Evidence for Opsin-Based Photosensitivity in Coral Larvae

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EVIDENCE FOR OPSIN-BASED PHOTOSENSITIVITY IN CORAL LARVAE

By

Benjamin M. Mason

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EVIDENCE FOR OPSIN-BASED PHOTODETETRITY IN CORAL LARVAE

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Photosensitive behaviors and circadian rhythms are well documented in reef-building corals and their larvae, yet photoreceptive structures and opsins have not been described in these organisms. Here I provide evidence for red sensitivity in several species of coral larvae. Behavioral experiments with two Caribbean corals, *Porites astreoides* and *Acropora palmata* demonstrated that larvae settle and metamorphose at a greater frequency on red substrata than on similar substrata of other colors. Attachment to red substrata was not observed when larvae were maintained in the dark, suggesting that red sensitivity was responsible for the observed behavior. Extracellular recordings confirm photosensitivity and indicate that the peak sensitivity of coral photoreceptors are shifted towards the orange-red region of the visible light spectrum, similar to the spectra (fluorescence and reflectance) of preferred artificial (plastic) and natural (crustose coralline algae) settlement substrata. Using Blast analyses and a PCR-based approach, I have identified, sequenced and cloned two full-length opsin cDNAs from *A. palmata* larvae. One cDNA (Acropsin 1) encodes an opsin protein that is similar to a vertebrate melanopsin; the second (Acropsin 2) encodes a protein that is most similar to cephalopod rod opsin. I have successfully developed synthetic peptide antibodies against each Acropsin 1 and Acropsin 2. Western blots of adult *A. palmata* and *A. cervicornis* protein
detect a 37kDa and 40kDa band, corresponding to the predicted molecular weights of Acropsins 1 and 2, respectively. Immunohistochemistry confirms expression of both opsins in *A. palmata* larvae. Staining of sectioned larvae demonstrates that Acropsin 1 is localized in the larval gastroderm while Acropsin 2 is localized in solitary epithelial cells, scattered throughout the larval ectoderm but with a polarized distribution and higher concentration in the aboral epidermis. This research provides several lines of evidence to support the existence, and demonstrate one potential ecological function, of opsin-based photosensitivity in corals.
In memory of Matthew Beard, whose experiments as a NOAA summer intern provided the motivation for this study, but whose life was taken prematurely by the reckless actions of another.
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CHAPTER 1
INTRODUCTION: LIGHT, PIGMENTS, OPSINS, PHOTOSENSITIVITY IN CNIDARIANS, CORAL REPRODUCTION AND ECOLOGY OF CORAL LARVAE

Light

The sun is the primary source of energy and light for our planet. Gravitational forces at the core of the sun pull the sun’s mass inward creating intense pressure and causing the nuclear fusion of hydrogen atoms into helium. These reactions create a tremendous amount of heat and cause the atoms to expel photons, which are eventually emitted in the form of electromagnetic waves and freely travel through space until they reach Earth.

Solar radiation is composed of a continuum of electromagnetic waves ranging from the short-wavelength (\( \lambda \)), high frequency waves [gamma rays (\( \lambda: \sim 10^{-14} \text{ m} \)), x-rays (\( \lambda: 10^{-10} \text{ m} \)) and ultra-violet radiation (\( \lambda: \sim 10^{-8} \text{ m} \)], to long-wavelength, low frequency waves [infrared radiation (\( \lambda: \sim 10^{-4} \text{ m} \)), microwaves (\( \lambda: \sim 10^{-2} \text{ m} \)) and radio waves (\( \lambda: \sim 10^{0} \text{ to } 10^{2} \text{ m} \)). Most of the electromagnetic radiation emitted by the sun falls within the visible light band, centered at approximately 500 nm (Figure 1a-b) and only about 40% of the electromagnetic radiation incident upon our atmosphere actually reaches Earth’s surface. Gamma rays, X-rays, and short-wave ultraviolet radiation (\( \lambda: <200 \text{ nm} \)) are efficiently absorbed by oxygen and nitrogen in our atmosphere and much of the ultraviolet radiation between 200 to 300 nm is absorbed by ozone in the stratosphere. Carbon dioxide and ozone partially absorb infrared radiation, resulting in a distribution of wavelengths incident upon the earth’s surface that represents narrow
windows of the total electromagnetic spectrum. One of these windows, that between 350 and 900 nm, includes visible light (Friedman 1986; Figure 1a).

A significant portion of the visible light that reaches the surface of Earth penetrates the world’s oceans, where additional portions of the spectrum are rapidly absorbed. In the open ocean attenuation is biased toward the long and short wavelengths of the visible light spectrum. Red and ultraviolet light is attenuated most rapidly, creating a strong gradient and shift in the spectral power distribution of down-welling light and irradiance with depth (Levine and MacNichol 1982; Figure 1c).

Life as we know it would not exist without the availability of solar radiation and strong selective pressure, has resulted in the evolution of diverse mechanisms for harnessing the energy of visible light. Of primary importance may be the chlorophylls and other photosynthetic pigments and associated pathways that have evolved to enable the capture and conversion of solar energy to chemical energy used to drive the fixation of organic carbon. However, life has also evolved to utilize visible light for various other biological processes, including phototaxis, the regulation of biological and circadian clocks, the regulation and synchronization of reproductive cycles, and that with which we are most familiar – vision.
Figure 1.1. Availability and spectral distribution of electromagnetic radiation. (a) Types of electromagnetic radiation emitted from the sun, showing the position of the visible light spectrum and attenuation of electromagnetic radiation in Earth’s atmosphere; (b) Wavelengths and relative positions of different electromagnetic waves; (c) Attenuation of visible light with depth in the open ocean. [Images from: http://amazing-space.stsci.edu (a), http://www.antonine-education.co.uk (b), and adapted from Levine and MacNichol 1982 (c)].

Pigments: The Light Harvesting Molecules

At the most fundamental level, vision or photoreception depends on the absorption of light by a pigment molecule or system of molecules. Pigments enable an organism to capture photons of light and convert this energy into biochemical signals. A necessary property of a pigment is its ability to absorb and utilize, thereby quickly dissipating, light energy. The absorption and dissipation of this energy results in conformational change or degradation of these molecules, and once degraded these
molecules must be regenerated or reactivated. The key to this ability is found in the common chemical structure of the light-harvesting molecules. All pigment molecules consist of carbon atoms linked by alternating single and double bonds. This configuration enables photo-isomerization of the molecule and subsequent regeneration via a simple reaction, aided by enzymes that catalyze the conversion of $2\text{O}_2 + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}$ (Wolken 1995).

While pigments share a basic chemical, structural elements, side groups and the formation of complexes with other molecules give rise to the diverse and unique chemical properties (i.e. reflectance, absorption spectra, solubility) and determine the biological functions of various classes of pigment. The major classes of pigments include:

**Chlorophyll.** A light-harvesting molecule synthesized by all green plants, algae, and photosynthetic bacteria. Chlorophyll a displays two absorbance peaks (in the blue and red regions) and its green color is derived from magnesium.

**Bilins and phycobilins.** Degradation products of hemoglobin, hematin compounds or chlorophyll, the phycobilins include phycocyanins (blue pigments) and phycoerythrins (red pigments). They are found in red and green algae where they serve as accessory pigments for photosynthesis. These pigments are chromoproteins (molecules consisting of a chromophore (pigment molecule) and protein). They differ from chlorophyll and carotenoids in that they are water, rather than fat-soluble soluble.

**Phytochrome.** A chromoprotein that absorbs red light and is responsible for photoperiodism in plants.
**Pterines.** Red and yellow pigments associated with the photoreceptor systems. They are responsible for phototactic behavior in fungi, algae and protozoa (Galland et al. 1990). Pterines and their derivatives are also found in insect eyes where they serve as screening pigments, but they are not known to play a primary role in visual photoreception (Stravenga 2002).

**Melanins.** A principle pigment of vertebrates and a ubiquitous class of biological pigments of which tyrosine is a precursor. Melanins are common in and around photoreceptive structures and in eyes. Melanins absorb wavelengths extending from infrared to UV with increasing with the greatest absorption in the UV range of the spectrum. While their exact role they serve in eyes is not known it has been proposed that they may serve a photo protective function by absorbing damaging UV radiation or as screening pigment, by absorbing reflected or stray light (Hu et al. 2008)

**Flavins and flavoproteins.** (e.g., riboflavin - vitamin B12), are yellow photosensitive pigments that absorb strongly in the blue. Flavins are responsible for chloroplast movement, participate in chloroplast electron transport systems during photosynthesis, are associated with phototactic behavior, phototropism, and phototaxis of fungi, algae, and protozoa. A flavin is one of two chromophores associated with the chromoprotein cryptochrome (photopigment responsible for circadian rhythms in plants, and recently described in corals) that it responsible for regulating circadian rhythms. While flavins are not the photoreceptor molecule responsible for animal vision, they are found in the retinas of frog, rabbit, rat and bovine retinas where they function in visual processes.
**Carotenoids.** These are yellow, orange, and red pigments that are widely distributed among living organisms. Their structure consists of a system of forty carbon atoms linked by alternating single and double bonds. This structure allows carotenoids to exist in either cis- or trans- geometric configurations. The most familiar of these pigments is carotene of which the most abundant form is all-trans-B-carotene. Animals are unable to synthesize carotenoids and therefore need to obtain them by ingesting plants. Animals convert B carotene to vitamin A, an alcohol whose terminal aldehyde is retinal. Retinal is forms a complex with a diverse class of proteins – the opsins - and forms the basis for visual (and non-visual) photoreception found throughout the animal kingdom.

**Rhodopsin: The Universal Photopigment**

Since its initial discovery rhodopsin has subsequently been discovered in nearly all phyla including microorganisms such as halobacteria (Lenci and Guetti 1989), euglena (Walne et al. 1998), dinoflagellates (Hollingsworth 2005), sponge larvae (Leys et al. 2002), and all higher invertebrate (Gartner and Towner 1995) and vertebrate organisms (Wolken 1995). Despite conservation of the basic molecular structure and mechanism of activation, slight changes in key functional amino acids, gene duplication and organization of this trans-membrane receptor into different types of photoreceptor cells and structures has given rise to dramatic variation in form, function and visual capacity among the photo- and visual systems of living organisms.

Rhodopsin is a chromoprotein (protein chromophore complex) consisting of the protein opsin and a retinoid chromophore - retinal. It is a member of the G protein-coupled receptor (GPCR) superfamily, by far the most abundant class of trans-membrane
receptors which consists of approximately 950 genes in the human genome, including approximately 500 sensory GPCRs (e.g., Palczewski 2006; for summary see Figure 2). All GPCRs are characterized by the same seven alpha-helical trans-membrane

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**Figure 1.2.** Summary of major GPCR subfamilies. (a) Diagram indicating the major structural differences and positions of ligand binding sites and representative members of GPCR subfamilies 1-3; (b) Simplified phylogeny (nearest-neighbor) analysis of representative proteins from each of the five GPRC subfamilies (from Bockaert & Pin 1999).
structure (seven trans-membrane subunits, three cytoplasmic loops, three extracellular loops, a cytoplasmic C-terminus tail, and a extracellular N-terminus). The environmental signal for most GPCRs are small molecules (diffusive ligands) that bind to the receptor causing a conformational change on the intracellular surface which enables the binding and activation of anywhere from several to hundreds of heterotrimeric guanylate nucleotide-binding molecules (G proteins; reviewed by Palczewski 2006). There are four recognized families of G-proteins involved in GPCR signaling (Gs, Gi, Gq, and G12). Go and Gt (transducin), also discussed below, are members of the Gi subfamily. Activation of G proteins results in GDP/GTP exchange (at the binding site on the alpha subunit), followed by dissociation of the receptor subunits and subsequent activation of secondary messengers resulting in signal transduction and effecting some type of biological response (Downes & Gautam 1999; Figure 1.3).

The visual pigment rhodopsin, is unique among the GPCR superfamily in that conformational change of rhodopsin results from the capture of photons of light by a chromophore. Both vertebrate and invertebrate photosensitive opsins utilize 11-cis-retinaldehyde (11-cis-retinal) or a variant as their chromophore (Hardie and Raghu, 2001). This chromophore is covalently bound via a protonated Schiff base linkage to a Lysine residue located in the seventh trans-membrane domain of the receptor (Figure 1.4). The absorption of a photon by the chromophore results in photoisomerization of cis-retinal to all-trans-retinylidene, accompanied by a simultaneous conformational change in rhodopsin to its active form, metarhodopsin. Metarhodopsin then binds intracellularly to the to a heterotrimeric G proteins, resulting in a visual signal cascade.
Figure 1.3. Diagram modeling the mechanisms of activation, signaling pathways and biological responses linked to GPCR signaling. Shown are the various ligands (or stimuli - light) known to bind GPCRs, a general model of a GPCR indicating the conserved structure - N-terminal tail (N), seven trans-membrane helical domains, three cytoplasmic loops (I1, I2, I3), three extracellular loop regions (E1, E2, E3) and a cytoplasmic tail (C). Also shown is a general model of a heterotrimeric G protein with alpha (a), beta (b) and gamma (γ) subunits, which dissociate upon binding GTP. The four families of G proteins (Gs, Gi, Gq and G12) classified by their α subunits each contain multiple subtypes. The different subunits interact with different effectors signaling different pathways that ultimately result in some type of biological response. The β and γ subunits which remain bound after dissociating from α, diffuse into the cytoplasm where they effect additional messengers and ion channels (from Dorsham & Gutkind 2007).
**Figure 1.4.** Models of bovine rhodopsin and its chromophore retinal. (a) Two-dimensional model of bovine rhodopsin. “In” and “Out” indicate cytoplasmic and extracellular sides of the membrane, respectively. Highly conserved amino acid residues are shaded grey. The retinal-binding lysine (K296; located in the seventh transmembrane domain) and the glutamate counterion (E113) are circled in bold; (b) Structural diagrams of the 11-cis- and all-trans- isomers of retinal; (c) Three-dimensional structural diagram of bovine rhodopsin indicating the position of trans-membrane domains I-VII and showing retinal positioned within the chromophore pocket; (d) Structural diagram of 11-cis-retinal bound to K296 and its interaction with the counterion (E113) (from Terakita et al. 2008).
**Ciliary and Rhabdomeric Photoreceptors**

Within the animal kingdom there exists amazing diversity in visual capacity and function of photosensitive structures, from eyespots and solitary epithelial cells responsible for phototaxis and photosensitive reflex behavior to complex, image-forming eyes. There is general agreement that the ancestral photoreceptive organelle evolved from among ciliated (flagellated) protists in connection with the cilium (Wolken 1977) and early recognition of two general types of photoreceptive cells led Eakin (1963) to propose two main lines of evolution, a ciliary line in coelenterates and deuterostomes and a rhabdomeric as an offshoot, in protostomes. However, since this proposal there have been numerous examples of evidence to refute the two-line hypothesis. We now know that (1) some protostomous phyla (Gastrotricha, Kamptozoa, Bryozoa) possess ciliary photoreceptors; (2) both types can be found in several phyla and often in the same animal; (3) examples of mixed ciliary/rhabdomeric receptors exist (e.g., in Cnidarians); and (4) many rhabdomeric receptors possess ciliary structures. These discoveries have led to controversy over the origins and diversification of photoreceptor cell types (reviewed by Vanfleteren 1982), yet despite controversy surrounding the their evolution, two main types of photoreceptor cells, ciliary and rhabdomeric, form the basis most visual systems.

The ciliary type is derived from modifications (surface enlargement via folding and projections) of the plasma membrane of a cilium or flagellum where as the rhabdomeric are derived from similar modifications of the distal cell membrane. The terms ciliary and rhabdomeric are also used to describe the respective types of opsins. Although exceptions do exist, vertebrates typically possess ciliary types while most invertebrates possess rhabdomeric types. In the ciliary opsins, light causes the formation of
metarhodopsin which activates heterotrimeric Gt (transducin) by GTP-GDP exchange. Active Gtα binds to and activates phosphodiesterase (PDE), which hydrolyzes cyclic GMP (cGMP) to GMP. The hydrolysis of cGMP results in the closing the cyclic-nucleotide-gated (CNG) channels, that remain open in the dark state, resulting in hyperpolarization of the photoreceptor cell (Figure 1.5).

**Figure 5.** Diagram showing the construction of the mammalian retina, light activation and signaling mechanisms of vertebrate (Gt-coupled) opsins. The retina is composed of the inner (retina horizontal, bipolar, and amacrine cells) and outer (rods and cones) retina. Rod outer segments consist of stacked disc membranes rich in rhodopsin. Once bound to 11-cis retinal (in the ground state), absorption of photons causes isomerization of retinal to all-trans-retinal resulting in a conformational change in the opsin protein and allowing binding of the G protein (Gt). Once bound to opsin, Gt is activated by GDP/GTP exchange. The G protein subunits dissociate and Gtα binds to and activates phosphodiesterase (PDE), which hydrolyzes cyclic GMP (cGMP) to GMP. The hydrolysis of cGMP results in the closing the cyclic-nucleotide-gated (CNG) channels, that remain open in the dark state, resulting in hyperpolarization of the photoreceptor cell and signal propagation (from Shichida & Matsuyama 2009).
In rhabdomeric cells typical of invertebrates, absorption of a photon by rhodopsin converts it to the thermostable metarhodopsin. Metarhodopsin then activates heterotrimeric Gq by GTP-GDP exchange, and Gaq dissociates and binds to and activates phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bis-phosphate (PIP2) to inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG). Two classes of light-sensitive ion channels (TRP and TRPL) are then activated by PLC by a still-unknown mechanism resulting in depolarization of the photoreceptor cell (Yau & Hardie 2009; Figure 1.6).

Figure 1.6. Simplified schematic comparing cell structure and differences in signaling pathways between vertebrate ciliary (Gt) opsin receptors and invertebrate rhabdomeric (Gq) opsin receptors. In ciliary cells the photosensitive surface is derived from modification of the cilia membrane, whereas in rhadomeric receptors it is derived from modification of the distal cell membrane. C-opsin signaling proceeds as described in Figure 5. In rhadomeric cells, absorption of a photon by r-opsin converts it to the thermostable metarhodopsin. Metarhodopsin then activates heterotrimeric Gq by GTP-GDP exchange, and Gaq dissociates and binds to and activates phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bis-phosphate (PIP2) to inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG). Two classes of light-sensitive ion channels (TRP and TRPL) are then activated by PLC by a still-unknown mechanism resulting in depolarization of the photoreceptor cell (from Fernald et al. 2006).
The Retinoid Cycle and Rhodopsin Regeneration

In vertebrates, regeneration of rhodopsin from metarhodopsin involves phosphorylation by rhodopsin kinase and subsequent binding of arrestin, while the regeneration of 11-cis retinal is catalyzed by enzymes of the retinoid cycle (Wang and Montell 2007). Since cis-retinal is required for photoactivation of rhodopsin, the maintenance of photosensitivity requires a continual supply of retinoids (Vitamin A and its derivatives). Animals are unable to synthesize these pigments and must acquire them from their diet (plants, fungi, or bacteria) as vitamin A or its precursors. Once acquired, multiple enzymes are involved in the processing of these pigments and transport to the chromophore attachment site on membrane bound opsin (reviewed by von Lintig et al. 2005). Carotenoids are highly lipophilic and therefore require enzymes for uptake into cells and transport. Recent research indicates that multiple class B scavenger receptors (e.g., ninaD, SANTA MARIA) are involved in the uptake and synthesis of retinoids (Wang and Montell 2007). Among the key enzymes in Vitamin A formation are the β-carotene oxygenases (BCO; β, β-carotene-15,15’-monooxygenase) that catalyze the centric cleavage of β-carotene to produce retinaldehyde (e.g., Kiefer et al. 2001; Paik et al. 2001). Since β-carotene is the most abundant carotenoid in nature, this enzyme is of critical importance in chromophore formation. In vertebrates, a separate enzyme pathway known as the retinoid or visual cycle is responsible for the regeneration of 11-cis retinal from bleached (photoisomerized) all-trans retinal. This cycle occurs in the retinal pigment epithilium (RPE) and involves two key enzymes, lecithin-retinol acyl transferase, (Lrat) and Rpe65 that convert trans-retinol to trans-retinyl ester and trans-retinyl ester to cis-retinol, respectively (Lucas 2006). While much of what is known
regarding pigment regeneration is based on study of the pathway of the retinal pigment epithelial cells (RPE) a second pathway, dedicated strictly to regeneration of cone pigment and residing in Muller glial cells, also exists. In this pathway, all-trans-retinol is re-isomerized directly to 11-cis-retinol which is taken up by the cone cell body before being converted to 11-cis-retinal (Jones et al. 1989). In invertebrate rhabdomeric receptors the metarhodopsin state is typically thermostable (i.e. all-trans-retinal does not dissociate from the opsin). Even though invertebrates possess the cellular machinery for pigment regeneration (Wang and Montell 2007), the rhodopsin active state is typically restored by photo-isomerization. The metarhodopsin absorbance peak is red-shifted, for example, *Drosophila* metarhodopsin’s peak absorbance occurs at ~570 nm compared the ~480 nm peak of the active rhodopsin state (Stravenga 2002).

**Figure 1.7.** Diagram representing pigment cycles in: (a) vertebrate rods and cones; (b) invertebrate (*Drosophila*) rhabdomeric receptors (from Yau and Hardie 2009).
Upon absorption of light, invertebrate rhodopsin forms a thermostable metarhodopsin which prevents the dissociation of the chromophore from opsin. Since metarhodopsin is still bound to its chromophore it is able to be photo-converted back to rhodopsin. As a result, a continuous equilibrium between metarhodopsin and rhodopsin is established in the light (as long as the spectral distribution is centered between the absorbance maxima of the rhodopsin and metarhodopsin states). One consequence of thermostable metarhodopsin is the inability of invertebrates to dark-adapt (restore photopigment content of photoreceptors to 100% rhodopsin in the dark). Most invertebrates seem to lack the enzymes required for the direct regeneration of rhodopsin from metarhodopsin (Cronin 1986). Thus, in order to completely restore photopigment to rhodopsin at least some arthropods and mollusks (and likely other invertebrates) must regularly replace photoreceptor membranes and insert newly synthesized rhodopsin. Such membrane turnover events are tuned to light dark cycles and therefore could be tied to mechanisms for measuring photoperiod. Another consequence of the rhodopsin-metarhodopsin equilibrium is its affect on the spectral sensitivity of rhodopin. While only the rhodopsin to metarhodopsin transition results in visual excitation (Cronin 1986), metarhodopsin has a slightly red-shifted absorbance maximum and requires longer wavelengths to photoisomerize retinal and restore rhodopsin. Other photostable pigments in the vicinity of the photoreceptor cells are capable of assisting with the photorestitution of rhodopsin by altering the spectra of incident or reflected light (e.g., Stravenga 2002; for more detail see “Spectral Sensitivity” below).
Extraocular Photoreception

Despite the complex construction and diversity of visual structures found among the metazoans, photosensitive structures are not required for the expression of opsins or opsin-based photosensitivity. In many animals, even those with complex image-forming eyes, photosensitivity is mediated through extra-ocular surfaces, cell and tissues. Such sensitivity, referred to as extra-ocular or extra-retinal, is widespread throughout the animal kingdom, occurring in both invertebrates and vertebrates (e.g., Wolken 1995). In extra-ocular photosensitivity, photoreceptive cells are not organized into sensory structures. Instead, they may be solitary or grouped populations of cells, often occurring in the vicinity of pigment or non-photosensitive pigmented cells (Singla 1974). Without an eye, image formation is not possible, but extra-ocular photoreceptors are still capable of regulating biological processes, including phototaxis, cilia or flagellar motion, expansion and contraction of pupils or chromophores, self-shading as well as controlling hormone regulation and rhythmic/circadian behavior.

Since the expression of opsins is not restricted to visual structures, the identification of extra-ocular opsins and populations of photosensitive cells has been slow and has relied on immunological and molecular biological techniques. Early examples of extraocular photosensitivity were described in association with epithelial cells, but also less likely cells and tissues including neurons, muscle cells, and glands (Yoshida 1979, Arkett 1989, Sawyer et al. 1994, Wolken 1995). However, in recent years, advancements in molecular biology tools and the availability of genomic databases has aided in the discovery and characterization of novel opsins from a taxonomically diverse range of organisms and, in many cases, from surprising locations within these organisms.
Different classification schemes have been proposed based on biochemistry (G protein coupling and signaling pathways), protein sequence homology and intron/exon structure of opsin genes, (eg. Terakita 2005 - Figure 1.8; Shichida and Matsuyama 2010 - Figure 1.9). Here, I follow the six-subfamily scheme proposed by Schichida and Matsuyama (2010). The six subfamilies include:

**Vertebrate visual and non-visual opsin (Gt/ciliary opsins) subfamily.** In addition to their localization in visual structures and well-documented involvement in visual processes, members of the ciliary (Gt) subfamily also occur non-visual photoreceptor cells (cells other than rods and cones). The vertebrate visual opsins can be subdivided into five groups including 4 cone opsins and one rod opsin. In the retina, cone opsins occur in different populations of cone cells and are characterized by their spectral sensitivities. The groups of vertebrate cone opsins include the S group (short-wavelength/UV or violet absorbing), the M1 or B group (medium wavelength; blue sensitive), the M2 or G group (medium wavelength; green sensitive), and the L or R group (long wavelength; red or green sensitive). The rod opsins (Rh), which occur in rod cells, are blue-green sensitive (Okano et al. 1992). In addition to their localization in rods and cones, the vertebrate visual opsins are also expressed non-visual cell types. They occur in pineal photoreceptor cells (Wada et al. 1998) where a rod-like opsin in a teleost (carp *Carla catla*) shows both diurnal and seasonal fluctuations in expression with peaks at mid-day during seasonal temperature and photo-period maxima which also corresponds to the fish’s spawning phase (Seth & Maitra 2010). Immunohistochemistry
and PCR analysis also suggests that opsins may be expressed in the human epidermis (Tsutsumi et al. 2009).

**Figure 1.8.** Phylogenetic tree of seven recognized opsin subfamilies. Shown are clades corresponding to: 1) Gq-coupled opsin/melanopsin subfamily; 2) Encephalopsin/TMT-opsin subfamily; 3) Vertebrate visual and non-visual opsin subfamily; 4) Go-coupled opsin subfamily; 5) Neuropsin subfamily; 6) Peropsin subfamily; 7) Photoisomerase subfamily. Six of the seven opsin subfamilies are known to occur in humans (from Terakita 2005).
Figure 1.9. Diagram demonstrating diversification and phylogenetic relationship of the six opsin subfamilies relative to the GPCR subfamilies and G protein subtypes from which they are derived. Similarity among opsin types suggest six distinct subfamilies: 1) Vertebrate visual and non-visual opsins/encephalopsins; 2) Go coupled opsins; 3) Gs coupled opsins; 4) Gq coupled opsins (invertebrate r-opsins and melanopsins); 5) Photoisomerases; 6) Neuropsins (from Shichida & Matsayuma 2010).

An additional class of non-visual, vertebrate (Gt) opsins has been identified in the pineal photoreceptor cells of most non-mammalian vertebrates. These include the pinopsins, parapinopsins, vertebrate ancient (VA) opsins and parietopsins. Pinopsin has been localized in the pineal organs of birds (Okano et al. 1994) and lizards (Tanaguchi et al 2001). The closely related UV-sensitive and bi-stable parapinopsin has been found in
teleosts (Blackshaw & Snyder 1997) and jaw-less fish (Koyanagi et al 2004). VA-opsin is found in the inner retina (amacrine and horizontal cells) and brain of teleosts (Soni & Foster 1997), while a splice valiant of VA opsin called VAL opsin (named for its elongated cytoplasmic tail) is localized to deep parts of the brain and the horizontal cells of the zebrafish (eg. Kojima et al. 2000; Kojima et al. 2008). VAL and VA appear to have a broader taxonomic distribution and were recently discovered in the hypothalamic neurons of birds where they are believed to mediate photoperiodic response (Halford et al. 2009).

One of the most intriguing examples of non-visual photoreception is that found in the parietal or “third eye” (an eye complete with a cornea, lens and retina found on the top of the head) of reptiles. These cells contain an opsin (parietopsin) that is closely related to the vertebrate visual opsins (Su et al. 2006), but they also possess pinopsin. The two opsins have different sensitivities and are coupled to antagonistic signaling pathways. Pinopsin is blue-light sensitive and causes hyperpolarization of the cells through a gustducin signaling pathway, while parietopsin is sensitive to green light and operates via a Go signaling pathway leading to cell depolarization. The function of this chromatic antagonism is not yet known.

