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# Evolutionary Expansions and Neofunctionalization of Ionotropic Glutamate Receptors in Cnidaria

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EVOLUTIONARY EXPANSIONS AND NEOFUNCTIONALIZATION OF

IONOTROPIC GLUTAMATE RECEPTORS IN CNIDARIA

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by

Ellen Grace Dow

2019

To: Dean Michael R. Heithaus
  College of Arts, Sciences and Education

This dissertation, written by Ellen Grace Dow, and entitled Evolutionary Expansions and Neofunctionalization of Ionotropic Glutamate Receptors in Cnidaria having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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DEDICATION

I dedicate this to my nurturers.

To my mother, Janet Kvanvig Sappington, who reminds me why I am passionate about each thing I do and treats me as her equal.

To my father, Roger Dow, the mighty oak who has shown me my roots and given me the support and spirit for this acorn to sprout so far away from home.

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ABSTRACT OF THE DISSERTATION

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Professor Mauricio Rodriguez-Lanetty, Major Professor

Reef ecosystems are composed of a variety of organisms, transient species of fish and invertebrates, microscopic bacteria and viruses, and structural organisms that build the living foundation, coral. Sessile cnidarians, corals and anemones, interpret dynamic environments of organisms and abiotic factors through a molecular interface. Recognition of foreign molecules occurs through innate immunity via receptors identifying conserved molecular patterns. Similarly, chemosensory receptors monitor the environment through specific ligands. Chemosensory receptors include ionotropic glutamate receptors (iGluRs), transmembrane ion channels involved in chemical sensing and neural signal transduction. Recently, an iGluR homolog was implicated in cnidarian immunological resistance to recurrent infections of bacterial pathogens. I postulate that iGluRs in cnidarians may act as danger-sensing and/or pathogen recognition receptors adjacent to immune defense and nervous system signaling. In Chapter One, I explain the exploration of diversity and divergence within cnidarian iGluRs, complimented with predicted functions in the context of correlated response to biological and environmental signals, setting the groundwork for functional characterization. In Chapter Two, I characterized

the divergence of cnidarian iGluRs in comparison to other metazoans through maximum likelihood phylogenetic analyses, which revealed greater evolutionary expansion of cnidarian iGluR lineages, including a Cnidaria-specific class. Gene expression differentiation implies select iGluRs respond transcriptionally to bacterial challenge, supporting the hypothesis that cnidarian iGluRs respond to pathogen signals. In Chapter Three, I investigated a putative endogenous rhythm to iGluR expression, as chemosensory receptors may have the capacity to anticipate daily environmental fluctuations. While a circadian rhythm does not appear to be a primary contributor to biological rhythms in iGluR gene expression, symbiosis and diurnal fluctuations are implicated factors. In Chapter Four, I chromogenically localized *Exaiptasia pallida* iGluR expression to the epidermis and concentrated within sensory tentacles, alongside cnidocytes. Expression of iGluRs in proximity of sensory cells is consistent with the putative function of iGluRs in cnidarian neural signaling. In the final chapter, I synthesized my research in its entirety; highlighting that cnidarian iGluRs expansions indicate cnidarian-specific neofunctionalization towards functions of chemosensory cnidarian-environmental signaling. New hypotheses and future research are presented to continue the study of iGluRs as chemosensory receptors within the cnidarian nervous system.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

AMPA alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA analysis of variance

ATD amino terminal domain (also known as NTD as N refers to any amino acid)

BLAST basic local alignment tool

C1 phylogenetic clade 1 from Fig. 2.1 and 2.S1

C2 phylogenetic clade 2 from Fig. 2.1 and 2.S1

C3 phylogenetic clade 3 from Fig. 2.1 and 2.S1

C4 phylogenetic clade 4 from Fig. 2.1 and 2.S1

C5 phylogenetic clade 5 from Fig. 2.S1, exclusive to IRs

C6 phylogenetic clade 6 from Fig. 2.S1, exclusive to GluD

C7 phylogenetic clade 7 from Fig. 2.S1, exclusive to *Trichoplax adhaerens* (Placozoa) sequences

C8 phylogenetic clade 8 from Fig. 2.S1, exclusive to GluK

CNS central nervous system

CX phylogenetic clade 1 from Fig. 2.1, exclusively *Mnemiopsis leidyi* (Ctenophora) sequences

DAMP damage-associated molecular pattern

DD 12 hr dark : 12 hr dark diurnal cycle, also constant darkness

EtOH ethanol

FISH fluorescent *in situ* hybridization

GluA        AMPA-specific ionotropic glutamate receptor, protein

GluD        ionotropic glutamate receptor delta, protein

GluK        kainate-specific ionotropic glutamate receptor, protein

GluN        NMDA-specific ionotropic glutamate receptor, protein

GluR        refers protein sequence of *E. pallida* iGluR subunit

gluR        refers to gene sequence of *E. pallida* iGluR

GR        gustatory receptor

HSD        honestly significant difference, associated with Tukey post-hoc

iGluR        ionotropic glutamate receptor

IL-1R        interleukin-1 receptor

IR        ionotropic receptor

ISH        *in situ* hybridization

LBD        ligand binding domain

LD        12 hr light : 12 hr dark diurnal cycle

MAMP        microbe-associated molecular pattern

ML        maximum likelihood phylogenetic analysis

NMDA        N-methyl-D-aspartic acid

NTD        see ATD

OR        olfactory receptor

PAMP        pathogen-associated molecular pattern

PCR        polymerase chain reaction

PRR        pattern recognition receptor

qPCR        quantitative polymerase reaction (also RT-qPCR)

RNA        ribonucleic acid

TIR        toll/interleukin-1 receptor

TLR        toll-like receptor

TMD        transmembrane domain

CHAPTER I. INTRODUCTION


Organisms sense immediate environments through an interface of essential barriers and

receptors designated to defend against harmful infectious agents. Immune systems

differentiate between self and non-self (1), which is observed from microscopic single-

living prokaryotes i.e., bacteria retaining innate immune mechanisms recognizing viral

infections (2,3) to complex multicellular organisms harboring additional learning-based

recognition pathways (4,5). There are two primary systems immunity relies upon, innate

and adaptive. Innate immunity has evolved across pathways for organisms to inherently

recognize foreign entities through a genome-based system (4,6–8). Adaptive immunity

prolongs specific resistance against pathogens over longer periods; however, evidence of

adaptive immunity is not observed in early-diverged metazoans (8–11). Current findings

support the theory of simultaneous evolution of innate and adaptive immune systems,

rather than a previous interpretation that put forth expanding adaptive immunity upon the

foundation of an innate system (5). The observation of conserved gene sequences

suggests each pathway has independently evolved across phyla. Invertebrates primarily

rely on innate functions, coded within genomes, for identifying microbial and/or

chemical cues to distinguish pathogenic agents (8,11–14). The growing availability and

understanding of '-omics' data in early-diverged metazoans allows for gaps to be filled

within complexities of innate immunity, one group of particular interest is Cnidaria.

**1.1 Introduction to Cnidaria**

Cnidarians are named by their distinctive stinging cells referred to as cnidocytes, which are used in defense and prey capture (15–18). The importance of accumulating genetic data from cnidarians comes from the ecological importance of reef-building corals that form the highly productive framework of diversified reef ecosystems, which provides essential habitats for fish and marine invertebrates (19–22). Reefs are known for a number of canonical symbioses. In general, symbiosis between diverse organisms provide the basis for fitness success across environments (23–25). Corals provide habitat for microorganisms, creating specific microbiomes for different species and habitat ranges (26–28). A unique endosymbiosis that has driven molecular and signaling research within Cnidaria occurs between cnidarian hosts and photosynthetic algal dinoflagellates from the family Symbiodiniaceae (29–38). The cnidarian-Symbiodiniacea mutualism is the main source of primary productivity for coral reef habitats in tropical and subtropical regions (38–41). The host cell provides dissolved inorganic carbon, nitrates, phosphates, and essential compounds to endosymbiotic Symbiodiniaceae in exchange for organic carbon and nitrates from metabolic output of dinoflagellates (33,35,37,42–47). Immune regulation is hypothesized to serve for continuation of winnowing, or selecting and filtering specific symbionts for mutualism (48–50).

Cnidarian hosts differentiate pathogenic and mutualistic symbionts using various signaling pathways. Signaling and receptor pathways have been outlined in Cnidaria predominantly by identifying homologs of established proteins from model systems within cnidarian genomes and transcriptomes. Sequenced cnidarian genomes include

*Hydra magnipapillata*, a hydrozoan, and *Nematostella vectensis*, an aposymbiotic

anthozoan, *Acropora digitifera* and *Stylophora pistllata*, scleractinian corals, and

symbiotic and aposymbiotic of *Exaiptasia pallida* (colloquially referred to as Aiptasia)

(51–56). Additional transcriptomes have been established along with these genomes, to

identify various homologous innate immune genes between cnidarians and higher

metazoan systems (57–61).

## 1.2 Innate immunity within cnidarians and the importance of pattern recognition

The primary knowledge of innate immunity in cnidarians is based on reception, followed

by predicted pathways from genetic data. Recognition occurs in cnidarians through

detection of microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs)

by pattern recognition receptors (PRRs) as a function of innate immunity

(7,50,56,59,62,63).  The group of PRRs identified in early-divergent metazoans includes

Toll-like receptors (TLRs) and related Toll/Interleukin-1 Receptors (TIR domains)

located within sponges (64) and cnidarians (65–67). The TLR signaling pathway in the

anemone *Nematostella vectensis* follows a bilaterian metazoan path, including the

presence of an intracellular TIR domain connected to extracellular LRRs (8,67). The

occurrence of five TIR-containing proteins in *N. vectensis* (13), three of which contain

multiple immunoglobin domains and resemble mammalian interleukin-1 receptors (IL-

1Rs) (68), is consistent with the hypothesized development of the TLR pathway prior to

the divergence of cnidarians. Furthermore, immune genes identified in *Hydra*, include

four TIR-domain-containing proteins of which two have similar features to MyD88,

including a death domain, and two TIR homologs lacking LRRs (68–71). The TLR

signaling pathway appears to be essential for recognizing different microbes in Cnidaria, as suggested from evidence of differential host gene expression, altered bacterial colonization, and susceptibility to pathogens within MyD88-knockdown *Hydra* polyps (72,73). The MyD88-mediated TLR signaling cascade in *Hydra* does not appear to follow the canonical bilaterian pathway, while it is hypothesized to be have a role in establishing bacteria colonies (72). However, substantial gene-loss may have occurred between hydrozoans and cnidarians, due presence of canonical Toll/TLR pathway in cnidarians, but lack thereof in hydrozoans (65,72). Gene-loss within cnidarian innate immunity appears to happen randomly within immune pathways through a loss of Toll, TLR, and IL-1R receptors in *Hydra*, while pathway intermediates remain present (Miller et al., 2007). This brings to the forefront the importance of identifying alternative immune or signaling pathways within Cnidaria.

Prospective signaling pathways and organismal systems may have crucial involvement in innate immunity as regulators during the early stages of the onset of symbiosis or pathogen response, as microbial infections activate transcriptional activity in immune pathways (74,75). Pathways that use receptors similar to PRRs may have a higher likelihood to identify infectious agents because of conservation across bacteria and other signaling pathways. Potentially, chemical signals could be used as a molecular pattern for receptors similar to PRRs, such as chemosensory proteins. A family of chemosensory proteins has been correlated to immune response through up-regulation in response to coral pathogenic bacterium *Vibrio coralliilyticus* (76), implying

environmental chemical signals could serve in cnidarian recognition of conserved chemical patterns and ligands.

**1.3 Chemosensation as a proposed signaling network within anemones**

Chemosensing is defined as the transduction of a chemical signal by a receptor, tangential to the nervous system and built upon behaviors leading to a biological response (77,78). Organisms require continuous input of sensory information for behaviors that contribute to acquiring resources for survival and reproduction (79,80), which includes receiving chemical signals to interpret the environment. Chemosensory receptors, such as ionotropic glutamate receptors (iGluRs), within invertebrates distinguish changes in the environment and send consequent signals through the nervous system (79,81). Within the cnidarian *Exaiptasia pallida,* an iGluR protein fragment was found to be up-regulated post-exposure to *V. coralliilyticus* (76).

Ionotropic glutamate receptors recognize glutamate or a receptor-specific ligand in the receptor domain, which opens the transmembrane channel to transport calcium ions into the cytoplasm of the cell and elicit consequent physiological responses (80,82). Receptor cells will outnumber central neurons responsible for processing sensory information within the nervous system, requiring many types of signals to interpret and distinguish various cues (79). Aside from iGluRs, other chemoreceptors i.e., odor receptors (ORs) and gustatory receptors (GRs), may offer cnidarians an additional path to recognize external chemical cues (77,83–87). These receptors aid in prey capture, predator evasion and other roles essential to survivorship (77). The use of chemosensing

in early-diverging metazoans (88) and *Caenorhabditis elegans* (89) is linked to behavior patterns, as any response to an external stimulus has the potential to trigger a conscious or unconscious physiological and/or behavioral change. The chemosensory response begins with a receptor with ligand specificity, similar to PRRs.

### 1.4 An introduction to the function of iGluRs

The family of iGluRs are receptors known for neuronal communication in sensory responses to environmental factors (77,80,88,90,91). However, iGluRs are conserved across metazoans and within plants. In *Arabidopsis thaliana*, iGluRs are responsible for root signaling and implicated in the danger theory response to tissue damage accrued by the presence of a pathogen (92–98). Expanding into metazoans, iGluRs are present within the sponge phyla, Porifera, and are implied to have a role in sponge environmental sensing (14,99). In Ctenophora, iGluRs are important in signaling and development of the uniquely complex nervous systems of comb jellies (87,100,101), while the role of iGluRs within bilaterian invertebrates expands to chemosensing. A flavor of iGluRs, known as Ionotropic Receptors (IRs) have an important role in olfaction and identifying food sources in insects (77,88,102). Chemosensory organs characteristically have IRs localized to their specialized tissues within invertebrates, including insect antennae and mollusk rhinophores (77,79,103). Within vertebrate systems, iGluRs are responsible for synapses within the central nervous system (CNS) particularly receiving signals within the brain (81,103–105). The consequence of iGluRs is clear in deregulation of glutamate leading to neurodegeneration (106,107). Conversely, in cnidarians the role and extent of diversity of iGluRs is unknown, however there presence has been observed (77,108).

**1.5 A brief review of the dispersed nervous system and defensive cells in Cnidaria**

Cnidarians do not possess a CNS that would traditionally interpret iGluR signals. Rather, cnidarians have a nerve net, or diffused nervous system composed of sensory and ganglionic neurons, and their respective processes interspaced among epithelial cells (87,109,110). The nervous system present in anthozoans, such as the non-symbiotic anemone *Nematostella vectensis*, is composed of linear tracts of neurites, or a nerve cord, within the parietal region and between mesenterial endomesoderms (78,85,111). Directional neural conduction triggers local muscle contraction during neuro-myoepithelial synapses (78,111–113). The nervous system of sensory cells and neurons are interwoven throughout the epidermis to connect with mechanical and chemical defense mechanisms.

Cnidarian tentacles concentrated with defensive stinging cells of nematocytes and spirocytes, have microscopic harpoons called nematocysts and spirocysts, respectively (114–117). Synaptic modulation controls by spirocyst discharge by mutliple neurotransmitters (85,114,118). Glutamate, a known neurotransmitter is known to aid in triggering a response to prey and self-defense (85,115,117) In non-symbiotic *Hydra*, iGluRs modulate signal coordination and increase of nematocyst launching or discharge (85). The connection between iGluRs with and bacteria is implied the presence of poly-gamma-glutamic acid that could trigger nematocyst discharge (117), which is also consistent with the hypothesis of iGluR involvement in defensive chemosensory pathways. While initial theories postulated mechanosensory stimulus, current findings

suggest chemosensory-triggered discharge of nematocytes opposed to solely physical

contact (115). The correlation of iGluRs involved in signaling from the perspective of

neurosensory system within Cnidaria to the cnidocyte glutamate response; connecting

immunity and nervous system through fast signaling.


## 1.6 Dissertation aims

Recent evidence of iGluRs in anthozoans comes from *E. pallida* from an iGluR protein

sequence identified as up-regulated in response to a bacterial challenge (76). Cnidarian

iGluRs are implicated in putative chemosensory and/or immune responses to bacteria,

therefore I used *E. pallida* as a model system (56,59) to understand the role of iGluRs in

cnidarians and their potential as chemoreceptors correlated with innate immunity (Fig. 1).

I used several methods to begin characterizing the iGluR protein family within Cnidaria.

In Chapter Two, I used a phylogenetics approach to determine the extent of cnidarian

diversity and homolog conservation as a starting point for predicting putative functions.

The available genome and transcriptome of *E. pallida* was important in the molecular

framework for measuring transcriptional expression. Analyzing the response of a model

lab cnidarian, a sea anemone to bacteria challenges and across diel treatments as an

introduction to correlating iGluR response to the environment within Chapters Two and

Three. Chromogenic localization of gene expression within Chapter Four was then used

to lay the groundwork for dispersal of expression among cells, particularly in relation to

sensory cells and the nervous system.

**1.7 Questions and overview of each data chapter**

<u>Chapter II</u>

- What is the extent of cnidarian iGluR diversity?

- Are the protein structures of *E. pallida* iGluRs reflective of functional characteristics through conserved domains?

- What level are iGluRs involved during the response to microbial challenges?

The aim of this first data chapter was to determine the extent of cnidarian iGluR diversity and propose putative functions for iGluRs within *E. pallida*. As few iGluRs have been noted or compared to model species iGluRs (77,108,119,120), the necessity to explore homolog diversity and begin exploration into putative functions was the first step in characterizing cnidarian iGluRs. Through comparing reference proteomes (defined by Uniprot as a subset of proteomes selected following criteria to represent model organisms and species of interest) and predicted proteomes from Reef Genomics database, I traced cnidarian iGluRs among other metazoan lineages of classically described iGluRs. From the lineage specific expansions of iGluRs within *Exaiptasia pallida*, the conserved domains were compared to predict functions and the potential of functional protein units based on completeness. Bioinformatic inferences were coupled with correlative analysis of the transcriptional response of iGluRs to pathogenic bacterial challenges within the lab model cnidarian *E. pallida.* The reasoning behind the initial approach was the identification of an iGluR peptide fragment as highly up-regulated in response to a pathogenic challenge to *Vibrio coralliilyticus* (76,121).

Chapter III

- Is there a daily expression cycle for cnidarian iGluRs?

- Do cnidarian iGluRs have a role in biological cycles?

The aim of the second data chapter was a continuation of the first predictive exploration of iGluRs within *E. pallida*. The transcriptional levels of iGluRs within *E. pallida* varied across time points, regardless of treatment and including the control anemones, thus a continuation into the gene expression of iGluRs within a 24-hr cycle was investigated. The purpose of this chapter was to explore the expression cycles of iGluRs in the perspective of biological rhythms using a temporally robust experiment with independent biological replicates. Analysis of this experiment continued beyond diel cues of light and into the presence of Symbiodiniaceae within another cnidarian species from prior published, but not addressed, transcriptomic data (122). Integrating contributions of diel and symbiotic factors to iGluR expression patterns lead to hypotheses proposing metabolites from Symbiodiniaceae putatively affect rhythms of iGluR expression.

Chapter IV

- Where are iGluRs localized within *E. pallida*?

The aim of the third data chapter was to localize iGluR expression within *E. pallida* using *in situ* hybridization. This is a significant contribution to the field, first through establishing a protocol for *in situ* hybridization within *E. pallida* that is robust and replicable, which can be used in further experiments within collaborating labs and the

community at large, and second for genes with low expression. While the working hypothesis stated iGluRs were likely to be located in sensory tentacle cells and gastrodermal tissues that phagocytize pathogenic agents, the results indicated that iGluRs localized solely within the epidermis of anemones. Expression was concentrated within tentacles; however, some localization of iGluR expression had punctate patterns throughout the column of the anemone body plan. Localization of iGluRs has suggested future experiments for pursuing the function of cnidarian iGluRs.

