

5.3.1 RNA-seq Transcriptome assembly

Sequence Read Archive (SRA) files were downloaded from NCBI using SRAToolkit command-line utilities (v2.8.0). The raw reads were assembled using Trinity (v2.3.2, Grabherr et al. 2011) using default parameters. Briefly, Trinity calls Trimmomatic (Bolger et al. 2014) to trim the raw reads with quality scores below 20, normalizes the raw read counts *in silico* before assembling. Longest amino acid coding sequences were predicted from the Trinity contigs using Transdecoder (v3.0.0 Grabherr et al. 2011).

5.3.2 Finding r-opsin phototransduction cascade genes using BLAST and Phylogenetically Informed Annotation

Because gene expression levels can vary among samples for both biological and technical reasons, and because most of our transcriptomes were made from a single mantle sample per species rather than multiple biological or technical replicates, we decided to simply count a gene as expressed if we were able to find it in the transcriptome and validate it as a good hit using a custom script to run the Phylogenetically Informed Annotation pipeline via commandline (Speiser et al. 2014). First we used BLAST (v. 2.6.0, Gish and States 1993) with pre-established sets of gene-specific sequences from Speiser et al. (2014) as baits, and a cutoff threshold of 1e-7. We combined the blast hits for each gene from all species into a single file, then aligned the hits into a fixed PIA alignment for each gene with MAFFT-profile (Katoh and Standley 2013). We then placed the newly aligned blast hits into a fixed topology PIA gene tree using RAxML (v. 8.0, Stamatakis 2014). The gene trees contain landmark sequences that have been functionally implicated in phototransduction as well as other closely related sequences. Once blast hits were placed within the fixed gene tree, we only counted a hit as validated if it fell within the clade containing the validated landmark sequences, and counted phylogenetically validated hits as 'present' for

each species. For the mantle-specific analysis, transcriptomes that had under 1000 core orthologs (identified using HAMSTR) were considered to be of too low quality and excluded from further analysis, unless we recovered at least one opsin candidate of any kind. From the r-opsin phototransduction cascade, we looked for expression of arrestin, G-protein subunits alpha, beta and gamma, diacylglycerol kinase, G-protein receptor kinase 1 and 2, protein kinase C, rdgb, rdgc and transient receptor protein (TRP).

We took additional steps to validate and categorize opsin blast hits before including opsins in the full presence-absence table. We created a new opsin phylogeny with a large dataset of metazoan opsins and non-opsin GPCRs from Feuda et al. (2012) and Ramirez et al. (2016) to use as the backbone tree for PIA. The non-opsin GPCRs were added to help sort candidate opsins from sequences of closely related, but non-opsin, GPCRs. Adding distant non-opsin GPCRs is known to destabilize in-group relationships among opsin clades (Feuda et al. 2012), which changed the tree topology from published trees, but is still useful for sorting most of the blast hits into their appropriate clades. We also incorporated opsin blast hits from Xenoacoelomorpha, the hypothesized sister clade to all other bilaterians, and which are not currently present in any opsin phylogeny. We aligned using SATe-II (same configurations from Chapter 4 and Ramirez et al. 2016; Liu et al. 2012) and created a new opsin phylogeny with Ultrafast bootstrap values using IQ-TREE (1.4.0; Nguyen et al. 2015). Some sequences fell into inappropriate phylogenetic clades in the resulting gene tree, which could represent either real mollusc sequences that were ambiguously placed, or else contamination of the sample transcriptome from other sources. While some of these may reflect true mollusc opsins that were poorly placed because of sequencing or assembly artifacts, we excluded them to be conservative.

5.3.3 Counting and visualizing presence or absence of phototransduction genes

We used a custom python script called LOIC to programmatically create lists of gene presence (Swafford, 2017), based on the landmarks for each opsin clade. For categorizing opsins, we used opsin paralogs previously described in Ramirez et al. (2016). We created a presence-absence table of these PIA-validated hits using the 'presabs' function from the R package 'fuzzySim' (Barbosa and Márcia Barbosa 2015). Finally, we visualized gene presence-absence across the mollusc phylogeny using Evolview (Zhang et al. 2012).