Another group of opsins recognized as a subgroup within the vertebrate opsin subfamily is the encephalopsins, which can be further subdivided into the encephalopsin (panopsin)/teleost multiple tissue (tmt) opsin group and the Platyneris c-opsin/pteropsin group. Mouse encephalopsin (also called panopsin due to its widespread distribution) is strongly expressed in the brain and testes but is also weakly in the heart, lung, liver, kidney, muscles, pancreas and the retina (Halford et al. 2001). A homologous opsin (tmt)
is widely expressed in neuronal and non-neuronal tissues of teleosts and is thought to regulate photic entrainment of the peripheral biological clocks (Moutsaki et al. 2003). Two other, opsins - *Platynemis c-opsin* and pteropsin - with similarity to vertebrate opsins, have recently been described in the, brains of the rag worm Platynemis (Arendt et al. 2004) and insects (Verlarde et al. 2005), respectively. The functions of these are unknown.

**Gq-coupled/melanopsin subfamily.** Gq-coupled (rhabdomeric) opsin are the visual opsins of arthropods and molluscs and are very similar to the non-visual, vertebrate melanopsins (Provencio et al. 1998, 2000). Both vertebrate melanopsin and invertebrate rhabdomeric opsins couple to a Gq and activate a phospholipase-C (PLC) cascade (e.g., Panda et al. 2005). Similar to vertebrate visual opsins, the invertebrate Gq opsins can be spectrally tuned to support color vision (e.g., Marshall et al. 2007; Koyanagi et al 2008; see also, *Spectral Tuning* below). While similar in structure and molecular function, vertebrate melanopsins plays only a minor role in vision, but has other important biological functions. Mouse melanopsin is known to play a role pupil constriction (Hattar et al. 2003b), the photo-entrainment of circadian rhythms (i.e. keeping biological rhythms synchronized to changes in day length; Hattar et al. 2003a), and modulation of sleep (Altimus et al. 2008). Two distinct isoforms of melanopsin (Opn4L, a 521 amino acid protein & Opn4S, 466 amino acid protein) are expressed in different anatomical subtypes of photosensitive retinal ganglion cells (pRGCs) of the mammalian retina and may play different roles in generating the variety of known light responses (Pires et al. 2009). Melanopsins have also been localized in intrinsically photosensitive retinal
horizontal cells (pRHCs) of fish retinas, which play a role in creating center-surround receptive fields of visual neurons, representing the first non-rod/cone photoreceptor cells involved in image-forming vision (Cheng et al. 2009).

**Go opsin subfamily.** While rhabdomeric cells are the dominant photoreceptor cell type found among the invertebrates, examples of ciliary photoreceptors also occur. The scallop has both rhabdomeric and ciliary photoreceptor cells, and its ciliary photoreceptors contain a novel opsin different from either the Gt-coupled (*Platyneris c*-opsin) or the classic invertebrate Gq-opsin. The opsin in the ciliary photoreceptors of the scallop co-localizes with a large amount of Go-type G protein and is thought to activate Go (Kojima et al. 1997). A similar Go opsin has been found in the chordate amphioxus, but appears to be absent from other vertebrate or invertebrate genomes (Koyanagi et al. 2002). Activation of scallop Go-coupled opsin elevates the intracellular cGMP which subsequently opens K+ selective channels, leading to hyperpolarization of the cells (Gomez & Nasi 2000).

**Gs opsin subfamily.** The most recent additions to the opsins is a subfamily found in ciliary photoreceptor cells cnidarians (hydra, the sea anemone *Nematostella vectensis*, and in jellyfish; Suga et al. 2008). One member of this group, an opsin from the box jellyfish *Tripedelia cystophora*, was shown to bind Gs resulting in an intracellular increase of cAMP (Koyanagi et al. 2008). This subfamily of opsins appears to have both visual (occurring in the compound eyes of jellyfish; Koyanagi et al. 2008) and non-visual
(since they must occur in non-visual in epithelial or endothelial cells of hydra and *N. vectensis*) functions.

**Photoisomerase subfamily.** The photoisomerases include retinochromes, retinal-G-protein-coupled receptor (RGR) opsins, and the peropsins. The primary function of this subfamily is the regeneration (photoisomerization) of 11-cis-retinal from all-trans-retinal. The members bind all-trans-retinal (Figure 2b) as their chromophore and regulate the stereospecific photoisomerization of all-trans- to 11-cis-retinal (Hara & Hara 1968, Hao & Fong 1999, Koyanagi et al. 2002). Retinochrome and RGR opsins are not coupled to G proteins and are unable to generate a cellular response. Retinochrome is known to supply 11-cis-retinal to the visual cycle in molluscs (Hara & Hara 1973). RGR opsins, do not appear to donate 11-cis-retinal to other opsins, but are localized to the retinal pigment epithelium (RPE) and Muller cells in vertebrates (Jiang et al. 1993) where they are believed to regulate retinoid traffic and pigment regeneration during the retinoid cycle (Terakita et al. 1989; Chen et al. 2001). A similar all-trans-retinal-binding molecule, peropsin, is also found in the RPE of vertebrates, suggesting that it may function as a photoisomerase. However, peropsins contain sequence motifs that are conserved among and integral to the function of opsins, but that are lacking in retinochrome and RGR opsins, such as the D/ERY motif located at the cytoplasmic surface of the second transmembrane domain and NPXXY motif of the seventh trans-membrane domain (Shichida & Matsuyama 2010), and is not clear whether peropsins bind a G protein. A protostome homologue of peropsin, with photosomerase-like behaviour in vivo, was recently discovered in non-visual cells of spider retina, evidence that peropsin existed before the
deuterosome protosome split (Nagata et al. 2010; for more detail on the evolution of opsins see Chapter 6).

**Neuropsin subfamily.** Tarttelin et al. (2003) were the first to report the expression of a novel opsin (neuropsin; Opsin5) in eye, brain, testes and spinal cord of mammals, but at the time the function was unknown. Neuropsin has since been identified in the deep brain - paraventricular organ and fibers adjacent to the pituitary gland, which translates photoperiodic stimuli into neuroendocrine responses. The heterologously expressed pigment has sensitivity peak at 420 nm and is believed to play a role in regulation of seasonal reproduction in birds (Nakane et al. 2010).

**Spectral Sensitivity**

Despite conservation of its basic components (retinal and opsin), rhodopsins (retinal/opsin complex) display a wide range of absorbance maxima. Rhodopsins extracted from vertebrate and invertebrate eyes display spectral sensitivities ranging from 340 (UV) to beyond 700 nm (infrared; Wolken1995). The spectral absorption of isolated retinal and opsins consistently have absorbance maxima centered at approximately 370 and 500 nm, respectively, regardless of the absorbance maxima of the complex from which they were extracted. This and protein mutation studies are evidence that variation in spectral sensitivity of the retinal/opsin complex is largely a result in differences in key, amino acids (of the opsin protein) that line the chromophore pocket and interact with retinal. Substitutions of only a handful of few key amino acid residues can shift the absorption maxima of opsin pigments (Deeb 2005; Figure 1.10).
Figure 1.10. Two-dimensional model of human cone opsins indicating the five sites necessary to shift spectral sensitivity from green (M2) to red (L) (from Yokoyama 2000).

Since the primary structure of opsin and single amino acid substitutions are capable of modifying the spectral sensitivity of the rhodopsin, the opsin protein and absorption spectra of rhodopsin are subject to the pressures of natural selection. As a result, spectral sensitivities of rhodopsins are typically tuned to the photic environment of the organism. In marine environments, where red light is rapidly attenuated and blue wavelengths dominate, blue sensitivity is the norm. Sensitivity in marine invertebrates is typically between 450 and 550 nm. However, long wavelength and UV sensitivity is common in shallow, tropical waters, especially among coral reef fish (e.g., McFarland and Munz 1975, Losey et al. 2003), but also among crustaceans (e.g., Goldsmith 1990; Jokela-Maata et al. 2005).
Selective pressure has also given rise to multiple opsins with varying spectral sensitivity that are simultaneously expressed and differentially localized within organisms. This has acted to broaden the spectral sensitivity of organisms and more importantly has allowed color discrimination and color vision. For example, humans contain rod cells and three types of cone cells. The rod cells contain rod opsin (Rh) with an absorption maxima centered at ~500 nm, while the cones possess three distinct opsins [a blue-sensitive (M1), a green-sensitive (M2) and red-sensitive (L)] with absorption maxima centered 420, 530, and 560, respectively (Deeb 2005; Figure 1.11a). Other mammals, such as dogs and mice, have only two cone photoreceptors, blue and green. As a result they are not sensitive to light as far toward the infrared region as we are, and they do not discriminate colors as well. In contrast, birds have highly acute color vision based on at least five pigments – Rh plus four cone pigments (S, M1, M2, and L; Hart and Hunt 2007). Color vision is not restricted to vertebrates, and some of the most advanced mechanisms for discriminating colors are found among the arthropods. An extreme example is found in the mantis shrimp where tiered rows of cells and multiple pigments combine to produce a 12-channel color vision system (Marshall et al. 2007; Figure 1.11b).

Although important in determining the absorbance of isolated retinal/opsin complexes, amino acid substitutions are not the sole mechanism responsible for spectral tuning and influencing the sensitivity of photoreceptors. Screening pigments also play a role in color discrimination by filtering light incident upon photoreceptor cells and visual structures (Goldsmith et al. 1990). Pigment oil globules or granules are common in the distal region of vertebrate cone cells (Hart and Hunt 2007) as well as eyes and
photo receptors of invertebrates. The most common pigments found in oil globules include carotenoids (e.g., Wakakuwa et al. 2004; Stavenga 2002) and melanins (e.g., in

Figure 1.11. Comparison of the spectral sensitivities of human, bird and arthropod visual pigments. (a) Spectral sensitivity curves for the three human cone opsins (M1, M2, and L) and four cone pigments found (S, M1, M2, and L) found in the European starling [From Rowe 2000]; (b) Spectral sensitivities of cells located in the lower tier of the mantis shrimp eye compound eye. Two tiers of cells each with narrow absorbance curves provide the mantis shrimp with 12-channel color vision (from Marshall et al. 2007).
cnidarians; Martin 2002). These pigments act as long-pass cut-off filters and, as a result, can shift the spectral sensitivity peak of a photoreceptor to a wavelength longer than the absorbance maximum of the underlying pigment. They can also effectively narrow the spectral sensitivity curve and reduce overlap of the absorbance spectra of the underlying pigments (Bowmaker 1977). The diversity and prevalence of screening may be greatest among the arthropods. Pigments in the eyes of insects, have been shown to play roles in spectral tuning (e.g., Stravenga 2002), photoconversion of metarhodopsin to rhodopsin (by reflecting and focusing red wavelengths of light upon the photoreceptors), ensuring that photoreceptors only capture light from a narrow axial direction (thereby enabling orientation), and removing stray light that could create noise (reviewed by Stravenga 2002).

The form of retinal bound to opsin can also influence the absorbance maximum of the rhodopsin complex. A1 retinal is the form preferred by most invertebrates and vertebrates, but A2 (3,4-dehydroxyretinal) is also common in fish, amphibians and reptiles (Shichida & Matsuyama 2010). Substitution of A2 retinal for A1 results in a red shift in the absorbance maxima. Fish have been shown to shift from A1 to A2 in response to changes in their light environment and seasonal variation in the use of A1 and A2 has been reported in some fish as well as crayfish (Suzuki & Eguchi 1987). A3 retinal (3-hydroxyretinal has been observed in some insects (Seki & Vogt 1998) and fish (Minamoto & Shimizu 2002) and a forth type (A4; 4-hydroxyretinal) has been found in squid which also use A1 and A2 to create rhodopsin complexes with different absorbance maxima from a single opsin protein (Michinomae et al. 1994).
Photoreception in Cnidarians

Photosensitivity has been observed and described in nearly all kingdoms and phyla of organisms, and the cnidarians are no exception. A variety of photodetectors ranging from simple eyespots to complex, multicellular light-detecting organs, and simple eyespots to complex camera-type eyes complete with corneas, lenses and retinas occur within the phylum cnidaria (reviewed by Martin 2002). Interestingly, the photosensitive cells of cnidarians are ciliary, resembling dominant cell type found in vertebrates rather than the rhabdomeric cells that are dominant among the invertebrates (Eakin 1982).

It is not surprising that light influences many behavioral activities of ocelli-bearing cnidarians (hydrozoans and scyphozoans). These animals undergo diel vertical migrations (e.g., Arkett 1989), display phototaxis (e.g., Hamner et al. 1994) and shadow responses to rapid changes in light intensity (e.g., Singla 1974; Arkett & Spencer 1986), gamete maturation and release are stimulated by light and dark (e.g., Mills 1983; Arkett 1989), and in cubomedusae with complex eyes, vision may play a role in prey capture (Larson 1976) and mating behaviors. More surprising is the fact that many of these same photosensitive behaviors are also observed among the non-ocellus-bearing cnidarians. Light is known to play a role in the expansion and contraction of sea anemones (e.g., Fleure and Walton 1907; Banthim and Pantin 1950; Gladfelter 1975), and corals (e.g., Lasker 1979; Sebens and DeRiemer 1977). Phototactic behavior has been observed in these organisms (Kawaguti 1944; North 1957; North and Pantin 1958; Pearse 1974; Shick 1991), and the synchronous spawning of corals is tuned to both lunar phase and changes in daylight (e.g., Fadlallah 1983; Bull 1986; Szmant 1986; Gorbunov and
Falkowski 2002). Despite the abundant examples of photosensitive behavioral modifications among the cnidarians, the first cnidarian photopigments have only been described within the last couple of years and photopigments and specific photosensitive cells have still not been identified in anthozoans.

Early evidence for cnidaria photopigments were based primarily on immunohistological and electrophysiological evidence. Musio et al. (2001) reported a rhodopsin-like protein in ectodermal cells of *H. vulgaris* and Martin (2002) used antibodies directed against blue-, green-, and ultra-violet sensitive zebrafish opsins to localize opsin-like proteins in the complex eyes of cubozoan jellyfish, *Carybdea marsupialis*. Similarly, Ekstrom et al. (2008) used nine different opsin antibodies to screen the eyes of *C. marsupialis* and *T. cystophora* and concluded that multiple opsin-based photosystems are present in these organisms. Electroretinograms recorded in the hydromedusa *Sarsia tubulosa* indicated an absorbance peak at 540 nm while *P. penicillatus* has a peak at 530 (Weber 1982; Arkett 1985). A more recent studies by report spectral sensitivities for the lens eyes of several cubomedusae ranging between 485 and 512 nm (Coates et al. 2006; Ekstrom et al. 2008).

Finally, within the last couple of years, cnidarian opsins (cDNAs and protein sequences) have been described (e.g., Suga et al. 2008). As many as 18 full-length complementary DNAs (cDNAs) have been cloned from *Cladonema radiatum* (a jellyfish with eyes) and two from *Podocoryne carnea* (a jellyfish lacking eyes). Exploration of the genomes of the hydra *H. magnipapillata* and the sea anemone *Nematostella vectensis*, revealed as many as 63 and 31 opsin genes, respectively (Suga et al. 2008; Putnam et al. 2007). Analysis of sequence homology suggests that the cnidarian opsins form a distinct
group but are more closely related to vertebrate, ciliary opsins than Gq/invertebrate melanopsins (e.g., Suga et al. 2008; Terakita et al. 2008) and preliminary biochemistry indicates that at least one of these opsins, from the lens eye of *C. marsupialis* couples to Gs and initiates a cAMP signaling cascade (Koyanagi et al. 2008). Despite the recent advancements made in identifying and describing opsins from these groups, opsins still have not been described or photosensitive cells identified in corals.


The maintenance of genetic diversity and persistence of sessile marine invertebrates relies on the successful dispersal and recruitment of their propagules. As a result most of these organisms have a pelagic larval stage that is specialized for dispersal and settlement. Due to the structural simplicity of most invertebrate larvae (the lack of complex sensory organs and brain), pelagic larvae rely on environmental cues for navigation and the location of suitable settlement habitat. Environmental stimuli responsible for navigation and settlement include both chemical (organic compounds, pheromones, odors; reviewed by Pawlik 1992) and physical (texture, surface energy, and light; e.g., Hills. 1999, Mullineaux & Butman 1990, Mundy and Babcock 1998, respectively) cues.

The scleractinian corals are a classical example of a sessile invertebrate with a highly specialized dispersal and settlement phase. Modes of sexual reproduction in these corals generally fall into one of two categories: brooding (corals with internal fertilization that release developed larvae) or spawning (release eggs and/or sperm that fertilize and develop externally) (e.g., Fadlallah 1983; Szmant 1986). With both reproductive modes,
the end result of sexual reproduction is a free-swimming, semi-passive planula larva.

Aside from variation in size (brooded planulae tend to be larger), most coral planulae are morphologically similar regardless of whether they were brooded or developed externally. They are generally pear or barrel-shaped but can alter their shape, quickly and become elongated or spheroid (e.g., Atoda 1951; Harrigan 1972). The body of planulae consists of several layers: an outer epidermis or ectoderm, a central layer of mesoglea, and an inner gastrodermis or endoderm, which lines the coelenteron. The epidermis consists of columnar, ciliated or flagellated epithelial cells, mucous cells, secretory cells, cnidocytes and also contains pigment granules (Fadlallah and Pearse 1982). Planulae are polarized with an oral and aboral end. The aboral epidermis is forward during swimming and substrate exploration. It tends to be thickened and contains a complex nerve layer and sensory cells, but not discrete nerve cells (e.g., Vandermuelen 1974). The gastroderm is composed of spheroidal or polygonal shaped cells that are rich in lipid bodies (Lyons 1973; Vandermuelen 1974). Symbiotic zooxanthellae are usually found within the gastrodermal cells of brooded larvae.

The pelagic life of coral larvae can range from hours to days (Harrison and Wallace 1990), after which they must locate habitat suitable for attachment and metamorphosis – the first phase of coral recruitment. Successful recruitment (identification and inclusion in a population) is the result of success during three sequential early life-history phases: planktonic larval, settlement (larval settlement and metamorphosis), and post-settlement growth and survival (Ritson-Williams et al. 2009). Habitat selection by settling larvae plays a critical role in determining post-settlement survival (e.g., Mundy and Babcock
Figure 1.2. Micrographs of swimming and sectioned coral planulae. (a) Mature planula from a brooder, *Agaricia agaricites*; (b) Mature planulae from the broadcast spawner, *Acropora palmata*; (c) A confocal micrograph of a longitudinal section through an *A. palmata* planula, indicating the aboral and oral ends; (d) Enlarged view (box from panel c) of a cross section through *A. palmata* indicating the larval ectodermal/epithelial, endodermal/gastrodermal, and mesoglea layers.
Evidence suggests that multiple sensory cues are involved in habitat selection, settlement, and metamorphosis (e.g., Raimondi and Morse 2004), yet many details regarding the link between sensory biology and settlement ecology of coral larvae remain elusive.

Chemical cues are known to influence larval behavior, settlement, and metamorphosis in many species. For example, *Porites astreoides* planulae are responsive (swimming downward) to water-soluble cues originating from the reef (Gleason et al. 2009), and many species settle and metamorphose in response to crustose coralline algae (CCA) and/or microbial films (e.g., Morse et al. 1988; Harrington et al. 2004; Webster et al. 2004). Many species of larvae also display substratum preferences that are related to the depth and habitat distribution of adults. For example, Baird et al. (2003) found that two species of *Goniastrea*, with an adult distribution restricted to reef flat environments, preferred to settle on terracotta tiles conditioned at 2m depth while two species of *Fungia* whose adults distributions are restricted to the base of reef slopes preferred to settle on tiles conditioned at 12m. Golbuu and Richmond (2007) found that *Goniastrea retiformis*, which is common on the shallow reef front areas dominated by CCA, showed a significant preference for *Hydrolithon reinboldii*, while *Stylaraea punctata*, abundant on inner reef flats where CCA coverage is low and carbonate rubble is the dominant substrate, preferred to settle carbonate rock covered in biofilm.

While chemical cues are important in influencing settlement and metamorphosis, light is also known to influence behavior and habitat selection by coral larvae. For example, Kawaguti (1941) observed positive phototaxis in four species of zooxanthellate planulae, noting that several-fold difference in sensitivity among species. Similarly,
azooxanthellate *Montastraea faveolata* larvae are also positively phototactic. In the laboratory they were observed swimming at the surface of graduated cylinders when kept in the light but were positively geotactic, remaining at the bottom of cylinders when kept in the dark (Szmant and Meadows 2006). In addition, *Porites astreoides* larvae prefer environments sheltered from UV radiation (Gleason et al. 2006), species-specific preferences for light intensity (Babcock and Mundy 1996; Mundy and Babcock 2000) at settlement have been shown in *Goniastrea aspera, A. tenuis, Oxypora lacera* and preference for light quality (Mundy and Babcock 1998) is apparent in *G. favulus* and *Montipora peltiformis*.
CHAPTER 2

LARVAL SETTLEMENT EXPERIMENTS: PORITES ASTREOIDES AND ACROPORA PALMATA USE SPECTRAL LIGHT CUES FOR FINE-SCALE HABITAT SELECTION DURING SETTLEMENT

Background

Coral recruitment is the result of success during three sequential early life-history phases: planktonic larval, settlement (larval settlement and metamorphosis), and post-settlement growth and survival (Ritson-Williams et al. 2009). Habitat selection by settling larvae plays a critical role in determining post-settlement survival (e.g., Mundy and Babcock 2000; Harrington et al. 2004; Birrell et al. 2005). Evidence suggests that multiple sensory cues are involved in habitat selection, settlement, and metamorphosis (e.g., Raimondi and Morse 2004), yet many details regarding the link between sensory biology and settlement ecology of coral larvae remain elusive.

Chemical cues are known to influence larval behavior, settlement, and metamorphosis in many species. For example, Porites astreoides larvae are responsive (swimming downward) to water-soluble cues originating from the reef (Gleason et al. 2009), and many species settle and metamorphose in response to crustose coralline algae (CCA) and/or microbial films (e.g., Morse et al. 1988; Harrington et al. 2004; Webster et al. 2004). Many species of larvae also display substratum preferences that are congruent with the depth and habitat distribution of adults. For example, Baird et al. (2003) found that two species of Goniastrea, with an adult distribution restricted to reef flat environments, preferred to settle on terracotta tiles conditioned at 2m depth in contrast to two species of Fungia whose adult distributions are restricted to the base of reef slopes
and preferred to settle on tiles conditioned at 12m. Golbuu and Richmond (2007) found that *Goniastrea retiformis*, which is common on the shallow reef front areas dominated by CCA, showed a significant preference for *Hydrolithon reinboldii*, while *Stylaraea punctata*, abundant on inner reef flats where CCA coverage is low and carbonate rubble is the dominant substrate, preferred to settle on carbonate rock covered in biofilm.

While chemical cues are clearly important in determining settlement and metamorphosis, light is also known to influence behavior and habitat selection by coral larvae. Phototactic behavior has been observed in coral planulae. For example, Kawaguti (1941) described positive phototaxis in four species of zooxanthellate planulae, noting a several-fold difference in sensitivity among species. Similarly, azooxanthellate *Montastraea faveolata* larvae were observed swimming at the surface of graduated cylinders when kept in the light but were positively geotactic, remaining at the bottom of cylinders when kept in the dark (Szmant and Meadows 2006). In addition, light quality and intensity have been shown to influence settlement. *Porites astreoides* larvae prefer environments sheltered from UV radiation (Gleason et al. 2006) while species-specific preferences for light intensity (Babcock and Mundy 1996; Mundy and Babcock 2000) at settlement have been shown in *Goniastrea aspera, Acropora tenuis, and Oxypora lacera*, and preference for light quality (Mundy and Babcock 1998) is apparent in larvae from *Goniastrea favulus* and *Montipora peltiformis*. Lastly, a previously undocumented photosensitive behavior was observed by the current authors while conducting experiments with *P. astreoides* larvae (BM and MB, personal observation). Larvae settled on a red, fluorescent, plastic cable tie (Figure 1) but failed to settle on other colors
of identical cable ties, unconditioned coral rubble and surfaces of plastic containers, prompting the study described in this paper.

**Figure 2.1.** Images and fluorescent spectra of substrata. (a) *Porites astreoides* spat attached to red/fluorescent orange cable tie (middle tie shown in panel (c)) used in Exp I; (b) Three-channel (405, 546, and 588 excitation) fluorescent confocal micrograph of CCA (*T. prototypum*). (see (d)) combine to produce a bright orange fluorescence; (c) Beaded cable ties used in Exp I, as they appear under white light; (d) Fluorescence emission spectra of CCA, including chlorophyll fluorescence and phycoerythrin fluorescence, and the red-beaded cable tie (corresponding to images a & b).

The color of an object is a function of the light emerging from the subject. The emerging light can result from addition (e.g., bioluminescence and fluorescence) and/or
subtraction radiances (e.g., pigment colors) (Lythgoe 1979). Fluorescence contributes little to coloration in the terrestrial environment, but in aquatic environments, where water spectrally filters both the incident and the reflected light, fluorescence appears to play a more significant role. Fluorescent pigments contribute to the coloration of many corals and sea anemones (e.g., Dove et al. 2001; Mazel and Fuchs 2003; Oswald et al. 2007), and fluorescence has been shown to enhance the brightness and visibility of signaling in mantis shrimp (Mazel et al. 2004). Unlike red and orange color resulting from subtraction radiances (pigments absorbing blue and green wavelengths but reflecting red or orange), red and orange fluorescence can be brighter than the incident light of those wavelengths and, at deeper depths, can create vivid color displays that contrast dramatically with the predominantly blue surroundings (Lythgoe 1979). Therefore, if substrate color is influencing the settlement ecology of coral larvae the effective stimulus may result from both fluorescence and reflectance of potential settlement habitat.

The aim of this study was to experimentally test the influence of substrate color on the settlement of coral larvae. Specific objectives were: 1) to investigate whether the preferential settlement on red substrata, previously observed in *P. astreoides* larvae, is a response to color; 2) to determine whether similar behavior exists in spawned larvae of *Acropora palmata*; 3) to determine the reflectance and fluorescence spectra of experimental substrata in order to characterize the spectral signature responsible for cuing larval settlement.
Materials and Methods

In this study, larval settlement preferences were tested by conducting choice experiments with two species – *Porites astreoides* and *Acropora palmata*. In experiments, larvae were offered plastic substrata representing a range of color choices. Separate experiments were conducted with each species in 2006 and again in 2009; each experiment had a slightly different design and employed different substrate types. Following the experiments, reflectance and fluorescence spectra were measured for a subset of the settlement materials to characterize and compare the spectral signatures of stimuli provided in the individual experiments.

**Larval collection.** Eight adult *Porites astreoides* colonies were collected from Little Grecian reef in Key Largo, FL, two days prior to the new moon in June, 2006 (Experiment I) and in June, 2009 (Experiment III). Colonies were transported to the University of Miami and maintained in a flow-through seawater table. Upon release, larvae were collected by pipet and transferred to containers with filtered seawater until experiments were set up. Larvae were less than 24h old when introduced to the experimental treatments. Gametes were collected from *Acropora palmata* in Key Largo, FL in Aug 2006 (Experiment II) and Aug 2009 (Experiment IV) by placing weighted nets over adult colonies immediately prior to spawning. Upon release, positively buoyant gamete bundles were collected in plastic containers secured to the top of the nets. Intact bundles were returned to the research vessels and gametes from colonies representing three different genotypes were combined to facilitate fertilization. Larvae were reared to competency following the methods described by Miller and Szmant (2006).
Settlement experiments

Experiments I & II. The design of the Experiment I was based on the apparent selection of red, fluorescent nylon cable ties in ancillary observations of P. astreoides larvae. In both Experiments I and II, larvae were offered arrays of nylon cable ties representing three colors secured around white fiberglass rods. In Experiment I, 10 P. astreoides larvae were placed in each of 12 replicate 100 ml dishes containing one each of red, green and white cable ties, and 1μm filtered seawater. The order of the colors was randomized and fiberglass rods were positioned in the center of each dish. Six dishes were kept in the dark and six were placed under 120 cm fluorescent strip light (GE Ecolux®) and maintained on a 12h:12h light/dark cycle. Partial water changes were conducted daily to minimize effects of evaporation and respiration. After 48 h, substrates were examined under a dissecting microscope and the number of settled (attached and metamorphosed larvae) was recorded. Metamorphosis here was defined as larval transition to an orally-aborally flattened primary polyp with secretion of a basal plate.

In Experiment II (conducted in August 2006), the experiment was repeated with A. palmata using a different type of plastic tie, different colors (red, orange and blue), and a longer duration (five days). Larvae were 5 d old when they were introduced to the experimental treatments.

Experiment III. The goal of this experiment was to provide P. astreoides larvae with a choice of plastic substrates (buttons) representing a broader range of the visible light spectrum. Colors included red, pink, orange, green, blue and purple. One button of each color was placed in each of 10 plastic petri dishes containing 25 ml of 1μm filtered seawater. The positions of the buttons were randomized within dishes, 10 larvae were
added to each dish and dishes were positioned on a lab bench beneath two 120 cm fluorescent strip lights (GE Ecolux®). Lights were maintained on a 12h:12h light/dark cycle, and the larvae were allowed 48 h to settle.