**1.8 Relevance and significance of cnidarian iGluRs**

Current understanding of cnidarian immunity primarily relies on putative function extrapolations derived from homologs well characterized in other invertebrate or vertebrate model systems. The attention to understanding immune defense evolution (8,13,123) remains biased toward vertebrate models with direct applications to human medicine (4,6–9). Pattern recognition receptors, components of the complement system, and alternative pathway are well-researched pathways known to contribute to innate immunity within cnidarians. However, there are knowledge gaps and unexplored signaling pathways within cnidarian immunity, including the role of chemosensory receptors.

Chemosensory proteins respond to specific ligands within the environment to evoke physiological changes and behaviors. One well-characterized chemosensory protein lineage within diverged metazoans is ionotropic glutamate receptors (iGluRs). Receptor proteins have diverse functions; known for receiving signals between synapses

in vertebrate brain and serving sensory roles for food detection in flying insects. Putative iGluRs have yet to be characterized within cnidarians. Through characterizing cnidarian iGluRs within a model symbiotic cnidarian, *E. pallida,* current hypotheses of iGluR evolution will be challenged, having occurred earlier in evolutionary history than previously predicted. The current project utilized several molecular applications to explore the diversity of iGluR lineages within cnidarians and putative functions of cnidarian iGluRs within the context of response to environmental factors. The following chapters outline expression patterns of cnidarian iGluRs, which opens up the possibility of an interrelationship between neural signaling and innate immunity, and expands our understanding of the role of iGluRs in cnidarians.

**1.9 Figures**
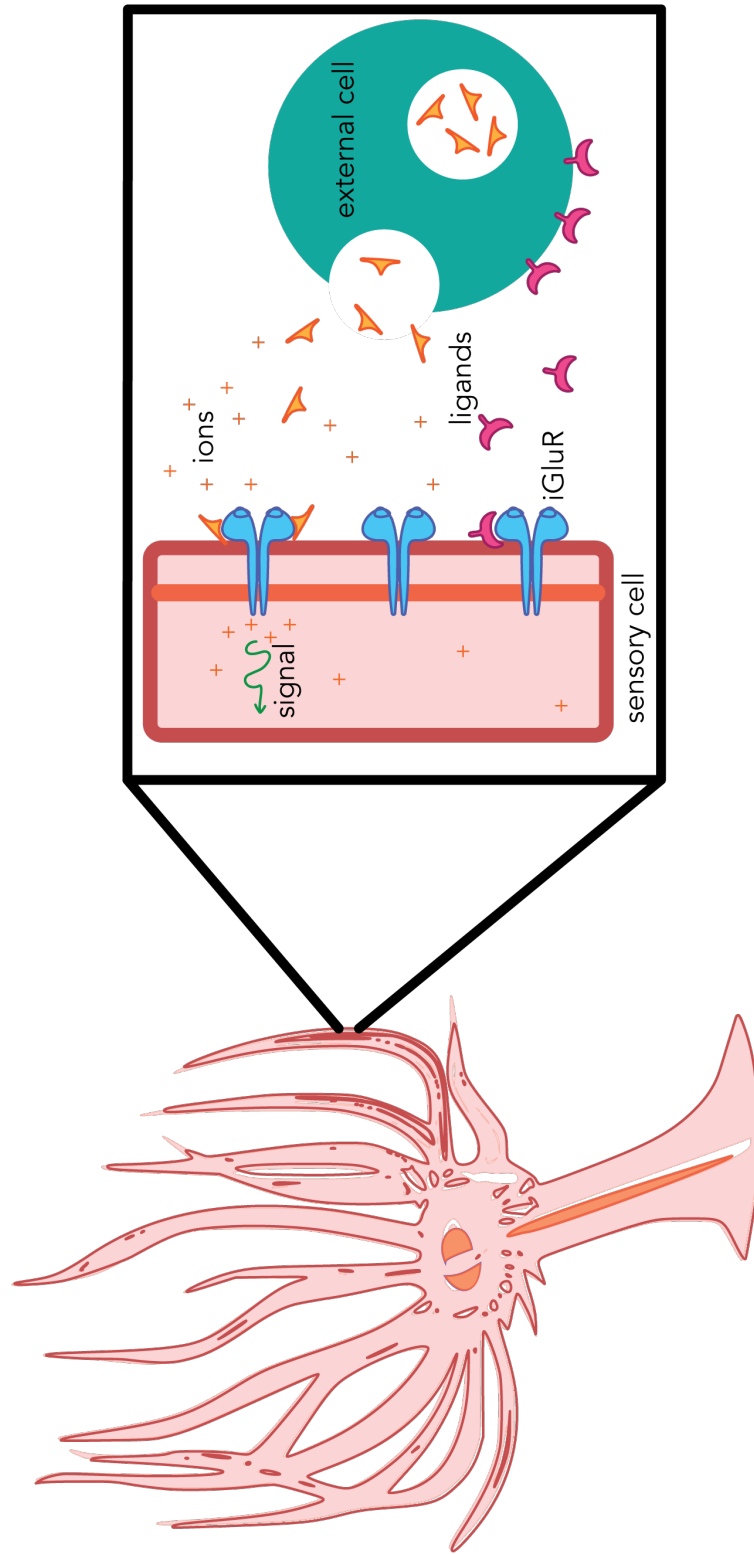


Figure 1.1 Infographic on the putative nature of iGluRs within the sea anemone *Exaiptasia pallida*. Within epidermal tissues, iGluRs are predicted to respond to ligands from the environment and propogate signals via the transmembrane ion channel.

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CHAPTER II. CNIDARIAN-SPECIFIC EXPANSION OF IONOTROPIC
GLUTAMATE RECEPTORS PROMOTE NEOFUNCTIONALIZATION WITH
IMPLICATIONS FOR CHEMOSENSORY IMMUNITY

**2.1 Abstract**

Ionotropic glutamate receptors (iGluRs) are transmembrane proteins involved in a variety
of biological processes from chemical-mediated neuron communication within vertebrate
brains to chemosensation within insect antennae. Recently identified iGluRs in cnidarians
have been postulated to function as danger-sensing and/or pathogen receptors in immune
defense. To address the hypothesis, we explored the extent of phylogenetic diversity of
iGluR proteins in available cnidarian sequenced genomes with particular interest on the
cnidarian-dinoflagellate model system, the sea anemone *Exaiptasia pallida*. Furthermore,
functional gene expression of multiple iGluR genes was measured from *E. pallida* in
response to bacterial pathogen exposure.  Our findings revealed a greater evolutionary
expansion of cnidarian iGluR lineages than recently reported, and the discovery of a new
cnidaria-specific class. Some cnidarian iGluRs maintain conserved domains while others
have a reduced domain composition indicative of functional divergence in the cnidarian
iGluR protein family. Gene expression differentiation of iGluRs suggests select genes
respond transcriptionally to bacterial challenge, supporting the hypothesis that cnidarian
iGluRs respond to pathogen signals. Altogether, cnidarian iGluRs expansions indicate
cnidarian-specific neofunctionalization towards functions as chemosensory receptors,
including immune chemosensing specific to bacterial infectious agents.

**2.2 Keywords:** Ionotropic Glutamate Receptor; iGluR; chemosensory*;* Cnidarian*;*

*Exaiptasia pallida*; coral immunity.


## 2.3 Introduction

Organisms have an ecological interface provided through immunity; the designated

system composed of essential barriers and recognition proteins, which works defensively

against harmful, infectious agents. Even the minutest microscopic single-living

prokaryotes i.e., bacteria, possess innate immune mechanisms to defend against viral

infections (1,2). In recent years, interest to understand how invertebrate immune defense

systems evolved has grown (3–7), but the focus remains heavily biased towards

vertebrate models with direct applications to human health. Unlike vertebrates, early-

diverged metazoans lack of an adaptive of immune system and rely upon innate

functions, coded within their genomes, to identify and respond to microbial and/or

chemical cues to recognize pathogenic agents (6–10). The dependence on an innate

immune system for defense against the countless microorganisms encountered during a

lifespan among invertebrates poses one of the greatest conundrums in comparative

immunology (6,11).


A widely used approach to understand invertebrate innate immunity is identifying

homologous genes of well-characterized genes in the vertebrate immune system.

Although the transfer of information from vertebrates to invertebrates derived from

homology is informative for understanding the functionality of orthologous genes, it is

less informative in gene families that have undergone several rounds of lineage-specific gene duplication and limited in its application of immune mechanisms unique to early-diverged metazoans (9). While homologous genes implicated in innate immunity has been identified in invertebrates (3,4,6,7,12–14), the detection of homologs in established functional pathways alone, without experimental evidence, does not lead to full comprehension of gene function. A complementary approach to elucidate immune functionality is by identifying expression changes of genes during pathogenic or antagonistic elicitor of immune defense. Using this approach, a number of genes involved in the defense response of the sea anemone, *Exaiptasia pallida* (Agassiz in Verrill, 1864)*, to the coral-pathogenic bacterium *Vibrio coralliilyticus* were identified (15). One of the discovered genes with increased expression encoded a chemosensory ionotropic glutamate receptor (iGluR) concurrent to improved resistance against repetitive bacterial challenges (15). The novel discovery of iGluR involvement in immune response of cnidarians aligns with similar findings in plants (16,17).

One important function of the immune system of marine organisms is to recognize potentially infectious agents, and for that chemosensory machinery might play an essential role. Ionotropic glutamate receptors (iGluRs) represent one particular "flavor" of chemosensory receptor proteins, which are embedded within the cell membrane to send external signals into the cell and elicit a physiological reaction or behavior (18,19). Chemosensing-associated behaviors include prey captures, predator evasion and other functions essential to survivorship described in early-divergent metazoans (20–22). Glutamate or a specific ligand binds to the ligand-binding region of

an iGluR and activates the opening of its ion channel to allow transport of calcium ions into the cytoplasm of the cell and further system responses (19,23). The iGluR protein family is known for neuronal communication in sensory responses to environmental factors (21,24); without a central nervous system (CNS), cnidarians use a nerve net, or diffused nervous system of sensory and ganglionic neurons spaced between epithelial cells to interpret iGluR signals (25–27). The nerve net present in anthozoans i.e., non-symbiotic anemone *Nematostella vectensis*, is an arrangement of linear nerve cords that send directional conduction to trigger local muscle contractions (28–30). The iGluRs identified in non-symbiotic *Hydra vulgaris* modulate nematocyst discharge as a response to prey or self-defense (29), supporting the hypothesis that iGluRs act consequently to triggered defense signals. Alternatively, if acting similarly to chemosensation in plants, cnidarian iGluRs could function as a component of the danger recognition response to tissue damage accrued by the presence of a pathogen rather than an antigen response to modulate chemical stresses and wound healing (13,31–33).

To characterize gene diversity and infer evolution of iGluR proteins in cnidarians, we explored homologs in available sequenced genomes including actinarians and scleractinian corals with particular interest on the cnidarian-dinoflagellate model system, the sea anemone *Exaiptasia pallida* (34). Moreover, we investigated through gene expression assays the involvement of *E. pallida* iGluRs in the immune response to infectious bacterial challenges. This study aids in interpreting the evolution of cnidarian iGluRs and putative functional role in bacterial chemosensing.

**2.4 Materials and methods**

**2.4.1 General metazoan dataset and phylogeny:** Ionotropic GluR sequences were
extracted from sequence databases to carry out phylogenetic analyses with the objective
of inferring the evolution of iGluRs across model metazoans with particular interest on
cnidarians. Based on isoform 1 from the previously identified cnidarian iGluR gene for *E.
pallida* (KXJ14767), additional iGluR homologs were identified from the Reference
Protein database for *E. pallida* (34) using default NCBI BLAST with an e-value cutoff of
$1\times10^{-10}$. In the case of multiple protein isoforms from the same gene, the longest isoform
was chosen. The sequences from *E. pallida* were numbered in order of blastp hits (i.e.,
GluR (query), GluR3, GluR4, GluRn, etc.). The two isoforms from GluR (KXJ4767)
were labeled as gluR.1 (XP_020899710) and gluR.2 (XP_020899711). The resulting *E.
pallida* protein sequences and the complete canonical proteomes from Uniprot containing
one primary protein isoform from each gene from one Chlorophyta, and 15 metazoans
species were combined into a local blast database. Sequences were labeled with species
genus initials and accession number (Table 2.S1, S2). A local blastp (BLAST 2.2.30+)
was run against the database using GluR.1 as a query with a cutoff e-value of $1\times10^{-10}$.
Sequences shorter than 200aa in length and sequences with less than 20% identity were
excluded while remaining sequences were aligned using the iterative refinement method
(L-INS-i), incorporating local pairwise alignment information for accuracy in Mafft
v7.221 in Terminal (35). The best model of evolution (LG +G6 +I +F) was determined
with Smart Model Selection from PhyML (36). BlackBox v8.2.4 RAxML performed
Maximum Likelihood analysis using the best model of evolution and defaults within

CIPRES portal (37). The resulting phylogeny of iGluRs was rooted with the two sequences from outgroup *C. reinhardtii* and is referred to as the general metazoan iGluR phylogeny.

**2.4.2 Cnidarian-specific dataset and phylogeny:** Using the general metazoan phylogeny, sequences from select clades were extracted and built upon with additional cnidarian sequences to improve the resolution of the evolution of iGluR in cnidarians. Sequences from nine scleractinian coral species from the Reef Genome project and from two non-cnidarians *Oscarella carmela* (Porifera) and *Mnemiopsis leidyi* (Ctenophora) species were used to build a second database (Table 2.S1 and 2.S2). Through a local blastp search against this database with gluR.1 as the query, sequences with an e-value of $1 \times 10^{-10}$ that were at least 200aa long, and had at least 20% identity were added to the extracted dataset from the general metazoan phylogeny. The resulting set of sequences was aligned using the iterative refinement method (L-INS-i) in Mafft v7.221 (35). The best model of evolution (LG +G6 +I +F) was determined with Smart Model Selection from PhyML (38). BlackBox v8.2.4 RAxML performed Maximum Likelihood analysis using the best model of evolution and defaults within CIPRES portal (37). The resulting phylogeny of iGluRs was rooted with the two sequences from outgroup *C. reinhardtii* and is referred to as the cnidarian iGluR phylogeny.

**2.4.3 Modeling the molecular structure of GluRs for functional insights:** Protein domains across all GluRs were identified within Pfam (39). Homology modeling of E. pallida iGluRs was performed to compare structure and putative molecular function

29

across the phylogeny. SWISS-MODEL from ExPASy was run with defaults to identify templates for subunit 3D models (40–46). Template searches were performed through BLAST and HHBlits against the SWISS-MODEL template library (SMTL) (40,41,43–49). Models were built from target-template alignment using ProMod3 defaults. Templates with highest quality predicted from target-template alignment features were selected using GMQE, while global and per-residue qualities of the models were evaluated through the QMEAN scoring function in SWISS-MODEL (50). The PyMol v2.2 program was used for viewing 3D model structures (51).

**2.4.4 Investigation of *Exaiptasia* iGluRs transcriptional expression under bacterial challenge:** To investigate the putative functional involvement of *Exaiptasia* iGluRs in immune response, changes of transcriptional expression was assessed in anemones exposed to established bacterial pathogens. For this, we performed a similar experiment conducted within our laboratory designed to study immunological priming from which we discovered the first iGluR implicated immune defense (15,52). The bacterial challenges were performed with known coral pathogens *V. coralliilyticus* and *Serratia marcescens* (52,53) in sub-lethal conditions in order to trigger an immune defense response without compromising survivorship.

**2.4.5 Animal maintenance:** Clonal symbiotic *E. pallida* anemones (CC7) were used in the experiment and maintained in artificial seawater at approximately 27 °C. Populations were kept on a day/night cycle of 12 hours light: 12 hours dark with 30 to 60 μmol photons $m^{-2} s^{-1}$ of light intensity and fed freshly hatched brine shrimp *Artemia* nauplii

twice a week. *Exaiptasia pallida* anemones were approximately 3mm in diameter and 8mm in length.

**2.4.6 Culturing bacteria (*Vibrio coralliilyticus* and *Serratia marcescens*) and experimental design of bacterial challenges:** The strains *V. coralliilyticus* BAA 450 (ATCC) and *S. marcescens* PDL 100 were used for challenge experiments. Bacteria were streaked from glycerol stocks stored at -4 °C and grown overnight on Marine Agar (Difco, USA) at 30 °C. The following day, a single colony was picked with an inoculating loop and grown to logarithmic phase overnight at 30 °C while shaking at 100 rpm in Marine Broth-2216 (Difco, USA). Cultures were centrifuged at 10,000 xg for five minutes, washed and re-suspended in sterile seawater before starting the infection trials. Bacterial cells were prepared to the designated final concentration of $10^8$ CFU mL$^{-1}$ calculated from growth curves generated by absorbance (optical density readings at 600 nm) plotted against known CFU mL$^{-1}$. Infection experiments were conducted at 30 °C and at a final concentration of $10^8$ CFU mL$^{-1}$ of *V. coralliilyticus* or *S. marcescens* following the protocol set forth by Brown and Rodriguez-Lanetty (15). Individual *E. pallida* anemones were acclimated to the experimental temperature (30 °C) for 24 hr prior to experiment and placed into a single well of a twelve-well tissue culture plate containing artificial seawater. Anemones were sub-lethally challenged for 72 hr with *V. coralliilyticus* or 48 hr with *S. marcescens* as described in Brown & Rodriguez-Lanetty (15) and Brown (52) (Fig. 2.S2). Sub-lethal exposures allow animals to engage in immunological defense without causing mortality. After completion of challenge exposures, anemones were transferred to new 12-well tissue culture plates with clean

seawater. Anemones were sampled at six time points (0, 24, 48, 72, 96 hr, and 4 wk).

Zero hours marks the time of inoculation. Control anemones were moved between 48 and

72 hr to new 12-well plates to mirror the bacteria-challenged treatments transfer and kept

at 30 °C for the entire experiment and not challenged with bacteria. Anemones were

snap-frozen in liquid nitrogen and stored at -80 °C until processed for RNA extraction.

**2.4.7 RNA extraction and cDNA synthesis:** We used the RNeasy mini kit (Qiagen,

Valencia, CA) to extract RNA. The samples were applied to spin columns from the

RNeasy kit and washes performed following the manufacturer's protocol using the on-

column DNase treatment (Qiagen, Valencia, CA) and two additional 80% ethanol

washes. RNA was eluted in a final volume of 30μL of nuclease-free water. Quantification

of extracted RNA was conducted using a NanoDrop 2000 (ThermoFisher, Carlsbad, CA)

and RNA integrity assessed visually on a 1% TAE bleach gel (54). Samples with a

spectrophotometric 260/230 ratio of less than 1.5 underwent additional cleanup using

10% 3 M sodium acetate (pH 5.2), RNA-grade glycogen at a final concentration of 0.05–

1.0μg/mL, and 2.5 volumes 100% ethanol to the sample. Following precipitation

overnight at −80 °C, samples were centrifuged for 20 min at 9500 rpm (4°C) to pellet

RNA. The pellet was washed with 250μL 70% ethanol, centrifuged for 5min at the same

conditions listed above, and dried before re-suspending in 20μL nuclease-free water.

Complementary DNA synthesis was performed with Superscript III first strand synthesis

system (Life Technologies, Eugene, OR) using 120 ng of RNA and Oligo dT primer.