5.3.4 Ancestral state reconstruction of opsin phototransduction gene expression

We identified orthologs in all of our mantle transcriptomes using a lophotrochozoan gene set from Kocot et al (2016) implemented in HaMStR (Ebersberger et al. 2009). If a transcriptome had no opsin BLAST hits and had less than 1,000 core orthologs found using HaMStR, we excluded that sample from the ancestral state reconstruction. To create our data matrix, we required that each gene ortholog set contain at least one of the species with the fewest number of core orthologs. This requirement was necessary because a few species (*Corbicula fluminea, Mercenaria campechiensis*, and *Margaritifera margaritifera*) had less than 100 core orthologs from HaMStR, but had at least one opsin. To align sequences for each gene, we used MAFFT with default settings, except a 'maxiterate' value of 1000 (Katoh et al. 2005). To clean up the alignments and remove ambiguously aligned sites, we used Aliscore (Misof and Misof 2009) and Alicut (Kück 2009), and we used trimAl (Capella-Gutiérrez et al. 2009) to remove very short (>10 bp) or empty sequences with the '-resoverlap' and '-seqoverlap' functions. We made single gene trees for each alignment

using FastTree (v. 2.1.9; Price et al. 2010) and removed paralogous sequences using PhyloTreePruner (Kocot et al. 2013). After all the filters, we retained 55 orthologs and used FASconCAT-G (Kück and Meusemann 2010) to create the final data matrix. We used RAxML (v. 8.2.9; Stamatakis et al. 2014) to create a maximum likelihood tree for our species, partitioning the matrix on each gene. We used the setting 'PROTGAMMAAUTO' to find the best-fitting protein model for each partition.

Using the presence/absence table and species tree generated above, we used the R package corHMM to infer the probability of r-opsin phototransduction expression in the mantle at each node in the species tree. We varied the number of rate categories (1 or 2), and the root probability calculation (equal or 'maddfitz'- which allows the root probability to be set using the transition rates calculated from the tree) and compared the models using AICc weights using the 'akaike.weights' command in the package 'qpcR' in R. To construct the ancestral state of r-opsin expression, we made two key assumptions for these data. First, because non-canonical r-opsins have only been described recently (Pankey et al. 2014, Ramirez et al. 2016), we do not know whether they function as the canonical, visual r-opsin clade does. Given this ambiguity, we decided that because they fall sister to the canonical r-opsins with high support, we would lump together the counts of canonical and non-canonical r-opsins. Additionally, because the only known function of retinochrome is as a photoisomerase integral to r-opsin phototransduction in molluscs, we counted the presence of retinochrome as evidence of r-opsin phototransduction in the mantle, even if we were unable to recover either a canonical or non-canonical r-opsin from that species. Finally, we visualized the ancestral state reconstruction of mantle-specific r-opsin expression using Evolview (Zhang et al. 2012).

5.4 Results

5.4.1 Mantle-specific opsin phototransduction cascade gene expression among molluscs

We recovered a total of 32 new candidate opsins from the mollusc mantle transcriptomes we analyzed, in addition to recovering 14 opsins from transcriptomes that had already been analyzed and previously described (see Figure 1). We found 13 new opsins in chiton mantles, including xenopsins, canonical r-opsins, retinochromes and Go-opsins. We recovered multiple opsin-like sequences from aplacophorans, a small class of shell-less, benthic, deep sea molluscs. However, the aplacophoran candidates were difficult to identify using our phylogenetically informed annotation, and so we were unable to determine which opsin clades they belong to. Many of the aplacophoran sequences fell in between known opsins and non-opsin GPCRs, and some appear to be contamination from cnidarians, the food source for most aplacophorans. Only one aplacophoran sequence, from *Tegulaherpia* spp., fell within the opsins. We recovered 7 opsins from 3 eyeless bivalves. We also recovered 6 candidate opsins from 2 different snail species.

We recovered at least one opsin sequence from 20 of the 28 mantle transcriptomes that met our minimum quality cutoff (see Figure 1). In total, the distribution of opsin types in this dataset is consistent with the types characterized by Ramirez et al (2016) in Chapter 4, namely that molluscs as a phylum have 6 of 9 hypothesized bilaterian opsin paralogs. Retinochromes were recovered most often (18), followed by canonical r-opsins (12), then xenopsins (9), Go-opsins and non-canonical r-opsins (4), and finally neuropsins (2). We found r-opsins (including both canonical and non-canonical r-opsins) expressed in mantle of 18 mollusc species, from 4 major mollusc classes, including chitons, bivalves, gastropods, and cephalopods. Retinochromes were similarly distributed, overlapping with expression

of either a non-canonical or canonical r-opsin in all but one case. Xenopsins also had broad distribution, found in the same 4 major mollusc classes are r-opsins. Go-opsins are present in bivalve and chiton mantles, but not detected in cephalopods, consistent with their known absence from the *Octopus bimaculoides* genome. Interestingly, no transcriptome expressed all 5 of the opsins known from mollusc genomes. Our bioinformatic mining also found many genes known from arthropods and vertebrates to be part of the r-opsin phototransduction cascade, including Gqα and TRP ion channels (see Figure 1).