Experiment IV. In this experiment, settlement substrates consisted of 5cm x 5cm square acrylic tiles cut from 0.8 cm thick acrylic sheet. Four intersecting grooves (a tic-tac-toe pattern) were carved into the upward-facing surfaces of all tiles using a soldering gun to provide texture known to facilitate settlement of some species (e.g., Peterson et al. 2005). Limestone tiles of equivalent dimension were cut from travertine floor tile and textured as above. Colors of substrates included red, orange, yellow, green, blue, and stone (limestone). One tile of each color was haphazardly arranged in the bottom of each of ten 20-liter aquaria, 12 liters of filtered seawater (1 µm) and 30 larvae were added to each. Water was lightly aerated and salinity was monitored daily during the course of the experiment. Salinity was adjusted by the addition of distilled water and maintained between 35 and 37 ppm. The ten aquaria were arranged beneath two ballasts (five aquaria beneath each) containing two 120 cm, fluorescent strip lights (one GE Ecolux; one All-Glass Aquarium 32W/8000K). Lights were maintained on a 12hr:12hr light/dark cycle during the experiment and larvae were allowed one week settle.

Spectral measurements of settlement substrata

Fluorescence Spectra. Fluorescent emission spectra were measured using a Leica TCS SP5 confocal microscope. Fluorescent emission spectra were generated for all plastic substrata (cable ties, heart-shaped buttons and acrylic squares) and CCA (Titanoderma
prototyopum, a species preferred by settling A.palmata larvae Ritson-Williams et al. 2010) by scanning materials from 415-700 nm at 10nm interval using the xyλ. scan mode (Leica Application Suite) with 405nm (UV) excitation. Fluorescence spectra were normalized as percent change in fluorescent emission (percent change relative to the baseline detection).

Reflectance Spectra. Diffuse reflectance of plastic buttons and acrylic squares was measured using a diffuse reflectance measurement system (Optronic Laboratories (OL), LLC). Components of the system included an integrating sphere reflectance attachment (OL 740-70), a diffraction grating monochromator (OL 750-M-D), and an OL 200/IR source attachment. A 150W quartz halogen lamp served as the radiation source during measurements. A digital motor control interface and drive motor were used to position diffraction gratings and control the wavelength of incident radiation with 0.1 nm precision. Reflected radiation was measured using a silicon photodiode detector and data was collected using OL 750/754 DOS Application Software. The diffuse reflectance of materials was measured at 10 nm intervals from 400 to 700 nm. Data was transformed to relative diffuse reflectance (diffuse reflectance at each interval/maximum reflectance from 400-700 nm).

Statistical analysis. Since aggregative settlement has been observed in both P. astreoides and A. palmata (personal observation) the settlement of individual larvae within experimental dishes was not considered independent. The pooling of larvae from multiple colonies likely reduced any bias resulting from this aggregative settlement effect.
but dishes were considered to be the smallest independent units and statistical analyses were performed only on the counts of dishes with or without settlement for each color. This count frequency data was analyzed using a categorical (contingency table) model to determine whether settlement was contingent upon substrate color. Pearson chi-squared tests were used to determine whether color had a significant effect on settlement within each experiment.

**Results**

Larvae of *P. astreoides* and *A. palmata* showed a higher settlement response to red (or red and orange) substrata (Table 1). When kept in the light, *P. astreoides* settled on all red cable ties but failed to settle on green or white. In the same experiment, larvae failed to settle when dishes were kept in the dark (Table 1, Exp I). In Experiment III, a total of 24 *P. astreoides* larvae settled on red buttons, greater than the total number that settled on all other colors combined. *A. palmata* larvae also preferred red (or red and orange) substrata. In Experiment II, *A. palmata* larvae settled in equal numbers on the red and orange cable ties but failed to settle on blue and in Experiment IV, the total number of settlers observed on red acrylic squares was greater than twice the number observed on any other color (Table 2.1).
Table 2.1. Summary of larval settlement experiments<sup>a</sup>

<table>
<thead>
<tr>
<th>Year</th>
<th>Spp.</th>
<th>Substrate</th>
<th>larvae</th>
<th>dishes</th>
<th>l/d&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Red</th>
<th>Green</th>
<th>White</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>2006</td>
<td>Pa</td>
<td>cable ties</td>
<td>60</td>
<td>6</td>
<td>1</td>
<td>32(7)</td>
<td>0(0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>6</td>
<td>d</td>
<td>0(0)</td>
<td>0(0)</td>
</tr>
<tr>
<td>II.</td>
<td>2006</td>
<td>Ap</td>
<td>cable ties</td>
<td>50</td>
<td>5</td>
<td>1</td>
<td>Red</td>
<td>Orange</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>5</td>
<td>d</td>
<td>8(3)</td>
<td>8(4)</td>
</tr>
<tr>
<td>III.</td>
<td>2009</td>
<td>Pa</td>
<td>buttons</td>
<td>100</td>
<td>10</td>
<td>1</td>
<td>Red</td>
<td>Orange</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>10</td>
<td></td>
<td>24(10)</td>
<td>3(2)</td>
</tr>
<tr>
<td>IV.</td>
<td>2009</td>
<td>Ap</td>
<td>squares</td>
<td>300</td>
<td>10&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1</td>
<td>Red</td>
<td>Orange</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td>10&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
<td>16(4)</td>
<td>6(3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total(maximum) # settled

<sup>b</sup> Species – P. astreoides (Pa)/A. palmata (Ap)

<sup>c</sup> Light environment – light(l)/dark(d)

<sup>d</sup> Experiments conducted in 20L aquaria

Since larvae of these species display aggregative settlement, the settlement of individuals on different treatments within each dish was not considered independent. Treating dishes as independent replicates, the number of dishes in which settlement was observed on red (or red and orange) substrata was consistently greater than on other colors (Figure 2.2). Chi-square tests of contingency, between substrate color and the frequency of dishes in which settlement was observed, indicated that settlement was contingent upon color in Experiment III (Figure 2.2b) with P. astreoides ($\chi^2=19.41$, p=0.0035) and Experiment IV (Figure 2.2d) with A. palmata ($\chi^2=11.77$, p=0.0381) (Figure 2.2). Statistical analysis was not performed on the results from Experiments I and II (Figure 2.2a,c) since differential settlement response was evident by inspection and low replication made the application of Chi-square tests inappropriate.
**Figure 2.2.** Results of settlement experiments. Bars represent the number of replicate dishes in which settlement was observed for each color of substrata. (a) Experiment I, cable tie experiment conducted in June 2006 (dishes kept in the light); (b) Experiment III conducted in June 2009 (plastic buttons); (c) Experiment II, cable tie experiment conducted in August 2006 (dishes kept in the light); (d) Experiment IV conducted in August 2009 (acrylic squares).

Spectral measurements revealed that a natural, preferred CCA spp., and experimental plastic substrata had common spectral attributes (Figure 2.1). The fluorescence emission spectra of CCA (*Titanoderma prototypum*) and the red, beaded cable tie used in Exp. I had similar fluorescent emission peaks at 580 nm and 590 nm, respectively (Figure 2.1d). Due to the size and surface irregularities of the CCA and the
cable ties, measurement of the diffuse reflectance was not possible using the instrumentation available. However, reflectance and fluorescent emission spectra were obtained for the plastic buttons and acrylic squares. The preferred substrata from each of these experiments, the red buttons and red squares, had reflectance spectra dominated by radiation greater than 600 nm and little or no reflectance or fluorescence of wavelengths in the blue or green spectral regions. Substrata experiencing little or no settlement in experiments, including the pink button, had reflectance spectra dominated by, or fluorescent peaks with, wavelengths between 400 and 550 nm (Figure 2.3). While some colors of the buttons had strong fluorescent peaks (Figure 2.3b), the red buttons did not exhibit detectable fluorescence and the acrylic squares were only weakly or non-fluorescent (Figure 2.3d). The percent change in emission of these materials only ranged from <1 (blue) to ~14% (red) and likely contributed little to their overall spectral signatures (Figure 2.3).

Discussion

These results demonstrate that larvae of at least one brooding, *P. astreoides*, and one spawning coral, *A. palmata*, settle and metamorphose at a greater frequency on red substrata than on similar substrata of other colors. Since this tendency was observed on a variety of plastic materials and only when experiments were conducted in light, it suggests the larvae are responding to color rather than a chemical unique to dyes in a particular material. Elevated rates of settlement and metamorphosis also were not restricted to red fluorescent materials, as the red buttons used in Experiment III with *P. astreoides* were not fluorescent, and red acrylic squares used in Experiment IV were only
**Figure 2.3.** Reflectance and fluorescence emission spectra of settlement substrata. (a) Relative diffuse reflectance plastic buttons (Exp. III); (b) Percent change in fluorescence emission of plastic buttons, generated with UV (405 nm) excitation; (c) Relative diffuse reflectance of acrylic squares (Exp IV); (d) Percent change in fluorescence emission of acrylic squares, generated with UV (405 nm) excitation.

weakly fluorescent with peaks in the yellow region of the spectrum. However, the response observed in Experiment I (Figure 2.2a) may have been enhanced by fluorescence, since the strong fluorescent peak of the red cable tie likely contributed to the overall red-orange stimulus provided by this substrate.

The induction of metamorphosis by long-wavelength light is not unprecedented. Incubation in red light resulted in more rapid metamorphosis of frog tadpoles (Joshi and Mohinuddin 2003). The acceleration of metamorphosis occurred in both intact and
experimentally blinded tadpoles indicating that the response was mediated by extraocular photoreceptors. Opsins are the only long-wavelength sensitive photopigments known to occur in animals (van der Horst and Hellingwerf 2004) and are likely responsible for the accelerated metamorphosis observed in tadpoles. While opsins are traditionally associated with visual structures (rod and cone cells of the retina), other non-ocular types of opsins (e.g., melanopsins, vertebrate ancient opsins, peropsins, neuropsins, pinopsins), with biological functions other than vision (e.g., cell division, photoentrainment of circadian clocks, the regulation of reproduction) have been described in various cell types (other than rods and cones) in both vertebrates and invertebrates (e.g., Terakita 2005; Shichida and Matsuyama 2009).

The only photosensitive molecules described in corals to date are cryptochromes (Levy et al. 2007), but these are blue-light sensitive molecules that function at the terminal end of circadian gene networks and are unlikely to play a role in this long-wavelength, settlement response. However, opsins have been discovered in other cnidarians (for example, the starlet sea anemone, Nematostella vectensis, Hydra magnapapillata and two hydrozoans; Suga et al. 2008) and a melanopsin-like transcript is present in the Acropora millepora larval transcriptome (Vize et al. 2009). Opsins are likely responsible for the settlement behavior observed in the present study and efforts to identify and describe opsins in A. palmata larvae are currently in progress.

Red-photosensitivity (and/or avoidance of spectral environments dominated by blue) could serve as a mechanism for locating habitat suitable for the survival of juveniles and adults. Although substrate color previously has not been hypothesized to influence settlement or metamorphosis in corals, some species show preference for specific light
qualities (Mundy and Babcock 1998), or intensities (Babcock and Mundy 1996; Mundy and Babcock 1998). These authors suggested that a possible ecological function of light-dependent settlement observed in some species is to concentrate larvae at a depth optimal for adult survival. Chromatic antagonism, (e.g., mechanism for simultaneous attraction to red but avoidance of UV or short-wavelength blue light) is necessary in order for photosensitivity to serve as a depth gauge in aquatic organisms (Nilsson 2009). The larvae in this study demonstrated chromatic antagonism - preference for substrata that reflected or fluoresced strongly in the red but weekly in the short-wavelength regions of the spectrum (Figure 2.3). If this photosensitivity influences swimming behavior it could serve to concentrate *A. palmata* and *P. astreoides* larvae within the depth zone inhabited by the adults. *A. palmata* and *P. astreoides* (green morph; the type collected for these experiments) are abundant in shallow reef environments (<10 m), where long-wavelength light remains abundant (e.g., Levine and MacNichol 1982). However, the settlement behavior demonstrated here suggests that these larvae use photosensitivity for fine-scale discrimination and selection of substrata, or microhabitats, that are conducive to post-settlement survival.

The composition of the plastic materials used in these experiments is unlike substrata that naturally occur on coral reefs, but red surfaces are common components of the coral reef benthos. CCA are conspicuous and important functional components of coral reefs, and can be especially abundant on shallow, high-energy reef crests (reviewed by Steneck 1986). While CCA is not required for the induction of settlement and metamorphosis in *P. astreoides* (personal observation) and *A. palmata* (Ritson-Williams et al. 2010), both species settle in greater densities on the surface of CCA when provided
with a choice between CCA and other substratum types. These algae have spectral characteristics that closely resemble the fluorescent emission and/or reflectance spectra of the red plastic substrates used in this study (Figure 2.1 & 2.3) and may provide similar spectral cues that enable fine-scale habitat selection during settlement. This behavior may be an evolutionary adaptation to strong selective pressure resulting from high rates of post-settlement mortality. While settlement on CCA or in CCA-dominated habitat does not necessarily increase a larva’s probability for survival, some species of CCA facilitate early post-settlement survival (e.g., Harrington et al. 2004; Ritson-Williams et al. 2009), and the distribution of CCA is inversely correlated with (e.g., Fabricius and De’ath 2001) other known sources of juvenile coral mortality (for example, macroalgae and sediment; e.g., Birrell et al. 2005).

The coral species investigated here are common in shallow reef habitats and fluorescence was not a consistent characteristic of the preferred substrata, but long-wavelength photosensitivity and the intense orange phycoerythrin fluorescence of CCA could combine to provide a mechanism for habitat selection capable of functioning at depths beyond the extinction of red and orange light. The fluorescence of phycoerythrin, an accessory pigment abundant in Rhodophytes, including CCA, is maximally excited by green light with a wavelength ~546 nm but is also strongly excited by blue (French and Young 1952). In oligotrophic water, blue and green wavelengths of light penetrate well below 50m (Levine and MacNichol 1982). Down-welling light dominated by blue and green wavelengths would be absorbed by and excite fluorescence of phycoerythrin pigments in CCA which would then emit light in the orange/red region of the spectrum. If spectral cuing of settlement and metamorphosis in coral larvae involves a
photopigment spectrally tuned to absorb in the orange/red region of the spectrum, the orange phycoerythrin fluorescence of CCA could serve as a beacon against an otherwise dark background.

As research continues to reveal more about the sensory biology of coral larvae, increasingly complex mechanisms are emerging. Recent results suggest that some species of coral larvae may be capable of using sound to locate coral reefs (Vermeij et al. 2010), and once larvae are near reef habitat, water-soluble cues may be responsible for inducing settlement behavior (Gleason et al. 2009). At least some species of larvae appear capable of using light cues to locate and position themselves at the depth optimal for their survival (Mundy and Babcock 1998), but once they contact the reef, many coral species still require cues associated with CCA to induce metamorphosis (e.g., Morse et al. 1988; Raimondi and Morse 2000). The results of these experiments suggest the red color and/or orange fluorescence of these algae may serve as cues, by creating a light environment with a spectral distribution capable of inducing settlement and metamorphosis.
CHAPTER 3

THE MOLECULAR CLONING AND SEQUENCE ANALYSIS OF CORAL OPSINS

Rationale

Opsin is the universal photopigment of the animal kingdom. While other photosensitive molecules do exist (e.g., blue-light sensitive cryptochromes: Levy et al. 2007), opsins form the basis of all visual and most non-visual photosensitivity. Opsins have been found in organisms ranging from bacteria to humans and in model organisms of almost every taxa in between. Abundant examples of photosensitive behaviors (e.g., UV avoidance, shadow response, synchronous spawning, and preference for red substrata during settlement in coral larvae; Chapter II), complex lens eyes (e.g., box jellyfish), and spectral sensitivities (both behavioral responses and ERGs recorded from the lens eyes of jellyfish), provide evidence for the existence of opsins in cnidarians, yet, until recently, these photopigments have remained elusive.

For years the identification of opsins in ancestral, basal metazoans, especially in organisms lacking eyes or photosensitive structure, has proven difficult. As members of the rhodopsin family of GPCRs, opsins have highly conserved 7-trans-membrane (7tm) structure, and several highly conserved functional motifs. The single feature that distinguishes opsin from other GPCRs is the presence of a retinal (light absorbing chromophore) binding lysine (K) residue within its 7th trans-membrane (TM7) domain.

Attempts to clone opsins from more basal metazoans, and “exotic” organisms, relied heavily on degenerate PCR– a method which exploits the conservative nature of these functional regions and the genetic code, by designing “cocktails” of PCR primers
which include all combinations of nucleotides that could potentially encode the template amino acid sequence. Unfortunately, the phylogentic position of the cnidarians (i.e. separate line diverging prior to the protostome/deuterostome split), and lack of homology to other invertebrate opsins, likely contributed to the lack of success with this approach (see discussion below).

However, within the past decade, advances in molecular techniques and cnidarian genome projects have produced the first cnidarian opsin sequences. Opsins sequences are now available for a sea anemone (*Nematostella vectensis*), *Hydra vulgaris*, and two jellyfish (Suga et al. 2008), and a project to sequence the larval transcriptome of the Indo-Pacific coral, *Acropora millepora*, while still in development, has produced >99,000 unigenes.

This chapter describes identification and sequencing coral opsins of the first coral opsins - full-length cDNAs from *Acropora palmata* larvae, and analysis of these sequences and their inferred proteins.

**Materials and Methods**

**DNA/RNA isolation**

Adult coral genomic DNA (gDNA) samples used in PCR reactions were prepared by R. Carter (Carter et al. 2004). Due to the age of these samples, quality was estimated prior to their use by running aliquots on a 1% agarose gels.

Total larval RNA was isolated from samples larvae that had been preserved in either RNAlater® or Trizol reagent and frozen at -80°C. Isolation of total RNA was achieved by Phenol:Chloroform:IAA, Acid-Phenol:Chloroform extraction following the
ToTALLY RNA™ (Ambion) protocol for samples stored in RNALater® and frozen at -80°C. Following extraction, RNAs were precipitated using isopropanol, collected by centrifugation and re-suspended in nuclease-free water.

**cDNA synthesis**

Larval cDNA was synthesized by reverse transcription (rtPCR) of larval total RNA (tRNA). Reverse transcription reactions were used to create random-primed cDNA, 3’ or 5’RACE-ready cDNA. Random-primed cDNA was synthesized using random hexamers, following the protocol in the First-Strand cDNA Synthesis Kit (Roche), 3’ RACE-ready cDNA was synthesized by reverse transcription using an oligo-dT (Flajnik poly-T) primer and M-MLV-reverse transcriptase. Initially 5’RACE-ready *M. faveolata* cDNA (during amplification and cloning of *M. faveolata* GPCRs – see below) was synthesized following the FirstChoice® RLM RACE Kit (Ambion) small rxn protocol. First, 5’ phosphates were removed from RNA with calf intestinal phosphatase (CIP). Tobacco acid pyrophosphate (TAP) was then used to de-cap the full-length mRNA, and a 5’RNA adapter (5’-GCUGAUUGCCAGAAUGAAACACUGCG UUUGCUGGCUUUUGAUAGAA-3’) was ligated to the de-capped mRNA using T4 RNA ligase. Later (during amplification of *A. palmata* Acropsins – see below) 5’RACE-ready cDNA was synthesized using a SMART™ RACE cDNA amplification kit (Clontech). 5’-RACE CDS Primer A (5’-(T)25VN-3’: V=A, G or C; N=A, T, G or C) was used in combination with SMART™ A oligonucleotide (5’-AAGCAGTGCTAT CAACGCAGAGTACGCGGG-3’), an oligonucleotide adapter that gets incorporated into
the 5’ end of the RNA template (forming an RNA/DNA hybrid) and allowing RT template switching.

3’/5’ RACE

Amplification of 3’RACE cDNAs was achieved using gene-specific forward (upper) primers (Tables 3.1, 3.2) and a reverse primer complementary to the oligo-dT primer used during the RT reaction. 5’RACE cDNAs were amplified using gene-specific primers (Tables 3.1, 3.2) in combination with RLM 5’RACE Outer Primer (5’-GCTGATGGCGATGAATGAACACTG-3’) or SMART™ RACE Universal Primer A Mix (long: 5’-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3’; short: 5’-CTAATACGACTCACTATAGGGCGAGATCGGCCGCT-3’). PCR reaction conditions and cycling conditions varied. PCR products were resolved on a 1% agarose gel. Bands were excised from the gel, filtered through siliconized glass wool, precipitated with isopropanol, re-suspended in PCR grade H$_2$O and either directly sequenced (Genewiz Inc) or cloned. When cloning was necessary, amplified DNAs were ligated with PCR2.1 TOPO cloning vector (Invitrogen) using T4 DNA ligase. E.coli (Top10 cells; Invitrogen) were transfected by electroporation. Cells were streaked and grown on LB(Kan30) plates, and subsequently purified. Cloned PCR products were sequenced and contigs were assembled using Seqbuilder software (Lasergene V7). Once full-length cDNAs were assembled, gene-specific primers were designed to regions external to the ORF and the full-length cDNAs were amplified by PCR and cloned as above. Cloning of the 5’ regions was determined by sequence analysis (presence of a 5’ UTR, open reading
frame including a start codon that overlapped with known internal sequence and alignment with other (non-coral) full-length opsin sequences).

**Degenerate PCR: Round 1**

Initial attempts to amplify opsin-like sequence employed degenerate primers used by Santillo et al. (2006) to amplify a small internal region of an opsin-like gene from Hydra. In designing these PCR primers, the BLASTX program was used to align 12 invertebrate rhodopsin sequences with the following GenBank accession numbers: scallop, AB006455; flying squid, X70498; European squid, X56788; octopus, X007797; crayfish, AF003543; horseshoe crab, L03791; fly(mela)-1, K02315; fly (pseudo)-1, X65877; crab-1, D50583; crab-2, D50584; locust-1, X80071. The regions selected for primer design were helix I (GNGVVIY) for the forward primer (5’-GG(AC) AA(CT) GG(CT) (AG)TT GTC AT-3’) and Loop CII (DRYNVI) for the reverse primer (5’- GT(GT) AC(AG) TTG TA(AT) CGA TC-3’). All PCR reactions were 50ul reactions and used a GotTaq® Flexi DNA Polymerase kit. During PCR, various reaction (manipulating buffer, MgCl2 and primer concentrations) and cycle conditions (manipulating annealing temperatures between 50-60°C) were used.

**Amplification and cloning of M. faveolata GPCRs**

Initial database screening (before the SymBioSys database was available; see below) was conducted by C. Voolstra (postdoc in M. Medina lab). *M. faveolata* and *A. palmata* larval EST databases were blasted using squid rhodopsin protein (Genbank Accession CAA40108). Blasts identified two opsin–like ESTs (Mfav_3738893 and
Mfav_3739163) in *M. faveolata*. Gene-specific primer pairs were designed for each of these transcripts (Table 3.1). These primers were used in 50 µl, standard PCR reactions using GoTaq® Flexi DNA polymerase kit (Promega). The cycling conditions used during PCR were: 94°C(5min), 80°C, add Taq; 30 cycles: 94°C(30s), 55°C(30s), 72°C(90s); 72°C(10min);

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Application</th>
<th>Primer Sequence</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mf_OP_EST_8998_1U</td>
<td>Internal PCR</td>
<td>5'-GCAAATGCTTAATGGTTGTC-3'</td>
<td>1.031kb</td>
</tr>
<tr>
<td>Mf_OP_EST_8998_2L</td>
<td>Internal PCR</td>
<td>5'-GGACTTCTCGAATTCTCC-3'</td>
<td></td>
</tr>
<tr>
<td>Mf_OP_EST_8998_2L</td>
<td>3'RACE</td>
<td>5'-GGAGAAATGAGGAAGTCC-3'</td>
<td></td>
</tr>
<tr>
<td>Mfav_8998_5RACE</td>
<td>5'RACE</td>
<td>5'-TGACGGATCGTAACAAGCG-3'</td>
<td></td>
</tr>
<tr>
<td>Mf-8998_START</td>
<td>Complete ORF</td>
<td>5'-CAGGTAAGAAAAAACAAGTTTCA-3'</td>
<td>1.074kb</td>
</tr>
<tr>
<td>Mf-8998_END</td>
<td>Complete ORF</td>
<td>5'-GGCAACAGAACCATTCTTT-3'</td>
<td></td>
</tr>
<tr>
<td>Mf_OP_EST_9163_1U</td>
<td>Internal PCR</td>
<td>5'-GCCATAAGCATCGATAGG-3'</td>
<td>467bp</td>
</tr>
<tr>
<td>Mf_OP_EST_9163_2L</td>
<td>Internal PCR</td>
<td>5'-GGATTTAAAGAAGATAA-3'</td>
<td></td>
</tr>
<tr>
<td>Mf_OP_EST_9163_2U</td>
<td>3'RACE</td>
<td>5'-TTAATCTTCTTTAAATCC-3'</td>
<td></td>
</tr>
<tr>
<td>Mfav_9163_5RACE</td>
<td>5'RACE</td>
<td>5'-TGCTAAGCAACTTAGGC-3'</td>
<td></td>
</tr>
<tr>
<td>Mf-9163_START</td>
<td>Complete ORF</td>
<td>5'-CATCGCAGTCAACGCCGA-3'</td>
<td>912bp</td>
</tr>
<tr>
<td>Mf-9163_END</td>
<td>Complete ORF</td>
<td>5'-CATTCTGACAACGTGAC-3'</td>
<td></td>
</tr>
</tbody>
</table>

4°C hold. Resulting PCR products were gel-purified, isopropanol precipitated and sequenced (Geneway). 3'/5'RACE PCR was performed as above. Resulting sequences were assembled using Seqbuilder and coding regions identified by translation of the contigs using Editseq software (Lasergene V7). Gene-specific primers were designed to
the ends of the coding regions of each full-length cDNA, and coding regions amplified by PCR using Phusion High-Fidelity PCR kit (NEB) following recommended guidelines for basic reaction and cycling conditions.

Full-length open-reading frames (PCR products) were cloned into TA vectors (TOPO2.1 – Invitrogen) by overnight incubation with T4 DNA ligase at RT. *E.coli* (Top10 cells; Invitrogen) were transfected by electroporation. Cells were plated and grown on LB(Kan30) plates containing Xgal and IPTG, to allow blue-white screening. Individual colonies were picked and grown on LB(Kan30) plates, cells were collected and minipreps were performed to isolate plasmid DNAs. Purified plasmids were digested with EcoR1 and fragments were resolved on 1% agarose gels to identify plasmids containing inserts. Plasmids containing inserts were sequenced.

**Degenerate PCR: Round 2**

During this project Anctil et al. (2008) published a description of four orphan GPCRs, similar to members of the Class A (rhodopsin) family of receptors, from *Acropora millepora*. These sequences, and the GPCRs I cloned from *M. faveolata*, contained several highly conserved regions that provided adequate templates for degenerate primer design.

Three of four *A. millepora* GPCRs showed a high degree of similarity. Alignment (ClustaW) of these proteins identified highly conserved regions (YAFVFA and NPFVYAW) at approximate positions 170 and 285, respectively (Figure 3.1). Degenerate primers (170 - Fwd: 5’-TAYGCNTTYGTNTTYGCNCA-3’; Rev: 5’-TGNGCRAA NACRAANGCRTA-3’) and (285 – Fwd: 5’-AAYCCNTTYGTNTAY GCNTGG-3’;
Rev: 5’-CCANGCRTANACRAANGGRTT-3’) were designed for each of these regions.

Alignment of *M. faveolata* GPCRs (Figure 3.2) also identified a highly conserved region AIS(I/L)DRY that was not found in the *A. millepora* proteins. Forward (5’-GCCATAAGCHTNGAYMGNTAC-3’) and reverse (5’-GTANGKRTCNADGCTTATGGC-3’) primers were designed for this region. PCR reaction and cycling conditions were the same as those used in *Degenerate PCR: Round 1* (above).
Screening of larval transcriptome databases

In 2008, an open access, coral EST database (http://sequoia.ucmerced.edu/SymBioSys/index.php) was launched. Included in the database were the larval transcriptome of the Indo-Pacific coral *A. millepora*, larval ESTs for three Caribbean species corals *A. palmata*, *M. faveolata* and *M. faveolata* and ESTs from adult *P. astreoides* (for a summary of database contents see Table 3.2). These databases were blasted (tblastn) with full and partial (putative opsin, 7th trans-membrane domains) invertebrate (e.g., squid) opsin protein sequences. Blast hits were then manually screened and opsin-like sequences that did not contain the putative opsin 7th TM, retinal-binding domain were excluded from further analysis. This screening process produced five opsin-like singletons all from the *A. millepora* larval transcriptome (Figure 3.3).