**2.4.8 Quantitative RealTime-PCR assays:** Primers with a length of 18 to 25 base pairs and an optimal melting temperature (Tm) of 60°C were designed using PrimerQuest (IDT, Coralville, IA). Through comparing to the *Exaiptasia* genome (34), primer pairs were designed across introns to prevent genomic DNA amplification. Nine working primers were utilized for qPCR amplifications targeting six of the identified *E. pallida* iGluRs (GluR) and three reference genes (Table S3). Primer concentrations were optimized by comparing qPCR reads using 0.5 nM and 0.25 nM of primer in order to minimize primer dimer formation and maximize output read. For qPCR analysis, a master mix of SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA), 0.34 nM of forward and reverse primers and nuclease-free water was added in 19.5μL aliquots to each well with 0.5μL of sample template. Plates for each gene were run on Bio-Rad CFX Connect systems at a thermocycle of initial polymerase activation of 30 s at 98°C, followed by 38 cycles of 15 s denaturation at 95°C, then annealing for 30 s at 54°C, followed by extension for 30 s at 72 °C with a plate read, and final Melt-Curve analysis. Plates had an Inter-Run Calibrator to control for variation of reads between runs. Negative controls for No Reverse Transcriptase were run for all samples with primers to confirm no genomic DNA contamination or bias. Raw output was corrected for variations between each run using an inter run/plate control within the BioRad program. PCR amplification efficiency was corrected through LinReg v.2016.1 (55). Gene expression was normalized using the geometric means of three reference genes, ribosomal proteins L10 and L12, and poly (A)-binding protein (56,57). Fold change of relative quantities was found by subtracting control average of each time point from treatment replicates to measure difference in expression caused by the bacterial challenge. Statistical analysis to

compare bacterial challenges effect on fold change expression across time was completed

by linear model 2-way ANOVA using Tukey HSD post hoc multiple comparisons of

means with a 95% family-wise confidence level R v3.5.0 (58). Data normality and

homoscedasticity were tested using Shapiro-Wilks test (59) or Levene-Bartlett's test,

respectively. In cases where ANOVA assumptions were not fulfilled, data was

transformed using Tukey's Ladder of Power transformation and analyzed using White's

adjusted ANOVA in R v3.5.0 using car, MASS, and rcompanion, glue, and

"userfriendlyscience" packages (60–63).

## 2.5 Results

**2.5.1 Identification of iGluR homologs:** One hundred sequences from Cnidaria met the

criteria to be identified as iGluR homologs in our Reef Genome proteome database. An

additional 265 homologs were identified in our assembled Uniprot reference proteome

database (Table 2.S1, 2.S2).

**2.5.2 Phylogenetic inferences of cnidarian iGluRs to representative iGluR groups:**

To explore the evolutionary history of iGluRs in Cnidaria, two phylogenies were built.

The first phylogeny covered a selection of metazoan taxa using *Chlamydomonas*

*reinhardtii* as the outgroup (Fig. 2.S1). For all taxa, except *E. pallida*, the Uniprot

reference proteomes were used to compile an assessment of the presence or absence of

iGluRs (Table 2.S1). For *E. pallida*, iGluRs were identified from the Reef Genomics

protein database, resulting in 22 paralogs (Table 2.S1). The reference proteome tree

revealed the presence of eight clades. Clades 1 and 2 diverged earlier than the rest of the clades. Clade 1 was represented by orthologs of NMDA-specific GluN1 (sensu Collingride et al. 65) found in most metazoans (Fig. 2.S1). Clade 2 was composed of NMDA-GluN2 and NMDA-GluN3 orthologs, which were well represented across Metazoa with major lineage-specific expansion in Cnidaria and to a lesser extent in mollusks and tardigrades (Fig. 2.S1). Clade 3 was conformed by non-NMDA iGluR orthologs from Placozoa (*Trichoplax adhaerens*) and Cnidaria, each with their own lineage-specific expansions (Fig. 2.S1). Clade 4 held another cnidarian-specific expansion of non-NMDA iGluRs with strong node support (Fig. 2.S1). Clade 5 was represented by ionotropic receptors (IR) that are missing from chordates, but highly duplicated within mollusks. Clades 6, 7, and 8 demonstrate broad lineage-specific expansions (Fig. 2.S1). Notably, clades 5 to 8 are devoid of cnidarian sequences.

To improve resolution of the cnidarian part of the tree, sequences from clades 1-4 were extracted and combined with an expanded set of sequences from Cnidaria, Ctenophora, and Porifera (Tables 2.S1, 2.S2). The resulting tree overall confirmed the clades in the previous larger tree and further placed ctenophore sequences in one major clade that shows a high level of lineage specific expansion (Fig. 2.1; CX). Porifera fell within C3, together with *T. adhaerens* and Cnidaria, suggesting that this clade was present in early Metazoa. As in the previous tree, C4 held only cnidarian sequences. Although the sequence set from *Acropora millepora* and other corals from the Reef Genomics project is incomplete, their placements within the phylogeny strongly suggest they follow the pattern of *E. pallida, N. vectensis*, and *S. pistillata* (Fig. 2.1).

**2.5.3 Molecular modeling of *Exaiptasia pallida* GluRs with functional insights:** Three

Pfam domains (39) are characteristic of iGluRs, an amino (or N-) terminal domain

(ATD), a ligand-specific binding domain (LBD), and a transmembrane domain (TMD)

that is an ion channel. Conserved domains for iGluRs identified within Pfam (39) showed

multiple *E. pallida* iGluRs contain all three conserved domains (Fig. 2.2). The ATD was

identified as atrial natriuretic factor receptor family ligand binding region

(PF010904/IPR001828) in GluN, GluA, GluK and IR8a and 25a, but it is missing within

select GluR sequences (Fig. 2.2). The LBD often characterized as ligand channel

glutamate binding domain (PF10613/IPR019594), contains two highly conserved regions,

2.S1 and 2.S2, which form a clamp for binding solutes or ligands (Fig. 2.2). The LBD

includes the sequence motif SYTANLAAFL, conserved in all chordate iGluRs (65–67),

but where threonine substitutes serine in GluRs. The LBD region is also identified as

bacterial extracellular solute-binding protein family 3 (PF00497/IPR001638) that is

closely associated to glutamate-binding domains (Fig. 2.2) (68). The TMD region

identified as the ligand-gated ion channel domain (PF00060/IPR001320) is mostly

conserved within GluRs (Fig. 2.2). In GluR22 (C1) and GluR6 (C3), the TMD

correspond to a calmodulin-binding domain of NMDA receptor 1 subunit

(PF10562/IPR018882) (Fig. 2.2). GluR19 (C2) has one domain that is not characterized

within Pfam (Fig. 2.2). Of all GluRs, the sequences that include all domains are within

C2: 11, 18, 16, 13, 14, within C3: 7, within C4: .1, .2, 3, 9, 5, and 4, although some

domains are incomplete.

Homology models were built for two representative cnidarian iGluRs using the SWISS-MODEL server (40–46). The two representative proteins with all three typical iGluR domains were GluR11 (C2) and GluR.1 (C4) (Fig. 2.1 & 2). The homology models were constructed based on two different templates, identified from the global GMQE values (normalized values of 0.58 and 0.56). For GluR11 (C2), the template was a structure of the activated GluA2 from a chimeric sequence *Rattus norvegicus* and *Mus musculus* (PDB ID: 5WEO, chain A). The sequence identity for the target and template was 32% and 84% of the target was modeled (Fig. 2.3A). For GluR.1 (C4) the template was an apo structure representing the resting state of GluA2 as determined from *R. norvegicus* (PDB ID: 4U2P, chain B). Sequence identity for the target and template was 31% and 86% of the target was modeled (Fig. 2.3B). Both proteins were modeled as homotetramers to represent the functional iGluR unit (Fig. 2.3). The local QMEANs of the models were -4.93 and -4.87, respectively for GluR11 (C2) and GluR.1 (C4) (Fig. 2.3). The QMEAN values indicate low quality models for globular protein models, but can be expected for transmembrane receptors (45,50).

**2.5.4 Transcriptional analysis of iGluR gene expression following bacterial challenge:** To test whether the transcriptional expression of discovered *E. pallida* iGluR proteins responded to infectious agents, gene expression was measured through qPCR analyses over six time points (0, 24, 48, 72, 96 hr and 4 wk) following bacterial exposure (Fig. 2.S2; Fig. 2.4). Out of designed iGluRs primers, six provided successful amplifications. Primers targeted gluR.1 (C4), gluR.2 (C4), gluR4 (C4), gluR8 (C4), gluR21 (C2), and gluR23 (C3) (Table S3). Designed primers targeting the sole C1

sequence (gluR22) did not provide successful amplification; therefore, no transcriptional data was produced for C1 *Exaiptasia* ortholog. Expression of genes gluR23 (C3) and gluR21 (C2) showed significant differences as a function of bacterial challenge (White's adjusted ANOVA, p=0.0003 and p=2.28e-5, respectively; Fig. 2.4C-D; Table 2.S4). *Exaiptasia pallida* gluR21 (C2) transcription regulation was affect by the interaction of time and treatment (2-way ANOVA Tukey HSD, p= 0.0190). *Exaiptasia pallida* gluR.1 (C4) and gluR.2 (C4) (isoforms of GluR differing by 24 sequential base pairs) also showed transcriptional responses to bacterial challenge (2-way ANOVA, p=0.0414 and p=0.0016, respectively; Table 2.S4), however, patterns differed over time. *Serratia marcescens*, significantly affect gluR.1 expression trends, with up-regulation at 96 hr (2-way ANOVA Tukey HSD, p=0.0319, p=0.0299; Table 2.S5). Up-regulation of gluR.1 (C4) under challenge of *V. coralliilyticus* appeared at 96 hr and 4 wk, but was not measurably significant (2-way ANOVA, p=0.401; Fig. 2.4A; Table 2.S5). *Exaiptasia pallida* gluR.2 (C4) showed a similar trend of expression over time in both bacterial treatments. Expression appeared to increase at 48 hr under the challenge of *S. marcescens* and transcriptional expression remained high during the subsequent times points (2-way ANOVA Tukey HSD, p=0.00112; Fig, 4B; Table 2.S5). *Vibrio coralliilyticus* had a borderline significant affect on expression regulation of gluR.2 (2-way ANOVA Tukey HSD; p=0.0746; Fig. 2.4B; Table 2.S5). *Exaiptasia pallida* gluR4 (C4) and gluR8 (C4) were not differentially expressed.

**2.6 Discussion**

We have performed to date the most comprehensive phylogenetic analysis of cnidarian ionotropic glutamate receptors that has permitted us to develop a model for their evolution within metazoan. Our study also revealed functional differences based on protein domains *in silico* and the involvement of certain iGluRs groups in the recognition of biological danger *in vivo*, as part of the defense response of *Exaiptasia pallida*.

**2.6.1 Evolutionary expansions of iGluRs in Cnidaria:** Our findings revealed a greater expansion of cnidarian iGluRs than recent metazoan iGluR phylogenies reported (21,69). The 22 *Exaiptasia pallida* iGluR genes are comparable in number to other Cnidaria and greater than most other metazoan species in the study (Table 2.S1). Only *Lottia gigantea* and *Crassotrea gigas* have more iGluR homologs, 45 and 31 genes, respectively (Table 2.S1). Our phylogenies confirm lineage-specific iGluR gene expansions across metazoan (21,23,67,69–71) and reveal a distinctive pattern of iGluR expansion within the recently sequenced *E. pallida* genome (34) and other Cnidaria that resolved in four phylogenetic clades (Fig. 2.1 and 2.S1). We interpret our findings in the context of a recent phylogenetic hypothesis of the evolution of iGluR in metazoans, in which four sub-families (Lamda, NMDA, Epsilon, and AKDF) and 10 classes were proposed (69). The GluN sequences from our phylogenetic trees were represented in clades C1 and C2, and both include cnidarian sequences along with other metazoan orthologs (Fig. 2.1 & 2.S1). However, our tree topology is not congruent with the recent NMDA iGluR phylogeny (69). Ramos-Vicente and colleagues proposed four classes containing NMDA-specific

(GluN) orthologs where two of them included cnidarian sequences (NMDA1: GluN1 and a cnidarian-specific NMDA class). Moreover, the cnidarian NMDA class was proposed to be an early-diverged sister clade of the other three NMDA-specific classes (GluN1, 2 and 3; sensu 70). On the contrary, in both our phylogenetic trees, GluN1 (C1) is the sister group of C2 that includes GluN2/3+cnidarian. Further, the branch lengths in C1 are shorter than in C2, suggesting that the C1 with GluN is closer to the ancestral state and the C2 with GluN2/3+ cnidarian more derived.

In regard to the other three resolved clades (C3, C4 and CX; Fig. 2.1) represented by non-NMDA iGluRs, we noted that two *Nematostella* paralogs (NV_A7RPU4 and NV_A7T1G4) in C3 have been identified as belonging to the subfamily Epsilon (GluE1_Nve and GluE2_Nve, respectively; sensu Ramos-Vicente et al. (69) and two other *Nematostella* paralogs (NV_A7S4J5 and NV_A7S4J7) in C4 belong to the subfamily AKDF (GluAKDF1_Nve and GluAKDF_Nve2, respectively; sensu 70). This implicates that Cnidaria (in particular *E. pallida*) also possess a diverse representation of iGluRs in these two non-NMDA subfamilies. However, our findings suggest that C3 and C4 are phylogenetically more related to one another than to the monophyletic CX, represented by ctenophore iGluRs. Ramos-Vicente and colleagues suggested that ctenophores only have one type of iGluR from the subfamily Epsilon (69), which further implies that C3 and C4 also belong to subfamily Epsilon, the closest to subfamily NMDA. The two *Nematostella* sequences from the subfamily AKDF within C4 seem to be a misclassification by Ramos-Vicente et al. (69). This could be attributed to difference in sequence composition, such as insect IRs (C5) that allowed us to separate GluA-

AMPA and GluK-Kainate orthologs (C8) and GluD-Delta (C6) from C3, C4 and CX. We proposed based on our phylogenetic analyses that the cnidarian specific Clade 4 is an expansion of non-NMDA iGluRs within subfamily Epsilon and should be considered a cnidarian class on its own within this subfamily (Fig. 5).

**2.6.2 Neofunctionalization and subfuctionalization of cnidarian iGluRs:** In cnidaria, numerous gene duplications are evident. The GluR co-orthologs within the four main clades may have similar functional characteristics but given the amount of lineage-specific gene duplications, the amount of amino acid substitutions as indicated by branch lengths, and altered domain compositions, this may not always be the case. The common scenario following gene duplication is that the resulting functional redundancy allows for relaxed selective pressures. Consequently, the different gene copies often show increased amino acid substitution rates and a more rapid exploration of sequence and function space. The majority of gene duplicates become pseudogenes (72), but those that are retained either do not change in function or go through a process of neo- and subfunctionalization (73,74). For *E. pallida,* several sequences in our dataset lack all characteristic iGluR domains (Fig. 2.2), which is consistent with sub- and neofunctionalization. These sequences may be partial sequences in the database and full-length in the actual genome, but they may also have functionally diverged by mechanisms beyond amino acid substitutions such as domain loss. Like co-receptor ionotropic receptors (IR) within insects, GluR22, 19, 17, 6, and 15 (Fig. 2.2) lack of ATD (18,20,23). Loss of the ATD appears to have happened in GluRs in all four clades at different times, suggesting that this loss may benefit fitness. Amino acid substitutions of

key residues in LBD or TMD can alter specificity to ligands or ions, respectively. The changes within regions of functional domains have potential to differentiate the functional roles via environmental ligands or signal pathways. The *E. pallida* sequences containing all representative iGluR domains may still function as archetypal iGluRs.

**2.6.3 Certain iGluR clades in *E. pallida* are functionally implicated in the response to bacterial exposure:** The first indication that iGluRs had a role in cnidarian immune defense came from findings that revealed that immunological primed anemones showing resistance to repetitive bacterial infections had a significant increase of protein expression of GluR (C4) compared to naïve anemones that encountered the pathogen for the first time (15). In this study, we further examined the functional conservation in immune defense from the other iGluRs clades unveiled from our phylogenetic analyses by exploring whether bacteria exposure would elicit transcriptional expression changes across these GluRs. Through a functional exploration of six *E. pallida* iGluRs, the findings indicated that GluRs from other clades (apart from C4) also responded to bacterial challenges such was the case for iGluRs in clades C2 and C3. This implicates an immunological functionality of *E. pallida* iGluRs from both NMDA (at least C2) and Epsilon subfamilies (C3 and C4). However, the patterns of expression through the time-course of this study differed among iGluRs and in some cases showed to be dependent on the bacterial pathogens. In general, the response of these iGluRs was more evident to bacterium *Serratia marcescens* than to *Vibrio coralliilyticus*, which might be associated with difference in infectivity dynamics between the two bacterial pathogens.

The transcriptional increase of the isoforms gluR.1 and gluR.2 from C4 to a

bacterial pathogen confirmed similar results for the same gene at proteomic level within

the same experimental time-course (15). However, the other two tested iGluRs also from

C4 (gluR4 and gluR8) did not change expression under the bacterial challenge indicating

that not all *Exaiptasia* iGluRs in C4 are implicated in the response to bacterial infections,

which suggests that some of them may be involved in other functional processes. While

gluR8 may not be functional due to the lack of several iGluR functional domains (Fig.

2.2), gluR4, on the other hand, had all three characteristic iGluR domains and is expected

to function as an iGluR. Moreover, it is worth noting that both these two gluR4 and

gluR8 grouped in different sub-clades within C4 compared to the immunologically

involved GluR (i.e. gluR.1/R.2) (Fig. 2.1), and this differential phylogenetic signature

might relate to possible functional divergence within Epsilon (GluE) C4 (Fig. 2.5).


Initially, iGluRs were thought to be strictly involved with the nervous system (in

synapses) but recent findings have revealed that even in mammals these glutamate

receptors are implicated in the regulation of innate immune responses (75,76) and in

boosting adaptive immune T-cells (77). In plants, knockout mutants have shown that

some iGluRs homologs, glutamate-like receptors (GLR), play important roles in defense

response (32). Furthermore, a recent proposed model speculates that plant GLR ligands

might in some cases function as danger-associated molecular patterns (DAMPs) and

suggested that the sensing of different combinations of host and non-host derived

components by GLRs during infections may be integrated to regulate alternative plant

defense pathways (16). Our findings draw some parallels to what we have learned from

plants; and provide new light on the functional property of iGluRs from early-diverged metazoans in immune defense and chemosensory associated to danger. Future studies using the *Exaiptasia* model system to determine the localization of expression of these receptors along with the identification of ligand specificity will provide further evidence to explain the mechanism in immune defense regulation and also to reveal other novel functions from other cnidarian iGluR clades.