5.4.2 Ancestral state reconstruction of mantle-specific r-opsin expression

The best model according to Akaike weights was the reconstruction with 1 rate category and the root probability calculated based on the transition rates, rather than equally weighted (AICc weight = 0.55 vs 0.44 for the equal weighting model). Our ancestral state reconstruction for r-opsin expression in mollusc mantles suggests that the last common ancestor of molluscs most likely expressed r-opsin in its mantle, and that this pattern of expression was conserved throughout the evolution of different mollusc lineages (see Figure 2). Consistent with the lack of opsins recovered from aplacophoran transcriptomes, the ancestral state reconstruction suggests a loss of r-opsin expression in the aplacophorans.

5.5 Discussion

We considered the macroevolution of octopus LACE, a novel behavior, by tracing the evolutionary history of one of its components, an r-opsin based, dispersed light sense in its skin. Our question was when the dispersed light sense originated relative to the origins of the other major component underlying LACE, the evolutionarily novel chromatophore or-

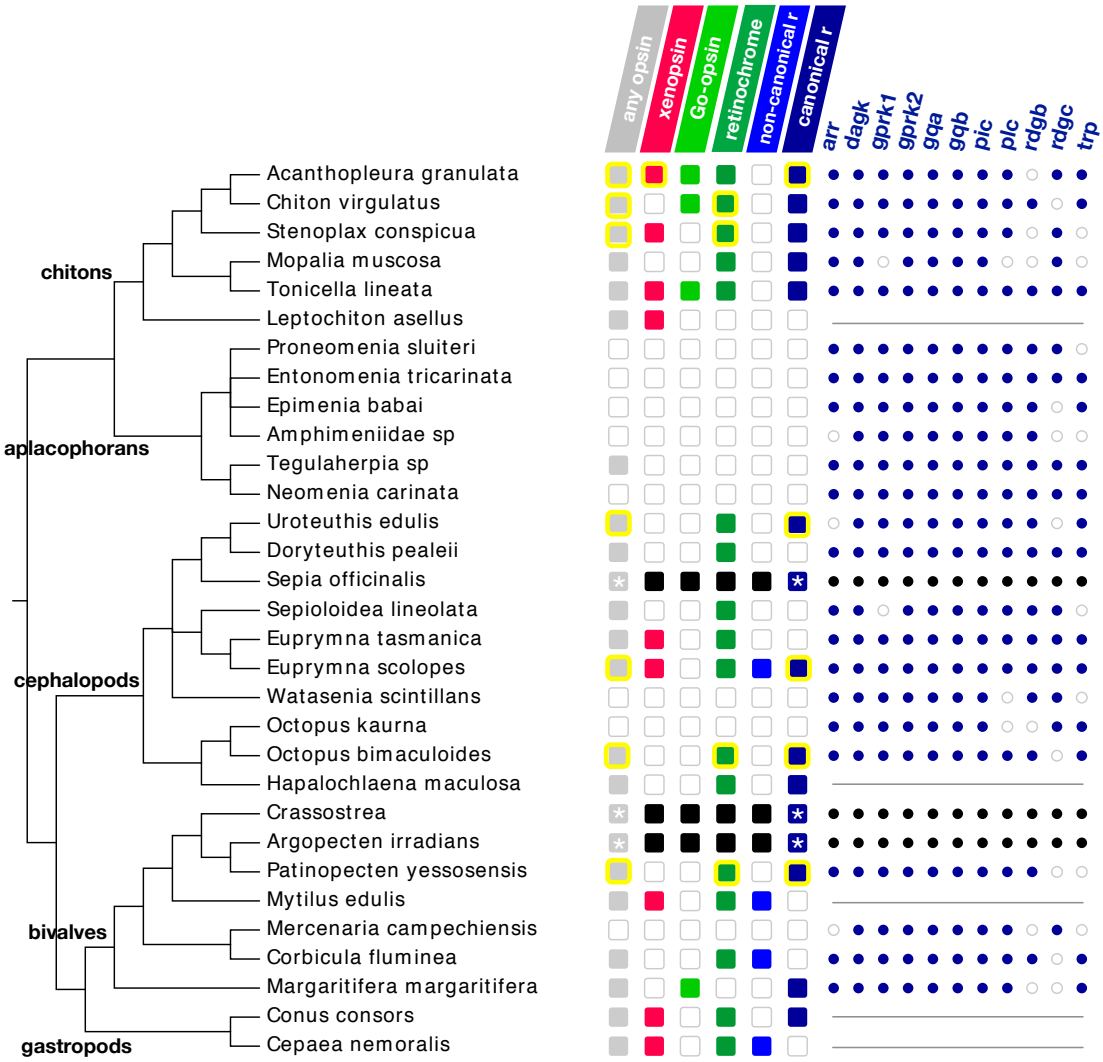

Figure 5.1: The distribution of opsins recovered from the mantles of 28 mollusc species from 5 major mollusc classes, including chitons, aplacophorans, cephalopods, bivalves and gastropods (top to bottom), with an additional 3 species from the literature only. Dark pink = xenopsin, light green = Go-opsin, dark green = retinochrome, light blue = noncanonical r-opsin, dark blue = canonical r-opsin. Blue dots represent the presence of r-opsin phototransduction cascade genes in mantle transcriptomes. Empty squares and circles represent absences from our dataset. Black squares are missing data from species that are known to express r-opsin in mantle, but weren't part of our transcriptomes. Yellow boxes are opsins that were previously described in the literature and recovered in our analysis. Those squares with white asterisks are only included as present from the literature because we lacked transcriptome data from those species for this analysis.