Table 3.2. Summary of coral sequences available for screening in the SymBioSys EST database.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>ESTs</th>
<th>Contigs</th>
<th>Singletons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acropora millepora</td>
<td>larvae</td>
<td>99455</td>
<td>42208</td>
<td>57247</td>
</tr>
<tr>
<td>Acropora palmata</td>
<td>mixed</td>
<td>56683</td>
<td>6793</td>
<td>7854</td>
</tr>
<tr>
<td>Montastrea faveolata</td>
<td>mixed</td>
<td>32391</td>
<td>5084</td>
<td>6527</td>
</tr>
<tr>
<td>Montastrea annularis</td>
<td>larvae</td>
<td>4184</td>
<td>310</td>
<td>2078</td>
</tr>
<tr>
<td>Porites astreoides</td>
<td>adult</td>
<td>12863</td>
<td>2030</td>
<td>4651</td>
</tr>
</tbody>
</table>
Figure 3.2. Five opsin-like ESTs from the larval Acropora millepora transcriptome, identified by screening of the SymBioSys database. The top blast hit (blastx of the Genbank nr sequence database) is shown below each transcript.

Amplification & cloning of coral opsin-like transcripts

Gene-specific PCR primers were developed for each of the A. millepora ESTs (Table 3.3) and used to amplify A. palmata larval cDNA. Isolation of A. palmata total RNA and cDNA synthesis was performed as described above. Internal regions of the A.
palmata transcripts were amplified using gene-specific primer sets, sequenced and aligned (ClustalW) with A. millepora ESTs in order to confirm that primers were

Table 3.3. Gene-specific primers designed against Acropora millepora opsin-like ESTs and used to amplify internal PCR products from A. palmata larval cDNA

<table>
<thead>
<tr>
<th>Primer name</th>
<th>EST size</th>
<th>Primer Sequence</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amill_23454_fwd</td>
<td>256 bp</td>
<td>5'-TATGGGGTGCTCCATTGTATGT-3' 5'-GAGAGGAAGAAGGCAAGAC-3'</td>
<td>150 bp</td>
</tr>
<tr>
<td>Amill_23454_rev</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amill_23452_fwd</td>
<td>187 bp</td>
<td>5'-AAGGAGCAATGGGGAAGACA-3' 5'-GCCTCGATCAAGGAATCGA-3'</td>
<td>156 bp</td>
</tr>
<tr>
<td>Amill_23452_rev</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amill_7493_fwd</td>
<td>367 bp</td>
<td>5'-AGATGGCCCTATTGGAGATGA-3' 5'-GGATGTACGCTCAGATGA-3'</td>
<td>160 bp</td>
</tr>
<tr>
<td>Amill_7493_rev</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amill_7494_fwd</td>
<td>226 bp</td>
<td>5'-AGGGAAACACGTCCTGTCTAC-3' 5'-TAGGCCACAAATGAGAGGA-3'</td>
<td>170 bp</td>
</tr>
<tr>
<td>Amill_7494_rev</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amill_1711_fwd</td>
<td>235 bp</td>
<td>5'-CGAAGAATGAAACACAGGCA-3' 5'-GCAAGAAGAAATGCCATTACCT-3'</td>
<td>325 bp</td>
</tr>
<tr>
<td>Amill_1711_rev</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

amplified the targeted gene products. New A. palmata-specific primers were designed from internal regions. The 3’ ends were amplified using a classic 3’ RACE protocol (after Scotto-Lavino et al. 2006). 5’ ends were amplified using a SMART RACE kit (Clontech), as described above. PCR products were resolved on a 1% agarose gel. Bands were excised from the gel, filtered through siliconized glass wool, precipitated with isopropanol, re-suspended in TE and either directly sequenced (Genewiz Inc) or cloned. When cloning was necessary, amplified DNAs were ligated with PCR2.1 TOPO cloning vector (Invitrogen) using T4 DNA ligase. E.coli (Top10 cells; Invitrogen) were transfected by electroporation. Cells were streaked and grown on LB(Kan30) plates, and
subsequently purified. Cloned PCR products were sequenced and contigs were assembled using Seqbuilder software (Lasergene V7). Once full-length cDNAs were assembled, gene-specific primers (Table 3.4) were designed to regions external to the ORF and the full-length cDNAs were amplified by PCR and cloned as above.

Table 3.4. Gene-specific PCR primers used to amplify full-length Acropsin cDNAs from *A. palmata* larval cDNA

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP1_51_fwd</td>
<td>5'-TGGAGTGTTGGTGGTTTTTCA-3'</td>
<td>1.219 kb</td>
</tr>
<tr>
<td>OP1_1270_rev</td>
<td>5'-GCATTGGTCAACCATAATTGTG-3'</td>
<td></td>
</tr>
<tr>
<td>NEW_21_fwd</td>
<td>5'-AGTGGTATCAACCGAGCAA-3'</td>
<td>1.326 kb</td>
</tr>
<tr>
<td>NEW_1347_rev</td>
<td>5'-CATGAGTCAACAACGGATCG-3'</td>
<td></td>
</tr>
<tr>
<td>OP3_FULL_fwd</td>
<td>5'-GTCGGAGGGGAGAGTGA-AA-3'</td>
<td>972bp</td>
</tr>
<tr>
<td>OP3_FULL_rev</td>
<td>5'-GCCCTTAGCTACCGTGAGG-3'</td>
<td></td>
</tr>
</tbody>
</table>

OP1_51_fwd/1270_rev = Acropsin 1; New_21_fwd/1347_rev = Acropsin 2; OP3_FULL_fwd/rev = Acropsin 3

All sequences (whether cloned or directly sequenced from PCR products) were analyzed by blastx (against the Genbank nr sequence databases) in order to determine homology with known proteins. Sequence processing and editing was done using the Lasergene V7 software package. Inferred amino acid sequences were determined by translation of cDNAs using Editseq. Protein alignments were constructed using Megalign (ClustalW, slow/accurate using a the Gonnet protein weight matrix).

Predictions of protein topology (transmembrane domains and loop regions) were based on Kyte Doolittle hydrophobicity analysis (Kyte and Doolittle 1982; window=19 amino acids) and Emini surface probability (Emini et al. 1985) analysis using the Lasergene
(DNASTAR) Protean module V8. Predictions were then manually adjusted based on the position of conserved functional domains known from x-ray crystallography of squid (Shimaura et al. 2008) and bovine opsins (reviewed by Palczewski et al. 2006). Two-dimensional models of were created using TOPO2, Transmembrane protein display software (Johns S.J.; http://www.sacs.ucsf.edu/TOPO2/). The model was created using TOPO2, Transmembrane protein display software (Johns S.J., TOPO2, Transmembrane protein display software, http://www.sacs.ucsf.edu/TOPO2/) based on predictions of transmembrane domains, and additional annotations and decorations were added manually.

**Results**

*Degenerate PCR: Round 1*

Attempts to amplify internal regions (~250bp products) of opsin-like DNA from gDNA of adult *Porites astreoides, Acropora palmata, Diploria strigosa*, and *Montastrea faveolata* and larval cDNA from *P. astreoides*, *A. palmata*, and *M. faveolata*, using the Op-Helix and Op-Loop1 primers failed to produce PCR products bearing any similarity to opsin. While PCR products of approximately the correct size (200-300bp) were amplified, blast analysis (blastx) of these sequences against the Genbank databases never revealed any similarity to opsins or other GPCRs. In some cases these products were similar to other genes, but more often, blasts of these sequences did produce any significant hits.
**Amplification and cloning of M. faveolata GPCRs**

Internal, standard PCR with gene-specific primers designed against *M. faveolata* opsin-like ESTs (3738998 and 3739163) amplified products of the expected size. Sequencing confirmed that the products represented the targeted transcripts. 3’ and 5’RACE PCR successfully amplified products that overlapped with the internal regions of the cDNAs. Contig assembly resulted in full-length cDNAs with open reading frames (ORFs) of 1.074kb and 912bp for Mfav_EST_3738998 and Mfav_EST_3739163, respectively. Gene-specific PCR primers (Table 3.1) successfully amplified the complete ORFs of each opsin-like cDNA, and these products were successfully cloned.

The cloned *M. faveolata* opsin-like cDNAs encode 358aa (transcript 3738998) and 304aa (transcript 3739163) proteins (Figure 3.3). Blast analysis (blastp of the Genbank nr protein database) revealed that these proteins are 7-pass trans-membrane proteins belonging to the Class A (rhodopsin family) of GPCRs and most similar to trace amine, beta adrenergic, melanocortin, and octopamine receptors (Figure 3.4). Alignment of these sequences with squid (*Loligo forbesi*) rhodopsin demonstrates that, while they contain the conserved functional residues that are diagnostic of the rhodopsin family of GPCRs, they lack the lysine (K) residue in the 7th trans-membrane domain that is essential for binding the chromophore 11-cis-retinal, and diagnostic of opsins (Figure 3.4).
Figure 3.3. Inferred amino acid sequences of two *Montastrea faveolata* GPCRs corresponding to ORFs of ESTs 3738998 and 3739163. Blastp analysis of the proteins indicates that these are putative GPCRs and members of the rhodopsin family of receptors. While they do not share strong homology with other known GPCRs they are most similar to trace adenosine, trace amine, melanocortin and octopamine receptors.
**Figure 3.4.** Alignment of the two cloned *Montastrea faveolata* GPCRs with *A. millepora* orphan GPCRs and squid rhodopsin. The centers of trans-membrane domains are indicated above the aligned sequences, boxes do not accurately represent lengths of these regions. Residues highlighted in green represent amino acids that are conserved among members of the rhodopsin family of GPCRs, indicating that these proteins are all members of the rhodopsin family of receptors. Residues in red indicate a conserved region (part of the D/ERY motif) that is highly conserved among members of the rhodopsin family but absent in *A. millepora* receptors. The purple lysine (K) residue highlighted in the Squid (*Loligo forbesi*: CAA40108) rhodopsin sequence but absent from the coral GPCRs, is the retinal-binding lysine residue found in the 7th trans-membrane domain of opsins. The double cystine (CC) residues highlighted in blue are typically conserved among members of the rhodopsin family of receptors. These are palmitoylated and function by anchoring the protein in the cell membrane. While these occur in the *M. faveolata* receptors and squid rhodopsin, they are absent from three of the four *A. millepora* receptors.
Degenerate PCR: Round 2

Degenerate PCR primer pairs, designed against conserved regions of *A. millepora* orphan GPCRs (Amill_170, Amill_285) and against a highly conserved DRY (AIS(I/L)DRY) motif present in two *M. faveolata* GPCRs, were used to successfully amplify, sequence and clone partial, opsin-like transcripts, including two different transcript from *A. palmata*, one from *Diploria strigosa* and two from *Favia fragum* larval cDNA. Amino acid sequences inferred from these transcripts resemble *A. millepora* and or *M. faveolata* GPCRs. ClustalW alignments indicates a high degree of similarity among sequences, and all contain conserved Class A GPCR residues, however, none of these possess the seventh trans-membrane lysine that is diagnostic of opsin (Figure 3.5).

**Figure 3.5.** Alignment (ClustalW) of amino acid sequences inferred from partial, coral larval cDNAs, amplified using degenerate PCR primers, with known coral GPCRs and squid rhodopsin. The partial larval cDNAs align well and show a high degree of similarity to full-length coral GPCRs. All contain residues that are conserved among the rhodopsin class of receptors (highlighted in green), but all are lacking the 7th trans-membrane lysine (K; circled in red).
**Amplification and cloning of coral opsin-like transcripts**

Gene-specific PCR primers designed from the five opsin-like *A. millepora* ESTs successfully worked across species to amplify equivalent transcripts from *A. palmata* larval cDNA. Alignments of sequenced PCR products with *A. millepora* ESTs confirmed that I had amplified the same gene products in *A. palmata*. Two of these products, those amplified with primers to *A. millepora* Seqindexes 7943 and 24352, overlapped to form a single contig. 3’ and 5’ RACE PCR was used to successfully amplify of 3’ and 5’ cDNAs for three of the four unique transcripts, and gene-specific primers, were used to amplify the entire coding region of all three, which from here on I will refer to as Acropsin 1, 2, and 3.

The coding regions of each Acropsin cDNA were successfully cloned into TA vectors and sequenced. Acropsin 1 contains a 1.073 kb ORF that encodes a 343aa protein with a predicted molecular weight of 38.7kDa (Figure 3.6). Acropsin 2 contains a 1.053kb ORF that encodes a 349aa protein with a predicted molecular weight of 39.5kDa (Figure 3.7) and Acropsin three (a full-length cDNA of 972bp) contains 387bp ORF that encodes a 129aa protein with a predicted molecular weight of 14.5kDa (Figure 3.8).

Protein blasts (blastp) against the Genbank non-redundant (nr) protein database indicate that all three Acropsins are opsins. Acropsin 1 is most similar to melanopsin, while Acropsin 2 and 3 show greatest homology to cephalopod (e.g., squid and octopus) rod opsins (Table 3.5).

Alignment of Acropsins 1-3 with human long-wavelength sensitive opsin and squid rhodopsin demonstrates significant conservation (identity and semi-conservative substitution) of key functional residues and the putative opsin domain (Figure 3.9). The
7th trans-membrane domains, containing the retinal-binding lysine residue, are particularly well conserved.

Hydrophobicity (Kyte Doolittle) and surface probability (Emini) analysis accurately predicted secondary structures of both bovine and squid rhodopsins as confirmed by comparison with x-ray crystallography data for these proteins. 2D models indicate the 7-transmembrane structures and conserved functional residues (Figure 3.10-11). Hydrophobicity and surface probability analysis predicted similar structures for Acropsin 1 and 2 (Figure 3.12-13). Key functional residues (counterions, 7tm lysine, di-sulfide bridge-forming cysteines) are also present in the coral opsins. While Acropsin 3 is too small to be a seven transmembrane protein, analysis suggests that it is a partial opsin, containing two transmembrane domains, one of which is a putative opsin domain (Figure 3.14).

**Table 3.5.** Summary of top three blast hits retrieved with Acropsin 1 and 2.

<table>
<thead>
<tr>
<th>Hit</th>
<th>E Value</th>
<th>Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acropsin 1 novel protein similar to opsin 4 (melanopsin) <em>Danio rerio</em></td>
<td>3.0e-3</td>
<td>34%</td>
</tr>
<tr>
<td>parietopsin <em>Xenopus (Silurana) tropicalis</em></td>
<td>1e-36</td>
<td>32%</td>
</tr>
<tr>
<td>melanopsin <em>Coryphaenoides armatus</em></td>
<td>2e-35</td>
<td>33%</td>
</tr>
<tr>
<td>Acropsin 2 rhodopsin <em>Octopus dofleini</em></td>
<td>7e-27</td>
<td>29%</td>
</tr>
<tr>
<td>rhodopsin <em>Sepia officinalis</em></td>
<td>2e-26</td>
<td>32%</td>
</tr>
<tr>
<td>opsin <em>Loligo subulata</em></td>
<td>2e-25</td>
<td>31%</td>
</tr>
</tbody>
</table>
Figure 3.6. Coding region (cDNA) and inferred amino acid sequence of Acropsin 1. The transcript results in a 343 amino acid protein with a predicted molecular weight of 38.7 kDa.
Figure 3.7. Coding region (cDNA) and inferred amino acid sequence of Acropsin 2.
The transcript results in a 349 amino acid protein with a predicted molecular weight of 39.5 kDa.
**Acropsin 3** – full-length cDNA (972 bp)

cagtgtactcaacgagatcagccggtgtctgtgctgtgctgtgctgagggagagtgaaact 60
gaaatctcggttttatcacacagccgaagccagagctgtaacacacccatgaaagcctgg 120
aaagctctagctctgcaaatctctacagatagctgcacctattatctacttctgcgac 180
aaaccaaaacttcagaatcagataaaaccggaaatctctgctggctagctgcgatctgta 240
atatattcggtacacttatggctgttgtgatgtaaccgaactgtgctgttctcttata 300
tcgttctattgaacttattaagttcaacttaatgtgatccacacttatgctggcagagtaat 360
ttagactactctgtcctgtctcttctttgtcctttgtctgtaacagacactttcgcgca 420
gatgtctgtcaggaagagactcgtgaatcaaatattgaggagagagacagtggcagatgaaaact 480
cccggcctgtacagcagctgctctccattactccgtctctacgctgctctcgcctcctcg 540
ctcgtctcgttcctcctaattgcagagccagagaacaagacgcttaagcagactctgt 600
cccggcctttatctgctgtaaatggactagttataatcattacgtaaatgtctactgtcagttaac 660
agggcctctcgcagcacttggtctgttagtctgctctactgactgactggtctgctctcgcctg 720
ctcgtctcgttcctcctaattgcagagccagagaacaagacgcttaagcagactctgt 780
gttcagatgtaccatctctcaattgctctctctgtctctctgcctctctgcctctctgcct 840
ttcgaccagactctctcctctacagtggatagctgctctctgcctctctgcctctctgcct 900
cttggagctgtctgtgcyyagagcactctcagtggatagctgctctctgcctctctgcctctctgcct 960
aaaaaaaaaa 972

**Acropsin 3** – Inferred amino acid sequence (129aa; mw=14.5kDa)

MKLLRLTAITAFMLSWSYPMLSLSIFRGNSVSTEGAEVPALMAKASVYNIYVT 60
VMRRFRRRTLHRHIVSCMCRLSLSFVWTPHGEQETKRRVTSTVTSTTVSIPAPENFGFPEI 120
LVSLHQVPF. 129

**Figure 3.8.** Full-length cDNA and inferred amino acid of Acropsin 3. The 972 bp transcript contains a 390bp open reading frame (position 471-861; capital letters), resulting in a 129amino acid, 2-pass trans-membrane protein.

**Figure 3.9.** Partial alignment of Acropsins with squid, and human long-wavelength sensitive, opsin proteins. Sequences were aligned by ClustalW (Slow/accurate; Gonnet protein weight matrix) using Lasergene (DNASTAR) Megaline module. Shown region of the alignment containing the 6th and 7th transmembrane domains. Conserved, functional opsin residues are highlighted in green. Retinal-binding lysine residues occur (highlighted in red) in the same position within the 7th transmembrane domains of each sequence.
Figure 3.10. Two-dimensional structural model of bovine rhodopsin. The model represents the seven transmembrane structure of bovine rhodopsin. N-terminal tail, cytoplasmic loops (CL1-3), extracellular loops (EL1-3), and C-terminal tail are labeled. EL2 re-enters the chromophore pocket of the protein and is thought to form a “plug” allowing conserved cysteine residues (shaded green) to form a disulfide bridge. Two highly conserved, glutamic acids (E113, E151), are shaded red. Orange residues indicate functional motifs (E/DRY in TM3; NPxxY in TM7) that are highly conserved among GPCRs of the Class A family of receptors, and thought to play a role in G protein interactions. The purple residue (K272) indicates the highly conserved retinal-binding lysine residue.
Figure 3.11. Two-dimensional structural model of squid rhodopsin. The model represents the seven transmembrane structure of Acropsin 1. N-terminal tail, cytoplasmic loops (CL1-3), extracellular loops (EL1-3), and C-terminal tail are labeled. EL2 re-enters the chromophore pocket of the protein and is thought to form a “plug” allowing conserved cysteine residues (shaded green) to form a disulfide bridge. The highly conserved invertebrate counterion, glutamic acid (E180) is shaded red. Orange residues indicate functional motifs (E/DRY in TM3; NPxxY in TM7) that are highly conserved among GPCRs of the Class A family of receptors, and thought to play a role in G protein interactions. The purple residue (K305) indicates the highly conserved retinal-binding lysine residue. TM5 and TM6 of squid rhodopsin are each >40 amino acids in length. While this model represents this as broader transmembrane domains, a three dimensional model (lower left; from Shimamura et al. 2008), based on the crystal structure of squid rhodopsin shows that these transmembrane helices extend into the cytoplasm.
Figure 3.12. Two-dimensional structural model of Acropsin 1. The model represents the seven transmembrane structure of Acropsin 1. N-terminal tail, cytoplasmic loops (CL1-3), extracellular loops (EL1-3), and C-terminal tail are labeled. EL2 re-enters the chromophore pocket of the protein and is thought to form a “plug” allowing conserved cysteine residues (shaded green) to form a disulfide bridge. The highly conserved counterion, glutamic acid (E151) is shaded red. Orange residues indicate functional motifs (E/DRY in TM3; NPxxY in TM7) that are highly conserved among GPCRs of the Class A family of receptors, and thought to play a role in G protein interactions. The purple residue (K272) indicates the highly conserved retinal-binding lysine residue.
**Figure 3.13.** Two-dimensional structural model of Acropsin 2. The model represents the seven transmembrane structure of Acropsin 1. N-terminal tail, cytoplasmic loops (CL1-3), extracellular loops (EL1-3), and C-terminal tail are labeled. EL2 re-enters the chromophore pocket of the protein and is thought to form a “plug” allowing conserved cysteine residues (shaded green) to form a disulfide bridge. The highly conserved counterion, glutamic acid (E151) is shaded red. Orange conserved residues of functional motifs (E/DRY in TM3; NPxxY in TM7) that are highly conserved among GPCRs of the Class A family of receptors, and thought to play a role in G protein interactions. In this protein cysteines (C; yellow) are found in place of tyrosine (Y) in both motifs. The purple residue (K272) indicates the highly conserved retinal-binding lysine residue.
Figure 3.14. Two-dimensional structural model of Acropsin 3. The model represents the two predicted transmembrane domains and single extracellular loop. Even though this protein has only two membrane domains, the second transmembrane domain contains a putative opsin functional motif - the NPxxY (Orange) functional motif found in the TM7s of Class A GPCRs and a lysine (K; purple) residue (K272).

Discussion

Degenerate PCR: Round 1

Analysis the coral GPCRs and Acropsins provides insight into why original attempts to amplify coral opsin-like cDNAs likely failed and why Cnidarian opsins remained elusive for years. The more ancestral coral opsins lack strict conservation of functional motifs that are highly conserved among model invertebrates. They are actually more closely aligned with vertebrate opsins, but even here we see sequence
divergence at some of the sites that would otherwise serve as good templates for
degenerate PCR primer design. While they contain the key functional residues of Class
A GPCRs and have highly conserved opsin domains, this opsin domain, the NPxxY motif
is the only region that is both conserved and provides an adequate starting template for
primer design. The GNGVVIY (in TM1) and DRYNVI (in CL2) motifs, which served as
the sites for degenerate primer design are highly conserved in invertebrate opsins, but are
largely absent from coral sequences. The corresponding motifs in coral GPCRs vary
from 33% identity (DRxxxx (A. millepora GPCRs), xRYxxx (Acropsin 1) and xRxxxI
(Acropsin 1)) to 50% identity (DRYxxx (M. faveolata GPCRs)) with DRYVVI motif.
and the coral GPCRs and Acropsins possess only ERxxxx, or in the case of the two
Montastrea GPCRs, DRYxxx. Similar identity is observed between the other motif
(GNGVVIY) and the coral GPCRs.

Another problem of the degenerate PCR approach has likely been the position of
the conserved domains, and flexibility of GPCR loops. Most of the conservation occurs
in membrane helices, and large regions of flexible (non-conserved) sequence can exist
between conserved motifs. The GNGVVIY and DRYNVI primers were designed to
regions that would amplify only 250nt encoding a flexible region of the protein. Even if
these successfully amplified GPCR or opsin cDNA, it would have been difficult to
identity of these amplified sequences as GPCRs by blast analysis or alignment.

*M. faveolata GPCRs*

Sequence analysis indicates that the two full-length *M. faveolata* GPCRs cloned
from larval cDNA are members of the Class A (rhodopsin) receptor family, but are
unlikely to function as opsins. Blast analysis identified these as putative 7tm receptors of the rhodopsin family. While they possess highly conserved Class A family, functional domains, DRY (in TM2) and NPxxY (in TM7), these proteins lack the 7th trans-membrane lysine residue, required for the binding of retinal and the formation of functional opsins (Figure 3.4).

The possession of DRY motif by these two receptors is significant. The E/DRY motif is highly conserved among members of the Class A GPCR family. These residues have been proposed to function either in G protein coupling/recognition or by maintaining GPCRs in their ground state (preventing constitutive activity) or in the recognition of G proteins (Rovati et al. 2007). While the DRY motif occurs in the two *M. faveolata* GPCRs it is absent from the four orphan, *A. millepora* GPCRs (Antcil et al. 2008), and may provide insight into the evolution of the Class A GPCR family.

Blast analysis (blastp) of the Genbank nr protein database indicates that these proteins have approximately equal similarity to trace amine, adenosine, beta adrenergic, melanocortin and octopamine receptors. The diverse range of functions represented by these blast hits, makes inference of the *M. faveolata* GPCR functions difficult. Biochemical analysis will be required to demonstrate the function of these receptors.

**Degenerate PCR: Round II**

The cloning of the two *M. faveolata* GPCRs and four orphan GPCRs from *A. millepora* (Antcil et al. 2008), enabled the design of new, more effective degenerate PCR primers. Using these primers, I was able to amplify and clone partial cDNAs from larval cDNA of several coral species (*Acropora palmata, Diploria strigosa*, and *Favia fragum*;
Figure 3.5). The placement of the primers, one at the NPxxY motif in TM7 and two others upstream at AISIDRY (CloopII) and YAFVFA (TM6) motifs and, resulted in amplification of an internal region GPCR sequences containing the TM7 domain. While partial larval cDNAs aligned well with other coral GPCRs and possess diagnostic features of Class A receptors, none of these sequences possessed retinal binding lysine residues in TM7 (Figure 3.5) and are unlikely to function as opsins. For this reason they will not be considered further here.

Since degenerate PCR failed to yield transcripts containing a retinal binding, I was forced to revise my approach. Fortunately, a larval EST library, developed for *A. millepora* enabled the identification of several transcripts encoding putative opsin, 7th transmembrane domains. Working from these transcripts, I was able to clone the Acropsins.

**Coral Opsins: Acropsins 1-3**

Sequence analysis of Acropsins 1 and 2 provides several compelling lines of evidence that these gene products form functional opsins:

Blast analysis (blastp of the Genbank nr protein database) predicts that both Acropsins are putative 7tm receptors belonging to the rhodopsin family, and both proteins contain the putative opsin domain (7th TM, retinal binding motif). These proteins are also most similar to putative opsins. The top blast hit (Table 3.5) and 24 of the top 100 blast hits retrieved by Acropsin 1 were melanopsins. The remainder of the top 100 blast hits were also opsins, mostly other non-visual opsin types (parietopsin, teleost multiple tissue opsin, vertebrate ancient opsin and pinopsin). The top four (squid and
octopus rhodopsins; Table 3.5) and 30 of the top 100 blast hits returned by Acropsin 2 were rhodopsins. Like Acropsin 1, all blast hits were opsins, and in addition to rod opsins, non-visual opsins (e.g., melanopsins and peropsins) were common. These results indicate that both Acropsins are opsins, but determination of the opsin subtype (i.e. biochemistry or the G protein that they couple) is not possible given the diverse subfamilies of opsins represented among the blast hits.

Sequence alignments and two-dimensional models demonstrate that Acropsin 1 and 2 possess functional motifs and residues critical to opsin formation and function. Most importantly, Acropsin 1 and 2 both contain highly conserved 7TM domains (Figures 3.9, 3.12-13), including a retinal binding lysine (K) residue. In addition, they both possess at least a partial E/DRY motif in the same location, (cytoplasmic end of TM3) as in other opsin (e.g., squid and bovine; Figures 3.10-11). An ERY motif is found in Acropsin 1 but Acropsin 2 contains ERC in this position. The substitution of cysteine for tyrosine in Acropsin 2 may influence the G protein recognized and coupled by this receptor (Rovati et al. 2007). This is a semi-conservative substitution in regard to amino acid functional group (one polar amino acid for another), but the substitution also introduces a sulfhydryl (SH) and could alter the function of this motif. A similar substitution (C for Y of the NPxxY motif) is observed in the otherwise well-conserved 7TM domain of this receptors. Y306, the tyrosine in TM7 of bovine rhodopsin is known to form hydrogen bond with N73 in TM2 (Palczewski et al. 2006) and these motifs are involved in linking clusters of water molecules that occur within the transmembrane region of opsins (Okada et al. 2002). The substitution of cysteines in these motifs may allow di-sulfide bridge formation between the extracellular surfaces of TM3 and TM7.
X-ray crystallography, biochemical analysis and point mutation studies will be necessary in order to determine the effect that these substitutions have upon receptor 3D structure and function.