## 2.7 Acknowledgements

## 2.8 Tables and Figures

Table 2.S1 Taxa included within analysis and abbreviations used along with the number of sequences per taxa included in the iGluR phylogenies for both cnidarian and general metazoan trees.

| Taxa | Abbreviation | Cnidarian | General Metazoan |
|---|---|---|---|
| *Exaiptasia pallida* | EP | 22 | 22 |
| *Nematostella vectensis* | NV | 19 | 19 |
| *Stylophora pistillata* | SP | 26 | 26 |
| *Acropora digitifera* | AD | 2 | - |
| *Acropora millepora* | AM | 9 | - |
| *Astreopora spp.* | ASP | 4 | - |
| *Fungia scutaria* | FS | 4 | - |
| *Favia spp.* | FSP | 2 | - |
| *Madracis auretenra* | MA | 2 | - |
| *Montastrea cavernosa* | MC | 2 | - |
| *Porites asteroides* | PAU | 7 | - |
| *Porites lobata* | PL | 1 | - |
| *Mnemiopsis leidyi* | ML | 19 | - |
| *Trichoplax adhaerens* | TA | 10 | 14 |
| *Oscarella carmela* | OC | 7 | - |
| *Drosophila melanogaster* | DM | 1 | 16 |
| *Homo sapiens* | HS | 7 | 18 |
| *Lottia gigantea* | LG | 6 | 45 |
| *Octopus bimaculoides* | OB | 3 | 20 |
| *Biomphalaria glabrata* | BG | 5 | 21 |
| *Crassotrea gigas* | CG | 5 | 31 |
| *Lingula unguis* | LU | 2 | 12 |
| *Chlamydomonas reinhardtii* | CR | 2 | 2 |
| *Ramazzotius varieornatus* | RV | 6 | 15 |
| *Hypsibius dujardini* | HD | 2 | 8 |
| *Strogylocentrotus purpuratus* | SPU | 0 | 9 |
| *Helobdella robusta* | HR | 0 | 15 |
| *Stichopus japonicus* | SJ | 0 | 13 |

Table 2.S2 Primer sequences with names and gene identification.

| Gene | Sequence ID | Primer Name | Primer Sequence |
|---|---|---|---|
| gluR.1 | 24461 | iGluR1f | GTGGGACGATCGAAGTGAATGT |
| | 24461 | iGluR1r | GGAGTCTGTGTTTGTCTTCTGCC |
| gluR.2 | 24462 | iGluR2f | ACGTGAGGAAGGAGAGATTGA |
| | 24462 | iGluR2r | AAGTGAAAAACCGGGCTGTG |
| gluR23 | 29220 | iGluR5f | GGAGCAATAGCAGAGGATGAC |
| | 29220 | iGluR5r | CCGTAAGCTATGGTGAGAACAG |
| gluR21 | 25547 | iGluR6f | GTGTTGACTGTGATGCTAAATCC |
| | 25547 | iGluR6r | CCACTTCAACGTCCAGTACAT |
| gluR4 | 5331 | iGluR7f | CTGATAGTCGCTGGAATCAATCT |
| | 5331 | iGluR7r | CAGGACTCCATTCACCAATCTG |
| gluR8 | 29007 | iGluR8f | TGCAGTTCTTGATGCCCTTTGA |
| | 29007 | iGluR8r | GACCAGAAGACGGCCTGATATAGA |
| l10 | 29093 | L10f | CTTGGACAGGAGCATCGATAATA |
| | 29093 | L10r | CCAGTGGGTTACGAAGACTTT |
| l12 | 8348 | L12f | TCACATGGTGTCAGATGAGTATG |
| | 8348 | L12r | CATACCAGTTTGGAGCCTATCA |
| pabp | 2230 | PABPf | AGAGAATTCCGCTTAGCAACTC |
| | 2230 | PABPr | GAAGCAAGCTGTCGCTTAGA |

Table 2.S3 Statistical significance of 2-way ANOVA between factors of time and treatment.

| Treatment | gluR.1 | gluR.2 | gluR23[+] | gluR21[+] | gluR4[+] | gluR8 |
|---|---|---|---|---|---|---|
| time | 0.033* | 0.0046* | 8.92e-6* | 1.22e-5* | 0.1734 | 0.1376 |
| treatment | 0.0414* | 0.0016* | 0.0003* | 2.28e-15* | 0.2428 | 0.5206 |
| time:treatment | 0.2376 | 0.0839 | 0.1638 | 0.0190* | 0.4737 | 0.7197 |

[+] White's adjusted ANOVA for unequal variance, * significant p-value < 0.05

Table 2.S4 Statistical significance of 2-way ANOVA Tukey HSD post-hoc using 95% family-wise confidence level on treatment factor.

| Treatment | gluR.1 | gluR.2 | gluR23[+] | gluR21[+] | gluR4[+] | gluR8 |
|---|---|---|---|---|---|---|
| Serratia – Control | 0.0319* | 0.0011* | 0.9542 | 0.4907 | 0.4965 | 0.4916 |
| Vibrio – Control | 0.4018 | 0.0746' | 0.7376 | 0.7043 | 0.9160 | 0.9912 |
| Vibrio – Serratia | 0.3854 | 0.2392 | 0.5569 | 0.1362 | 0.2830 | 0.4186 |

[+] White's adjusted ANOVA for unequal variance,  * significant p-value < 0.05
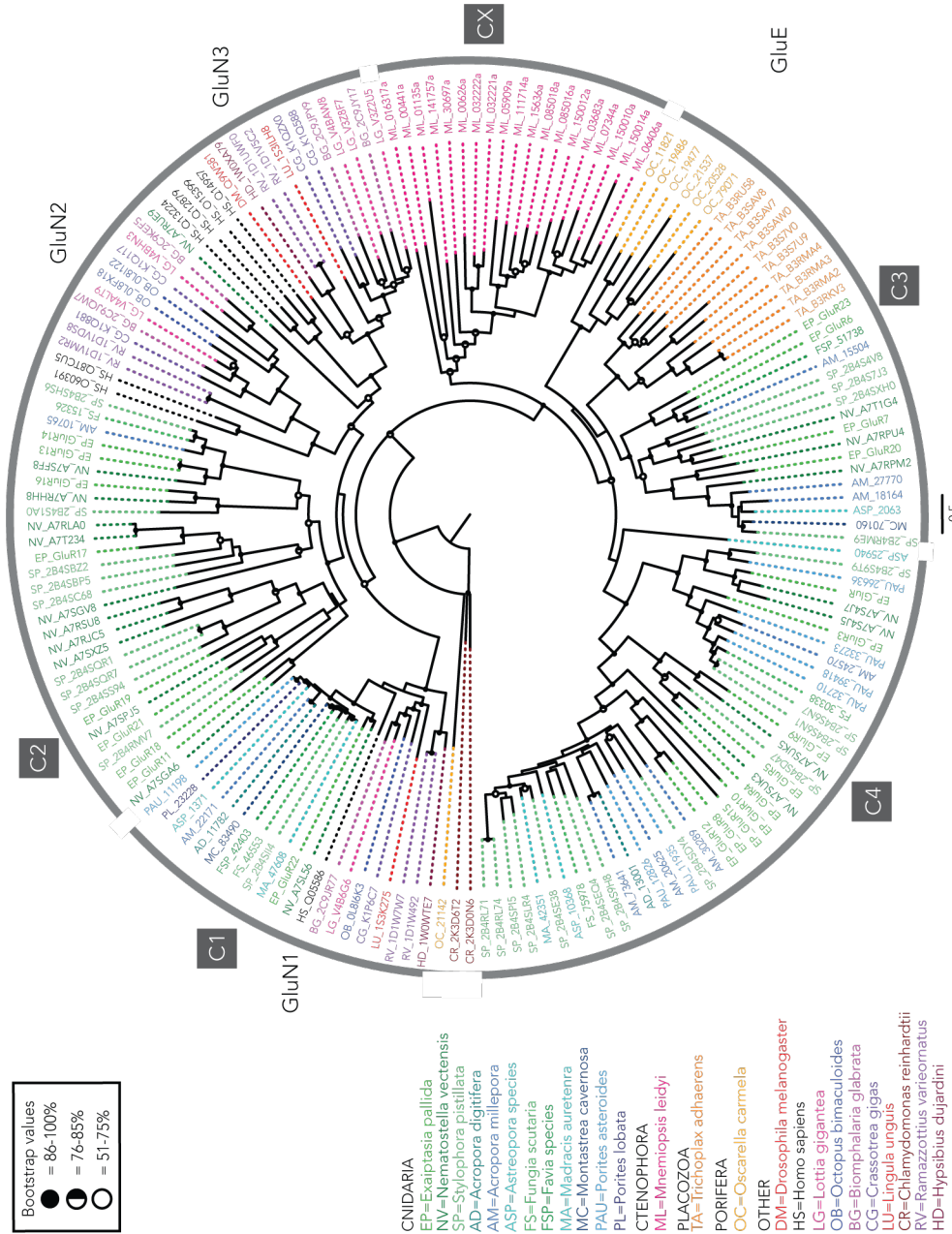
Figure 2.1 Maximum likelihood phylogenetic tree based on amino acid sequences of iGluR homologs from cnidarian species and NMDA-specific iGluRs (GluN). Tree viewed in FigTree v1.4.2 and rooted to *Chlamydomonas reinhardtii*. Node support based on 1000 rapid bootstraps using best-fit evolutionary model LG +G6 +I +F and conducted by RAxML BlackBox v8.2.4. Support marked by circles: empty 51-75%, half 76-85%, full 86-100%. Sequence labels colored by species, corresponding to key. Sequences are identified by first letter of genus, first letter of species and modified accession number. Clades are numbered in ascending order. Scale bar, 0.5 expected rate of substitution per site.

49
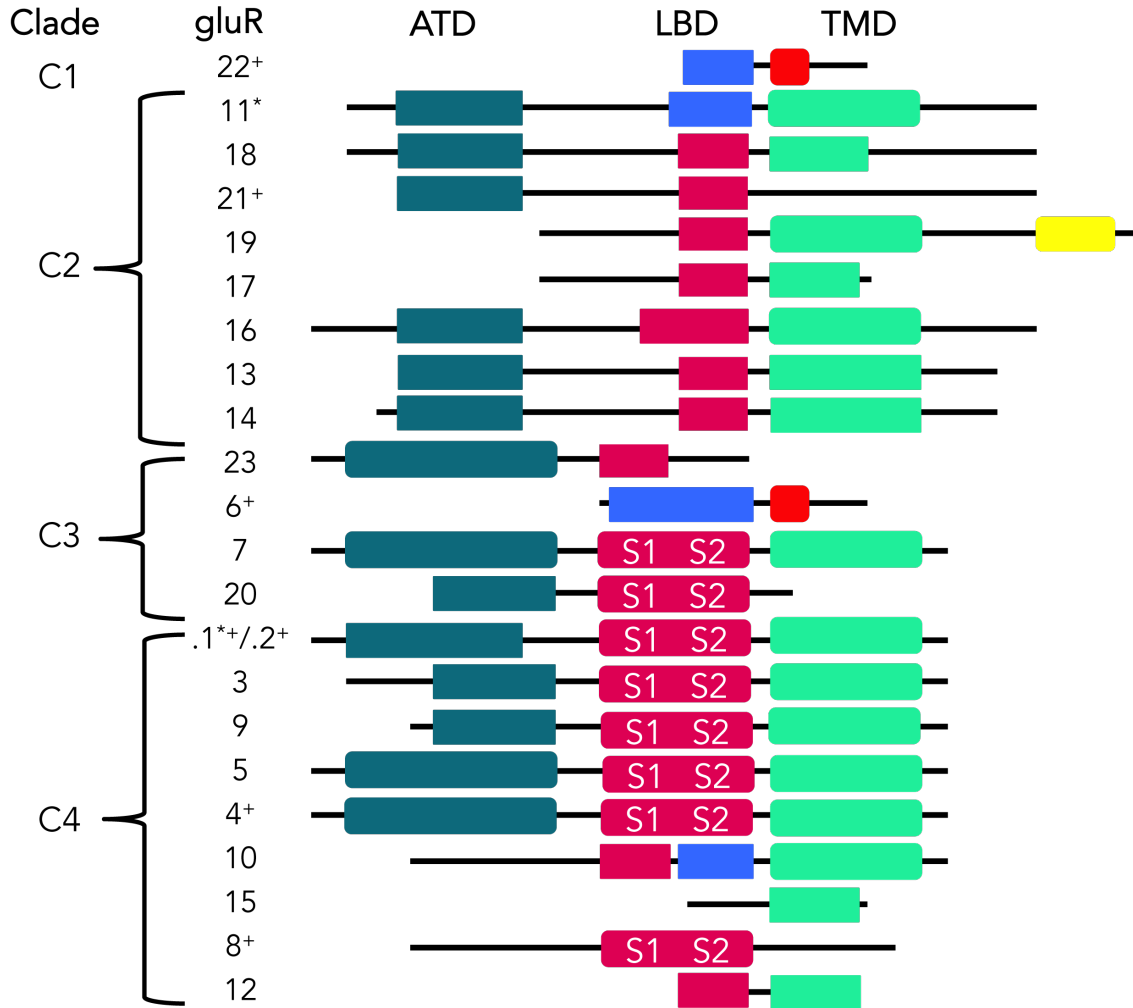
Figure 2.2 Conserved domains of *Exaiptasia pallida* ionotropic glutamate receptors, iGluRs. Amino- or N-terminal is ANF_receptor family ligand binding region (PF010904/IPR001828) colored turquoise. The ATD is known to not be conserved within IRs. The second conserved domain region is the ligand binding domain, usually specific for glutamate or glycine ligands. This region is colored a bright magenta if the conserved domain is ligand channel glutamate binding domain (PF10613/IPR019594) and contains conserved sequences of S1 and S2 if complete. This region is also conserved as bacterial extracellular solute-binding protein family 3 (PF00497/IPR001638), colored blue, and is closely related to the glutamate binding domain. The transmembrane region specific to ions for transmembrane channel is colored mint if it is the conserved sequence of the ligand channel domain ligand channel (PF00060/IPR001320). This region contains a conserved calmodulin-binding domain of NMDA receptor 1 subunit (PF10562/IPR018882) for 22 and 6, colored red. There is one non-identified region colored yellow in 19. These colors correspond to colors of domains in Figures 2.3. GluR sequences are sorted in the order as present within the phylogenetic tree, ascending from the root. Complete domains have curved corners, partial sharp corners. Superscript * denotes modeled in Figure 2.3. Superscript + denotes gene expression has been quantified in Figure 2.4.
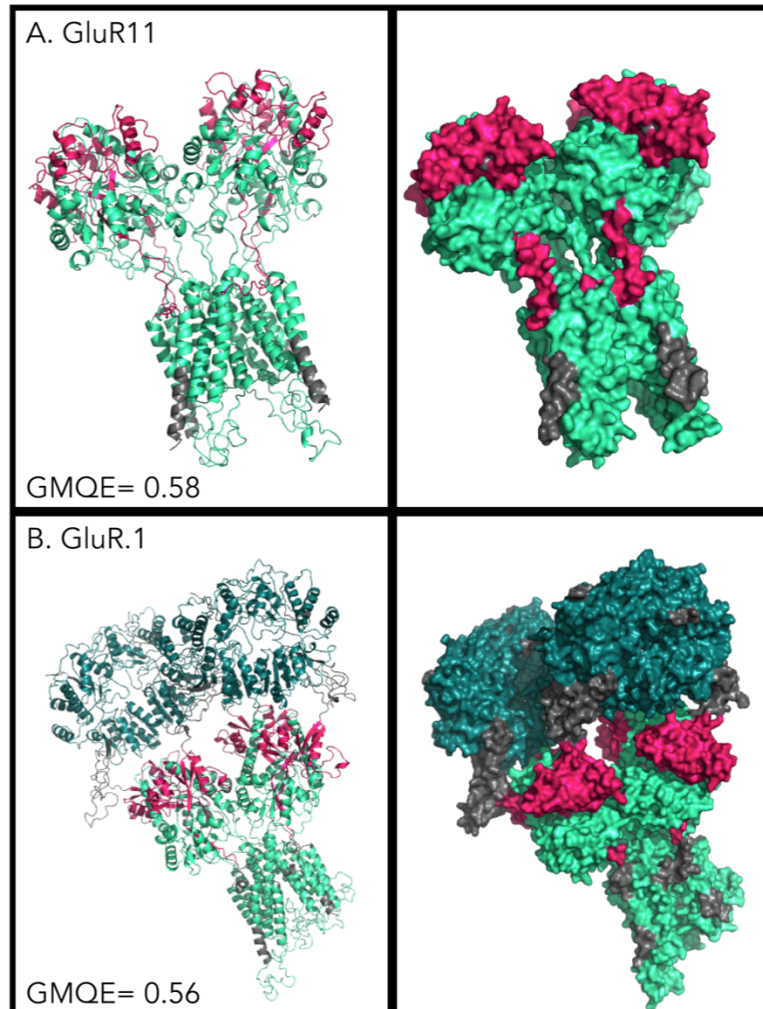
Figure 2.3 Homotetramers of *Exaiptasia pallida* GluR6 and GluR.1.
Predicted 3D structures (left) and predicted surface model (right) of select-
ed sequences from cnidarian GluR11 (A) and GluR.1 (B). The protein is a
tetramer with four subunits corresponding to one another. Predicted struc-
tures are colored by conserved domain regions (teal= amino terminal
domain, pink=ligand binding domain, green cyan=ion channel,
grey=non-conserved). An extracellular N-terminal domain (NTD) is
involved in assembly of iGluR subunits into heteromeric complexes. NTD
precedes the ligand-binding domain (LBD), whose half-domains (S2 and
S3) form a Venus flytrap-like structure that will close around glutamate and
related ligands when activated. The transmembrane domain follows these
two binding domains, forming a channel to allow calcium ions through the
membrane. GluR11, as deduced from the amino acid sequence of *E. pallida*
KXJ21128 aligned with Glutamate receptor, Voltage-dependent calcium
channel gamma-2 subunit chimera (PDB ID= 5weo.1.A) and is modeled as
a homotetramer (A, left). GluR11 predicted model has an overall QMEAN
score of -4.93(A, middle). GluR.1 predicted structure has only ligand
binding and ion channel conserved domains (A, right). GluR.1 protein, as
deduced from the amino acid sequence of *E. pallida* KXJ14767 aligned
with Glutamate Receptor 2 (PDB ID = 4u2p.1.B) and is modeled as a
homotetramer (B, left). GluR.1 predicted model has an overall QMEAN
score of -4.87. GluR.1 predicted structure shows all three conserved iGluR
domains, amino terminal, ligand binding site, ion channel (B, right).