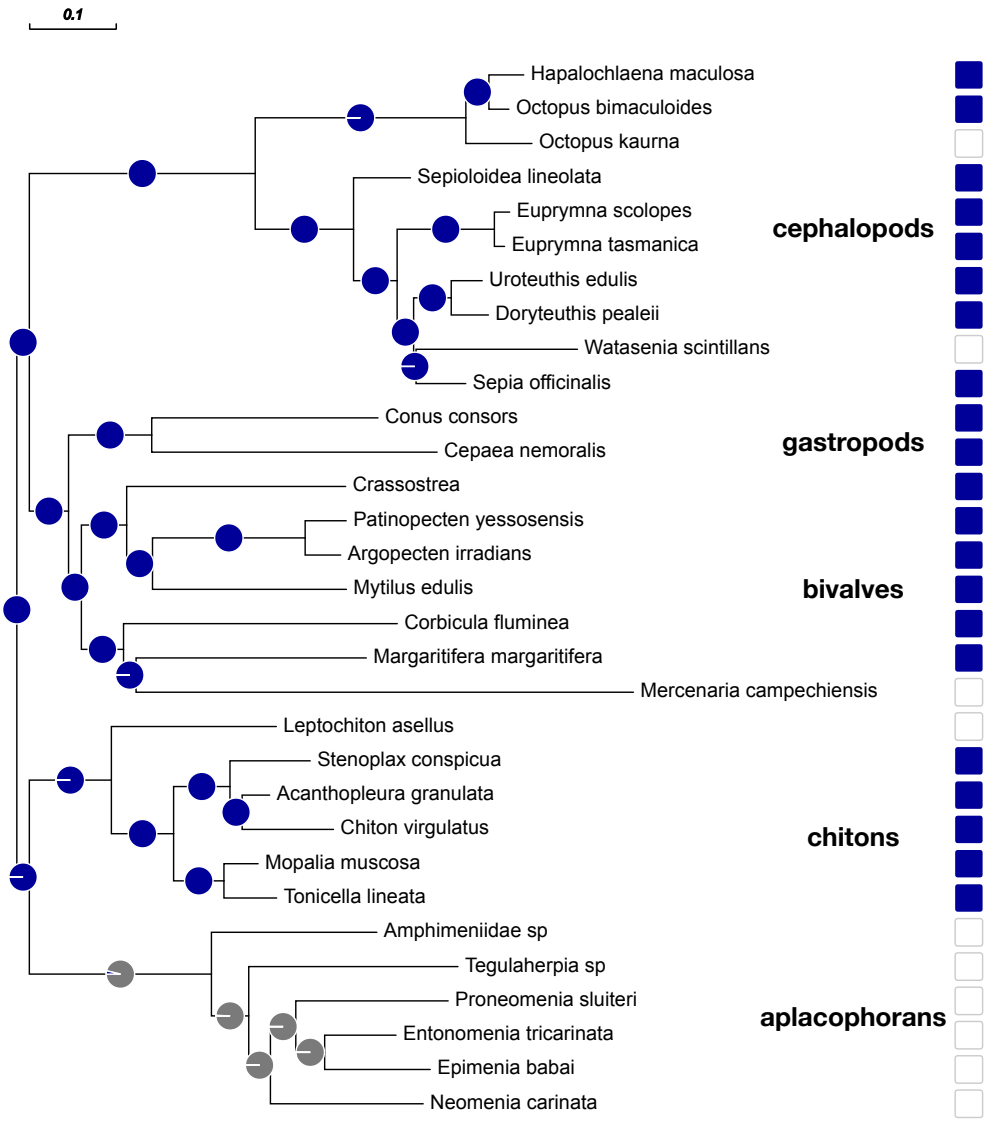

Figure 5.2: Ancestral state reconstruction of r-opsin phototransduction expression in mollusc mantle. Percentage of blue in each circle represents the probability that r-opsin was expressed in mantle at that node. Blue represents presence, grey absence at each node, estimated using a hidden Markov Model in corHMM. Squares next to species names represent the presence of any r-opsin cascade related opsins, which include canonical r-opsin, non-canonical r-opsin and retinochrome. Empty squares represent absences in our dataset, and we scored these as absent for the ancestral state reconstruction.

gans in cephalopod skin? Mantle tissue-specific r-opsin expression might represent a deep homology at both the molecular and sensory system levels that was inherited from a com-

mon molluscan ancestor, or a relatively recent innovation in cephalopods, and independent at the cellular level from the origins of the cells expressing r-opsin in the mantles of other mollucs.

Previous work in a handful of other molluscs had shown that opsins, and especially r-opsins, are expressed in mantle tissues in cephalopods, bivalves and chitons. The results from this analysis offer more data points in a wider assortment of molluscs, including the first sequence data for the expression of opsins, including r-opsins, in the mantle of two gastropods. A fundamental aspect of defining a homology is establishing phylogenetic continuity, in this case, of expression of opsins in mantle as our proxy for a dispersed light sensing system. We used ancestral state reconstruction methods to more formally test