The Acropsins also possess other key functional residues - disulfide bridge-forming cysteine residues and a highly conserved glutamic acid counterion (E180 in squid rhodopsin; Figure 3.11). Two cysteines, one located at the extracellular end of TM3 and one in EL2 are highly conserved among opsins (e.g., squid and bovine; Figures 3.10-11). From x-ray crystallography of bovine rhodopsin it is known that EL2 forms a hairpin loop (fold back upon itself) and enters the chromophore pocket of rhodopsin. The re-entry of this loop has two important consequences: 1) it brings together the cysteine residues allowing them to form a di-sulfide bridge; 2) it brings a conserved, negatively charged glutamic acid (E181 in bovine rhodopsin; E180 in squid rhodopsin; Figures 3.10-11) into the chromophore pocket and in contact with retinal, where it can balance the otherwise unstable positive charge (i.e. serve as a counterion) created by the protonated Schiff-base linkage of retinal to lysine (reviewed by Filipek et al. 2003). This glutamic acid serves as the counterion in invertebrate opsins. While E181 is also present in bovine rhodopsin, the role of counterion has been assumed by a glutamic acid in TM3 (E113 in bovine) in vertebrates (Terakita et al. 2004). Acropsins 1 and 2 possess conserved cysteines and the more ancestral, invertebrate, glutamic acid counterion (E151 in Acropsin 1 and 2; Figures 3.12-13), which should allow them to fold and behave in a manner similar to other invertebrate opsins.

Another highly conserved motif is missing from the Acropsin proteins - the double cysteines found in the C-terminal tail of rod opsins (e.g., squid and bovine
rhodopsins; Figures 3.10-11). The crystal structure of bovine rhodopsin demonstrates that these cysteines are anchored in the plasma membrane by palmitoyl substituents. The Acropsins all contain a single cysteine residue in their c-terminal tails but lack the double cysteine. Although conserved in rod opsins, the C-C motif is not an essential element of cone opsins. For example, vertebrate cone opsins often possess single or no cysteines in their c-terminal tails and mass spectroscopy has shown that these single cysteines may or may or may not be palmitoylated (Ablonczy et al. 2006).

Acropsin 1 and 2 (Figures 3.11-12) have dramatically reduced N-terminal tails compared to those found in squid and bovine rhodopsin. Acropsin 1 is actually lacks a N-terminal tail altogether and the tail of Acropsin 2 contains only 11 amino acids and one potential N-glycosylation (N9) while squid (Figure 3.10) and bovine rhodopsin (3.11) tails contain 30 and 24 amino acids, respectively, and 3 glycosylation sites. The N-terminal tail of rhodopsin forms a globular domain and is involved in “plugging” the chromophore pocket (Placzewski et al. 2006). The reduced tail of Acropsin 2 may limit its involvement as part of the chromophore pocket “plug”. This may force Acropsin 2, and Acropsin 1, without a N-terminal tail, to compensate for the absence of tail function through other protein modifications.

The most intriguing result from efforts to clone coral opsins is the discovery of what appears to be a retinal-binding two-pass transmembrane protein – Acropsin 3. The full-length cDNA, is consistently amplified from larval cDNA using gene-specific primers; contains a poly-A tail, 3’ and 5’ untranslated regions (UTRs), and a small open reading frame encoding Acropsin 3 (Figure 3.14). It is unlikely that this protein recognizes and binds a G protein since it lacks cytoplasmic loops typically involved in G
protein interaction, but it is possible since G protein coupling is largely determined by the helical region (helix 8) found between the TM7 and the C-C motif of the cytoplasmic tail (Palczewski et al. 2006). This protein could potentially form a functional dimer with other opsins or could serve as a retinal binding/cellular transport protein by imbedding in lysosomes involved in the intracellular transport of retinal in photoreceptors or adjacent cells.

The results described here provide evidence that Acropsins 1 and 2 are similar to and possess the elements required for the formation of functional photopigments – 7tm structure, retinal binding sites, E/DRY and NPxxY motifs, conserved cysteines in their EL2 and TM3, and glutamate counterions. Future research, biochemistry and functional expression studies will be needed to confirm whether these coral proteins form functional opsins, the G proteins that each couples and their spectral sensitivities.
CHAPTER 4

CONFIRMATION OF OPSIN EXPRESSION & IDENTIFICATION OF OPSIN-POSITIVE CELLS IN CORAL LARVAE: THE DEVELOPMENT OF CORAL OPSIN ANTIBODIES, WESTERN BLOTS AND IMMUNOLOCALIZATION

Rationale

Since visual organs are not present in corals, the photosensitivity of coral larvae, demonstrated here (see Chapter II) and observed in previous studies (see Chapter I), must result from extraocular photoreceptors. These photoreceptors may be solitary or occur in clusters, and be scattered or localized to particular regions or structures of an organism. The photopigments responsible for extra-ocular sensitivity were traditionally considered to exist as evolutionary precursors to ciliary or rhabdomeric opsins found in light-sensing organs, and were generally considered to occur in similar (ciliary or rhabdomeric) cell types. However, the recent discovery of diverse opsin subfamilies, with broad taxonomic distribution, and expressed in diverse range of cell types, have forced a revision of thinking about photosensitivity.

Cell morphology, ultrastructure (e.g., cilia, microvilli-structural features that determine the cell type) and the organization of supporting cells (e.g., pigment cells) or structures (e.g., oil globules) can suggest the presence of photopigments, but often these clues are not present and, even of they are, the identification and localization of photopigments relies on molecular and immunohistological methods.

This chapter describes: 1) the development of antibodies against two coral opsins, Acropsin I & II (see Chapter III); 2) immunological methods (western blots and immunohistology), and results from these, confirming the expression of Acropsin 1 and
and localizing these in coral larvae; 3) the results of transmission electron microscopic analysis of larval cell ultrastructure that may provide clues regarding the function of larval phhotoreceptors.

**Materials and Methods**

*Antibody development*

*Peptide design and synthesis.* Antibodies against Acropsin 1 and 2 were developed using a synthetic peptide approach. Two regions of high antigenicity, corresponding to the third cytoplasmic loop and cytoplasmic tail regions of each Acropsin 1 and 2 were identified. Selection of antigenic regions was accomplished following the guidelines advised by J. Stewart (Dept. of Biochemistry, U. of Colorado, Denver; co-author of Solid Phase Peptide Synthesis; posted on the ABRF bulletin board) and those available on the Genscript website (www.genscript.com). Secondary structure prediction was done for each opsin using the Chou-Fasman program to identify turns. Hydrophobicity (Hopp-Woods hydrophobicity data) was calculated using PROWL (http://prowl.rockefeller.edu/) and ExPASy Molecular Biology Server (http://us.expasy.org/), with a running average of 6 residues, to identify hydrophobic regions that were also predicted to be turns.

Glycosylation sites (N-linked CHO chains, N-x-T or N-x-S; O-linked CHO chains, S-x-x-P or T-x-x-P) were avoided. Cysteines, methionines, and tryptophans (C, M, W) were avoided since these are susceptible to oxidation and side reactions. Attempts were also made avoid multiple or adjacent residues of valine, isoleucine, tyrosine, phenylalanine, tryptophan, leucine, glutamine or threonine (V, I, Y, F, W, L, Q, T) in order to prevent beta sheet formation. The hydrophobic amino acid-tryptophan, leucine, valine,
methionine, phenylalanine and isoleucine (W, L, V, M, F, I)-content of the peptides was kept below 50% and at least one charged amino acid-arginine, glutamine, aspartic acid, lysine (R, Q, D, K) occurred per every 5 AAAs. The selected peptides (Table 4.1) ranged from 11-15 amino acids. Synthesis of the peptides was outsourced (Genscript Corporation; www.genscript.com). Peptides derived from regions of cytoplasmic loop III (internal region) were ordered with modifications (acetylation and amidation) to block the N and C-terminals, respectively, and C-terminal peptides were ordered with acetylation or acetylation and amidation. The specified purity was >70% but the actual purities, as determined by HPLC ranged from 80.2-97.0% (Table 4.1).

**Table 4.1.** Acropsin peptides (amino acid sequences) selected for antibody production.

<table>
<thead>
<tr>
<th>Opsin</th>
<th>Region</th>
<th>Length</th>
<th>Sequence</th>
<th>Modification</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acropsin 1</td>
<td>C-loop III</td>
<td>12aa</td>
<td>GRQSPFTKRTFV</td>
<td>N/C</td>
<td>95.7%</td>
</tr>
<tr>
<td></td>
<td>C-tail</td>
<td>12aa</td>
<td>DNYSNRPENI</td>
<td>N</td>
<td>97.0%</td>
</tr>
<tr>
<td>Acropsin 2</td>
<td>C-loop III</td>
<td>15aa</td>
<td>KNSQITKDGIVKRK</td>
<td>N/C</td>
<td>80.2%</td>
</tr>
<tr>
<td></td>
<td>C-tail</td>
<td>11aa</td>
<td>YNGKTIAFRRS</td>
<td>N/C</td>
<td>83.7%</td>
</tr>
</tbody>
</table>

Synthetic peptides were selected from sequence corresponding to highly antigenic regions of the third cytoplasmic loop (C-loop III) and cytoplasmic tail (C-tail) regions of each Acropsin 1 and 2. Lengths ranged from 11-15aa and purity from 80.2-97.0%. The N-terminal (N) or N&C terminals (N/C) were blocked by acetylation or amidation, respectively.

Test Bleed) using two rabbits for each peptide (2 Acropsins x 2 peptides x 2 rabbits = 8 total) was ordered. Peptides were submitted as lyophilized powder, and were conjugated to keyhole limpet hemocyanin (KLH), and mixed with Complete Freund’s Adjuvant (Incomplete Freund’s Adjuvant for boosts) prior to inoculation. An additional boost was performed prior to collection of the production bleed.

Antibody Screening. Screening of test bleeds was accomplished using glutathione S-transferase (GST) fused with the peptides used for antibody production. In order to create the GST fusion proteins, the region of coral cDNA encoding each peptide was amplified from *A. palmata* cDNA by PCR. Total RNA was isolated and cDNA prepared as above. PCR primers (Table 4.2) used for amplification included restriction sites to enable subsequent cloning of products into a bacterial expression vector. PCR products were run on a 2% agarose gel, excised and purified as above. Approximately 1µg of each product and 1ug of pGEX-2T vector (GST gene fusion system; GE Healthcare) were digested with BamH1/EcoR1. Digestions were carried out by incubation at 37°C for 6 hrs. Following digestion, the pGEX-2T reactions were treated with alkaline phosphatase – 1µl of alkaline phosphatase was added to the reaction mixture, tubes were incubated at 37°C for 30 minutes and then heated to 65°C for 15 minutes. Digestion products were gel purified, bands were excised, purified as above and re-suspended in 10µl of TE pH 8.0. Inserts were ligated to the linearized pGEX-2T vectors using T4 DNA ligase by
Table 4.2. PCR primers used for amplification of peptide-encoding regions of Acropsins cDNA during construction of GST-Acropsin peptide fusion proteins.

<table>
<thead>
<tr>
<th>Opsin</th>
<th>Region</th>
<th>Restriction Site</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acropsin 1</td>
<td>C-LoopIII</td>
<td>BamH1</td>
<td>F: 5'-GGGCACGATCCATAGCTAAAGTGATCTGGGAG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EcoR1</td>
<td>R: 5'-GCCGGAAATTCCGACAAAGGTCTTCTCGTAA-3'</td>
</tr>
<tr>
<td></td>
<td>C-Tail</td>
<td>BamH1</td>
<td>F: 5'-GGGCACGATCCGAAACAGCGACTACGTTCT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EcoR1</td>
<td>R: 5'-GCCGGAAATTCCGATAATTTCCAGGACGTTACG-3'</td>
</tr>
<tr>
<td>Acropsin 2</td>
<td>C-LoopIII</td>
<td>BamH1</td>
<td>F: 5'-GGGCACGATCCAAAAGGACTTGGGGGCAAAAATT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EcoR1</td>
<td>R: 5'-GCCGGAAATTCCATGAGTACCGCTTCTTTATTA-3'</td>
</tr>
<tr>
<td></td>
<td>C-Tail</td>
<td>BamH1</td>
<td>F: 5'-GGGCACGATCCATACATCCAGTCTCCTCAAGA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EcoR1</td>
<td>R: 5'-GCCGGAAATTCCGTCAACACAGCGATCGAACGA-3'</td>
</tr>
</tbody>
</table>

PCR primers used to amplify cDNA encoding the peptides used for antibody production. Peptides were selected from regions of the third cytoplasmic loop (C-LoopIII) or cytoplasmic tail (C-Tail) of each coral opsin (Acropsin 1 & 2). PCR primers contained BamH1 or EcoR1 sites to allow subcloning of the amplified PCR products into a pGEX-2T bacterial expression vector.

overnight incubation at 21°C. Resulting plasmids were used to transform *E. coli* (Top10 cells) by electroporation. Cells were collected in 720µl of LB and incubated with shaking for 1 hr at 37°C. Cultures were plated out on LB-Amp (100µg/ml) and incubated overnight at 37°C. The following day, ten colonies from each plate were picked and streaked on LB-Amp (100µg/ml) and grown overnight at 37°C. Cells were collected and mini-preps were performed.

Collected cells were placed in microcentrifuge tubes containing 200µl of lysis buffer (8% sucrose, 0.5% Triton X-100, 50mM EDTA, 10mM Tris HCl, pH 8.0), mixed 14:1 with 10mg/ml lysozyme in 10mM Tris HCl, pH 8.0. Cells were resuspended by vigorous pipetting and lysed by boiling tubes for 50s. Cellular debris was pelleted by centrifugation at 13,000 rpm for 1hr. Supernatant was transferred to clean tubes, mixed 1:1 with chloroform:isoamyl (24:1) and centrifuged at 13,000 rpm for 5 minutes. This
step was repeated before supernatant was transferred to clean tubes and plasmid DNAs were purified by isopropanol precipitation (as above).

Electrically competent BL21(DE3) cells (Novagen) were chemically transformed with purified plasmid DNAs. For transformation 1 µl of plasmid DNAs were added to 50 µl of cells and tubes were incubated on ice for 30 min. After incubation, cells were heat shocked by incubating tubes at 42°C for 90 s. Tubes were then returned to ice for 3 min and 450 µl of SOC media was added to each tube. Tubes were incubated with shaking for 1 hr at 37°C and plated on LB (Amp100) plates.

After overnight incubation, single colonies from each transformation were used to inoculate 2 ml of LB (Amp100). Cultures were incubated overnight with shaking at 37°C. The following day 200 ml of each starter culture was used to inoculate 500 ml of LB (amp 100). Cultures were grown at 37°C until they reached log phase (OD 0.5-1.0, determined using an Eppendorf Biophotometer. Once all cultures had reached log phase, 500 µl of 1000x IPTG was added to each and cultures were grown at 37°C for an additional 5 h. Cultures were transferred to 500 ml capacity Nalgene centrifuge bottles and cells were pelleted by centrifugation at 4000 rpm for 30 min. Cell pellets were washed once with lysis buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA + 1x protease inhibitor cocktail) and re-suspended in 1 ml of same buffer. Cell suspensions were transferred to clean microcentrifuge tubes and cells were lysed by freezing/thawing. Cellular debris was pelleted by centrifugation at 4°C for 20 min, the supernatants were then transferred to clean tubes and SDS PAGE protein loading buffer was added to each sample.
Affinity purification of Acropsin antibodies. The affinity purification of Acropsin antibodies was achieved by column purification of production bleed antisera using Acropsin peptides covalently linked to AminoLink Coupling Resin (Thermo Scientific). 10 ml Bio-Rad columns were used for purification. Columns were equilibrated with 20 mM sodium phosphate pH 7.5 + 200 mM NaCl and 2 ml of AminoLink resin (50% per volume) were added and allowed to run through the column. The column was then washed with 10x volume of sodium phosphate buffer. One mg of each Acropsin peptide was dissolved in 1 ml of dH2O and diluted 1:4 w/ Coupling Buffer (Sodium Phosphate + Cyanoborohydride). Peptide solutions were added to columns (saving 100µl for analysis of binding efficiency) and columns were incubated at 4°C overnight with gently rocking (inversion). The unbound peptide was run through the column, collected and saved for determination of binding efficiency, the column was then washed with 4 ml of 1 M NaCl and then quenched by running 10 ml of 1 M Tris HCl + 0.05% NaN3 through the column. Columns were washed 3 times with 1x PBS pH 7.4 + 0.05% NaN3 before 1 ml of antiserum was added and columns were place on a rocker at 4°C for 2-3 hours. After incubation, the antiserum was run off and unbound was collected. Columns were washed with an approximately 20x volume of PBS until clean as determined by spectrophotometric determination of the protein content of eluate.

A set of twelve microcentrifuge tubes per column, each containing 25 µl of Tris, was prepared and antibodies were eluted with 4 ml of 0.1 M glycine pH 2.5, collecting 5 drops per tube. Immediately following the collection of eluted antibody, columns were washed with 10x volume of 1x PBS pH 7.4 + 0.05% NaN3, capped and stored at 4°C. The protein content of collected fractions was determined by measuring the absorbance
of fractions relative to a glycine:Tris (4:1) blanking solution. The two samples with the highest protein content were combined for dialysis. For dialysis, antibody solutions were injected into Slide-A-Lyzer® Dialysis Cassettes (Thermo Scientific) using a syringe and cassettes were floated in beakers containing 2L of 1x PBS, covered and incubated overnight at 4°C with gentle stirring. The following day, antibody solutions were removed, aliquotted and stored at -20°C.

**SDS PAGE and western blots**

The expression of proteins (GST-coral peptide fusion proteins and coral opsins) was confirmed by SDS PAGE resolution of proteins followed by coomassie staining or western blotting. For preparation of coral protein samples, fresh or frozen coral larvae and adult tissue was homogenized on ice in lysis buffer containing 50 mM Tris, 100 mM NaCl, 5 mM EDTA, containing protease inhibitor cocktail (Roche Complete Protease Inhibitor Cocktail Tablets) by passing (~10x) the tissue through a 23g syringe needle. Samples were then centrifuged at 14,000rpm for 15 minutes. Supernatant was transferred to a clean microcentrifuge tube and SDS PAGE protein loading buffer (Laemmli buffer) was added to each sample. Samples were aliquotted and frozen at –20°C until use. Proteins were resolved on a 12% (w/v) polyacrylamide gel using using a SE 250 Mighty Small II, basic unit (Hoefer Scientific). Electrophoresis was carried out at constant amperage (30 milliamperes). The resolved proteins were either transferred to nitrocellulose membranes by electrophoretic transfer, for western blotting, or gels were stained with coomassie to estimate protein loading, quality or confirm expression.
Transfer to nitrocellulose membranes was conducted at a constant voltage (300 mV) in a TE 22 Mini Transfer Tank (Hoefer Scientific) containing 0.25% sodium bicarbonate buffer. Unoccupied sites on nitrocellulose membranes were blocked with 5% powdered milk in 1x TBS. Membranes were washed in blocking solution for 1 hr before incubating for 1 hr in primary antibodies, (Acropsin antibodies). Primary antibodies were diluted in 1xTBS containing 1% milk. Membranes were washed with 1xTBS for 60 min and incubated with secondary antibody (horseradish peroxidase-linked anti-rabbit IgG) diluted in 1:3000 in 1xTBS containing 1% milk (for regular detection) or 1:75,000 in 1xTBS containing 5% milk (for SuperSignal detection). After washing (as above) membranes were incubated in ECL Western Blotting Detection Reagent (Amersham Biosciences) or SuperSignal West Femto Substrate (Thermo Scientific) and immediately developed on X-OMAT LS Scientific Imaging Film.

*Immunohistochemistry and imaging of sectioned larvae*

The localization of coral opsins and identification of larval photoreceptors was achieved through immunohistochemistry and fluorescent confocal microscopic imaging of sectioned coral larvae. Larvae were fixed in 10% formaldehyde or 4% paraformaldehyde. Prior to embedding, samples were rinsed with distilled water. Larvae were embedded in 3.5% agar in 1x PBS and 150 micron sections were cut using a Lancer® Vibratome, Series 1000 Sectioning System. Sections were permeabilized in PBS containing 0.15% TWEEN and 0.15% TRITON for 20 minutes, incubated in BTGN (5% goat serum; 2% BSA; 0.1% TWEEN; 1x PBS) for 45 minutes to block non-specific binding, washed in PBS + 0.15% TWEEN and stained with primary antibodies, either (1)
rabbit polyclonal anti-opsin directed against squid opsin (Robles et al. 1986) or (2) rabbit-polyclonal anti-Acropsin antibodies diluted 1:300 in solution for antibody (2% BSA; 0.15% TWEEN; 1x PBS). Sections were incubated overnight at 4°C, washed 3 x 30 minutes in PBS containing 0.15% TWEEN. After washing, sections were incubated for 2 hours with Alexa Fluor® 546, goat anti-rabbit IgG (Invitrogen) diluted 1:1000 in solution for antibody. Sections were then washed as above and incubated with DAPI diluted 1:10,000 in PBS containing 0.15% TWEEN or mounted onto microscope slides using fluormount with DAPI (Southern Biotech). Preparations were examined using a Leica Inverted and Upright TCS SP5 Confocal Microscopy System, with a 4 fluorescent channel detection system. Images were captured and processed using the Leica software (Leica Application Suite).

**Fluorescence emission spectra of unstained larvae**

In order to determine patterns and spectra of endogenous fluorescence, emission spectra of sectioned, unstained *A. palmata* larvae, fixed in either 10% formalin or 4% paraformaldehyde, were measured using a Leica TCS SP5 confocal microscope. Fluorescence emission profiles were generated by scanning sections (range: 415-700 nm) at 10nm intervals using the xyλ scan mode (Leica Application Suite). Scans were repeated for all available excitation lasers (405, 458, 476, 488, 496, 514, 543 and 633nm). Fluorescence spectra were normalized as percent maximum fluorescence.
Transmission electron microscopy

Larvae were fixed in 2.5% gluteraldehyde in sodium cacodylate buffered seawater and kept at 4°C until they were transported to the University of Miami’s Center for Advanced Microscopy (CAM). Larvae were removed by pipet and washed 3 times (10-20 minutes per wash) with seawater buffer, followed by secondary fixation in 4% osmium tetroxide in seawater for 1-2 hours. Larvae were washed three times with distilled water, stained with 0.5% uranyl acetate at 4°C overnight, then washed twice with distilled water prior to dehydration. Dehydration was accomplished by consecutive 10 min incubations in 25%, 50%, 75%, 95%, 100% and 100% ethanol at room temperature. Dehydration was followed by two, 10-minute incubations in transition solvent. The larvae were then infiltrated by stepwise incubation of the sample in solutions containing an increasing ratio of resin to solvent (1:2, 1:1, and 2:1). All incubations were allowed to proceed overnight. Imbedding was accomplished by placement of the larvae in 100% resin for 15 minutes – 1 hour. After this time, the resin was polymerized at 60-70°C for at least 8 hours. The imbedded samples were then sectioned using a microtome and imaged using a Phillips EM-300 100keV Transmission Electron Microscope.

Results

Expression of GST-coral peptide fusion proteins and antibody screening

All four GST-coral peptide (GST-Acropsin1_cloopIII, Acropsin1_ctail, Acropsin2_cloopIII and Acropsin2_ctail) fusion constructs were successfully expressed in E. coli (Figure 4.1). Total protein lysates of bacterial cultures were used for western
Figure 4.1. Coomassie gel demonstrating expression of GST-acropsin peptide fusion proteins. From left to right, lanes represent total protein lysates from bacterial cultures expressing GST-Acrop1_loop, Acrop1_tail, Acrop2_loop and Acrop2_tail fusion proteins with (a) and without (b) IPTG induction.

Blot screening of Acropsin antibodies. Western blots revealed that rabbit antisera (from production bleeds) from at least one rabbit, raised against each Acropsin peptide (Acrop1_loop, Acrop1_tail, Acrop2_loop and Acrop2_tail) recognizes the corresponding GST-acropsin fusion protein (Figure 4.2).
Figure 4.2. Immunoblot screening of Acropsin antisera. Lanes were loaded with total protein lysate from GST-coral Acropsin peptide bacterial cultures and probed with the corresponding, unpurified antisera. Lanes, from left to right were probed with Acrop1_loop, Acrop1_tail, Acrop2_loop and Acrop2_tail, production bleed antisera from each rabbit (a & b). Positive results (~30kDa bands indicated by the arrow) are observed with all but Acrop2_tail (b).

Western blots

Affinity-purified Acropsin antibodies (Acrop1_loop, Acrop1_tail, Acrop2_loop and Acrop2_tail) were used for immunoblots of larval and adult coral protein. Blots were conducted with adult and larval *A. palmata*, and adult *A. cervicornis*. Acropsin 1_tail and Acropsin 2_loop antibodies gave positive results for both adults, labeling bands with molecular weights of ~37 and 40 kDa corresponding to the predicted molecular weights of Acropsin 1 and 2, respectively (Figure 4.3). On immunoblots of *A. palmata* larval protein, Acropsin antibodies consistently detected a large band with a molecular weight of ~50kDa (Figure 4.3). Positive and negative control blots (results not shown) suggest
this band may represent an IgG-binding protein expressed in the larvae or resulting from contamination of larval protein samples. A band corresponding to the molecular weight predicted for Acropsin 2 was detected in *A. palmata* larval protein by the Acropsin 2_loop antibody, but positive results were not observed for Acropsin 1 (Figure 4.3). Acropsin 1_tail and Acropsin 2_loop were used to probe these blots because these reacted most strongly with GST-fusion proteins. Probing with Acropsin 1_loop and Acropsin 2_tail antibodies was also attempted but these antibodies produced weak or no bands.

![Figure 4.3. Immunoblots of adult and larval coral protein. From left to right, lanes indicate total protein from *A. cervicornis* (adult), *A. palmata* (adult), *P. astreoides* (larvae) and *A. palmata* (larvae), probed with Acrop1_tail and Acrop2_loop antibodies. Bands of ~ 37kDa corresponding to Acropsin 1 are detected in *A. cervicornis, A. palmata* adults and *P. astreoides* larvae. A band at ~ 40 kDa corresponding Acropsin 2 is observed in all samples.](image-url)
**Immunohistochemistry**

Preliminary immunohistological results were obtained for sectioned larvae from several coral species (the mountainous star coral *Montastrea faveolata*, the brain coral *Diploria strigosa*, the mustard-hill coral *Porites astreoides*, and the golf-ball coral *Favia fragum*). Positive results were observed in all four species, when sections were probed with a rabbit polyclonal antibody directed against squid rhodopsin (Robles et al. 1986). In all species, positive staining was observed, and indicated the presence of an opsin-like protein localized in a sub-population of cells within the epithilium of these larvae.

Opsin-positive cells appeared to lack organization in the two spawning species, *D. strigosa* and *M. faveolata*. Instead, the antigen was localized in solitary cells scattered throughout the ectoderm (Figure 4.4 & 4.5). In the two brooding species (*P. astreoides* and *F. fragum*), a polarized distribution of cells was evident, and both species contained higher densities of opsin-positive cells within the aboral epidermis (Figures 4.6 & 4.7). The same antibody failed to produce positive results in *A. palmata* larvae (Results not shown).

The Acropsin antibodies produced positive results with multiple species of larvae. Acropsin 1_tail and Acropsin 2_loop antibodies labeled different populations of cells, in *A. palmata* larvae. Acrop1_loop ab revealed that Acropsin 1 is localized in the endoderm of *A. palmata* (Figure 4.8) while Acrop2_tail revealed that that Acropsin 2 is localized in a population of epithelial cells with a distribution (scattered throughout the ectoderm with additional concentration in the aboral epidermis) similar to that observed in *P. astreoides* and *F. fragum* larvae stained with anti-squid rhodopsin antibody (Figures 4.9-13). These antibodies also gave positive results when used with other coral species, identifying
opsin-positive cells in the epithilium of *A. cervicornis* (Figure 4.14), *P. astreoides* (Figure 4.15-16), and *M. faveolata* (Figure 4.17). However, the Acropsin antibodies did not consistently stain the same populations of cells when used across species (Table 4.1).