51
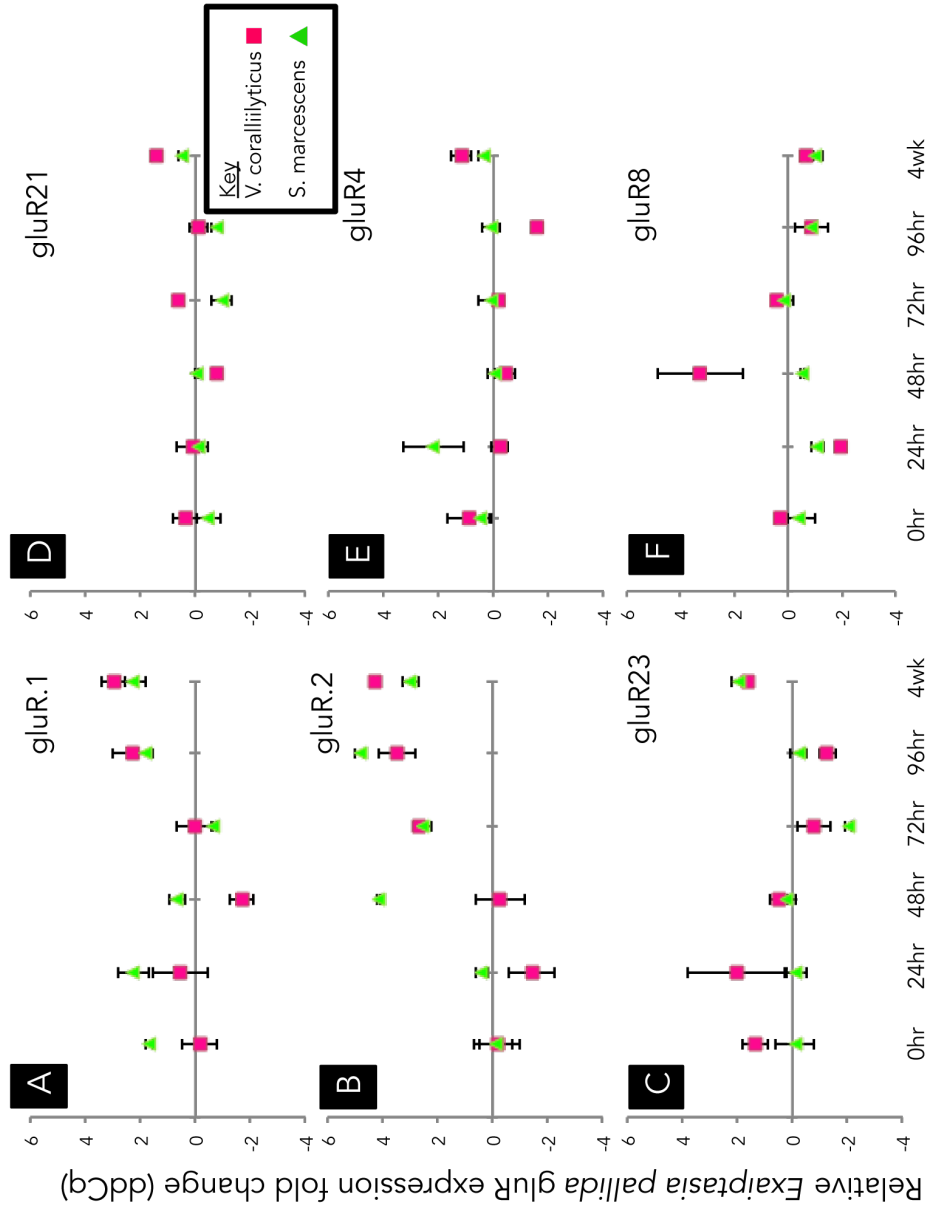
Figure 2.4 Relative fold difference (log2 scale) in gene expression of *Exaiptasia pallida* gluR variants upon treatment with bacterial challenges (*Vibrio coralliilyticus* and *Serratia marcescens*) across time. Gene expression was normalized to l10, l12 and pabp genes. A) gluR.1, B) gluR.2, C) gluR23, D) gluR21, E) gluR4, F) gluR8.
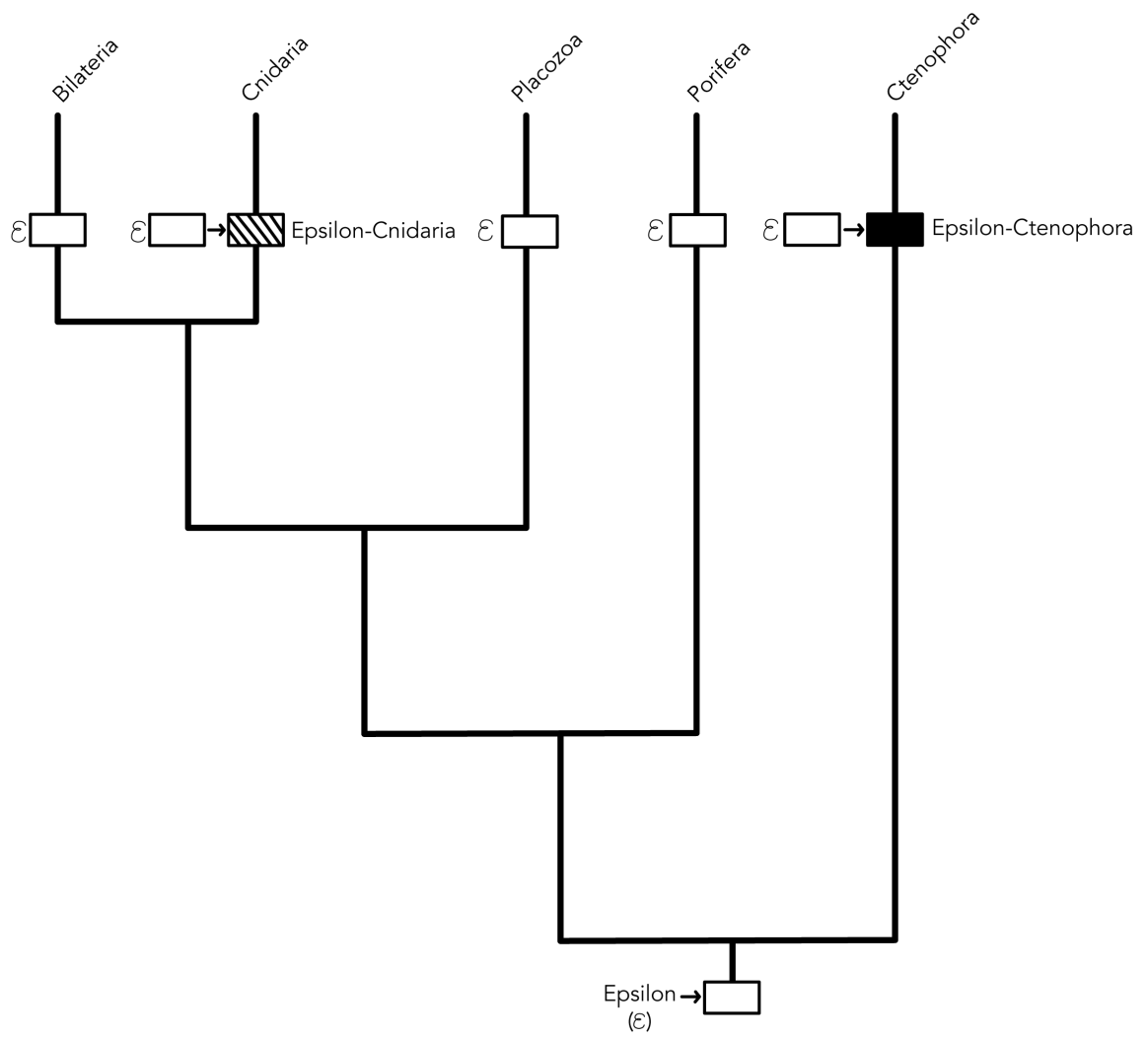
Figure 2.5 Proposed evolutionary tree for iGluR subfamily Epsilon to include cnidarian-specific divergent clade.
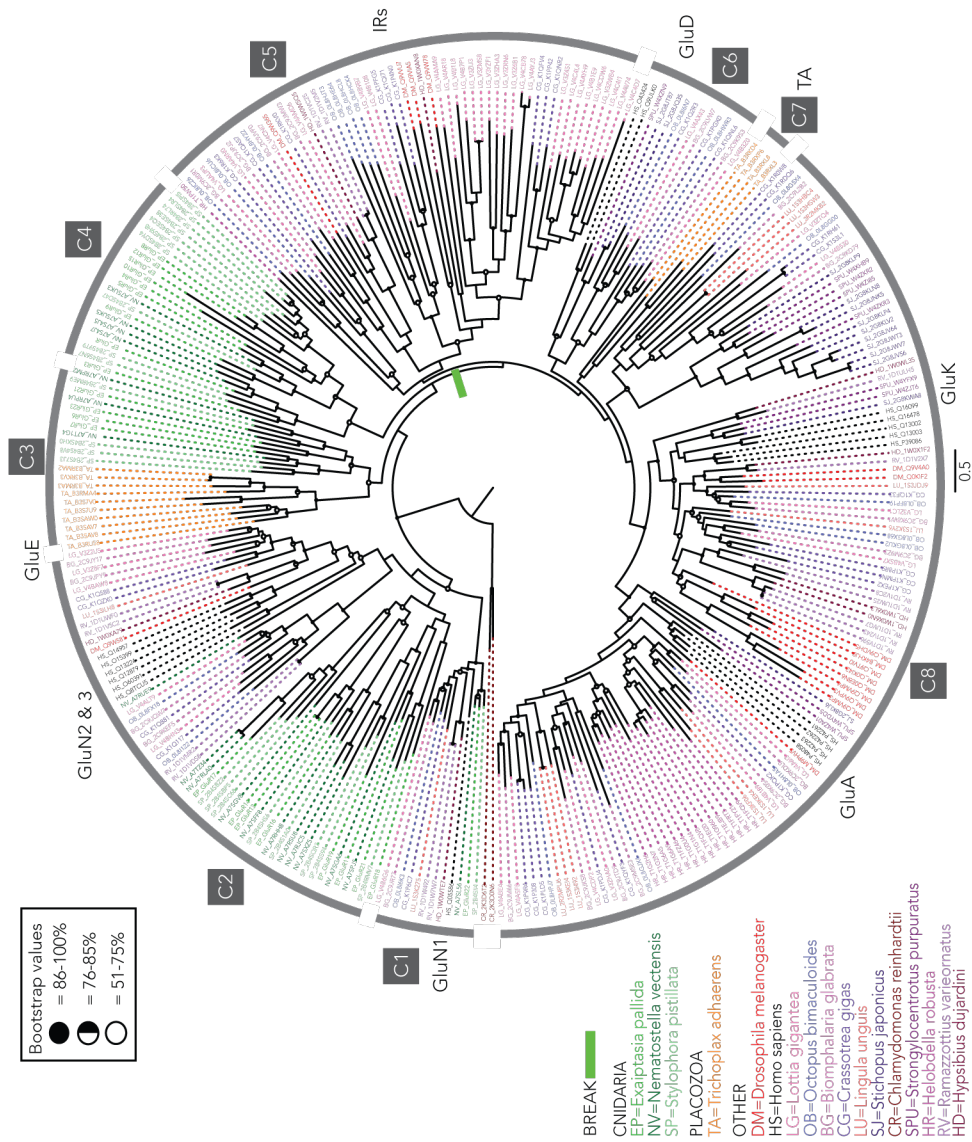
Figure 2.S1 Maximum likelihood phylogenetic tree based on amino acid sequences of iGluR homologs from *Exaiptasia pallida* and reference proteomes. Tree viewed in FigTree v1.4.2 and rooted to *Chlamydomonas reinhardtii*. Node support based on 1000 rapid bootstraps using best-fit evolutionary model LG +G6 +I +F and conducted by RAxML BlackBox v8.2.4. Support marked by circles: empty 51-75%, half 76-85%, full 86-100%. Sequence labels colored by species, corresponding to key. Sequences are identified by first letter of genus, first letter of species and modified accession number. Clades are numbered in ascending number to reference in text. Green break bar denotes sequences prior are included in the cnidarian-based tree. Scale bar, 0.5 expected rate of substitution per site.
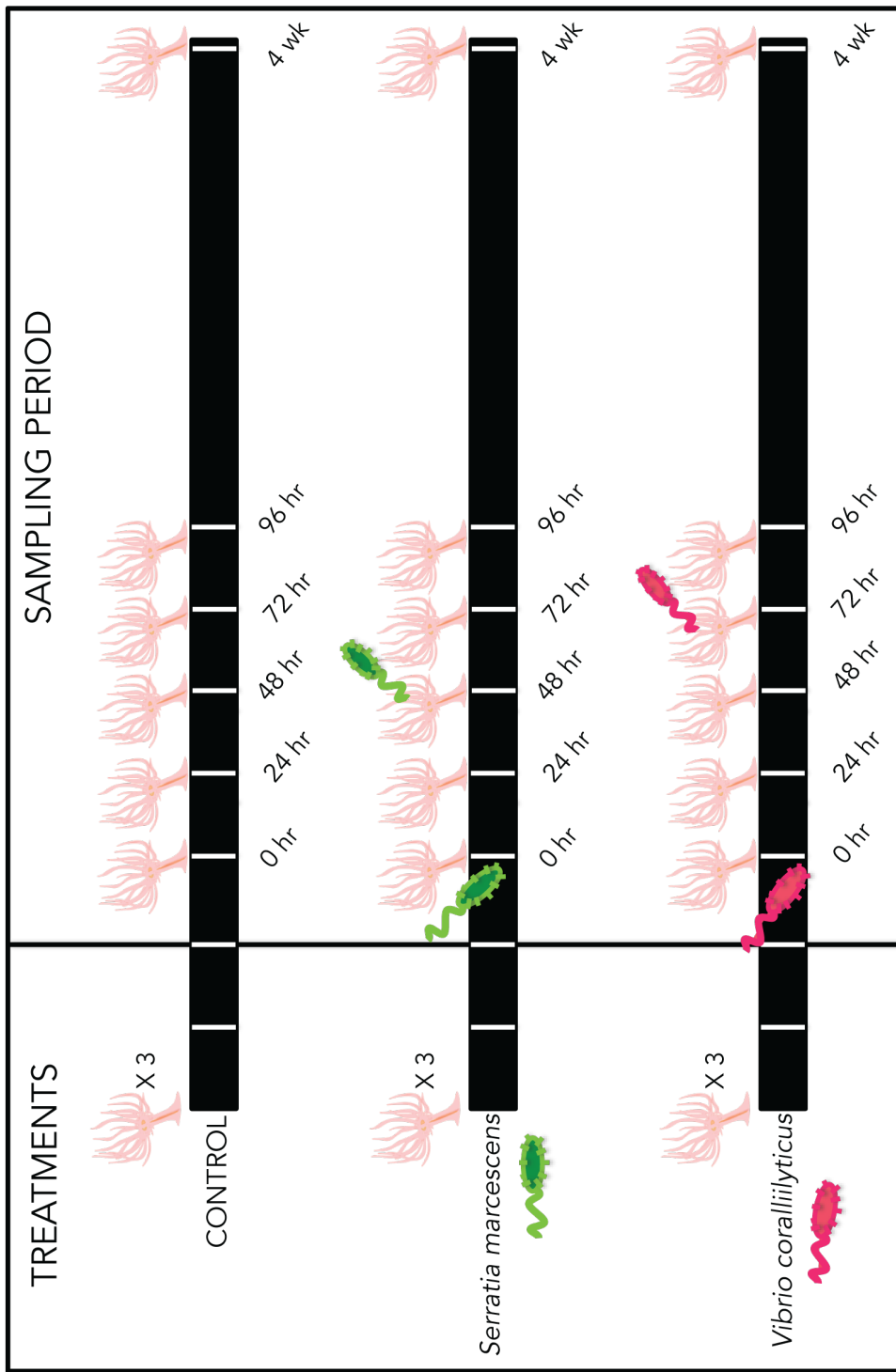
Figure 2.S2 Experimental setup of bacteria challenges on *Exaiptasia pallida* as described in Methods: Culturing bacteria (*Vibrio coralliilyticus* and *Serratia marcescens*) and experimental design of bacterial challenges.

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CHAPTER III. EXPRESSION OF CHEMOSENSORY IONOTROPIC GLUTAMATE
RECEPTORS UNDER THE INFLUENCE OF BIOLOGICAL AND
ENVIRONMENTAL OSCILLATIONS IN CNIDARIANS

## 3.1 Abstract

Environmental fluctuations of light, tide, and temperature influence physiology and
behavior of marine invertebrates, particularly sessile corals and anemones.
Accompanying environmental changes is the internal endosymbiosis of algal
dinoflagellates, which dominantly affects physiology of cnidarian hosts through diel
rhythms. Both intracellular and extracellular oscillations have been demonstrated to alter
host gene expression within corals and anemones, including products of metabolic
processing. Chemosensory gene regulation has not been explored in relation to diurnal
fluctuations and endosymbiosis. In Cnidaria, the chemosensory protein family, ionotropic
glutamate receptors (iGluRs), are of particular consequence as signaling proteins in
interpreting envrionmental cues. Here we quantified mRNA expression of two *Exaiptasia
pallida* iGluR genes (gluR.2 and gluR8) to compare the influence of a recurring light:
dark cycle and response to constant darkness on gene transcription. In the absence of
light, periodicity length of *E. pallida* gluR.2 and gluR8 expression increased. The change
in transcription periodicity as result of light changes paired with symbiotic versus
aposymbiotic periodicity changes from *Exaiptasia diaphana* transcriptomic data,
implicates regulation of iGluRs by Symbiodiniacea metabolic outputs. While an
endogenous circadian rhythm does not appear to be the primary contributor to

biorhythmic expression cycling in these chemosensory genes, symbiosis and diurnal fluctuations appear to have a role.

## 3.2 Keywords

*Exaiptasia*; chemosensing; iGluR; circadian; diurnal; diel; circatidal; cnidarian; symbiosis

## 3.3 Introduction

Corals build the highly productive framework of diversified reef ecosystems that provide essential habitat for fish and marine invertebrates, a foundational service for fisheries and ecotourism (1–3). Corals and closely related anemones are particularly impacted by environmental diel cycles of light and temperature because they are sessile (4–6). While both diel and circadian refer to a period of approximately 24-hrs, circadian rhythms are considered as endogenous, oscillating under constant conditions in absence of a stimulus whereas in diel rhythms are directed by external cues (7,8). There are also observations of diel rhythms for a suite of cnidarian behaviors and variation in gene expression (9–12). Daily light cycles regulate numerous physiological processes and behaviors, observed in wide-ranging physiological and even behavioral changes of coral from metabolism to tentacle retraction and expansion (13–16). Evidence of altered expression of genes related to growth is also consistent with a diurnal response (5,17). Light drives much of the physiological responses observed through the innerworkings between symbiosis and metabolysis.

Cnidarian diel cycles are strongly linked to central metabolism and cellular redox states through glycolytic enzyme cycles (12). Within symbiotic cnidarians, diurnal cycles produce alternating energy sources from nutrients delivered by a close-knit endosymbiosis with the algal dinoflagellate family Symbiodiniaceae (3,12,18–22). In daylight, energy is derived from photosynthesis by the Symbiodiniacea partner alongside consequent reactive oxygen species (ROS) production, while at night corals consume zooplankton (23–29). Heterotrophic feeding and continued respiration in the absence of photosynthesis can lead to hypoxia and decreased pH within tissues (12,24,30,31). A predicted consequence of nightly respiration-driven hypoxia is the upregulation of hypoxia inducible factor (HIF)-targeted genes i.e., glycolic enzymes aldolase, phosphoglycerate kinase, and phosphoglycerate dehydrogenase (32). The characteristic 24-hr periodicity occurred in facultative symbiotic anemones (32). It it proposed that the endosymbiosis of Symbiodiniaceae promotes the cycle of a diel or circadian 24-hr period (33).

The origins of circadian clockwork within cnidarians are suggested to include conserved molecular and functional components (10,15,16,33,34). These molecular components follow the 24-hr circadian cycle, but have two alternative regulating sources. Cnidarian clock, cycle, (11,15) and cryptochrome (14) are proposed to act as clock-controlled genes (CCG), meaning expression is regulated by an internal molecular clock to produce measureable circadian rhythm as a cycle (16). In contrast, diel cycle genes (DCGs) display a 24-hr period, but remain reliant on consistent diel oscillations. Cnidarian DCGs include some cryptochrome (blue light photoreceptor) genes (14). Select

photoreceptor DCGs i.e., cryptochromes and opsins, are hypothesized to interact with circadian genes as transcription factors to produce a response via physiological changes under circadian regulation (10,12,15,16,32). It appears these biogeochemical environmental conditions are the ultimate drivers behind physiological changes in molecular systems through entrainment of physiological rhythms. However, the reception and propogation of signals within the response to environmental cues in cnidarians is largely unknown and predominantly based on correlative findings.

Cnidarians have chemosensory receptors coded within their genomes to sense environmental changes (35,36) (Ch.2). Receptors include chemosensory gated ion channels that have the potential to propogate further signals. The expression rhythms associated with chemosensory genes that are responsible for near immediate reactions and responses, including the family of ionotropic glutamate receptors (iGluRs), are for the most part unknown. The function of iGluRs has yet to be identified within cnidarians and most organisms, aside from antennal ionotropic receptors within insects (37–40). These transmembrane proteins receive exogenous chemical signals and relay signals through a membrane-bound ion channel (39,41). Signals sent through the iGluR prompt responses in accordance with chemical environment changes, either as synaptic communication or feeding detection (42–45). The family of iGluRs have established roles within neuronal communication (46–48) and the potential for relaying signals in perpetuating biological rhythms. Genes regulated by biological rhythms are predicted to change expression in anticipation of environmental oscillations (15,36,49,50). Initial measurements of iGluR expression within the sea anemone *Exaiptasia pallida* (Agassiz

in Verrill, 1864) indicated variable expression responses to pathogenic bacteria; unknown if variation in gluR expression is the result of biological rhythms (Fig. 3.1; Ch. 2). This opened the possibility that variation in gluR expression reflects a biological rhythm. Examination of expression profiles further prompted exploration into the rhythmicity of chemosensory genes to determine if the circadian clock or another rhythm regulates gluR expression. Responses to environmental variations were previously reported in chemosensory iGluR, Ionotropic Receptor 25a (IR25a) (51). Clock protein oscillations are postulated to control temperature-dependent behaviors in low-amplitude envrionmental cycles within central clock neurons in conjunction with cycling expression of IR25a in *Drosophila* (51,52). Similar low-varying day-night fluctuations are found in some marine environments during certain seasons (3,6). We postulate intracellular cycles further perpetuate the influence of expression cycles via environmental, chemosensory receptors.