whether r-opsin phototransduction genes were expressed in the mantle of the last common mollusc ancestor. Our reconstruction strongly suggests that this is most likely the case, based on the distribution of r-opsin expression among the molluscs that we sampled. This is a clear result, but could be even better supported by a more targeted sampling regime. We assembled the only extensive survey of mollusc mantle tissue for opsin expression, using samples collected by others for other purposes, primarily phylogenomics. Many were not deeply sequenced and/or only one biological and technical replicate was sequenced contributed to the gaps in expression data. Also, the taxa that were sampled were typically for within-class phylogenomic studies, and so we are missing data from key outgroups, such as scaphopods and monoplacophorans. Future work should include these taxa and deeper sequencing to be able to have more confidence in absences in the dataset.

The fact that the last common ancestor of molluscs likely expressed r-opsin in its mantle suggests that the dispersed, dermal light sense underlying octopus LACE evolved prior to the evolution of chromatophores and LACE. Thus, the novel LACE behavior may be the result of the new combination of more recently evolved chromatophore organs with an older dispersed light sense in the an ancestral cephalopod. We are only just beginning

to be able to evaluate gene expression in an explicit rigorous comparative phylogenetic framework, which is important for moving beyond the assumption of homology of gene expression restricted to shallow comparisons among traditional model organisms.