**Figure 4.4.** Fluorescent confocal micrographs of sectioned *D. strigosa* larvae. (a) Longitudinal, cross sectional view through a *D. strigosa* planula, showing opsin-positive cells (red) scattered randomly throughout the larval ectoderm; (b) close up (40x oil) view of three opsin-positive cells within the larval ectoderm. Sections were stained with rabbit polyclonal anti-squid rhodopsin antibody. [Red: opsin-like protein; Blue: cell nuclei; Green: endogenous GFP]
Figure 4.5. Fluorescent confocal micrograph of a sectioned *M. faveolata* planula stained with anti-squid rhodopsin antibody. Exterior view of the surface of the larva showing opsin-positive cells scattered throughout the larval ectoderm. [Red: opsin-like protein; Blue: cell nuclei]
Figure 4.6. Fluorescent confocal micrographs of symbiodinium and sectioned F. fragum larvae. **Top Left:** Fluorescent micrograph of DAPI-stained (blue nuclei) Symbiodinium Clade A showing red chlorophyll autofluorescence and the spherical shape of the coral symbionts (image from Bob Moore, MMB, University of Sydney, Australia). **Top Right:** Cross section through a Favia planula stained only with DAPI. Blue = cell nuclei, Green = endogenous GFP localized in the larval epithelium, Red = chlorophyll autofluorescence of Symbiodinium residing within the larval endoderm; **Bottom Left:** Fluorescent confocal micrograph of a cross section through the aboral ectoderm of a Favia planula stained with anti-squid rhodopsin antibody and DAPI. Blue = cell nuclei, Green = endogenous GFP, Red = Symbiodinium/zooxanthellae (Z) and opsin-like protein. **Bottom Right:** Single channel (red) fluorescent confocal micrograph of a longitudinal cross section through a Favia planula stained with anti-squid rhodopsin antibody and demonstrating polarization of opsin-positive cells within the larval ectoderm (higher concentration of opsin-positive cells in the aboral ectoderm (AE) versus the oral ectoderm (OE). The white box indicates the region of enlargement (bottom left panel). Red = opsin-like protein.
Figure 4.7. Fluorescent confocal micrographs of sectioned *P. astreoides* larvae stained with anti-squid rhodopsin antibody. (a) Close up (40x-oil objective) image showing the morphology of opsin-positive cells found scattered throughout the larval ectoderm. (b) Longitudinal, cross section showing a polarized distribution of opsin-positive cells with a greater density in the aboral ectoderm. Spherical, red cells present in the larval endoderm are symbiotic zooxanthellae. [Red: opsin-like protein; Blue: cell nuclei; Green: endogenous GFP]
**Figure 4.8.** Fluorescent confocal micrograph of a sectioned *A. palmata* larva showing localization of *Acropsin 1* within the mesoglea (M) and larval endoderm (END). [Red = *Acropsin 1* (Acrop1_loop AB); Blue = cell nuclei; Green = endogenous GFP]
Figure 4.9. Fluorescent confocal micrograph of a sectioned *A. palmata* larvae showing localization of *Acropsin 2* within solitary epithelial cells scattered throughout the larval ectoderm. [Red = *Acropsin 2* (Acrop2_loop AB); Blue = cell nuclei; Green = endogenous GFP]
Figure 4.10. Fluorescent confocal micrograph of a sectioned *A. palmata* larva showing three Acropsin 2-positive epithelial cells within the larval ectoderm. The cells appear to terminate proximally in axon-like processes (A) within the mesoglea, the layer separating the ectoderm from the endoderm which is known to contain nerve cells. Red = *Acropsin* 2 (*Acrop2_loop AB*); Blue = cell nuclei; Green = endogenous GFP.
Figure 4.11. A single (red) channel fluorescent confocal micrograph of a sectioned *A. palmata* larva displaying two Acropsin 2-positive epithelial cells. Red = *Acropsin 2* (Acrop2_loop AB).
Figure 4.12. Fluorescent confocal micrograph of a double-stained section of an *A. palmata* larva showing Acropsin 2-positive epithelial cells within the larval ectoderm. Again, the cells appear to terminate proximally in axon-like processes (A) in the mesoglea, the layer separating the ectoderm from the endoderm which is known to contain nerve cells. Red = *Acropsin 2* (Acrop2_loop AB); Blue = cell nuclei; Green = endogenous GFP.
Figure 4.13. Fluorescent confocal micrograph of a longitudinal cross through an *A. palmata* larva, showing a polarized distribution of *Acropsin* 2-positive cells, with a dense population of these cells found in the aboral ectoderm. Red = *Acropsin* 2 (Acrop2_loop AB).
**Figure 4.14.** Fluorescent confocal micrograph of a longitudinal cross through an *A. cervicornis* larva, showing a polarized distribution of *Acropsin* 2-positive cells, with a dense population of these cells found in the aboral ectoderm. Red = *Acropsin* 2 (Acrop2_loop + Acrop2_tail AB).
Figure 4.15. Fluorescent confocal micrographs of a sectioned *P. astreoides* larva showing Acropsin 1-positive cells within the larval epithelium. Sections were stained with a cocktail of antibodies (Acrop1_loop & Acrop1_tail) against Acropsin 1. (a) Red = *Acropsin 1*; Blue = DAPI; Green = endogenous GFP; (b) Single-channel (red) fluorescent micrograph of an individual Acropsin1-positive cell. The arrow indicates what appears to be an axon body and axons.
Figure 4.16. Fluorescent confocal micrograph of a sectioned *P. astreoides* larva showing Acropsin 2-positive cells within the larval epithelium. Sections were stained with a cocktail of antibodies (Acrop2_loop & Acrop2_tail) against Acropsin 2. Red = *Acropsin* 1; Blue = DAPI; Green = endogenous GFP
Figure 4.17. Fluorescent confocal micrograph of a sectioned *M. faveolata* larva. Opsin-positive epithelial cells were labeled using a cocktail of Acropsin 1 antibodies (Acrop1_loop + Acrop1_tail). Red = *Acropsin 1*; Blue = DAPI; Green = endogenous GFP.

Table 4.3. Summary of Acropsin immunohistochemistry results

<table>
<thead>
<tr>
<th></th>
<th>Acrop1-loop</th>
<th>Acrop1-tail</th>
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<tbody>
<tr>
<td><em>A. palmata</em></td>
<td>x</td>
<td>Ubiq./End.</td>
<td>Sol./Ect.</td>
<td>x</td>
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<tr>
<td><em>A. cervicornis</em></td>
<td>Unclear</td>
<td></td>
<td>Sol./Ect.</td>
<td></td>
</tr>
<tr>
<td><em>M. faveolata</em></td>
<td>Sol./Ect.</td>
<td></td>
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x = Staining was not attempted
Ubiq./End = staining was observed in all cells of the endoderm
Sol./Ect. = staining was observed in solitary cells in the ectoderm
(-) No staining was observed
**Endogenous fluorescence: Localization and emission spectra of unstained larvae**

Fluorescence confocal micrographs and xyλ scans of sectioned *A. palmata* larvae revealed the presence of two fluorescent chromophores – one blue and one green fluorescent protein – with different patterns of expression. The blue fluorescent protein had an emission peak at ~465nm (excitation: 405nm) and was localized in cells of the larval endoderm, while the green had a peak at ~530 (excitation: 488nm; Figure 4.14). Red fluorescence was not measured as the appropriate excitation laser was not installed on the confocal used for imaging.

**Transmission electron microscopy**

TEM micrographs of 5d old larvae revealed the presence of pigment granules scattered throughout the larva epithelium of both *Montastrea faveolata* and *Acropora palmata* larvae (Figures 4.19 & 4.20). This tissue layer is thought to consist of tightly packed, columnar, mono-ciliated epithelial cells (Vandermulen 1974). Stacks of pigment granules were observed within the epithelium of *M. faveolata*. Two types of granules, one dark/black granule and another lighter/grey granule, were observed, but the black type is dominant in *M. faveolata*. Granules were less abundant than in *A. palmata*. While two types of granules were also observed in *A. palmata*, the granules in this species lacked the stacked arrangement observed in *M. faveolata*. Instead, granules appeared to have a random distribution within the distal regions of the larval epithelium (Figures 4.19 and 4.20).
Figure 4.18. Endogenous fluorescence of *A. palmata* larvae. A two-channel (405 and 488nm excitations), fluorescent confocal micrograph of sectioned *A. palmata* larva (fixed in 4% paraformaldehyde and imbedded in 3.5% agar) showing differential localization of two fluorescent chromophores: a blue fluorescent protein localized in the larval endoderm and a green, with a ubiquitous distribution, occurring in both the endoderm and ectoderm (Top). Curves represent the emission spectra of two fluorescent chromophores, one blue fluorescent protein with an emission peak centered at ~465nm (blue curve) and second green fluorescent protein with an emission peak centered at ~530nm (green curve). Excitation wavelengths were 405 (blue curve) and 488nm (green curve)(Bottom).
Figure 4.19. Micrographs of *Montastrea faveolata* larvae. (a) TEM micrograph (1830x) of a 5d old planula, highlighting the structure of the larval epithelium. This tissue layer consists of tightly packed, columnar, mono-ciliated epithelial cells. Stacks of pigment granules (similar to the melanin granules observed in other cnidarians) are observed in these cells (PG); (b) TEM micrograph (3400x) of the same region of the larval epithelium seen in (a), highlighting the stacked pigment and distal microvillar projections (MV); (c) Fluorescent confocal micrograph of an immunofluorescently labeled *M. faveolata* planula (whole mount) indicating a population of epithelial cells expressing an opsin-like protein. Red = opsin-positive cells, Green = endogenous GFP, Blue = DAPI.
Figure 4.20. TEM micrographs of *A. palmata* larvae. (a) Micrograph of the larval epithelium showing electron-dense pigment granules. At least two types of granules (PG1 = grey; PG2 = black) are observed. Granules are located in distal regions of the epithelial cells (distal to nuclei (N)); (b) Larger region of the larval epithelium showing pigment granules localized in the distal regions of epithelial cells; (c) Close-up of pigment granules within the distal regions of epithelial cells with microvillar processes (MV) and cilia (C).
Discussion

Western blots demonstrate that Acropsin 1 and 2 are expressed in both adult *A. palmata* and *A. cervicornis*. Acropsin 1 and Acropsin 2 were detected as bands of approximately 37kDa and 40kDa, respectively, similar to the molecular weights predicted from the amino acid sequences of each protein (Figure 4.3; see Chapter III). Acropsin 1 and 2 antibodies also detected similar molecular weight bands in *P. astreoides* larval protein. Unfortunately, only Acropsin 2 was detected by western blot in *A. palmata* larvae. Failure to detect Acropsin 1 was likely a result of inadequate sample storage or sample preparation, rather than lack of expression. Resolved *A. palmata* larval protein was not stained with Coomassie to assess protein quality because larval protein was in short supply, but larval protein used for western blots had been stored at -20°C for almost 4 years. Since *A. palmata* failed to spawn in 2010, I was unable to repeat these western blots with fresh larval protein.

Acropsin 1 and 2 mRNAs (see chapter II) are found in *A. palmata* larvae, immunoblots indicate that both proteins are expressed in *P. astreoides* and immunohistochemistry identifies Acropsin-positive cells in both species (Figures 4.8-16). Taken together, these results provide powerful evidence for functional larval photoreceptors. While these proteins likely play a role in known larval photosensitivities (e.g., light intensity or color preference during settlement, diel vertical migration, or UV avoidance), Acropsin 1 and 2 are also expressed in adults, suggesting that the function of these opsins is not restricted to involvement in early life history processes.

The expression of Acropsin 1 and 2 in both larval and adult stages is surprising. Schwarz et al. (2008) found that 92% of *A. palmata* unigenes were stage-specific,
occurring in only one of 5 developmental stages (spawned eggs, early-stage larvae, late-stage larvae infected with symbionts or uninfected, adults). Only 387 (out of 4980) unigenes were present in more than one of these stages. However, one recent attempt to sequence the larval transcriptome of a congener, an Indo-Pacific species, *A. millepora*, resulted in greater than 11,000 unigenes (Meyer et al. 2009), suggesting that the *A. palmata* library is largely incomplete. This is supported by the presence of Acropsin 1 and 2 in *A. millepora* EST database absence in the *A. palmata* library (see Chapter III).

Preliminary immunohistology using an anti-squid rhodopsin antibody indicated the presence of opsin-like proteins, localized in epithelial cells of several species of coral larvae (Figures 4.4-4.7). Other studies have used this same antibody and other anti-opsin antibodies, with mixed results, to identify opsin-like proteins in the eyes and photoreceptors cnidarians. Musio et al. (2001) used the same anti-squid rhodopsin antibody (Robles et al. 1986) that was used in this study to identify opsin-like proteins in epidermal cells of *Hydra vulgaris*, but was unable to confirm that these proteins were opsins or that they occurred in photoreceptors. Anti-zebrafish opsin antibodies have also been used to successfully label cells of jellyfish eyes. Martin (2004) labeled the complex eyes of the cubamedusae *Carybdea marsupialis* using antisera directed against zebrafish ultra-violet-, blue-, green- and red-sensitive zebrafish opsins. The red-sensitive opsin antisera failed to label any of the visual organs, but the ultraviolet-, blue- and green-sensitive opsin antisera each labeled a different population of photoreceptor cells, suggesting that the jellyfish *C. marsupialis* possesses a visual system based on multiple opsin photopigments. The antisera labeled populations of cells with different morphologies. Some cells contained parallel membranes forming discs within their outer
segments while others lacked these membrane modifications and had labeling of inner and basal segments (Martin 2004). Ekstrom et al. (2008) used nine different antibodies (UV-, blue-, green- and red-sensitive zebrafish opsin, a chicken cone opsin, a chicken LW sensitive cone opsin, zebrafish rhodopsin, bovine rhodopsin, and Drosophila rhodopsin antibodies) to screen the lens eyes of the box jellyfish Tripedalia cystophora and C. marsupialis, but only observed positive labeling with the UV-sensitive zebrafish antibody.

Few examples of immunofluorescence labeling of cnidarian visual structures with anti-cnidarian opsin exist since the first cnidarian opsins have only recently been described, but Koyanagi et al. (2008) used an antibody developed against a jellyfish opsin to label the outer segments of cells in the lens eye of the box jellyfish C. rastonii. The present study is the first to use antibodies raised against coral opsins to label photoreceptor cells in corals.

The differential localization of Acropsin 1 and 2 in A. palmata larvae (Figure 4.8-4.13), suggests that the two opsins have different functions and it also confirms the specificity of these antibodies, Acropsin 1, which, based on sequence homology, is most similar to vertebrate melanopsin, was localized in the larval endoderm; Acropsin 2, which is most similar to cephalopod rod opsin, is localized in epithelial cells. The localization of these two opsins is similar to the differential localization of melanopin and rod/cone opsins in the vertebrate retina. In the vertebrate retina (see Figure 4.18), rod opsins are localized in rod cells, which are derived from the ectoderm, and function in vision. Vertebrate melanopsin is found in horizontal cells (Chang et al. 2009) and retinal ganglion cells (e.g., Pires et al. 2009)) that are derived from the endoderm, and function
in the photoentrainment of circadian rhythms (Hattar et al. 2003a) and pupillary reflex (Lucas et al. 2003).

The larval epithelial cells labeled with Acropsin 2 (and Acropsin 1 in the case of *P. astreoides* and *M. faveolata*) antibodies are similar, in abundance, distribution and morphology, to the population of larval cells labeled with the anti-squid rhodopsin antibody in other species. The consistent labeling of what appears to be the same population of cells by different opsin antibodies lends confidence to hypothesis that these cells are in fact larval photoreceptors. While the ultrastructure of these cells has not been resolved, since TEM micrographs did not reveal any features (e.g., specialized outer segments) specific to photoreceptors, the Acropsin-positive epithelial cells appear to be morphologically similar to other cnidarian photoreceptors (Figure 4.21; Singla 1974).

The acropsin-positive epithelial cells are likely monociliated cells, since the epithelium of coral larvae is composed of monociliated or flagellated collar-like cells (e.g., Lyons 1973). If cilia were preent in Acropsin-positive epithelial cells, the cilia were not labeled by the acropsin antibodies and there was no evidence to suggest proliferation or folding of the ciliary membranes. Assuming these cells are photosensitive, they appear to be most like the hypothetical indistinct photoreceptors (A) or the cells of the primitive eyespot of the hydromedusae *Leuckartiara octona* (B) (Figure 4.21).
Figure 4.21. Illustration of various photoreceptor cell types found within phylum Cnidaria. (A) Hypothetical indistinct photoreceptor composed of ciliated photosensory cells and supporting cells; (B) Example of cells found in the primitive eyespot of the hydromedusa *Leuckartiara octona*, consisting of mixed ciliated (c) photoreceptor cells (r), and supporting pigment cells (p). Both cell types contain microvillar (m) processes in their distal membranes; (C) Cells of the cup-shaped ocellus of red-eyed jellyfish *Polychoris penicillatus*. Interdigitating microvilli (m) emerge from the membranes of photoreceptor (r) cilia (c) and from distal membrane projections of the pigment cells (p); (D) Cells of the cup-shaped ocellus of the anthomedusa *Bougainvillia principis*. Microvilli (m) of pigment cells (p) and photoreceptor cells (r) have differentiated and no longer interdigitate (from Singla 1974).

The larval photoreceptors expressing Acropsin 2 show little or no organization (polarization or aggregation) in *M. faveolata* and *D. strigosa*. Acropsin antibodies stained small population (<10%) of solitary epithelial cells that were randomly dispersed throughout the larval ectoderm. Meanwhile, the two species of brooded larvae (*P. astreoides* and *F. fragum*) and two spawning, sibling species (*A. palmata* and *A. cervicornis*) displayed a polarized distribution of photoreceptors.

The polarized distribution of Acropsin 2 (the higher concentration of Acropsin 2-positive epithelial cells in the aboral epithelium) in *A. palmata* (Figure 4.13), *A. cervicornis* (Figure 4.14), offers clues to the potential function of this opsin. The aboral epidermis tends to be thickened, contains a complex nerve layer and sensory cells (e.g.,
Atoda 1953; Vandermuelen 1974), is forward during swimming and is used to probe, and
attach to, the substrate during settlement (Harrison and Wallace 1990). Concentration of
Acropsin 2 in this region suggests that this opsin may play a role in orientation during
swimming, and/or a role in substrate exploration. Additional Acropsin 2-positive cells
scattered throughout the epithelium in these species may assist in orienting the larvae
toward the light. Of the two opsins, Acropsin 2 seems more likely to play a role in long-
wavelength cuing of settlement and metamorphosis in *A. palmata* (see Chapter 2), but
Acropsin 1 may also play a role, possibly as a second, complementary, UV or blue-
sensitive pigment enabling the chromatic antagonism demonstrated in larval settlement
experiments.

While immunoblots indicate that Acropsin 1 and 2 antibodies react specifically in
*P. astreoides*, immunohistochemistry suggested otherwise. Rather than labeling different
populations of cells, as was the case in *A. palmata*, Acropsin 1 and 2 antibody cocktails
both labeled epithelial cells in *P. astreoides*. Additional work is needed to determine
whether Acropsin 1 is actually expressed in epithelial cells of *P. astreoides*. It is possible
that Acrop1_loop AB may show cross-reactivity with another opsin or similar GPCR.
The labeling of *M. faveolata* epithelial cells by Acropsin 1 antibodies may also be an
issue with cross-reactivity. Since, antibodies were developed against flexible regions of
*A. palmata* proteins, sequence divergence in these regions in other species could result in
cross-reactivity.

The localization of two endogenous fluorescent proteins in sectioned *A. palmata*
suggests that incident UV, and longer wavelength, radiation may reach the larval
endoderm. A green fluorescent protein has a ubiquitous distribution, occurring
throughout both the endo-and ectoderm, while a blue fluorescent protein is localized in the endoderm of *A. palmata* (Figure 4.18). The blue chromophore is excited by UV radiation (405nm) and fluoresces with a maximum emission of ~465nm (Figure 4.18). UV excitation of this pigment would produce blue light that could be captured and utilized by photoreceptors in the larval endoderm. Acropsin 1 is localized in this tissue layer, but so are cryptochromes (Levy et al. 2007), a blue-sensitive photoreceptors involved in circadian gene networks. The blue fluorescent protein may provide a mechanism for converting potentially harmful UV radiation into blue light that can then be utilized by the coral. This would represent an indirect photosensitivity to UV radiation and would enable incident blue light to be harvested for other purposes (e.g., by overlying, more distal photopigments, chromophores, or the chlorophyll pigments of symbiotic algae).

In addition to fluorescent proteins and photoreceptor pigments (and in the case of brooded larvae, photosynthetic pigments), pigment granules also occur in coral larvae (Fadlallah and Pearse 1982). Shielding pigments are found in, or in cells adjacent to, photoreceptive cells (PRCs) in practically all known animal eyes or photoreceptive structures (Nilsson 2009). While melanin is the dominant shielding pigment found in vertebrate eyes, a greater diversity of pigments have been found to serve this function among the invertebrates. Pterins constitute the dark eye pigment of the polychaete *P. dumerilii* (Viscontini et al. 1970), and pterins and ommochromes are the pigments in eyes of *Drosophila* (Shoup 1966). The simple cup-like eyes of a basal lophotrochozoan, *Dugesia* contain melanin (Hase et al. 2006). In addition to possessing ciliary (vertebrate-like) opsins, the camera-type of the cubozoan jellyfish, *T. cystophora*, also contains dark,
melanin pigment granules, similar to those found in vertebrate eyes. However, unlike vertebrates the melanin pigment in *T. cystophora* occurs within, rather than adjacent to, the photoreceptive cells, consistent with an ancestral (basal) condition (Kozmik et al 2008; Figure 4.21). The organization and nature of *Tripedalia* PRCs and pigment granules is of interest here since *Tripedalia* is the closest cnidarian relative of scleractinian corals for which photosensitive structures have been described.

Transmission electron microscopy micrographs revealed that larvae of the scleractinian corals, *M. faveolata* (Figure 4.19) and *A. palmata* (Figure 4.20), possess pigment granules that resemble the melanin granules found in adult and larval *Tripedalia* (Figure 4.22). While the abundance and ubiquitous distribution pigment granules does not appear to match the abundance (<10% of ectodermal cells) and distribution of photosensitive epithelial cells (Figures 4.6-*M. faveolata* & 4.9-4.13-*A. palmata*), these pigment granules may still be serving a shielding function for the larval PRCs, allowing the larva to determine directionality of incident light, affecting the spectral sensitivity by filtering incident light or simply reducing signal noise by absorbing scattered photons. Pigment granules may be especially important in shielding the photoreceptors in the endoderm of *A. palmata* larvae. These granules occur throughout the distal region of the larval ectoderm (Figure 4.20), where they would provide an effective shield for both Acropsin 2 containing epithelial cells as well as the underlying endoderm containing Acropsin 1.
Figure 4.22. TEM micrographs of photosensitive cells and structures from adult and larval stages of the cubozoan jellyfish, *Tripedalia cystophora*. (a) Cross section through the camera-types eye of *T. cystophora* showing the ultrastructure of ciliated (C) photosensitive cells, containing melanin pigment granules (PG) concentrated in the distal region of the cell, and shielding the nucleus (N) from incident light [From Kozmik et al. 2008]; (b) Photoreceptor consisting of a pigment cup (cup of pigment granules) surrounding photosensitive microvilli in a *T. cystophora* larva; (c) Close-up of the pigment cup showing the cilia root (CR) and microvilli (MV) projecting into center of the cup (from Nordstrom et al. 2003).

Alternatively, the dark, melanin-like granules described here may not be involved in photoreception at all, but may instead provide protection from the damaging effects of solar radiation. Nilsson (2009) argues that while pigment granules are almost always occur in the vicinity of photoreceptors, pigment granules existed before photoreceptors and serve many other functions independent of photoreception. Spawned coral larvae develop at the ocean’s surface where they are subjected to intense ultra-violet radiation (UVR), and strong selective pressure has lead to the evolution of multiple mechanisms for dealing with UVR. Larvae contain microsporin-like amino acid sunscreen compounds (Wellington and Fitt 2003; Yakovleva and Baird 2005), photoreactivation
repair mechanisms for UV-induced DNA damage (Reef et al. 2001) and green fluorescent proteins, which may also serve a photoprotective function (e.g., Dove et al. 2001). In addition to its role as a screening pigment, melanin serves as a sunscreen compound in many vertebrates and could be serving the same role in *A. palmata* and *M. faveolata* larvae. Additional work is needed to determine if the pigment granules observed here are in fact melanin, if they occur in or adjacent to larval photoreceptors, and the potential role that they may be playing as screening pigments.
CHAPTER 5

ELECTROPHYSIOLOGICAL DEMONSTRATION OF PHOTOSensitivity: EXTRACELLULAR RECORDINGS FROM CORAL LARVAE

Rationale

The absorption of light by a photoreceptor causes a conformational change in the molecule leading to polarization or depolarization of the photoreceptor cell (See Chapter I) and post-synaptic activity as light-induced electrical activity is propagated through second and third-order neurons of the retina. An electroretinogram (ERG) is the measurement of this light-induced electrical activity.

In animals with lens-type eyes, ERG responses are recorded using an extracellular electrode positioned either on the cornea, in the vitreous or at different levels inside the retina. Extracellular recording of electrical activity is possible because electrical currents spread along an extracellular matrix with electrical resistance. In the vertebrate retina, one electrical current spreads from the inner segments to the outer segments of parallel the photoreceptors (rods and cones), while additional electrical currents generated in post-synaptic cells of the retina. These currents combine to produce an ERG response (Figure 5.1).

Here I describe the results of extracellular recordings made in several species of coral larvae. Even though coral larvae lack eyes or identifiable visual structures, extracellular microelectrodes were used to successfully record light (flash)-induced electrical current from five species, providing physiological evidence for functional photoreceptors in coral larvae.
**Figure 5.1.** Representation of a flash ERG and points of origin of ERG waves. ERG trace indicating the negative a-wave, positive b-wave and oscillatory potentials. The a-wave originates from photoreceptor cells – rods and cones - in the outer retina. The exact origin of the b-wave remains in dispute, but evidence suggests that it primary source of origin is second–order neural cells – primarily ON bipolar cells. Other cells of the inner retina – amacrine and ganglion cells - can contribute oscillatory potentials to ERG wave form but these contributions are typically not seen in flash ERGs of the dark-adapted eye (from Cameron et al. 2008).
Materials and Methods

Larval collection

Recordings were made in five species of larvae – two brooded, zooxanthellate species (*Porites astreoides*, and *Agaricia agaricites*) and three azooxanthellate spawning species (*Montastrea faveolata*, *Acropora cervicornis* and *Acropora palmata*). *P. astreoides* and *A. agaricites* were collected according to the methods described in Chapter 2 for the collection of *P. astreoides*. Collections, and recordings, of *P. astreoides* and *A. agaricites* ERGs were made in June 2009. Collection of gametes and laboratory culture of *M. faveolata*, *A. cervicornis* and *A. palmata* was accomplished following the methods described in Chapter 2 for *A. palmata*. Recordings of these species were made in August 2009.

Electrophysiology

Extracellular recordings were made in five species of coral larvae – two brooded species (*Porites astreoides* and *Agaricia agaricites*) and three spawned species (*Acropora palmata*, *A. cervicornis*, and *Montastrea faveolata*). Larvae were prepared for recordings by immobilizing individual organisms on Kimwipes® saturated with filtered seawater. Small sections of Kimwipes® were placed in glass finger bowls and saturated by dropwise addition of filtered seawater. A single larva was then transferred, by pipet, to the surface of the Kimwipe®, and placed inside a dark box. Differential recordings (using two electrodes – one recording and one reference) were made by inserting the recording electrode (an extracellular tungsten microelectrode with 100-µm shank, <1-µm tip; FHC Inc.) into the epithilium of larvae, with the aid of a micromanipulator and dissecting
microscope. The reference electrode (AgCl-coated wire) was grounded by submersion in seawater within the glass fingerbowl (adjacent to the specimen).

The light stimulus was provided by a 150W quartz halogen lamp. Light was directed onto the specimens via one branch of a bifurcated, randomized fibre optic light guide (EXFO). An electromagnetic shutter (Uniblitz, VS25) under computer control was used to provide a stimulus flash with a duration of 75ms. The spectral purity of test stimuli, with and without blocking filters was verified using a spectroradiometer (9 nm FWHM, 350–700 nm; Ocean Optics, USB4000). White light from the lamp was filtered using various combinations of filters: a red longpass, glasses filter (RG 630), a blue bandpass filter (Edmund Optics) and neutral-density filters (0.15, 0.3, and 0.5od). Quantal intensities of light stimuli were matched as closely as possible during recordings.

A fibre optic illuminator (TechniQuip, Model R150-BM) connected to the other branch of the light guide was used to provide accessory illumination during specimen preparation. AC recordings were digitized and stored in LabView (v. 6.1, National Instruments) for later analysis of peak-to-peak response heights.

Larvae were allowed to dark adapt for 5 min prior to the start of recordings, and for 2min in between flashes. Flashes of the white (unfiltered) stimulus were delivered to larvae until a stable response was achieved, indicating that the larvae were fully dark-adapted. Flashes of red, blue and white stimuli of varying intensities were then delivered in a random order, until at least two recordings were made for each type of stimuli (red, blue and white) of matched intensities. Recordings were made for between five and eight individuals of each species.
Results

The spectral power distribution of the halogen light source was skewed toward the long-wavelength region of the visible light spectrum with a peak at approximately 650nm (Figure 5.1). The irradiance (integrated from 350-750nm) of the unfiltered halogen light source was $7.99 \times 10^{16}$ photons cm$^{-2}$ s$^{-2}$ during recordings of *Agaricia agaricites* and $1.81 \times 10^{17}$ photons cm$^{-2}$ s$^{-2}$ during recordings of *Montastrea faveolata*, *Acropora cervicornis*, and *Acropora palmata* larvae. Blue bandpass and red longpass, glass filters created distinct blue and red stimuli with spectral power curves centered

![Figure 5.2.](image)

**Figure 5.2.** Spectral power distributions of light/color stimuli. White (black curve) = the spectral power distribution of the unfiltered halogen light source; Blue block (blue curve) = the spectral power distribution of the blue stimulus created using a blue bandpass filter; RG630 (red curve) = spectral power distribution of the red stimulus created using the red glasses filter (Edmunds Optic, RG630).
at approximately 550 and 670nm, respectively. The tails of these curves overlapped slightly between 600 and 700nm.