The chemosensory iGluR family includes receptors that respond to glutamate as an activating ligand (53). Cnidarian glutamate cycling, within cells, is observed in the conversion of glutamine to glutamate via glutamate synthase at night (Fig. 3.2) (5). Glutamine synthetase in *E. pallida* is predicted to fix inorganic nitrogen as a function of Symbiodiniacea-cnidarian endosymbiosis (54,55). The rhythm of gluR genes may be consequence of diurnal cycling or an endogenous source. In order to test the diel cycle and whether expression is influenced by diurnal factors or if expression has an endogenous rhythm, *E. pallida* anemones were subjected to two diel treatments to compare consequent gene expression of gluRs. To follow a circadian cycle, a peak and

trough would be clearly present for *E. pallida* gluR gene expression within both a control treatment and retained through a 48-hr period of darkness. However, another biological rhythm may be apparent if there is an endogenous expression regulator as it is possible gluR expression patterns are associated with clock genes (9). Alternatively, if gluR expression is driven by documented daily changes in glutamate metabolism (5,53,55), expression patterns would be expected to decrease at the end of day to middle of night. Expression of gluR genes may be sensitive to changing glutamate levels from Symbiodiniacea metabolism, which is predicted to lead to differentiating expression cycles between diel cycling (4). Hypotheses on the function of *E. pallida* gluR genes can be challenged through measuring the gene expression responses between the two diurnal states.

## 3.4 Methods

**3.4.1 Animal maintenance:** Clonal symbiotic *E. pallida* anemones (CC7) were used in the experiment to reduce genetic variation and maintained in artificial seawater at approximately 27 °C. Populations were maintained in a day/night cycle of 12 hr light: 12 hr dark with 40 to 60 μmol photons $m^{-2}\ s^{-1}$ of light intensity and fed freshly hatched brine shrimp *Artemia* nauplii twice per week. *E. pallida* anemones were approximately 3mm in diameter and 8mm in length.

**3.4.2 Diurnal experimentation:** Two light treatments were used to test how the diurnal light cycle affects gluR.2 (isoform of GluR) and gluR8 expression. Clonal CC7 symbiotic

anemones were placed into 12-well plates 72hrs prior to sampling for settlement and not fed throughout the experiment. Control anemones remained under 12 hr light: 12 hr dark (LD) with lights turning on at 0800 hrs and off at 2000 hrs (Fig. 3.3). In the DD treatment, anemones were placed in the dark for one complete 24-hr cycle prior to sampling to study if expression in temporal, displaying circadian rhythmic periodicity or diel influence without carry over from the first 24 hrs (Fig. 3.3) (56). Anemones were snap-frozen using liquid nitrogen every four hours for a 24 hr period (Fig. 3.3). The process of placing anemones in well plates and consequent LD and DD sampling was repeated four additional times, over the course of four weeks to collect a total of five biological samples per time point for each cycle treatment (Fig. 3.3) (56).

**3.4.3 RNA extraction and cDNA synthesis:** We used the Monarch Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA) to extract RNA. The RNA was eluted in a final volume of 50 μL of nuclease-free water. Quantification of extracted RNA was conducted using a NanoDrop 2000 (ThermoFisher, Carlsbad, CA) and RNA integrity assessed visually on a 1% agarose gel, including 11 samples randomly selected for BioAnalyzer (Agilent Technologies, Inc, Waldbronn, Germany) assessments. Three of the 60 samples with initial spectrophotometric 260/230 ratio of less than 1.5 underwent additional cleanup using 10% 3 M sodium acetate (pH 5.2), RNA-grade glycogen at a final concentration of 0.05–1.0 μg/mL, and 2.5 volumes of 100% EtOH to the sample. Following precipitation overnight at −80 °C, samples were centrifuged for 20 min at 9500 rpm (4 °C) to pellet RNA. The pellet was washed with 250 μL of 70% EtOH, centrifuged for 5min at the same conditions listed above, and dried before re-suspending

in 30 μL of nuclease-free water. Synthesis of cDNA was performed with Superscript III first strand synthesis system (Life Technologies, Eugene, OR) using 200 ng of RNA and Oligo dT primer.

**3.4.4 Quantitative RealTime-PCR assays:** To measure RNA expression using qPCR, a master mix of SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA), 0.34 μM of forward and reverse primers and nuclease-free water was added in 19.5 μL aliquots to each well with 0.5 μL of sample template. Primers with a length of 18 to 25 base pairs and an optimal melting temperature (Tm) of 60 °C were designed using PrimerQuest (IDT, Coralville, IA). Through comparing to the *E. pallida* genome (Baumgarten et al., 2015), primer pairs (Table 3.1) were designed across introns to prevent genomic DNA amplification. Primer sets for gluR.2 and gluR8 were verified by length using normal endpoint PCR. Primer concentrations were optimized by comparing qPCR reads using 0.5 nM and 0.25 nM of primer in order to minimize primer dimer formation and maximize target amplification.

Plates for each gene were run on Bio-Rad CFX Connect systems (BioRad, Hercules, CA) at a thermocycle of initial polymerase activation of 30 s at 98 °C, followed by 40 cycles of 15 s denaturation at 95 °C, then annealing for 30 s at 54 °C, followed by extension for 30 s at 72 °C with a plate read, and after 40 cycles a final Melt-Curve analysis. Plates had an Inter-Run Calibrator to control for variation of reads between runs. Negative controls for No Reverse Transcriptase were run for all samples with primers to confirm no genomic DNA contamination or bias. Negative controls for

primers and samples were also run on plates to confirm no bias or contamination. Raw

output was corrected for variations between runs with an inter-plate calibrator (57,58).

Gene expression was normalized using the geometric means of three reference genes,

ribosomal genes l10 and l12, and poly (A)-binding protein, which had minimal change in

expression among treatments or time points (59–64). Relative quantities were calculated

by subtracting the minimum normalized average from each biological replicate to

measure relative difference in expression (65–67).


Expression quantities were tested for normality, variance, heteroscedasticity using

the gvlma package (68) and Shapiro-Wilk test (69,70) in Rv3.5.0 (71). Statistical

analyses to compare light cycle effects on relative quantities of GluR.2 across time points

were completed by linear model ANOVA using Tukey Honestly Significant Difference

Test with a 95% family-wise confidence level along with two-way ANOVA using Tukey

HSD post-hoc or using the car package for White's Corrected ANOVA for

heteroscedasticity (72) along with a Tukey post-hoc test using glue (73) and

userfriendlyscience (74,75) packages in Rv3.5.0. Rhythmicity in gene expression profiles

was assessed using JTK_CYCLE in R (76–78). Analysis using JTK_CYCLE was chosen

as the program produces a robust reference curve that is suited for data with noise and a

sampling frequency of four hours or less (79,80). Settings included five biological

replicates for each sample time point, sample interval of every four hours and to look for

rhythms with a period range of eight to 24-hr. Relative quantities of gluR.2 and gluR8

were graphed in Rv3.5.0 (61,81,82).

**3.5 Results**

In the study transcriptional expression of two Exaiptasia pallida gluR genes was quantified to compare relative changes between the light cycles of 12hr light: 12 hr dark (LD) and 12hr dark: 12hr dark (DD). Time and light treatment were analyzed for gluR.2 and gluR8 gene expression, in addition to rhythm analysis for the 24hr sample period. The 24hr sample period compares LD to DD, the entrained LD cycle and constant darkness starting at 24-hrs within the DD cycle, to isolate endogenous versus diel-dependent expression patterns.

**3.5.1 qPCR assays of *Exaiptasia pallida* gluR.2 under light treatments:** The light treatment (LD vs. DD) did not significantly affect measured transcriptional expression of gluR.2 (Table 3.2; two-way ANOVA; p=0.1306). Time was a factor in significantly differentiating the expression (Table 3.2; two-way ANOVA; p=0.0007). In LD, gluR.2 exhibited relatively high expression at 1400 hrs with lowest expression at 1800 hrs, which increased again at 2200 hrs (Fig. 3.4). In DD, transcriptional expression appears to vary significantly. Expression was elevated at 1000 hrs, peaked again at 2200 hrs and had the lowest relative expression at 0200 hrs (Fig. 3.4). In both DD and LD, gluR.2 expression was relatively high followed by a decrease in relative expression quantity. The lowest point at LD 1800 hrs was significantly different from the peaks in expression at DD 1000 hrs and 2200 hrs (Fig. 3.4; two-way ANOVA; A p=0.0046, B p=0.0101). Within DD, 0200 hrs time point was significantly different to both 1000 hrs (Fig. 3.4, Table 3.2; two-way ANOVA; C p=0.0068) and 2200 hrs (Fig. 3.4, Table 3.2; two-way ANOVA; D p=0.0145). Between the diel cycles of LD and DD, the period changed from

8-hr (Table 3.3; p-value=0.0212, JTK_CYCLE) to 16-hr (Table 3.3; p-value=0.0039, JTK_CYCLE) and the amplitude of expression increased (Table 3.3; LD 0.9167, DD 1.5526, JTK_CYCLE).

**3.5.2 qPCR assays of *Exaiptasia pallida* gluR8 under light treatments:** Transcriptional expression of gluR8 appeared relatively more consistent over time and with less spread for variance at each time point compared to gluR.2 particularly in LD (Fig. 3.5). Expression of gluR8 did not significantly differ as a function of the light treatment in statistical analysis (Fig. 3.5, Table 3.2; White's corrected two-way ANOVA; p=0.3331). However, the factor of time did significantly influence the  expression patterns (Fig. 3.5, Table 3.2; White's corrected two-way ANOVA; p=0.0031). In LD, time points of 1000 hrs and 1800 hrs differed significantly from DD 2200 hrs (Fig. 3.5, Table 3.2; corrected Tukey HSD; A p=0.0006, B p=0.0046). Expression values of gluR8 DD were fairly consistent with a single up-regulation peak at 2200 hrs. The timepoint of DD 2200 hrs was significantly different compared to low expresion values measured at 0600 hrs and 1000 hrs (Fig. 3.5, Table 3.2; corrected Tukey HSD; C p=0.0310, D p=0.0059). Periodicity changed from 8-hr in LD (Table 3.3.; p-value=0.0421, JTK_CYCLE) to 24-hr in DD (Table 3.3; p-value=0.0074, JTK_CYCLE). Unlike gluR.2, the amplitude decreased from LD to DD diurnal cycles (Table 3.3.; LD 0.7178, DD 0.5428, JTK_CYCLE).

**3.6 Discussion**

From the innate function of iGluRs, the environment likely regulates gluR expression, whether external of the cnidarian host or via Symbiodiniaceae metabolism. Expression measurements imply gluRs were not predominantly acting in the role of 'pacemakers' for a circadian rhythm (83). Rather, the expression of gluRs followed an ultradian rhythm below 24-hr. The cycle measurements were produced from individuals with variation between biological replicates, opposed to aliasing from an observable consequence of the week or day sampled from potential lab conditions. Overall, the gluR.2 and gluR8 were observed to display statstically significant differences in rhythm periodicity between LD and DD.

**3.6.1 Rhythm of expression in gluR.2**: Expression of gluR.2 peaked at LD 1400 hrs and again at LD 2200 hrs, with lowest expression at LD 1800 hrs. In comparison to DD, gluR.2 expression peaked at 1000 hrs and 2200 hrs. While both gluRs increased in expression at 2200 hrs, in DD expression is up-regulated relative to the subsequent time point. The gluR.2 expression periodicity changes from LD 8-hr to DD 16-hr, which is consistent with a diel-controlled gene (DCG). The change in periodicity may signify constant LD oscillation is necessary to maintain expression (14) or an alternative biological rhythm. For instance, in marine intertidal areas, circatidal regulation is an influence of natural habit within a coastal environmental, in the intertidal zone (32). In this study, light or the affect of light on the biological factor of endosymbiosis with photosynthetic Symbiodiniaceae instigate variables for igluR.2 transcriptional response.

**3.6.2 Rhythm of expression in gluR8:** Expression of gluR8 is up-regulated at 1800 hrs in LD compared to 2200 hrs compared in DD (Fig. 3.4). Rhythm of gluR8 changes from an 8-hr period in LD to a circadian-like rhythm 24-hr in DD, implying gluR8 is a clock-controlled gene (CCG) as expression cycles are maintained under constant conditions. While CCG rhythm is not completely evident; Symbiodiniaceae are affected by diel cycles and in turn influence cnidarian gene expression, which is interpreted from the period shift in both genes. We postulate that dinoflagellate metabolic cycling affects the circadian nature of gluR8 while in constant LD cycling. Gene expression changes between LD vs DD are consistent with diel or light-dependent expression. In our experiment, nightly darkness occurred from 2000 hrs to 0800 hrs, placing midnight at 0200 hrs (Fig. 3.3). The expression of gluR8 peaked at 1800 hrs LD (Fig. 3.5).

**3.6.3 Robustness of detecting host rhythms:** *Exaiptasia pallida* gluR8 displays a 24-hr rhythm in DD, reminiscent of a circadian rhythm. While a 48-hour record is considered a standard, logistical and financial limitations presented a challenge in experimental design, which instigated the need for an alternative design (56). The study design provides a virgorously independent level of replication rather than a continuous series. In a continuous series experiment, an uncontrolled variable change over a single 48-hr experiment could have a very large impact on biasing the results. Through this method, we can observe for consistency in peak timing synchronized to the daily light cycle or within a 24-hour day. Failure to detect a rhythm may be due to aliasing, which is unlikely as anemones were entrained to the LD light cycle and experimental transitions to the dark remained the same for each replication. The detected cyclic patterns are more likely to be

true temporal rhythms, whether endogenous or light-driven, over the five days of replication over a four week period. While the brevity of the study does make it difficult to calculate the length of the period with much accuracy, the objective of the study was to identify the sources of gene expression variation. Light and time were the factors measured, which very well correlate with metabolic diurnal cycling of Symbiodiniaceae. The shifts in rhythm are hypothesized to be a result of the change in glutamate levels under LD and the facultative symbiosis. If there is an influence of symbiosis on *E. pallida* gluR expression, then there would be a measurable difference between aposymbiotic and symbiotic anemones in diurnal cycles consistent with findings in *Exaiptasia diaphana* (32).

**3.6.4 Expression of iGluRs in *Exaiptasia diaphana* from transcriptomic data published by Sorek, et al. 2018:** Physiological influences of cnidarian endosymbiosis have been studied across a range of symbiotic states including symbiotic reef-building corals, facultative symbiosis, and aposymbiotic cnidarians (5,9,12,14,15). A recent study of aposymbiotic and symbiotic anemones in *Exaiptasia diaphana* (Rapp, 1829) detected biological rhythms for seven genes containing conserved domains associated to ionotropic receptor activity or extracellular-glutamate-gated ion channel activity exhibited (32). From the available metadata, we identified the Interpro conserved domains for iGluRs including ionotropic glutamate receptor L-glutamate and glycine binding domain (IPR019594, IPR001320), receptor ligand binding region (IPR001828, IPR028082), and metazoan ionotropic glutamate receptor (IPR001508) (Table 3.4) (32). The *E. diaphana* iGluRs displayed expression cycle patterns around a circatidal 12-hr

period (Fig. 3.6). Four iGluRs, glutamate-ammonia ligase, and glutamate synthase have a 12-hr period (Table 3.4 and Fig. 3.6). One 12-hr period iGluRs (c160485) had no difference between the aposymbiotic and symbiotic states, which leads to a postulation that this particular iGluR transcription is not influenced by symbiosis. Three iGluRs had 24-hr periods (Table 3.4; Fig. 3.7). Two of these iGluRs were found in aposymbiotic anemones compared to one in symbiotic anemones. The differences between expression responses of iGluRs to symbiosis and to diurnal cycles lead to our hypothesis that genes identified as iGluRs have the capacity to function as a response to environmental signals from symbiotic metabolites. We speculate that cnidarian iGluRs can alter the potential to respond to specific ligands through acting in various pairs as co-receptors, while iGluRs with inverse expression patterns have different functions altogether. Another possibility for downstream physiological responses is multiple iGluRs must respond to two or more specific ligands, acting in concert to send a signal.

**3.6.5 Symbiodiniaceae and light influences on iGluR expression rhythms:** Biological rhythms are dynamic and complex, especially when attempting to identify the regulators or causation of gene expression cycles. From these findings and analysis the iGluR and related genes expression patterns from Sorek et al. (32), we hypothesize iGluR gene expression rhythms changed in response to diel stimulation. Cnidarian gene expression is altered by symbiosis, including metabolic changes influenced by light in symbiotic cnidarians (4,31,32). Symbiodiniaceae are tightly associated with metabolic cycles in corals and anemones (5,28,29). As light affects Symbiodiniacea metabolism, *E. pallida* gluR expression could change in response to the intercellular amount of glutamate

available (12,31,84). Conversely, gluR expression could decrease in preparation for the influx of glutamate with changing glutamate metabolism. In facultative symbiotic *E. pallida*, anemones with endosymbionts have documented up-regulated expression of glutamine synthetase and glutamate synthase, which is predicted to occur from host cnidarians altering nitrogen metabolism in accordance to Symbiodiniaceae (55). In this example, rather than acting in response to light entrainment, expression of glutamate transporters might respond to the diel-oriented glutamate concentration in cells to avoid overstimulation (85), which supports the hypotheses that *E. pallida* gluRs respond to environmental cues of pathogens and chemicals, or indirectly to diel rhythmicity through the symbiotic relationship with Symbiodiniaceae via light cues.

### 3.7 Conclusion

Diurnal expression analysis indicate that gluR.2 and gluR8 rhythms are influenced by time of day and effect of light on symbiosis. Along with symbiotic expression differences in *E. diaphana*, iGluR mRNA expression cycles are responsive to the light cycle. Expression of gluRs under LD is not consistent with predicted levels of levels of glutamate increasing at night from symbiosis. Internal molecular modulators of *E. pallida* may anticipate increases in glutamate synthesis when not under LD cycles and subsequent metabolic outputs from Symbiodiniaceae. These findings suggest that under normal operating conditions cnidarian, *E. pallida* and *E. diaphana,* iGluR transcription display innate ultradian biological rhythms, which we hypothesize is influenced by endosymbiosis with light-dependent Symbiodiniaceae .

While for the most part, chemosensory receptors like iGluRs are known to respond to environmental cues in the form of chemical ligands, however, putative functions of cnidarian gluRs are unknown. The hypotheses developed from our findings of characterizing cnidarian iGluRs create a basis for functional studies. Measuring the rhythmicity of expression improves the knowledge base on iGluR presence within genomes and expression within cnidarians: organisms without a central nervous system to process neural signaling.