5.5.1 Additional caveats and future directions

Unfortunately, despite our efforts to get as many and broad mollusc mantle samples as possible, our efforts to characterize opsin complements across the molluscs was hampered by the fact that many transcriptomes seemed to lacked opsin sequences. We believe it is unlikely that all of these absences of evidence for any opsin expression are true losses, given that almost half of our transcriptomes did show tissue-specific opsin expression. There are numerous reasons why opsins may be present but weren't detected in our transcriptomes. First, opsins outside of eyes may be relatively lowly expressed, driven in part by the cell distribution and morphologies described for extraocular photoreceptor cells in Chapter 1. Extraocular photoreceptor cells, especially those in skin, lack the elaborated surface membranes seen in photoreceptors in eyes, which limits how much opsin they can store. The cells also tend to be dispersed over a larger area and not found near each other, so that a piece of mollusc mantle contains far fewer photoreceptor cells than even a much smaller eye sample. Indeed, it has been difficult to locate the cells expressing opsins in mollusc mantle at all– they are not pigmented and so we have to rely on immunohistochemistry and in-situs for opsin to see them. Thus, sequencing depth may make a big difference in whether opsins are recovered. The steady decrease of sequencing costs may help partly overcome the low expression obstacle, since we should be able to sequence samples more deeply for less money. Target-enrichment during sequencing may also help find opsins by focusing on the opsins themselves, but requires at least some previous knowledge of opsin sequences to be successful.

Another major challenge with this analysis is accurately identifying sequences that are likely true opsins versus those that are not. This challenge arose from two sources. First, opsins are part of the largest group of GPCRs, the rhodopsin-A class. In our assemblies we found many sequences that bore a passing similarity the opsins that we used as baits for our BLAST search. Typically, true opsins all possess a conserved lysine at position 296 (of the bovine rhodopsin) that has been used as a diagnostic for opsins, as this residue and the residues that surround it are not found in other GPCRs. While we were able to find the conserved lysine for many of the opsins we recovered, the sequences of others were too short and did not cover that particular region of the protein. Most of the mollusc mantles that we found sequencing data for were from species for which those data were the only data. This is likely to be an issue going forward in comparative analyses. Using a Hidden Markov Model profile framework, such as that implemented in hmmer3 may work better then BLAST for separating true opsin candidates from closely related sequences, since it relies on a global profile rather than purely sequence similarity. In the future, I would like to create an HMM profile for opsins to be used in hmmer3, as well as a database of opsin motifs that are shared among opsins but not with other non-opsin GPCRs. MEME Suite (Bailey et al. 2009) is a set of tools that can search and create a number of motifs *de novo* from a set of training sequences. These motifs can then be used to search through a set of unknown sequences, and the order and placement of the motifs within the sequence can be visualized and directly compared.

Finally, because RNA-seq is relatively new, we are only just beginning to have datasets like the one I have analyzed in this chapter, transcriptomes from one specific tissue type across many species. While these data are important for unraveling the evolutionary history of organismal traits, we also do not yet have a very solid foundation for analyzing large-scale gene expression datasets in a phylogenetic context, though we are starting to establish best practices (like those in Dunn et al. 2013). Gene co-expression networks have

become more popular as a rough way to link co-expression with potential co-function, but are currently limited in a comparative context because of the myriad sources of both biologically relevant and also spurious variability in gene expression within a transcriptome (see Dunn et al. 2013). Simple comparisons of transcriptomes also suffer from very strong "species-signal", which may be the result of concerted evolution within a lineage, complicating direct comparisons of gene expression levels and networks (Musser and Wagner 2015; Ruan et al. 2016).

5.5.2 Updates for studying EOPs in mollusc mantles and beyond

For this analysis, we used opsin expression as a proxy for opsin function, but when there are multiple opsins expressed in a tissue, we do not have a lot of good options for figuring out which opsins are doing what. For example, aside from their role in light sensing, opsins may also function in other sensory modalities, like mechanoreception, and these roles might be independent of light. This possibility has really only been explored in *Drosophila melanogaster* so far, where r-opsins that function as light detectors in fly eyes also are important for both heat-sensing and mechanoreception in other parts of the fly body (e.g. Shen et al. 2011; Senthilan et al. 2012) . The ciliated r-opsin expressing cells I described in Chapter 3 are thought to be mechanoreceptors, again highlighting the importance of not only looking for expression, but being able to functionally test how opsins are used in mollusc mantles.

In Chapter 1, we outlined some of the technical issues in studying the function of extraocular, dispersed photoreceptor cells and opsins. Many technological advances have arisen since that paper was published in 2011, but fundamental issues still remain. The challenge of visualizing the dispersed cells expressing opsins raises another, namely that even when we can find cells expressing opsin, or find opsin expression in mantle tissue, we