Recordings (traces) from all five species displayed similar waves forms. At maximum irradiance, the traces had small, negative deflections (comparable to the ERG a-wave) followed by large positive deflections (similar to the ERG b-wave) (Figures 5.3-5.5; for diagram of wave forms see Figure 5.1). The negative deflection appeared to depend on the specimen preparation and flash intensity. This deflection was not observed in all individuals and as the intensity of the light stimulus was reduced, or filtered, the negative deflections disappeared while the positive deflection decreased in amplitude and broadened (Figure 5.3-5.5).

Time-to-peak measurements (time from the onset of the light stimulus to the peak of the negative a-(L_a; negative deflection) or b-wave (L_b; positive deflection); see Figure 5.5), indicated that the response speeds of larval photoreceptors were slow relative to other invertebrate photopigments. L_a's ranged from 6ms in A. cervicornis to 20ms in A. agaricites. L_b's were similar, 188, 195 and 207ms, in P. astreoides, A. palmata and A. cervicornis, respectively, but about approximately 25% longer in A. agaricites (254ms) and M. faveolata (247ms) (Table 5.1).
Figure 5.3. Results of extracellular recordings from *P. astreoides*. The black line represents the change in membrane potential observed in response to the white light stimulus; blue line = the response to the blue stimulus; red line = the response to the red stimulus; green line = shutter trace (depression indicates duration of shutter opening – 75ms). The quantal intensities of the red (2.66E+15 photons cm\(^{-2}\) s\(^{-1}\)) and the blue (2.73E+15 photons cm\(^{-2}\) s\(^{-1}\)) stimuli were matched during this experiment.
Figure 5.4. Results of extracellular recordings from an *A. agaricites* planula. The black line represents the electrical current generated in response to the white light stimulus; blue line = the response to the blue stimulus; red line = the response to the red stimulus; green line = shutter trace (depression indicates duration of shutter opening – 75ms). The quantal intensities of the red (2.68E+16 photons cm\(^{-2}\) s\(^{-1}\)) and the blue (2.74E+16 photons cm\(^{-2}\) s\(^{-1}\)) stimuli were matched during this experiment.
**Figure 5.5.** Results of extracellular recordings from azooxanthellate larvae of three spawning species (*A. cervicornis, A. palmata*, and *M. faveolata*). The black line represents the electrical current generated in response to the white light stimulus; blue line = the response to the blue light stimulus; red line the response to the red light stimulus; green line the shutter trace (depression indicates duration of shutter opening). The quantal intensity of the blue (7.08+16 photons cm$^{-2}$ s$^{-1}$) stimulus was approximately 20% greater than the red (5.54+16 photons cm$^{-2}$ s$^{-1}$) in this experiment. Despite the difference in these intensities, the larval responses to the two stimuli were not significantly different.
Table 5.1. Response speeds of coral larval photoreceptors

<table>
<thead>
<tr>
<th>Species</th>
<th>$I_{a}$ (ms)</th>
<th>$I_{b}$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Porites astreoides</em></td>
<td>na</td>
<td>188(±15)</td>
</tr>
<tr>
<td><em>Agaricia agaricites</em></td>
<td>20</td>
<td>253(±20)</td>
</tr>
<tr>
<td><em>Montastrea faveolata</em></td>
<td>na</td>
<td>247(±6)</td>
</tr>
<tr>
<td><em>Acropora cervicornis</em></td>
<td>6</td>
<td>207(±21)</td>
</tr>
<tr>
<td><em>Acropora palmata</em></td>
<td>10</td>
<td>195(±28)</td>
</tr>
</tbody>
</table>

Response speeds of larval photoreceptors. Time-to-peak estimates (time from the onset of light stimuli to peaks) for a- ($I_{a}$) and b-waves ($I_{b}$). $I_{a}$ is based on a single recording (n=1). $I_{b}$ is the average of 3 recordings (n=3), ±1SD. a-waves were not observed in *P. astreoides* or *M. faveolata* (na).

*A. agaricites* and *A. palmata* larvae displayed graded responses (amplitude of the b-wave) to changing light intensity (Figure 5.6). This data was not collected for the other species due to time constraints (larval viability and availability of the recording equipment), and could not be collected for the blue light stimulus due to limitations of the filter holders and neutral density filters available.

All species of larvae demonstrated equal or greater sensitivity to the red stimulus compared to the blue (Figures 5.3-5.5). When the intensities of the blue and red stimuli were quantally matched during experiments the responses to red and blue measured in *P. astreoides* were approximately equal (Figure 5.3), but *A. agaricites* consistently displayed a greater response to the red stimulus (Figure 5.4). During the recording of ERGs from the spawning species, the intensity of the blue stimulus was approximately 20% greater than the red (Blue-7.08 and Red-5.54E+16 photons cm$^{-2}$ s$^{-1}$) yet, despite this difference, equivalent responses were observed to both blue and red stimuli (Figure 5.5).
Figure 5.6. Graded responses (amplitude of b-waves) to changing light intensity. A linear relationship between the response and changing intensity of the red-light stimuli (RG630 filter with 0.5, 0.3 and 0.15 neutral density filters) was observed in *A. agaricites* (a) and *A. palmata* (b) larvae when recording with the red-light stimuli.

**Discussion**

The ERG is an electrical response of photoreceptors originating from extracellular currents generated in response to a light stimulus. In humans, and other mammals, extracellular flash ERGs (response to a single, brief pulse of light) consist of negative a-wave, a positive b-wave, and may or may not display oscillatory potentials as a component of the wave form (see Figure 5.1; Cameron et al. 2008). The recordings made here from coral larvae are not true ERGs but flashes of light generated similar wave forms in these organisms. The changes in electrical current measured in coral larvae
consisted of a small, negative deflection (a-wave) that was only observed in response to the most intense, white light stimuli, and varied among individuals, followed by a relatively large positive deflection (b-wave). The responses measured here are similar to those observed in mammalian eyes (Cameron et al. 2008) but are also similar to ERGs recorded from the lens eyes the jellyfish *Polyorchis penicillatus* (Weber 1982) and *Tripedalia cystophora* (Figure 5.7; Garm et al. 2007), organisms with ciliary, depolarizing photoreceptors that signal via cyclic nucleotide gated pathways.

**Figure 5.7.** ERG recordings from the lens eyes of the jellyfish *T. cystophora*. (a) Traces from a single eye demonstrating the change in response (disappearance of a-waves and diminishing b-waves) with decreasing stimulus intensity; (b) The average normalized response of five individuals. The response is similar to that observed in coral larvae – a small negative a-wave followed by a large positive b-wave (from Garm et al. 2007).
In a true ERG, the a-wave originates from the photoreceptors (rods and/or cones) as light absorbed by the visual pigments (opsins) in the outer segments of the photoreceptors reduces the dark current or resting potential of the dark-adapted eye. In humans and other mammals, this current is expressed as a negative wave when recorded extracellularly, from the vitreous or the cornea. The recording made here of larval photoreceptors were also extracellular recordings. The a-waves, when observed, were small negative waves, but increased with increasing light intensity (were largest with the unfiltered white light stimulus) (Figures 5.4-5.5). This is consistent with behavior observed in mammalian ERGs.

In vertebrate ERGs, the a-wave is affected by both the stimulus intensity and the species. The amplitude of a-waves tend to be proportional to light intensity until saturating intensity is reached (Figure 5.8), but is also related to the density of cones occurring in the retina of the subject. Nocturnal animals (e.g., mice) possess so few cones that they are often incapable of producing a measureable a-wave, while organisms with well-developed color vision (i.e. greater density of cones; e.g., humans) have a large a-wave (Cameron et al. 2008). Small a-waves were observed in some recordings (for example, the white light response in *A. agaricites* (Figure 5.4) and the white light response in *A. cervicornis* (Figure 5.5). The relatively small size of this wave, compared to the amplitude of the b-wave, and inconsistency in the presence of an a-wave across recordings within a species (i.e. a-waves were not observed in every individual of a given species) may have resulted from a combination of factors: 1) the random positioning of the recording electrode relative to photoreceptors (i.e. proximity to photoreceptors and position within supporting or post-synaptic cells); 2) the low density (scattered
organization) of solitary epithelial photoreceptors found throughout most of the larval epithelium (Chapter 4). In eyes, the density and parallel organization of rods and cones focuses light-induced current, resulting in a strong response, the lack of similar comparable density and abundance of photoreceptors likely contributes to the relatively weak and variable a-waves recorded here (akin to an mammal with a low density of cones).

The exact source of the ERG b-wave in vertebrate eyes is still in question, but appears to be the summed response of light-induced electrical activity in cells post-synaptic to the photoreceptors. The major source of the b-wave is light induced activity in ON-center bipolar cells (e.g., Sieving et al. 1994) that originates either directly from these cells or from changes in the membrane potential of surrounding Müller cells (Miller and Dowling 1970). Biochemical evidence suggests that the b-wave also is influenced by activity in OFF-center bipolar cells (Sieving et al. 1994), while oscillatory potentials can result from light-induced activity in 3rd-order retinal neurons (amacrine and ganglion cells - Dong and Hare 2000; Awatramani et al. 2001) (see Figure 5.5). Post-synaptic cells involved in photoreception have not been identified in coral larvae, but larval epithelial photoreceptors appear to possess processes which terminate in the vicinity of the mesoglea, known to contain the nerve net of cnidarians (Martin 2002) and which likely represents the site of signal integration and processing (see Chapter 4). The cnidarian nerve cells would likely function in a similar manner to horizontal, and bipolar cells of the mammalian retina (as second order neurons integrating and propagating the sensory stimuli). The b-waves recorded here likely represent the summation of synaptic
Figure 5.8. Relative ERG responses of a dark-adapted human measured over a range of light intensities spanning 4 log units. As light intensity increases so does the size of the a-wave until saturating intensity is reached. The responses observed in coral larvae most resemble the human ERG responses measured at intermediate light intensities (-4.0 and -3.0) (from http://webvision.med.utah.edu).

In addition to similarity in a and b wave forms, the coral larval ERGs also display another basic characteristic of ERGs from higher multicellular organisms – a graded response to changes in light intensity. Graded potentials, responses proportional to changes in light intensity, were measured *A. agaricites* and *A. palmata* in response to varying intensities of the red light stimulus (Figure 5.6). Graded potentials occur in
vertebrates (Sieving and Wakabayashi 1991), but have also been observed in jellyfish (Weber 1982; Arkett and Spencer 1986; Garm et al. 2007).

Graded responses are proportional to light intensity within the dynamic range of an organism, and enable the organism to detect and interpret slight changes in light intensity within this range (Martin 2002). The linear fit of the graded responses measured in *A. agaricites* and *A. palmata* suggests that these light intensities fall within the dynamic range of this species (Garm et al. 2007).

The response speeds of larval photoreceptors, measured here as time-to-peak (*L_a* and *L_b*; Table 5.1) were slow, but may be a result of stimulus intensity and density of photoreceptors. For perspective, time-to-peak estimates of the cone response measured in the living human eye range from 15-28ms (Hood et al. 2002; Friedburg et al. 2004). Response speeds (*L_b*) for the upper and lower lens eyes of the box jellyfish *T. cystophora* and *Chiropsalmus sp.*, measured at maximum, near-saturating light intensities, ranged from 47-81ms (Garm et al. 2007). However, the speed of these responses varied with light intensity. Lower and wider responses with *L_s* in the range of 200-300ms - similar to the speed of coral larval responses (Table 5.1) - were observed in response to light intensities approaching the lower end of the dynamic ranges of these species (Figure 5.7; Garm et al. 2007).

Slow vision is typically considered an adaptation for either dim light or high visual acuity (Warrant et al. 2004; Warrant and Locket 2004). Since coral larvae lack eyes and live in tropical waters where they are exposed to bright sunlight, neither of these represents a plausible explanation for the evolution of slow response times. A more likely explanation is that the slow responses of coral photoreceptors act as temporal low-
pass filters – essentially providing photoreceptors with a smoothing function – as proposed for the lens eyes of jellyfish (Garm et al. 2007). Shallow water environments experience a constant flicker of illumination (Maximov 2000). Slow photoreceptors may eliminate noise created by this flicker, enabling the sensing and interpretation of spectral cues by organisms (e.g., coral larvae) lacking image-forming eyes.

The inability to achieve saturating intensities, a result of the available light source, restricted my ability to determine the dynamic range (range of sensitivity) and response speeds of larvae but more importantly it prevented the measurement of spectral sensitivity over the full visible spectrum. When the incident light was processed by a monochromator the resulting stimuli were not strong enough to elicit a response. Alternatively, I only was able to compare the responses of larvae to the blue and red stimuli described above. The response of *A. agaricites* to red was consistently ~20% greater than the response to blue when the red and blue stimuli were matched, and when the blue stimulus was approximately 20% greater than the red (during the recording of responses in *M. faveolata, A. cervicornis*, and *A. palamata*), larva consistently displayed equivalent responses to the two stimuli (Figures 5.4-5.5). These results demonstrate that larval photoreceptors are sensitive to red light, but they also suggest that the larvae possess a pigment with an absorbance peak centered between the peaks of the blue (550nm) and red (670nm) stimuli. An opsin with an absorbance centered between ~610 would respond strongly to both the blue and red stimuli and could produce the relative responses measured here. Pigments centered at or below 550nm would overlap complete with, and respond strongly to the blue stimulus, but weakly to the red, while a pigment
with an absorbance maximum at 650nm or above would overlap with and respond to the red stimulus but weakly to the blue.

The spectral sensitivity of larval phoptoreceptors predicted from these larval recordings differs from spectral sensitivities measured in isolated pigments, from ERGs or from sensitivities predicted on the basis of behavioral studies, in other cnidarians. Jellyfish *Sarsia saltatrix* and *Polyorchis pencillatus* display a shadow response at 550nm and between 480 and 500nm, respectively (Yoshida 1969; Arkett 1985). Corals, *Montastrea cavernosa* display feeding behavior (tentacle expansion) in response to dim blue light (Gorbunov and Falkowski 2002). ERGs show maximum responses at 540 in the jellyfish *S. tubulosa* (Weber 1982), 504/512nm and 510/497 in the upper/lower lens eyes of the box jellyfish *T. cystophora*, and *Chiropsalmus sp.* respectively (Garm et al. 2007). Koyanagi et al. (2008) determined the absorbance spectra of an opsin isolated from the large lens eye of the box jellyfish *Carybdea rastonii* after heterologously expressing the protein in mammalian cells and determined that it absorbed maximally at 500nm.

The waveform and behavior of traces recorded in coral larvae demonstrate: 1) coral larvae have a light-induced electrical response (i.e. are photosensitive); 2) their response to light intensity is graded; 3) coral larvae possess long-wavelength photosensitivity. The photosensitivity demonstrated by these recordings could be responsible for the red substrate preference observed in *P. astreoides* and *A. palmata* (Chapter 2), but relating this response to a specific photoreceptor (i.e. Acropsin 1 or 2; Chapter 4) will require additional analysis.
CHAPTER 6

CONCLUSIONS AND BROADER IMPLICATIONS

Conclusions

The results presented here provide several lines of evidence for opsin-based photosensitivity in reef-building corals. Chapter 2 discussed the results of larval settlement experiments, demonstrating color preference during settlement in two species – the mustard-hill coral, *Porites astreoides*, and the elkhorn coral, *Acropora palmata*. Chapter 3 described two full-length opsin proteins encoded by full-length cDNAs that I cloned from *A. palmata* larvae. These proteins possess the structure and functional elements necessary for membrane localization, retinal binding and G protein signaling. Chapter 4 presented immunological evidence that these opsins are expressed in corals and localized in different larval cell types – one in sensory epithelial cells and the other in the larval endoderm. Lastly, Chapter 5 reported results of extracellular recordings, demonstrating light-induced electrical activity (photosensitivity) in several species of both zooxanthellate (containing symbionts) and azooxanthellate, coral larvae.

While this research has developed several lines of evidence to support the existence, and demonstrate one potential ecological function, of opsin-based photosensitivity in corals, it falls short of demonstrating: 1) that these opsin-like proteins form functional opsins; 2) that these proteins are responsible the red color preference observed during settlement.

Since the start of this project, several groups (e.g., Suga et al. 2008; Koyanagi et al. 2008) have cloned and described opsins from other cnidarians. These proteins and
progress made on the assembly and annotation of the _Acropora millepora_ larval transcriptome, offer the opportunity to explore the evolutionary position, and possible function/biochemistry of Acropsin 1 and 2.

In this sixth and final chapter I: 1) revisit the sensory ecology of coral larvae as I describe an evolving model of larval settlement ecology, including the apparent functions and relative importance of chemical and light cues in larval behavior and settlement; 2) explore additional evidence (other than that presented in Chapters 2-5) for the existence of functional opsins; 3) use additional sequence analysis predict G protein binding; 4) discuss the phylogenetic position of coral opsins and implications for the evolution of phototransduction pathways; 5) discuss selective pressures that have likely influenced spectral sensitivity, distribution and evolution of coral photopigments.

**The sensory ecology of coral larvae**

Even though coral larvae consist of only two tissue layers (ectoderm and endoderm), approximately 12 cell types, and lack a true nervous system (Harrison and Wallace 1990), selective pressure has apparently led to the evolution of surprisingly complex sensory capacities. While it is important to recognize that not all species of coral larvae behave in an identical manner, examples of chemosensory, photosensory and, recently, auditory (Vermeij et al. 2010) capacity has been shown to exist in coral planulae. Various cues function in a hierarchical nature, and over a range of spatial scales, to assist larvae in their location of, settlement and metamorphosis in, habitats conducive to their survival (Raimondi and Morse 2000).
Numerous examples of photosensitive behavior have been reported in larvae. Kawaguti (1941) in one of the first descriptions larval photosensitivity reported photosensitivity in several species of larvae, noting that larvae containing symbionts were attracted to light but azooxanthellate larvae were not. He also observed interspecific differences in sensitivity which he hypothesized could provide a mechanism means for vertical positioning in the water-column and concentration of larvae at depths suitable for survival. Szmant and Meadows (2006) reported diel vertical migration in azooxanthellate larvae (*Montastrea faveolata*). More recently, (Gleason et al. 2006) described UV avoidance behavior in *Porites astreoides*. Mundy and Babcock (1998) showed preferences for either light quality (light with a given spectral distribution) or intensity in several species, which were correlated with the depth distribution of the adults. Like Kawaguti (1941) they concluded that these preferences could function as a depth gauge to concentrate larvae in habitat favorable for the survival of adults. Finally, the results presented in Chapter 2 represent the first evidence that some species of coral larvae may use spectral cues (in this case objects whose spectra are dominated by orange and red wavelengths of light) for fine scale habitat selection during settlement.

While examples of photosensitivity have been reported in the literature, chemotaxis has been the primary focus of investigations of larval sensory biology. Metamorphosis in many species is induced by cell wall morphogens in crustose coralline algae (CCA) (e.g., Morse et al. 1988, Morse et al. 1996, Morse and Morse 1991; Raimondi and Morse 2000, Baird and Morse 2004), densities of settlers in the field have also show a positive correlation with CCA (Harrington et al. 2004).
The larval settlement/CCA paradigm has evolved slightly over the years as the strict requirement for CCA has been shown to vary among species (Baird and Morse 2004, Golbuu and Richmond 2010), and some species have been shown to settle in response to microbial biofilms (e.g., Negri et al. 2001, Webster et al. 2004). Recent studies have also shown that settlement on or adjacent to CCA can actually be detrimental to newly settled corals and only certain species of CCA actually facilitate survival (Harrington et al. 2004; Ritson-Williams et al. 2009; Ritson-Williams et al. 2010). However, preference for these “facilitating” CCA types has been demonstrated in some coral species (Ritson-Williams et al. 2010), and even a general preference for CCA may offer advantages over alternative substrate types, since CCA is negatively correlated with (e.g., Fabricius and De’ath 2001) other known sources of juvenile coral mortality (i.e. sediment, macroalgae; Birrell et al. 2005).

In addition to surface or membrane-bound settlement inducers associated with biofilms and/or CCA, water-soluble cues have also been shown to influence larval behavior. Gleason et al. (2009) demonstrated that seawater collected from near the reef induces downward swimming and substrate exploration in P. astreoides. While the cue was not identified, data collected during a settlement experiment with the same species suggests that it originates from microbial biofilm communities.

During this study, while attempting to document color preference during settlement, I observed phototactic behavior in P. astreoides larvae that was altered by the presence of microbial biofilm. In this experiment, I placed 10 P. astreoides larvae in each of 20 plastic petri dishes. Plastic substrata representing two microbial conditioning treatments, unconditioned substrata and substrata conditioned for one week in a
flowthrough seawater system, were added to dishes and dishes were positioned on a laboratory bench near a fluorescent strip light. After 24hrs, larvae in dishes with unconditioned substrata were all swimming at the surface and a significantly larger proportion were observed swimming in the third of the dish nearest the light. In dishes with conditioned substrates, larvae abandoned this phototactic behavior. Over half of the larvae were observed exploring the bottom of the petri dish and their distribution within the dish was random (Figure 6.1).

![Graph](image)

**Figure 6.1.** Results of a behavior experiment conducted with *P. astreoides* larvae. Larvae (10 per dish) were kept in plastic petri dishes on a lab bench adjacent to an external, fluorescent light source (position indicated by the suns). Petri dishes (10 of each) contained plastic cable ties that were unconditioned (left) or conditioned (right) in re-circulating seawater for one week. In dishes with unconditioned ties, all larvae (100%) were observed swimming at the surface of the dish and 2/3 (0.66) were located in the third of the dish closest to the light source. In dishes with conditioned ties, larvae abandoned their photosensitive behavior and demonstrated substrate exploration. Over half of the larvae 55% were observed crawling on the bottom of dishes and no longer phototactic, with almost half (0.46) found crawling in the middle 1/3 of the dishes.

A hierarchy of cues - including multiple photosensitivities, chemicals, and possibly chemically induced photosensitivity - appear to influence larval behavior prior
to and during settlement. At least some species of larvae are phototactic, but data presented here and in previous research (Gleason et al. 2009) indicate that water-soluble, chemical cues either supersede or induce an additional photosensitivity. It is not clear whether the substrate exploration that has been observed upon contact with reef water (Gleason et al. 2009), or, in this case, water containing lightly conditioned, biofilmed substrates, is the result of larvae abandoning positive phototaxis or whether there is an integration of light and chemical cues that induces a second, negative phototaxis with a different spectral sensitivity (e.g., avoidance of UV or blue) that results in vertical repositioning.

Chemical induced photosensitivity has been observed in other aquatic organisms. DeMeester (1993) showed that predator (fish) kairomones induce negative phototaxis in water flea (*Daphnia magna*). Similar integrations of chemical and light cues have been observed in larvae of the water mite (Edwards 1999), and in Chlamydomonas (Govorunova and Sineshcheckov 2003). Chemical-induced photosensitivity could result from contact with a specific carotenoid compound, whose metabolite forms the opsin chromophore (11-cis-retinal or similar isoform). While carotenoids are abundant in seawater, coral larvae may only have receptors (discussed below) for, or enzymes for converting, specific carotenoids that originate from microbial biofilms or algal communities.

While chemical cues result in bottom exploration in laboratory experiments, this may be an artifact of the light environment and experimental design constraints. Mundy and Babcock (1998) provide evidence that larvae may use light cues to position themselves within depth strata that would concentrate them at a desired depth. In petri
dishes, aquaria or graduated cylinders, where most larvae behavior experiments have been conducted, avoidance of certain light intensities or wavelengths could push larvae to the bottom of containers as they attempt to locate desired spectral environment (i.e. in experiments they are observed in the bottom of containers, but on the reef they may descend to 5 or 10 meters).

In order for photosensitivity to act as a depth gauge across various times of day, and weather conditions, organisms require chromatic antagonism (attraction to one region of the visible light spectrum and avoidance of another) (Nilsson 2009). Such mechanisms would require either two photopigments, each with different spectral sensitivities and associated with antagonistic responses, or a bistable pigment (see Chapter 1). Bistable (Gq/melanopsin/r-opsins) opsins are photo-converted between the ground and signaling states by the absorption of light, and the absorbance peaks of the two states are often separated by ~100nm. If the two states resulted in antagonistic behaviors, a bistable pigment could be used to position an organism at depth where the spectral distribution of incident light resulted in a balance between the two opsin states. This study presents evidence for at least two photopigments (Acropsin 1 and 2) in *A. palmata* that may provide chromatic antagonism required for depth positioning and enable fine-scale habitat selection during settlement. While I have no evidence at this time that the Acropsins are functioning as larval photoreceptors or during these settlement processes, results presented here offer clues to their functions.

The distribution of Acropsin 1 and 2 provide insight into their possible functions. Acropsin 1 is localized throughout the larval endoderm. The ubiquitous distribution of this receptor within the larval endoderm and nerve net suggests that this opsin is unlikely
to influence directional swimming (since its distribution does not provide any mechanism for orientation), and more likely plays a role in interpreting changes in the light environment (e.g., light intensity, day/night, spectral distribution of light environment). In vertebrates similar functions (e.g., diurnal rhythms, seasonal reproductive cycles) are allocated to non-visual Gq/r-opsin (melanopsins) or Gt/c-opsins (tmt opsins, panopsin, pinopsins, parietopsins …). Blast analysis of Acropsin 1 demonstrates that this opsin is similar to these non-visual opsin types. It also has similar localization (see Chapter 4). Acropsin 1 is found in endodermal and neural tissue underlying ciliary, epithelial cells, similar to the distribution of melanopsin (in the horizontal and bipolar cells) in the inner retina of vertebrates.

The distribution of Acropsin 2 also may offer insight into its function. This receptor is localized in solitary epithelial cells found throughout the larval epithelium, but also occurs at higher density in the aboral epidermis (Chapter 4), suggesting that Acropsin 2 has a phototactic function. The concentration of Acropsin 2 photoreceptors in the aboral epidermis could enable larvae to determine the directionality of incident light, or light and orient toward (or away from) that stimulus, or discriminate among different colors of substrate during settlement.

As our understanding of larval behavior and settlement ecology continues to evolve, a surprisingly complex cascade of environmental signals and sensory biology is emerging. It now appears that coral larvae remain positively phototactic when they are in open water (away from the coral reef). This behavior may serve to increase their dispersal, avoid predation and conserve energy reserves that are more critical later, during substrate exploration and settlement phases. Substrate exploration (or vertical re-
positioning) appears to be induced following contact with water-soluble cues originating from the reef (Gleason et al. 2009), after which time larvae appear to abandon positive phototaxis (or acquire a second, negative phototaxis that results in vertical re-positioning). At least some species appear to use spectral cues to concentrate themselves within a depth strata favorable for post-settlement survival, but even after reaching the reef, metamorphosis often still requires cues associated with CCA. While compounds extracted from CCA clearly induce metamorphosis of some species (e.g., Morse and Morse 1991, Morse et al. 1996), including species of Acropora, (e.g., Harrington et al. 2004), it appears as though the spectral signatures of these algae may also induce settlement and metamorphosis.

**Additional evidence that the Acropsins may form functional opsins**

Until experiments are conducted to demonstrate function and determine the biochemistry of the Acropsins proteins, I am unable to claim with certainty that Acropsin 1 and 2 form functional photopigments. However, the larval EST database for *A. millepora* provides a resource with which to investigate the possible pathways that may be involved in Acropsin signaling and additional evidence for their formation of functional photopigments.

Below I present the results of a transcriptome, phototransduction and pigment cycle pathway analysis of the *A. millepora* larval EST database. The database was blasted (TBLASTN) using representative vertebrate and invertebrate proteins as bait, in order to determine the signaling proteins and pigment cycle members (e.g., enzymes, chromophore binding proteins) expressed in these larvae. Signaling proteins included in
the search were G proteins, regulators of G protein signaling (RGSs), rhodopsin/G protein receptor kinases (GRKs), signal terminators (arrestin)). Retinoid cycle proteins included: retinal pigment epithelium specific 65kDa protein (RPE65), retinol-dehydrogenase (RDH), cellular retinol-binding protein (CRBP), cellular retinaldehyde-binding protein (CRALBP), lecithin retinol acyltransferase (Lrat), NINAB (neither inactivation nor afterpotential B), NINAD, NINAG, SANTA MARIA (scavenger receptor acting in neural tissue and the majority of rhodopsin is absent), PINTA (prolonged depolarization afterpotential is not apparent) (Table 6.1).