## 3.8 Acknowledgements

**3.9 Tables and Figures**

Table 3.1 Primer sequences for qPCR assay with sequence names and gene identity

| Gene | Sequence ID | Primer Name | Primer Sequence |
|---|---|---|---|
| gluR.2 | 24462 | iGluR2f | ACGTGAGGAAGGAGAGATTGA |
| | 24462 | iGluR2r | AAGTGAAAAACCGGGCTGTG |
| gluR8 | 29007 | iGluR8f | TGCAGTTCTTGATGCCCTTTGA |
| | 29007 | iGluR8r | GACCAGAAGACGGCCTGATATAGA |
| L10 | 29093 | L10f | CTTGGACAGGAGCATCGATAATA |
| | 29093 | L10r | CCAGTGGGTTACGAAGACTTT |
| L12 | 8348 | L12f | TCACATGGTGTCAGATGAGTATG |
| | 8348 | L12r | CATACCAGTTTGGAGCCTATCA |
| PABP | 2230 | PABPf | AGAGAATTCCGCTTAGCAACTC |
| | 2230 | PABPr | GAAGCAAGCTGTCGCTTAGA |

Table 3.2 Two-way ANOVA p-values for factors and significant time points of *Exaiptasia pallida* gluR.2 and gluR8 gene expression. White's Corrected ANOVA and Tukey HSD was used for gluR8 to account for heteroscedasticity.

| Two-way ANOVA gluR.2 | |
| --- | --- |
| FACTOR | p-value |
| LIGHT | 0.1306 |
| TIME | 0.0007 |
| LIGHT : TIME | 0.0306 |
| DD:1000hr-DD:0200hr | 0.0068 |
| DD:2200hr-DD:0200hr | 0.0145 |
| LD:1800hr-DD:1000hr | 0.0046 |
| DD:2200hr-LD:1800hr | 0.0101 |

| Corrected Two-way ANOVA gluR8 | |
| --- | --- |
| FACTOR | p-value |
| LIGHT | 0.3331 |
| TIME | 0.0031 |
| LIGHT : TIME | NA |
| DD:2200hr-DD:0600hr | 0.0059 |
| DD:2200hr-LD:1000hr | 0.031 |
| DD:2200hr-DD:1000hr | 0.0006 |
| DD:2200hr-LD:1800hr | 0.0046 |

Table 3.3 Statistical outputs of Jonckheere-Terpstra-Kendall analysis for *Exaiptasia pallida* gluR mRNA expression values. gluR.2 LD cycle as a period of 8 hours, amplitude of 0.9167, phase 6. BH.Q 0.0282 with a Bonferroni-adjusted p-value of 0.0212, while the DD cycle has a period of 16 hours, amplitude of 1.5526, phase of 8. BH.Q is 0.01497 with an adjusted p-value of 0.0039. gluR8 LD cycle has a period of 8 hours, amplitude of 0.7178, phase of 6. BH.Q is 0.0421 with an adjusted p-value of 0.0421. While the DD cycle has a period of 24 hours, amplitude of 0.5428, and phase of 18. BH.Q is 0.0149 with an adjusted p-value of 0.0074

| Gene | Cycle | BH.Q | Adj P-value | Period (hrs) | Phase | Amplitude |
|------|-------|------|-------------|--------------|-------|-----------|
| gluR.2 | LD | 0.0282 | 0.0212 | 8 | 6 | 0.9167 |
| | DD | 0.01497 | 0.0039 | 16 | 8 | 1.5526 |
| gluR8 | LD | 0.0421 | 0.0421 | 8 | 6 | 0.7178 |
| | DD | 0.0149 | 0.0074 | 24 | 18 | 0.5428 |

Table 3.4 iGluR homologs and related genes with biological rhythms identified in *Exaiptasia diaphana* in symbiotic (sym) and aposymbiotic (apo) states (data extracted from Sorek et al., 2008).

| contig | Gene identity | Conserved domains | Period |
|---|---|---|---|
| c129587_g1_i1 | iGluR | IPR013099;**IPR001320**;IPR003091;IPR028325 | 12 (apo) |
| c163181_g1_i1 | iGluR | IPR013099;**IPR001320**;IPR028325 | 12 (apo) |
| c164132_g2_i2 | iGluR | IPR013099;**IPR013320**;IPR003961;IPR013783;IPR001320;IPR003091;IPR001759;IPR000884 | 12 (apo) |
| c161677_g1_i2 | iGluR | IPR013099;**IPR001320**;IPR003091;IPR028325 | 24 (apo) |
| c162028_g1_i4 | iGluR | IPR013099;IPR003961;IPR013783;**IPR001320**;IPR003091 | 24 (apo) |
| c160485_g1_i1 | iGluR | **IPR001508;IPR001320** | 12 (apo) ; 12 (sym) |
| c155937_g1_i4 | iGluR | IPR013099;**IPR001320** | 24 (sym) |
| c149386_g1_i1 | glutamate-ammonia ligase | IPR008147;IPR014746;IPR008146;IPR027303;IPR027302 | 12 (apo) ; 24 (sym) |
| c165177_g1_i2 | glutamate synthase | IPR013785;IPR028261;IPR000583;IPR002489;IPR006982;IPR012220;IPR002932;IPR006005;IPR009051;IPR01604 0;IPR029055;IPR023753;IPR001327 | 12 (apo) |
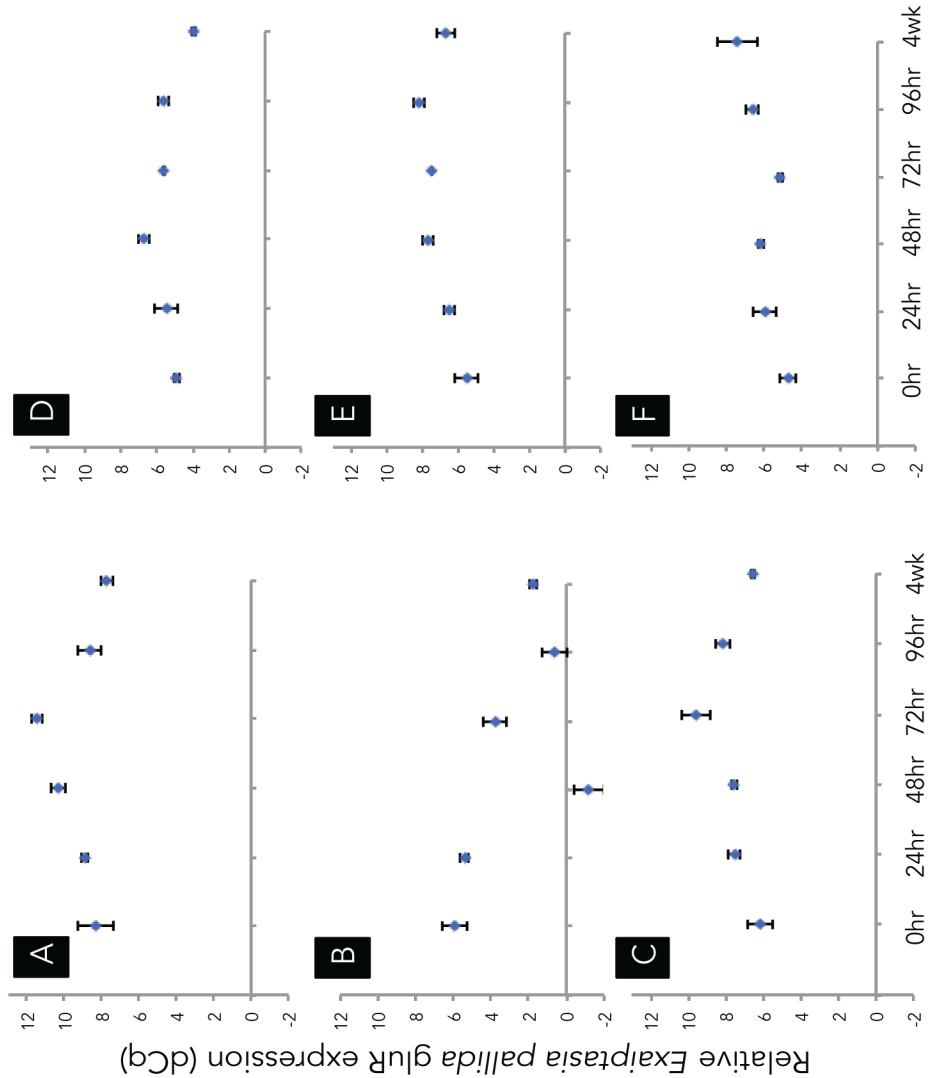
Figure 3.1 Preliminary data of relative gene expression of *Exaiptasia pallida* gluR variants across time. Gene expression was normalized to L10, L12 and PABP genes. A) gluR.1, B) gluR.2, C) gluR23, D) gluR21, E) gluR4, F) gluR8

84
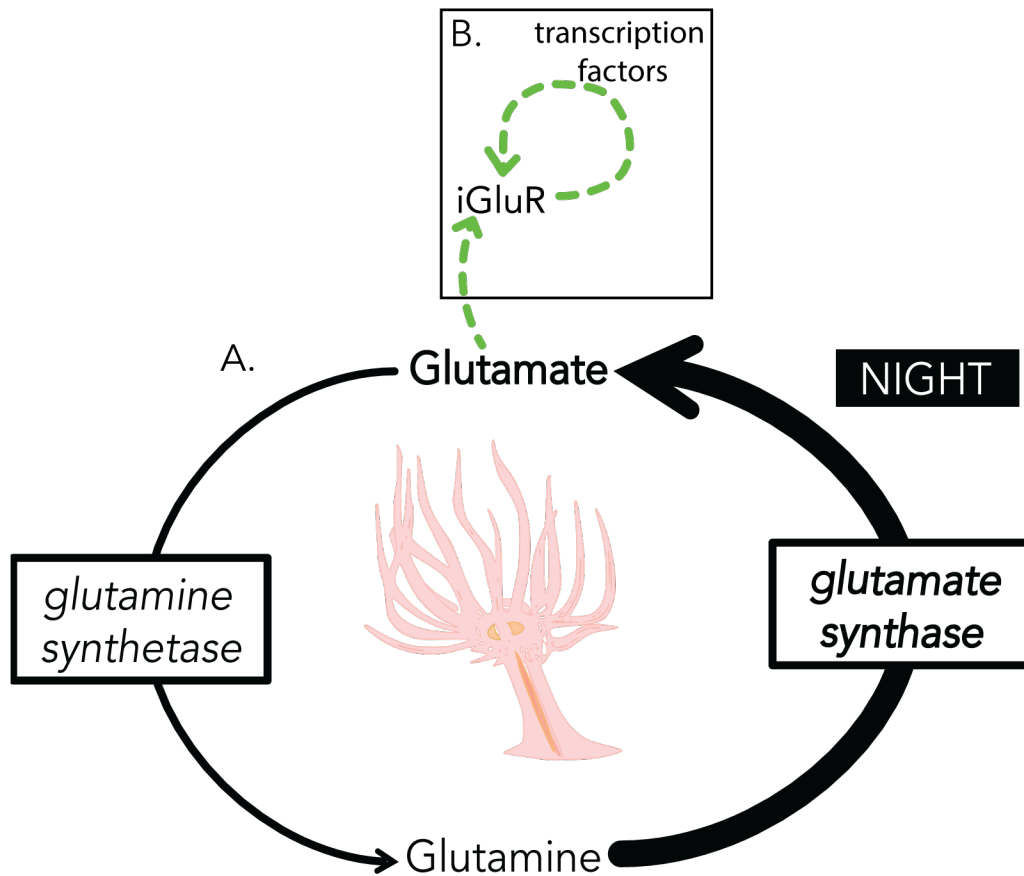
Figure 3.2 Representation of glutamate concentration cycling in cnidarians. A. levels of glutamate increase at night in consequence of the activation of glutamate synthase versus the shift to increasing levels of glutamine during the day with activation of glutamine synthetase in cnidarians. B. Putative response of iGluRs accompanied by the potential for downstream pathways affecting iGluR transcription from increased glutamate concentration.
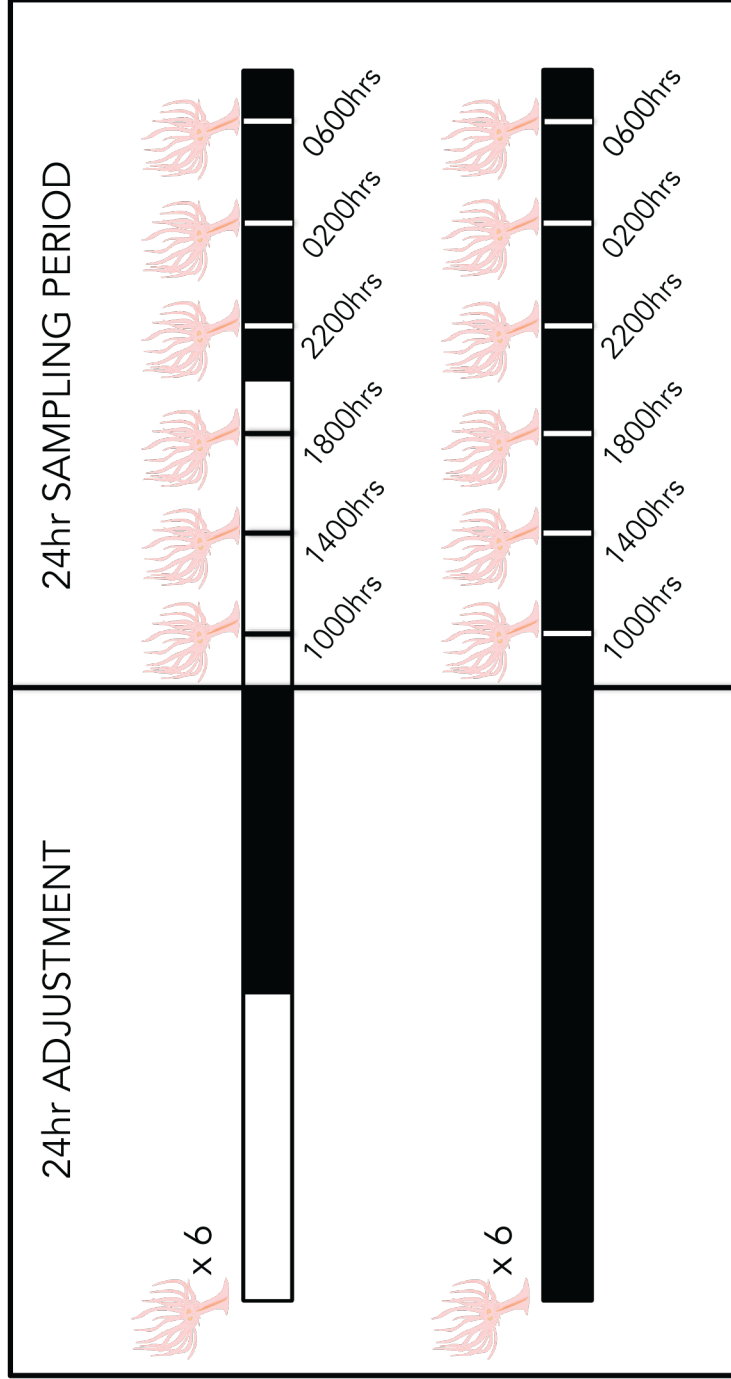
Figure 3.3 Experimental design and set-up of the light treatment cycles for *Exaiptasia pallida* anemones; 12hr light : 12hr dark (LD) compared to 12hr dark : 12hr dark (DD). Anemones in the constant dark treatment were given 24-hrs of adjustment in constant dark prior to sampling. In LD, lights turn on at 0800 hrs and off at 2000 hrs, indicated by the block of white Sampling by snap freezing in liquid nitrogen occurred every four hours at 0200 hrs, 0600 hrs, 1000 hrs, 1400 hrs, 1800 hrs, and 2200 hrs and took place for five 24 hour periods over four weeks to accumulate five biological replicates for each time point.
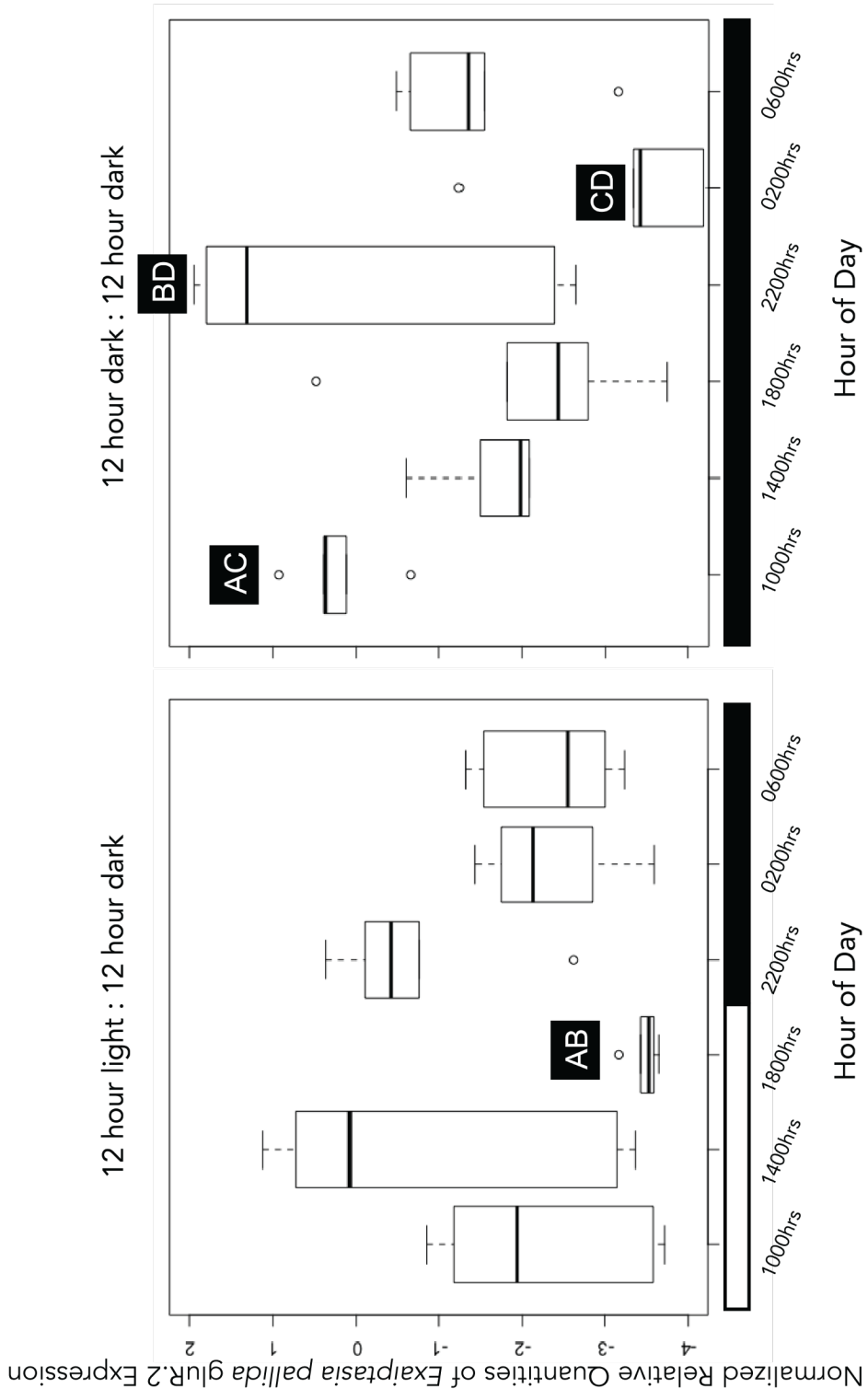
Figure 3.4 Boxplots of *Exaiptasia pallida* gluR.2 normalized relative quantities under 12hr dark: 12hr light (LD) and constant darkness (DD) over 24 hours. 2-way ANOVA between light and time p-value=0.0306; Tukey HSD post-hoc was run to determine significantly different time points. Time points denoted with the same letter are significantly different from each other. A) p-value=0.0046 B) p-value=0.0101 C) p-value=0.0068 D) p-value=0.0145
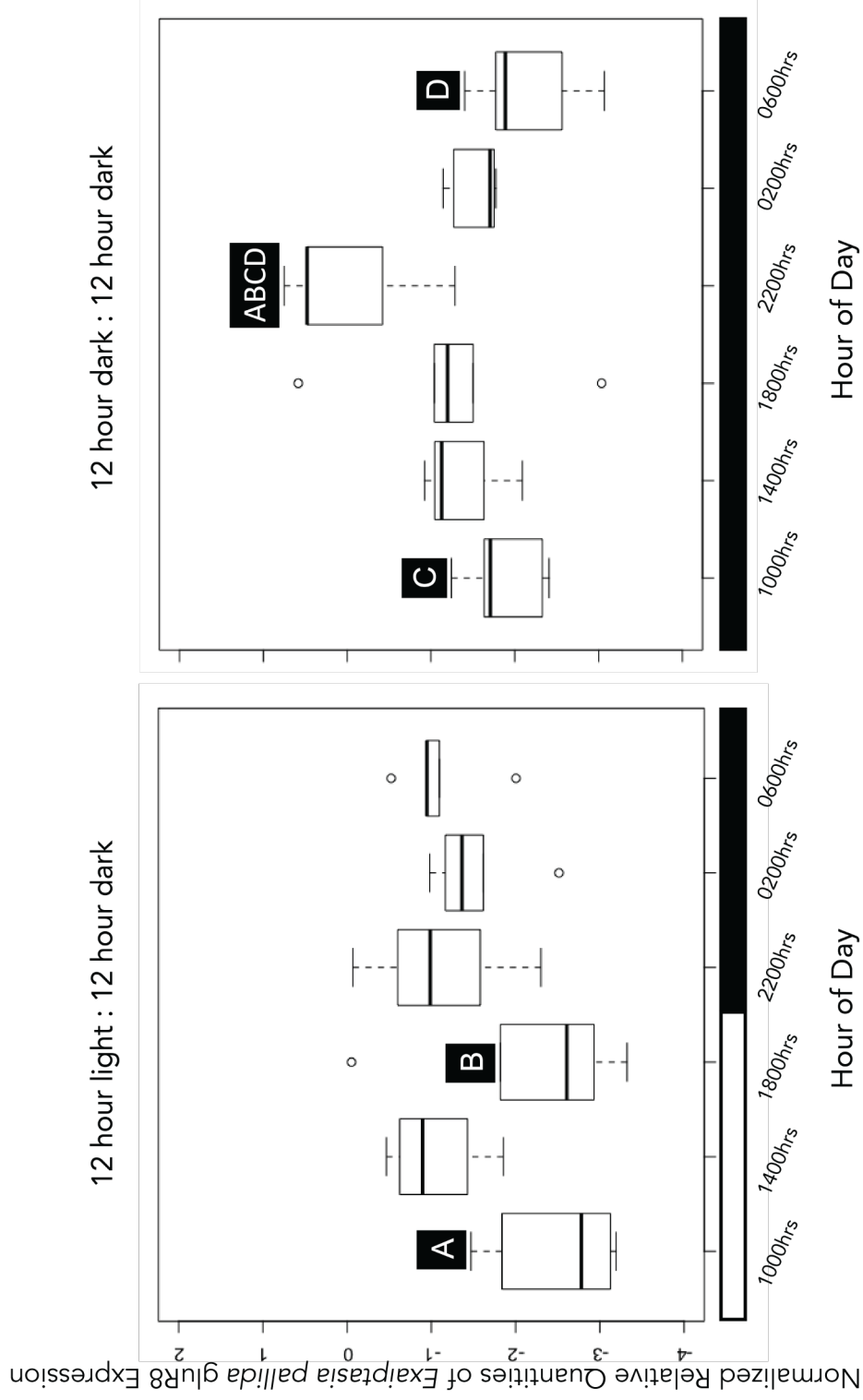
Figure 3.5 Boxplots of *Exaiptasia pallida* gluR8 normalized relative quantities under 12hr light (LD) and constant darkness (DD) over 24 hours. White's corrected ANOVA for time p-value=0.0031; Tukey HSD post-hoc was run to determine significantly different time points. Time points denoted with the same letter are significantly different from each other. A) p-value=0.0006 B) p-value=0.0046 C) p-value=0.0310 D) p-value=0.0059
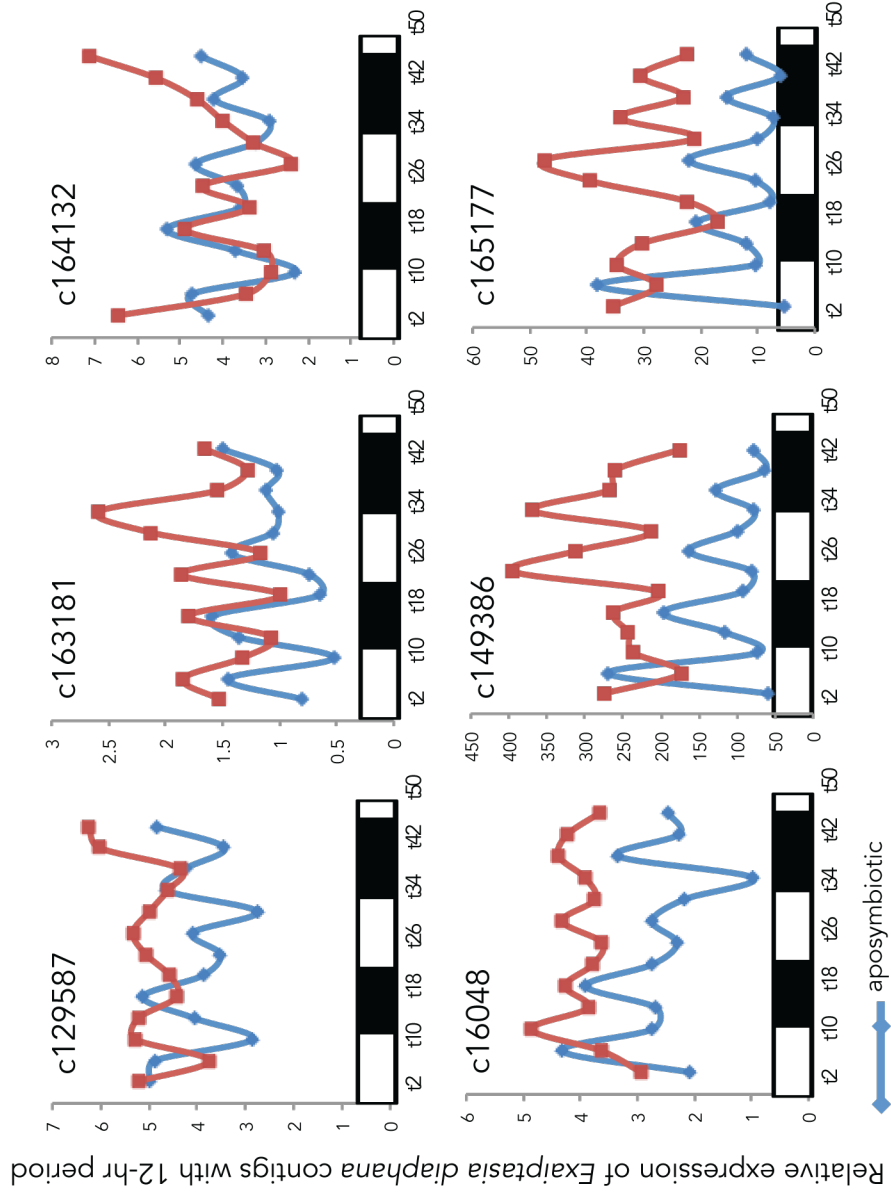
Figure 3.6 Relative expression of iGluR-related contigs with 12-hr periodicity identified in *Exaiptasia diaphana* to compare symbiotic and aposymbiotic rhythm patterns.