struggle to directly connect opsin expression with function. Electrophysiological recordings would be the gold standard at the level of the cell, but even when researchers have been able to record from dispersed photoreceptors to show that they sense light, there are few ways to experimentally manipulate opsins *in-vivo*. There are only two compounds that have been shown to affect opsin function: hydroxylamine and opsinamides. Hydroxylamine affects opsins that can be photo-bleached, like the c-opsins found in vertebrate eyes and vertebrate, arthropod and echinoderm nervous systems (Pepperberg and Okajima 1992). Many opsins are bistable, meaning that they are not thought to lose their chromophore when hit with photons of light, and hydroxylamine does not appear to alter the function of these opsins (Sexton and Van Gelder 2011). Opsinamides have been shown to ablate the function of melanopsin, a vertebrate r-opsin, *in-vivo*, and thus may be useful for altering the function of other r-opsins in other animals (Jones et al. 2013). There are other compounds that affect downstream components of the phototransduction cascade, but our inferences from these are dependent on our assumption that what we know of c- and r-opsin phototransduction from vertebrates and flies holds in other animals. This assumption must be tested going forward, because it is possible that differences in phototransduction mechanisms have evolved in these two lineages. All drug trials also suffer from the spectre of off-target effects, needing both negative and positive controls that can be difficult, if not almost impossible, to establish.

More recent technologies like heterologous expression, RNAi and Crispr-Cas9 are making it more possible than even to manipulate gene expression in even non-model organsims, and may hold the key to directly testing opsin properties, protein-protein interactions in the phototransduction cascade, and the function of particular opsins for particular animal behaviors. Opsins expressed in-vitro could be probed for their spectral sensitivity, and the sequences manipulated to test the impact of particular amino acid changes on function. Crispr has already been successfully applied to start manipulating gene expression during

the development of the slipper snail *Crepidula fornicata*, and could perhaps be applied in molluscs to both more easily visualize opsin expressing cells by driving GFP (as was done in for the marine worm *Platynereis dumerilii* in Backfisch et al. 2013), or in a knockdown to test for function in behavior (as in Guhmann et al. 2015).

Appendix A

A preliminary description of r-opsin protein expression in the Kölliker's tufts of paralarvae from *Octopus rubescens*

A.1 Background

Kölliker's tufts are distinctive, conical bristle structures found dispersed over the skin surface of recently hatched octopuses, including *Octopus rubescens* (reviewed in Brocco et al. 1974). The tufts consist of a large bundle of chitinous rods produced by and connected to a single, microvillar 'chaetoblast' cell at the base, as well as muscles which allow the tufts to move, all enclosed in sac protruding from the epidermis (Boletzky 1973; Brocco et al. 1974). While performing antibody staining for r-opsin in *Octopus bimaculoides* skin for Chapter 3, I also received a batch of *O. rubescens* paralarvae (pelagic stage before adult) and so performed a preliminary experiment to look for opsin expression in *O. rubescens* skin.

A.2 Methods

For antibody staining methods, see Chapter 3. I used the same tublin and opsin antibodies for this experiment. One difference is that I used a yellow-green Cy-3 secondary antibody to bind to the opsin primary, instead of the infrared Cy-5 secondary I used in Chapter 3. I mounted the paralarvae whole on slides and imaged using the same confocal microscope from Chapter 3.

A.3 Preliminary Results

I found that the Kölliker's tufts of *O. rubescens* paralarvae fluoresced strongly in the green channel I used to image the opsin antibody staining, particularly in the chitinous bundles and also what I presume are the chaetoblasts at the base of the tufts (See Figure 1). There was no tubulin staining associated with the Kölliker's tufts at all, although the

ciliated cells I found in Chapter 3 in *O. bimaculoides* skin could be seen clearly and also bound the opsin antibody. The antibody seemed to bind strongly to the chitinous 'rodlets' that form the tufts (see Figure 1A), as well as the basal chaetoblast from which the tufts are produced (see Figure 1B).

A.4 Preliminary Discussion

These results suggest that the Kölliker's tufts may express opsin. Given opsin's known role in light sensing, these results suggest that Kölliker's tufts may be sensitive to light. However, given that there was no tubulin staining, which acts as a neural marker, then the outcome of phototransduction might be different in Kölliker's tufts than what we know happens in neurons, namely an electrical impulse. Another possibility, as discussed at the end of Chapter 3 for the ciliated neurons in *O. bimaculoides* skin is that opsin is being used for mechanoreception, although this may have the same caveats in terms of being expressed in non-neural cells as for light sensing. A final possibility is that these preliminary results that look like opsin expression may be simply autofluorescence. The green channel is well known to cause autofluorescence in mollusc tissue, and I did not complete the necessary negative controls for opsin to compare to. Future experiments should perform the controls and image using a long-wavelength secondary antibody to avoid autofluorescence confounding the signal.