In addition to the opsin-like ESTs that I used for enabled the cloning of Acropsin 1 and 2, the analysis revealed that A. millepora larvae possess numerous homologues of proteins involved in G protein signaling. They have five different G proteins (two Gs, Gi, Gq, Go, and G12). Four of these G proteins are highly conserved in other invertebrates and vertebrates (Table 6.1). A. millepora appears to lack a Gt (transducin; the G protein involved in vertebrate c-opsin signaling; e.g., in human rod and cone opsins) homologue. The presence of G proteins but absence of Gt is consistent with previous bioinformatics studies demonstrating that G proteins existed prior to the origin of metazoan opsins (Plachetzki et al. 2010) but Gt arose later, during a vertebrate-specific block (chromosome) duplication event (Nordstrom et al. 2004).

Larvae also possess three RGS proteins (one RGS7, and two proteins similar to RGS19), one arrestin (similar to beta-arrestin 1) and two G protein receptor kinases (similar to human GPCR kinase 6, and beta-adrenergic RK6). These proteins are not homologous to opsin-specific isoforms found in higher organisms. However, since these genes had not diversified and been co-opted for specialized roles (e.g.,
phototransduction), inference of their function, either positive or negative, in *A. millepora* phototransduction pathways is limited.

*A. millepora* possesses at least some of the enzymes required for regeneration of retinal, but seem to lack pigment-binding proteins (e.g., CRBP, CRALBP) involved in uptake and transport of retinoids (Bok 1993). They also possess proteins similar to RPE65, beta-carotene oxygenase and retinol dehydrogenase, enzymes required for regeneration of 11-cis-retinal (e.g., Yau and Hardie 2009). While they lack putative, cellular retinoid-binding proteins, blast searches of NINAD and SANTA MARIA proteins identify *A. millepora* scavenger receptors and lysosome membrane proteins that may be involved in the recognition, uptake and intracellular (lysosome) transport of retinoids (Table 6.1).

**Prediction of Acropsin G protein coupling**

Until biochemical analysis can be completed to determine Acropsin/G protein coupling, sequence analysis is the best means by which to predict binding specificity. Two slightly different approaches were used to predict the G protein binding specificity of Acropsin 1 and 2: 1) a G protein coupling prediction program; 2) amino acid sequence alignment and phylogenetic analysis of G protein binding domains.
Table 6.1. *Acropora millepora* proteins with possible involvement in larval photoreception and pigment regeneration.

<table>
<thead>
<tr>
<th>Query</th>
<th>Function</th>
<th>Amii Seq</th>
<th>nt</th>
<th>Hits</th>
<th>E value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G protein</td>
<td>GPCR signaling</td>
<td>3582</td>
<td>1939</td>
<td>Gi subunit alpha_Astea(^a)</td>
<td>1.0E-177</td>
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<tr>
<td></td>
<td></td>
<td>5045</td>
<td>1887</td>
<td>Gq subunit alpha_Rat</td>
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<td></td>
<td></td>
<td>2413</td>
<td>1081</td>
<td>Go subunit alpha_Bovine</td>
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<tr>
<td></td>
<td></td>
<td>10430</td>
<td>966</td>
<td>Gs subunit alpha_Anoga(^b)</td>
<td>1.0E-124</td>
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<tr>
<td></td>
<td></td>
<td>3459</td>
<td>2628</td>
<td>G12 subunit alpha_Mouse</td>
<td>1.0E-118</td>
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<td></td>
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<td>Gs subunit alpha_Xenopus</td>
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<td>RGS</td>
<td>deactivate G proteins</td>
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<td>RGS7_Mouse</td>
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<td></td>
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<td>5623</td>
<td>1736</td>
<td>RGS19_Bovine</td>
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<td></td>
<td></td>
<td>1765</td>
<td>870</td>
<td>RGS19_Mouse</td>
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<td>Arrestin</td>
<td>terminates GPCR signaling</td>
<td>7443</td>
<td>1973</td>
<td>Beta-arrestin-1_Macfa(^c)</td>
<td>1.0E-127</td>
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<tr>
<td>GPRK1</td>
<td>terminates GPCR signaling</td>
<td>4094</td>
<td>1165</td>
<td>GPCR kinase 6_Human</td>
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<td></td>
<td></td>
<td>3858</td>
<td>580</td>
<td>Beta-adrenergic RK6_Human</td>
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<tr>
<td>RPE65</td>
<td>retinoid cycle</td>
<td>13704</td>
<td>1067</td>
<td>Beta, beta carotene oxygenase_Mouse</td>
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<td></td>
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<td>325</td>
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<td>RPE65_Chicken</td>
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<td>1081</td>
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<tr>
<td>CRBP</td>
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<td>CRALBP</td>
<td></td>
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<td>Lrat</td>
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<td>NINAB</td>
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<td></td>
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<td>784</td>
<td>Lysosome membrae protein 2_Human</td>
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<td>942</td>
<td>Scavenger receptor class B_Human</td>
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<td>PINTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NINAG</td>
<td></td>
<td>12136</td>
<td>451</td>
<td>Choline dehydrogenase, mito_Human</td>
<td>3.0E-40</td>
</tr>
</tbody>
</table>

\(^a\) *Asterina pectinifera* (starfish); \(^b\) *Anopheles gambiae* (mosquito); \(^c\) *Macaca fascicukaris* (macaque)

It is known that the cytoplasmic domains of GPCRs interact with G proteins to determine coupling specificity (or promiscuity; binding of more than one type of G protein) and, as a result, G protein binding specificity can be predicted from the amino
acid sequences of GPCRs. Predictions of G protein binding specificity were made for Acropsins 1 and 2 using PRED-COUPLE2 (http://athina.biol.uoa.gr/bioinformatics/PRED-COUPLE2/), a program that implements a library of refined profile Hidden Markov Models (pHMMs), trained by the intracellular domain sequences of 188 GPCRs with known coupling properties to the four families of G proteins. PRED-COUPLE2 produces outputs of four numbers, ranging from 0 to 1, which correspond to the probability that the GPCR in question couples to each of the four families of G-proteins. A cut-off of 0.3 has been found to discriminate between positive and negative predictions.

PRED-COUPLE2 predicted that both Acropsin 1 and 2 are most likely Gi/o coupled receptors (Table 6.2). However, the program also predicted a high probability of Gq/11 binding for both receptors, suggesting that these receptors could be promiscuous (bind multiple G proteins). Low probabilities (well below the 0.30 cut-off) of Gs and G12/13 binding were predicted for both Acropsin 1 and 2, suggesting that these two families (and three of the five G proteins present in the A. millepora transcriptome) are unlikely to function in Acropsin signaling. The replacement of the c-terminal tail of Acropsin 1 with Acropsin 3 led to a decrease in Gi/o binding probability and an increase in Gq/11 probability suggesting that, if Acropsin 3 does interact with a G protein, it is more likely Gq/11.

Only one cnidarian opsin has been characterized biochemically – an opsin from the lens eye of a box jellyfish (Koyanagi et al. 2008). This opsin binds Gs and activates a cAMP signaling cascade. Based on predictions of binding specificity, it is unlikely that either Acropsin 1 or 2 is coupled to Gs. Both sequence analysis (blast analysis indicating
similarity to Gt and Gq coupled receptors) and predictions of G protein binding specificity suggest that the Acropsins are Gi/o or Gq type opsins.

Table 6.2. Probabilities of G protein binding specificity predicted for Acropsin receptors

<table>
<thead>
<tr>
<th>Acropsin 1</th>
<th>Gi/o</th>
<th>Gq/11</th>
<th>Gs</th>
<th>G12/13</th>
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<tbody>
<tr>
<td>Acropsin 2</td>
<td>0.98</td>
<td>0.75</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Acropsin 1-3</td>
<td>0.99</td>
<td>0.61</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>(recombinant)</td>
<td>0.93</td>
<td>0.91</td>
<td>0.18</td>
<td>0.05</td>
</tr>
</tbody>
</table>

In the sequence alignment analysis the helix 8 (cytoplasmic loop 4) regions (a cytoplasmic, helical domain of opsin found immediately downstream from TM7 and known to interact with the Gα subunit of G proteins; Martin et al. 2000) of the Acropsins were aligned (by Clustal W using a Gonnet protein weight matrix) with representative opsin subfamily members. Analysis of sequence similarity predicted binding specificities for Acropsin 1 and 2 that are similar to those obtained using PRED_COUPLE2. A phenogram based on the ClustalW alignment of this G-protein binding region of the Acropsins estimates that the Acropsins fall in between the Go/Gt receptors (Gi/o G protein family) and Gq/melanopsin receptors (Gq/11 G protein family) (Figure 6.2).

The accuracy of predictions of binding specificity for the Acropsins may suffer from a vertebrate bias (use of vertebrate GPCRs in the construction of predictive models) or the Acropsins could be promiscuous receptors. It is known that many GPCRs, given the opportunity, are capable of binding multiple G proteins, within the same family or of completely unrelated families (reviewed by Hermans et al. 2003). Promiscuity of G
protein binding by the Acropsins could provide a means of functional diversification. In other words, a single Acropsin could function differently as a result of expression in different populations of cells or at different life history stages due to co-localization with, and differential expression of, G proteins, secondary messengers, downstream effectors etc... This may have been a common means of diversifying photoreceptor function in ancestral organisms, prior to the duplication and diversification of opsins.

Figure 6.2. Phenogram of Helix 8 (known G protein binding) domains of Acropsins and representative opsin types. Distinct groups are observed for Gt and Gq opsins. The Acropsins fall in between Gq and Go/Gt type opsins.
The evolution of opsins and phototransduction pathways: hints from the Acropsins

Light-sensitive transmembrane proteins employing retinal chromophores are found in bacteria (e.g., Mukohata et al. 1999), algae (Sineshchekov and Govorunova 2001; Nagel et al. 2002; Frassanito et al. 2010) and fungi (Bieszke et al. 1999). Although these receptors are generally referred to as rhodopsins, they do not bind G proteins, and function differently than metazoan rhodopsins, serving as light-driven ion pumps, ion channels, or sensory molecules. Recent bioinformatic evidence (Larusso et al. 2008) indicates that prokaryotic and metazoan opsins are not homologous, and their remarkable similarities (seven transmembrane structure and use of a retinal chromophore) are actually the result of convergent evolution. If metazoan opsins evolved independently, where and when did these receptors originate? The recent completion of several genomic databases for basal metazoan phyla has helped to pinpoint the origin of metazoan opsins.

While G proteins and GPCRs are present in choanoflagellates and sponges, opsins are not (Plachetski and Oakley 2007). Genomic databases, now available for the choanoflagellate Monosiga, and a sponge, Amphimedon, lack opsins (Plachetski et al. 2007), but opsins are found in fully sequenced cnidarian genomes (e.g., the sea anemone Nematostella vectensis, Hydra magnipapillata: Suga et al. 2008), suggesting that metazoan opsins likely originated from a GPCR that acquired light sensitivity in a common cnidarian-bilaterian ancestor. It appears that this gene then underwent duplication prior to the protostome-deuterostome split, giving rise to c-opsin (vertebrate/Gt opsin) subfamily and a second ancestral class that produced the r-opsin (Gq/melanopsin subfamily), Go opsins, neuropsins and photoisomerases (RGR opsins and peropsins) (Figure 6.3; Plachetzki et al. 2007).
Plachetzki et al. (2010) proposed that phototransduction evolved from an ancestral cyclic nucleotide gated (CNG) pathway that originated in a common pre-metazoan ancestor. The authors argued that the origin of the c-opsin/CNG phototransduction pathways would have involved alteration of only a single gene – the GPCR – since CNG channels and GPCRs pre-date the origin of opsins. As supporting evidence they demonstrated co-localization of CNG ion channels and opsin in the same population of cells in *Hydra*, and reported the absence of a canonical transient receptor potential (TRPC; channel involved in Gq/r-opsin signaling) gene in the *Hydra* genome.

While this may be true for *Hydrozoans* (e.g., hydra and box jellyfish), corals, or at least *A. millepora*, possess homologues of the components necessary for Gq-coupled phototransduction cascade (Gq and phototransduction pathway members – e.g., TRPC channel protein, phospholipase C (PLC), protein kinase C (PKC)). Blast searches of the *A. millepora* larval transcriptome retrieved transcripts of genes for both r- (Gq) and c-opsin (Gi/o; e.g., Gt) phototransduction cascades (Table 6.3; for reference see Figure 6.4).

As above, the presence of these pathway members does not demonstrate Gq phototransduction, but is certainly leaves open the possibility that one or more of the Acropsins may bind Gq. The high probability of Gq-binding predicted for the Acropsins, the possession of pathway members required for Gq signaling in *A. millepora* larvae, the homology observed between Acropsin sequences and members of the Gq/melanopsin family (Chapter 3), and the difficulty expressing Acropsin 1 in mammalian cells (a trait common among members of Gq/melanopsin subfamily; Chapter 4), all suggest that, at
Figure 6.3. Simplified metazoan phylogenetic tree and animal phylogeny depicting the proposed evolution of phototransduction pathways. (a) Simplified evolutionary tree of extant eumetazoan phyla. The arrow indicates the Cnidarian bilaterian split. Bilaterians comprise the protostomes and deuterostomes which are believed to have diverged ~550 million years ago. By this time a common ancestor had already evolved with both r-and c-opsin typephotoreceptors (from Yau and Hardie 2009); (b) Traditional view of animal phylogeny indicating the presence (shaded boxes) and absence (open boxes) of opsin, and (c) Same view showing the evolution of phototransduction pathways. GPCRs, G proteins and CNG channels are present in Choanoflagellates and sponges (Porifera). The presence of opsins in Cnidaria (and absence in more ancestral phyla) indicates that opsins originated in a metazoan ancestor prior to the cnidarian/bilaterian split. TRPC channel proteins are absent in hydrozoan cnidarians (Hydra and jellyfish), leading Plachetzki et al. (2010) to propose a post-cnidarian origin of r-opsin phototransduction cascade, which became the dominant pathway in protostome vision but relegated to non-visual photoreceptors in deuterostomes.
Table 6.3. r- and c-opsin phototransduction pathway members present in the *Acropora millepora* larval transcriptome.

<table>
<thead>
<tr>
<th>Protein</th>
<th>A. mill seq.</th>
<th>Hit</th>
<th>E score</th>
</tr>
</thead>
<tbody>
<tr>
<td>r-opsin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRPC channel</td>
<td>SEQINDEX15558</td>
<td>Trpc3 (melanopsin TRP; Rat)</td>
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<tr>
<td>PLC</td>
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<td>PLC1 (bovine)</td>
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<tr>
<td>PKC</td>
<td>SEQINDEX4863</td>
<td>Prkcb (mouse)</td>
<td>1.0e-135</td>
</tr>
<tr>
<td>INAD</td>
<td>SEQINDEX12195</td>
<td>InaD-like protein (human) protein</td>
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</tr>
<tr>
<td>c-opsin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNG channel</td>
<td>SEQINDEX4632</td>
<td>CNG-cation channel (catfish)</td>
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<td></td>
<td>SEQINDEX6216</td>
<td>CNGA3 (human)</td>
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</tr>
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<td>SEQINDEX1565</td>
<td>cGMP-specific PDE (PDE5A_rat)</td>
<td>1.0e-48</td>
</tr>
<tr>
<td></td>
<td>SEQINDEX15850</td>
<td>cGMP-specific PDE (PDE6; Drosophila)</td>
<td>3.0e-40</td>
</tr>
</tbody>
</table>

*Since diacylglycerol transferase (DAG) is not a gene product, a DAG kinase was used as evidence of DAG.*

least Acropsin 1, may signal using a Gq pathway. If a coral opsins is found to bind Gq and signal using a r-opsin-like signaling cascade, this would be the most ancestral known example of Gq phototransduction cascade.

Even if the Acropsins (or other coral opsins) don’t bind Gq, the presence of Gq phototransduction pathway members has implications for evolution of phototransduction, but also for the cnidarian families. The presence of these pathway members suggest that either: 1) they were present in a common ancestor prior to the Cnidarian/bilaterian split and subsequently lost in hydrozoans; 2) they are an example of convergent evolution having evolved independently in the anthozoans and a common deuterostome/protostome ancestor; 3) the traditional view of animal phylogeny (Cnidarian/bilaterian split; Philippe et al. 2009) requires revision (i.e. the hydrozoans and anthozoans diverged at separate times, the hydrozoans first and the anthozoans at a later date, after the origin of Gq signal
Figure 6.4. Diagrams representing ciliary (c-opsin) and rhabdomeric (r-opsin) phototransduction cascades. (a) Typical c-opsin cascade. Rhodopsin is converted to its active form, Rh*, which binds Gt causing it to be activated by GTP-GDP exchange. Active Gta (Gta*) binds to and activates phosphodiesterase (PDE), which hydrolyzes cyclic GMP (cGMP) to GMP, closing the cyclic-nucleotide-gated (CNG) channels that are open in the dark. Rh* is phosphorylated by GRK1 allowing Arrestin (Arr) to bind fully inactive Rh*. Red lines indicate negative-feedback (inhibitory) pathways via Ca²⁺.

(b) Typical r-opsin cascade. Absorption of light by rhodopsin (R) converts it to the active metarhodopsin state (M* or Rh*), binds Gq and activates it by GTP-GDP exchange. Active Gq binds to and activates PLC, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (InsP³) and diacylglycerol (DAG). Two classes of light-sensitive channels (TRP and TRPL) are activated by a still-unknown effect of PLC activity. Ca²⁺ influx feeds back positively and negatively at multiple sites, including PKC, NINAC/arrestin (Arr2), and the TRP/TRPL channels. Ca²⁺ is extruded by a Na/Ca exchanger. TRP, PKC, and PLC are assembled into a signaling complex by the scaffolding protein INAD, possibly linked to the F-actin core via NINAC, a CaM-binding class III myosin. INAD has 5 PDZ domains, associated preferentially with different targets. The precise composition of the native complex is uncertain (from Yau and Hardie 2009).
transduction pathways and from a common line that gave rise to the protostomes and deuterostomes). Hypotheses 1 or 3 are more likely and supported by the presence of Gq pathway members in the *Nematostella* genome (www.stellabase.org; data not shown).

While I have not examined the intron/exon structure of the Acropsin genes (gDNA), the proteins (amino acid sequences inferred from full-length cDNAs) offer insight into the relationship between the Acropsins, other cnidarian opsins and metazoan opsin subfamilies (Figure 6.5). When analyzed by ClustalW using the Gonnet method (taking into account semi-conservative amino acid substitutions) for estimating protein differences, Acropsins 1 and 2 align most closely with a single group (Group 1; Suga et al. 2008) of *Nematostella* opsins, while the other two anthozoan groups (*Nematostella* Groups 1 and 2), are more similar to the Gq opsins and peropsins. The Acropsins appear equally similar to both the Gt and Gq clusters, consistent with the Cnidarian-bilaterian split. However, if the same sequences are analyzed by ClustalW, using an Identity matrix (scoring similarity based solely on percent identity; percent matching amino acids) to determine protein differences, a different picture emerges. When unrooted, the Acropsins group together with Anthozoan Group 1 and form a larger clade with the Hydrozoan/Jellyfish opsins. All of these show greater similarity to the Vertebrate/c-opsin subfamily than the invertebrate/r-opsin subfamily. When rooted with bacterial rhodopsin, Anthozoan Groups 1 and 2 and the Acropsins form a clade with the Hydrozoan/jellyfish opsins, which is again, most similar to the Gt/c-opsin subfamily. Anthozoan Group 3 appears to be most similar to Go opsin and the Gs jellyfish opsin actually clusters with bacterial rhodopsin at the root of the tree.
Figure 6.5. Phenogram based on ClustalW (slow/accurate, Gonnet) showing the relationship of Acropsins 1 and 2 to the cnidarian (jellyfish and sea anemone) opsins and established opsin subfamilies. The tree displays five previously described opsin subfamilies: 1) Invertebrate Gq/melanopsins; 2) Peropsins; 3) Vertebrate Gt/c-opsins; 4) Go opsin; 5) Gs opsin. The neuropsin subfamily is not represented. Anthozoan opsins (from the sea anemone *Nematostella vectensis*) are highlighted with red boxes. These form three distinct groups that are labeled based upon Groups assigned by Suga et al. (2008). Two of the groups, 2 and 3 cluster with the Invertebrate Gq/melanopsin and peropsin groups, respectively. Group 1 clusters with Acropsin 2 and is also similar to Acropsin 1. The coral Acropsins fall in between the Hydrozoa/Jellyfish opsins and other opsin subfamilies.
Figure 6.6. Phenogram based on ClustalW (slow/accurate, Identity) showing the relationship of Acropsins 1 and 2 to the cnidarian (jellyfish and sea anemone) opsins and established opsin subfamilies. The tree displays five previously described opsin subfamilies: 1) Invertebrate Gq/melanopsins; 2) Peropsins; 3) Vertebrate Gt/c-opsins; 4) Go opsin; 5) Gs opsin. The neuropsin subfamily is not represented. Anthozoan opsins (from the sea anemone Nematostella vectensis) are highlighted with red boxes. Anthozoa groups 2 and 3 assigned by Suga et al. (2008) are split using this method. Here, the Acropsins cluster with Anthozoa Group 1 and the major Hydrozoa/Jellyfish opsin cluster and appear to share greater similarity with the vertebrate/c-opsin subfamily than the invertebrate/r-opsin, consistent with Suga et al. (2008).
**Figure 6.7.** Phenogram based on ClustalW (slow/accurate, Identity) as in Figure 6.6, but rooted with bacterial rhodopsin, showing the relationship of Acropsins 1 and 2 to the cnidarian (jellyfish and sea anemone) opsins and established opsin subfamilies. The tree displays five previously described opsin subfamilies: 1) Invertebrate Gq/melanopsin; 2) Peropsins; 3) Vertebrate Gt/c-opsins; 4) Go opsin; 5) Gs opsin. The neuropsin subfamily is not represented. The Acropsins consistently cluster with Anthozoa Group 1, but with the addition of bacterial rhodopsin, Anthozoa Groups 1 and 2 and the Acropsins form a clade with the Hydrozoan/jellyfish opsins, which is again, most similar to the Gt/c-opsin subfamily. Anthozoa Group 3 appears to be most similar to Go opsin and the Gs jellyfish opsins clusters with bacterial rhodopsin at the root of the tree.

While Acropsin 1 and 2 consistently cluster with Anthozoa group 1, their relationship to other cnidarian opsins and to the opsin subfamilies is less clear, and additional research is needed to determine the biochemistry and function of these
proteins. The diversity of opsins (three Anthozoan groups) found in the sea anemone *Nematostella* also suggests that additional opsins likely exist in corals. Coral EST databases have focused on larval stages. Additional coral opsins may be expressed exclusively in adults.

**Constraints and the evolution coral photoreceptors**

Two competing hypotheses have been proposed to describe the selective force acting upon visual pigments and structures of aquatic organisms. Munz (1958) argued that pigments evolved to maximize sensitivity, while Lythgoe (1979) argued that pigments and structures have evolved to maximize contrast. Munz and McFarland (1983) evaluated the strength of these hypotheses and determined that the rhodopsins of most mobile coral reef organisms have evolved to maximize contrast. Especially in shallow, tropical environments organisms appear to have developed complex color patterns, and sensitivity to UV an polarized light that support the maximization of contrast, likely in response to strong selective pressure resulting from the need to locate predators, prey, or for intraspecific communication (e.g., Losey et al. 1999; Marshall 2003; Chiou et al 2008). While these selective pressures have likely shaped the pigments and visual structures of mobile coral reef organisms, what selective pressures are operating to influence the evolution of photobiology in sessile organisms, for example corals? Do the physiological demands and composition of the coral holobiont offer insight into the evolution of coral photoreceptors and spectral tuning of photopigments?

In sessile organisms, contrast would be of little benefit, since predator avoidance and active prey capture are of limited importance. Without pressure to enhance the visual
detection of predators and prey, corals may have evolved primarily under pressure to maximize sensitivity. Reproductive success in sessile organisms requires synchronization of gametogenesis and spawning and coordination of these processes is arguably the greatest source of selective pressure operating on sessile organisms. Coral reproduction, like many other marine invertebrates is intimately tuned to lunar cycles (Harrison and Wallace 1990), but also triggered by darkness (e.g., Brady et al. 2009). The detection of light and dark does not require eyes or even simple visual structures, and pigments responsible for circadian entrainment, and the regulation of reproduction are often found in unexpected places (e.g., the brain, neural tissue, gonadal tissue; Shichida and Matsuyama 2009). As a result there has likely been little selective pressure for the development of visual structures in sessile organisms, as evidenced by the lack of eyes in coral and anemones but evolution of diverse and complex visual structures in other cnidarians (e.g., the box jellyfish *Tripedalia* and *Carybdea*) that have evolved pelagic, medusoid adult stages.

While the requirement for synchronous reproduction has exerted pressure on adults, it would not operate on pelagic, larval stages of corals. The selective pressures operating on larvae are likely those resulting from high rates of pre- and post-settlement mortality. Evidence of pre-settlement pressure is observed chemical defenses that some coral larvae have against fish and invertebrate predation (e.g., Lindquist 1996; Baird et al. 2001), and DNA damage, photorepair mechanisms (Reef et al. 2009). Post-settlement pressures would likely effect mechanisms that promote the location and selection of suitable settlement habitat. While these sensory mechanisms may be largely based on chemical cues, evidence suggests that photosensitivity has evolved to assist this process.
Mundy & Babcock (1998) showed that larvae demonstrate species-specific preferences for light intensities or qualities that correlate with adult habitat and may serve to position them at depths favorable for post-settlement survival. The present study demonstrates that some species can use color cues for fine scale habitat selection. In the latter case it appears as though some species have evolved orange/red sensitivity to facilitate the location of CCAs that may facilitate survival (Chapter 2). Preference for certain types of CCA has been shown in larvae from several species of *Acropora* (Harrington et al. 2004; Ritson-Williams et al. 2010).

At all stages, the physiological demands of the holobiont (the coral and its symbionts) likely impose constraints that influence the localization and the spectral sensitivity of photopigments. In adults, and in brooded larvae, symbiotic dinoflagellates reside within the coral endoderm. The chlorophylls a, c and various carotenoids present in dinoflagellates absorb visible light between 400-550 with a secondary peak between 650 and 700nm (e.g., Hennige et al. 2009; Figure 6.8), leaving a window between 550 and 650nm. Competition for light would likely favor the evolution of photopigments with sensitivities positioned within this window. In addition to photosynthetic pigments, corals also possess photoprotective pigments. Green fluorescent proteins (GFPs; e.g., Mazel et al. 2003; Alieva et al. 2008), and microsporine-like amino acids (MAAs; reviewed by Shick and Dunlap 2002), are found in host tissue of larval and adult stages, where they play important roles, absorbing ultraviolet radiation (UVR), and serving as antioxidants for the host (e.g., Fitt et al. 2009). While MAAs filter incident light, GFPs transform it - absorbing light centered at one wavelength and emitting radiation centered
at another. Some GFPs absorb maximally in the UV and short-wave blue regions of the spectrum, converting harmful forms of radiation into cyan or green light, but other GFPs (e.g., the green GFP in *A. palmata* larvae; Chapter 4) have absorbance maxima in green region and emit light of a longer (green, orange or red) wavelengths (reviewed by Alieva et al. 2008).

Coral fluorescent proteins and visual pigments may have co-evolved, with fluorescent proteins to serve a sensitizing function (transferring energy to a photopigment). Minke and Kirschfeld (1979) showed that a photostable, UV-absorbing pigment was responsible for transferring energy to fly rhodopsin. The leading candidate was a fluorescent lactoglobulin retinol complex (Kirschfeld et al. 1983). The fluorescent emission of coral fluorescent proteins (465 and 530) in *A. palmata* larvae (Chapter 4)
may offer insight into the spectral absorbance of the Acopsins. Fluorescent proteins could play a sensitizing role for a blue or green-sensitive pigment. This would also bestow upon corals a false or indirect UV photosensitivity (i.e. demonstration of UV sensitivity both electrophysiologically and behaviorally without the possession of a UV-sensitive opsin) and could explain the UV sensitivity observed in *Porites astreoides* larvae (Gleason et al. 2006).

Experiments that are currently underway will hopefully demonstrate: 1) whether the Acopsins form functional photopigments; 2) the type of G protein they bind and phototransduction cascade which they employ; 3) their individual spectral sensitivities. These pieces of information will provide further insight into the potential function(s) of these receptors, the role of photoreception in the ecology of coral larvae and the evolutionary origins of modern phototransduction pathways.
References


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