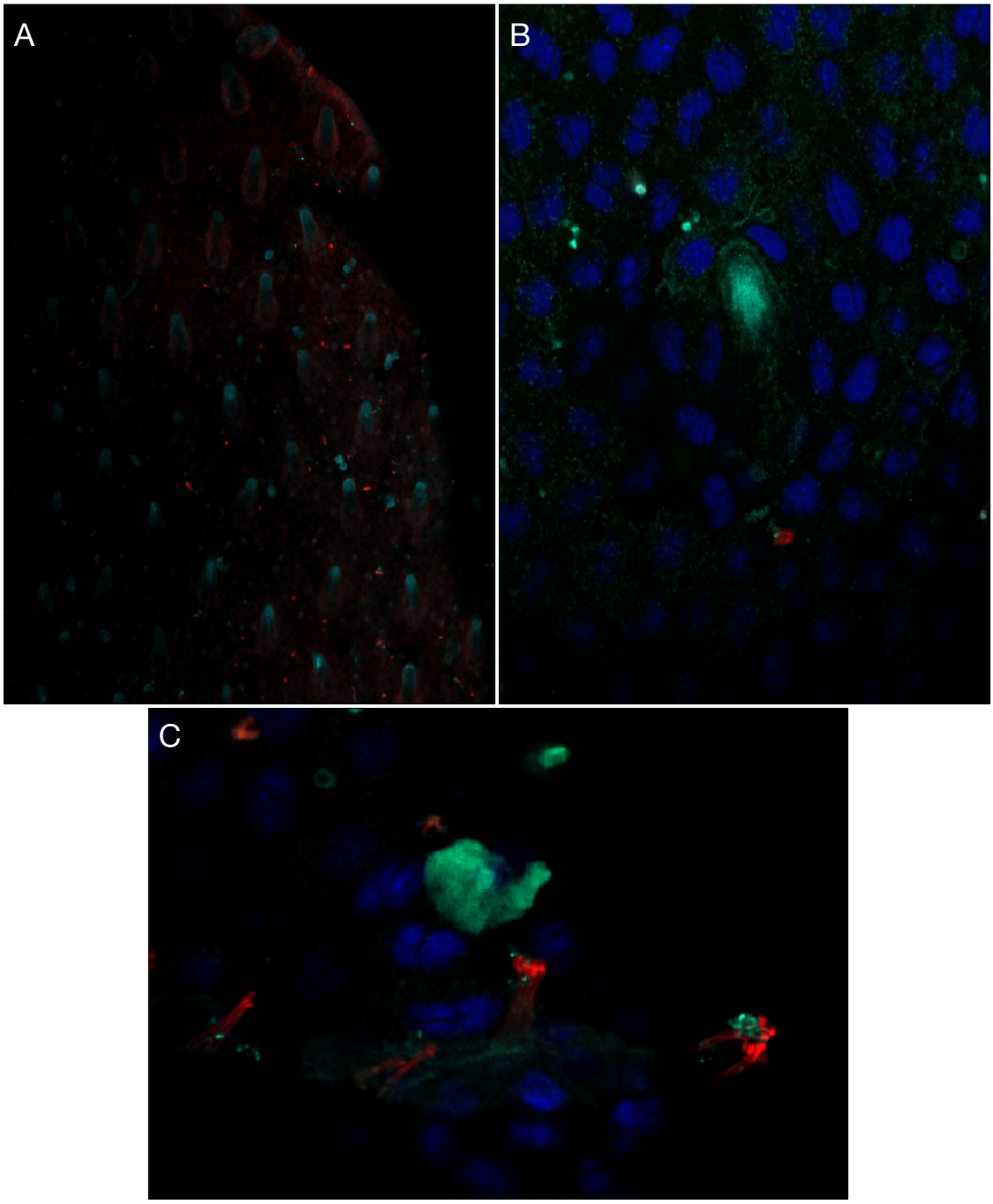

Figure A.1: Tubulin (red) and opsin (green) antibody staining of the Kölliker's tufts on the surface of *O. rubescens* paralarvae. A) 10x magnification of the mantle, where multiple Kölliker's tufts and multi-ciliated cells on the skin surface. B) 60x magnification of Kölliker's tuft, with chitinous rods C) 60x magnification of chaetoblast, below the chitinous rods.

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