89
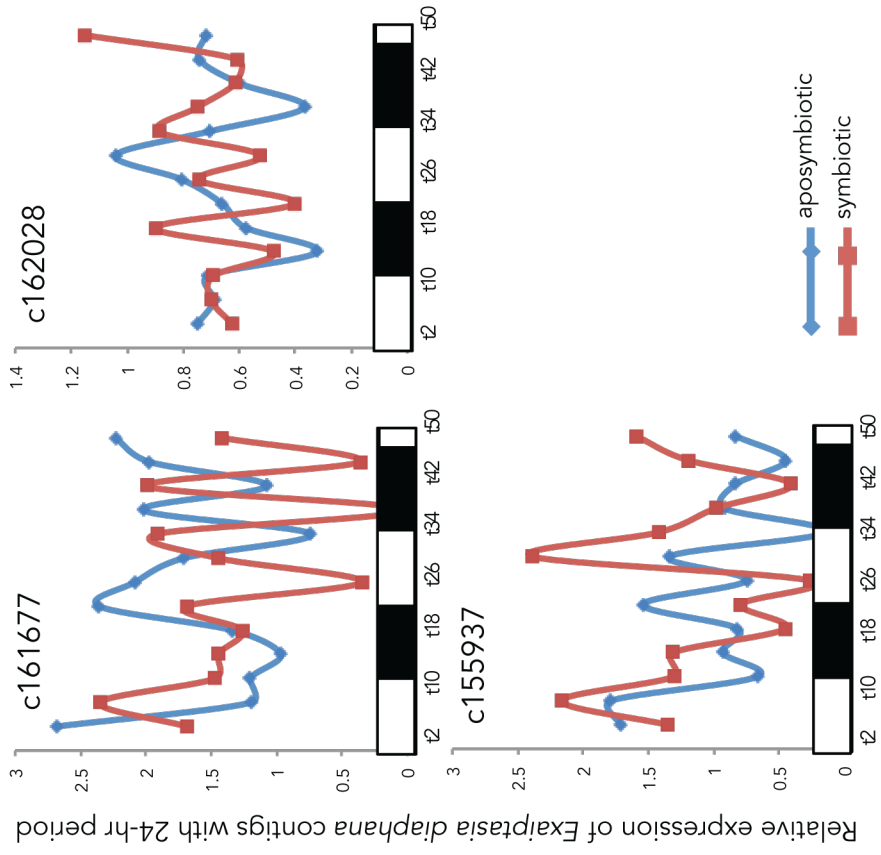
Figure 3.7 Relative expression of iGluR-related contigs with 24-hr periodicity identified in *Exaiptasia diaphana* to compare symbiotic and aposymbiotic rhythm patterns.

3.10 References

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CHAPTER IV. LOCALIZATION OF IONOTROPIC GLUTAMATE RECEPTORS

WITHIN THE CNIDARIAN *EXAIPTASIA PALLIDA* VIA *IN SITU* HYBRIDIZATION

## 4.1 Abstract

Ionotropic glutamate receptors (iGluRs) provide chemical sensory signals to various

metazoans, including a recently uncovered expansion of homologs within *Exaiptasia*

*pallida*. While the response of *E. pallida* iGluRs is correlated to bacteria-specific

signaling and environmental processing, it is important to now understand where these

genes are expressed. Cnidarian iGluRs are proposed to work by detecting external signals

from bacteria, potential extracellular ligands, and external environment cues. Defensive

or offensive spirocyst discharging may involve iGluRs via glutamate as a

neurotransmitter within the dispersed sensory system that lies within the epidermis.

Through developing mRNA *in situ* hybridization techniques for the sea anemone *E.*

*pallida*, iGluR gene expression was located primarily in tentacles, which contain high

densities of nematocytes and spirocytes, in addition to the polyp column for select

iGluRs. From these findings, gene expression of iGluRs is closely tied to the epiderm that

contains sensory cells and along the nerve net. In addition to cnidocytic protective

mechanisms within the anemone *E. pallida*, iGluRs are predicted to be involved in the

perpetuation of neural signals throughout the polyp column. *Exaiptasia pallida* iGluR

RNA expression was localized to the epidermis near sensory cells, congregated in the

tentacles in greater densities relative to the polyp column of anemones, which is

consistent with the chemosensory nature of this family of genes.

**4.2 Keywords**

*Exaiptasia pallida*, in situ localization, chemosensory, iGluR, Cnidaria

**4.3 Introduction**

Cnidarians, like many marine invertebrates, utilize various receptor pathways within their innate immune systems to recognize non-self and regulate interactions with organisms, such as microbes in the water column and symbiotic dinoflagellates (Symbiodiniaceae) (1–7). The phylum Cnidaria includes sessile organisms, corals and anemones, which introduces an obstacle to overcome with physiological responses (8,9). The first barrier in organismal defense includes recognition via the immune system. Immune-associated cells that identify non-self components i.e., immunocytes, are inhibited by immunosuppression chemicals, which prevent fusion and rejection during allogeneic experiments and in turn suppress the immune response (10,11). Immune gene expression in cnidarians has been associated with the gastroderm (2,8), which is consistent with recognition cells located in gastrodermal tissues that are often exposed to external organisms and chemicals taken in through the pharynx into the gastric cavity (Fig. 4.1A). To perpetuate communication and elicit defense responses from immune signals of changing environments, cnidarians require cells that function as receptors to recognize environmental factors.

Recognition receptors that distinguish infections may function differently within a dispersed nervous system in contrast to derived metazoans with bilateral body plans and centralized neural networks (12–14). These receptors may have multiple functional roles or diverged in evolution to serve as a connection between nervous systems and immune

99

recognition. A family of chemosensory receptors, ionotropic glutamate receptors (iGluRs) may have likely evolved in cnidarians to connect chemosensation and neural signaling with innate immunity (Ch. 2). The iGluR receptor family is responsible for perceiving signals, particularly from environmental chemicals within many invertebrates from insects to mollusks (15–17). While cnidarian iGluRs have been implicated in a chemosensory or immune response to bacteria (Ch. 2), the location of where iGluRs are expression is unknown within cnidarians (18,19). To better understand potential functions of cnidarian iGluRs, mapping the localization of iGluRs within the cnidarian nervous system is of utmost importance.

The cnidarian nervous system for anemones is a nerve net of interconnected nerve fibers, neurons and neurites that form synaptic contacts, dispersed between epithelial cells of the epiderm (20). The nervous system consists of sensory cells that receive information, ganglion cells that process information, from neuroglandular synapses within the pharynx to mechanically responsive muscle cells located at the base of the epidermal epithelium (20,21). Receptors send signals into cells via neural networks, beginning with apical cilium sensory cells, which lead to one- or two-way interneuronal, neuromuscular, and uniquely cnidarian neuro-nematocyte synapses (22–24). Cnidarian tentacles contain stinging cells: nematocytes and spirocytes, that have microscopic harpoons called nematocysts and spirocysts, respectively (Fig. 4.1; 16–19). Discharge of nematocysts was thought to be under local control by chemical and mechanical triggers, including neural control of spirocyst discharge (27,28); known as the independent effector hypothesis (without neural involvement), a three-cell pathway with a ganglion

cell integrated motor functions and sensory cells triggered spirocytes by vibratory and chemical stimuli from prey (25). However, synaptic modulation is involved in neural control of spirocyst discharge and more than one neurotransmitter can control spirocyst discharge with changes to mechanistic physiology (21,24,29). Sea anemones contain more ganglion than sensory types of neurons, which include dissociated neural cells within tentacles (22). The proximity of iGluRs to the nerve net, related ganglion and sensory neurons is unknown.

From our knowledge of iGluR function, putative specific ligands from phylogenetics (Ch. 2), and the involvement of glutamate in discharging spirocysts and nematocysts (25,27,29), there is a plausible connection of iGluRs and defense in cnidarians within the sea anemone *Exaiptasia pallida*. These connections have created speculation as to where iGluRs are expressed and involvement in the neurosensory network. By localizing expression through chromogenic mRNA *in situ* hybridization is the first step in understanding the function of iGluRs in *E. pallida*.

## 4.4 Methods

**4.4.1 Animal maintenance:** Clonal symbiotic *E. pallida* anemones (CC7) were maintained in artificial seawater at approximately 27 °C. Populations were kept on a day/night cycle of 12 hours light: 12 hours dark with 30 to 60 μmol photons $m^{-2}\,s^{-1}$ of light intensity and fed freshly hatched brine shrimp *Artemia* nauplii twice a week. *Exaiptasia pallida* anemones fixed for *in situ* hybridization were approximately 300 μm

in diameter and 750 μm in length, while anemones used for RNA extraction were larger at 2-3 mm in diameter and 6-8 mm in length.

**4.4.2 RNA extraction and cDNA synthesis:** The RNeasy mini kit (Qiagen, Valencia, CA) and Monarch Total RNA Miniprep Kit (New England Biolabs, Ipswich, MA) were used to extract RNA. The samples used on-column DNase treatment (New England Biolabs, Ipswich, MA) and 2 additional 80% EtOH washes. We eluted RNA in a final volume of 30 or 50 μL, respective of kit, with nuclease-free water. Quantification of extracted RNA was conducted using a NanoDrop 2000 (ThermoFisher, Carlsbad, CA) and RNA integrity assessed visually on a 1% TAE bleach gel (30). Samples with a spectrophotometric 260/230 ratio of less than 1.5 underwent additional cleanup using 10% 3 M sodium acetate (pH 5.2), RNA-grade glycogen at a final concentration of 0.05–1.0 μg/mL, and 2.5 volumes of 100% ETOH to the sample. Following precipitation overnight at −80 °C, samples were centrifuged for 20 min at 9500 rpm (4 °C) to pellet RNA. The pellet was washed with 250 μL of 70% ETOH, centrifuged for 5min at the same conditions listed above, and dried before re-suspending in 20-40 μL of nuclease-free water. cDNA synthesis was performed with Superscript III first strand synthesis system using Oligo dT primer (Life Technologies, Eugene, OR) and 200-400 ng of RNA.

**4.4.3 *Exaiptasia pallida* RNA probe design:** Full-length PCR primers between 18 and 25 bp in length were designed with an optimal Tm of 60 °C for *E. pallida* iGluR genes (gluR) using PrimerQuest (IDT, Coralville, IA). For PCR analysis, each targeted gene of interest was prepared with a 31 of H₂O, 5 μL of 10x Buffer for KOD polymerase, 5 μL

dNTPs, 3 μL MgSO$_4$, 1.5 μL of each primer, 2 μL cDNA from *E. pallida,* 1 μL KOD

polymerase (Billerica MA). Reactions were run on a thermocycle of initial polymerase

activation of 2 min at 95 °C, followed by 39 cycles of 20s denaturation at 95 °C,

annealing for 30s at a gradient from 52 °C to 59 °C, followed by extension for 60s at 70

°C. Products were checked for size on a gel using 10 μL of PCR product. The remaining

40 μL of PCR product was processed through the NEB PCR and DNA Cleanup (New

England BioLabs, Ipswich MA). For amplicon isolation, the low concentration reactions

were pooled and bands extracted from agarose gels. Concentrations were determined

using a NanoDrop 2000 (ThermoFisher, Carlsbad, CA).


  The NEB PCR Cloning Kit was used to ligate and transform PCR product into

competent *E. coli* cells following kit protocol (New England BioLabs, Ipswich MA).

Cloned products (Protocol Exemption IBC-17-017) were isolated using Plasmid

MiniPrep kit from NEB (New England BioLabs, Ipswich MA) and sequenced using

primers from the kit. Cloned genes are listed in Table 4.1. Once gluR sequences were

verified, PCR was used to re-amplify gluRs with additional pGEMHE overhangs (Table

4.2) and generate a linear for pGEMHE vector with the selected insertion site. Correct

band size of product was gel extracted and purified. The gluR inserts and pGEMHE were

assembled using the NEB HiFi Assembly kit (New England BioLabs, Ipswich MA) and

then transformed into competent cells. Transformations were screened using ampicillin

resistance, colony PCR, and sequencing. Plasmids were extracted using Plasmid

MidiPrep kit from NEB (New England BioLabs, Ipswich MA). Plasmids were then

completely digested as prescribed using SmaI or SbfI restriction digest enzymes (New

England BioLabs, Ipswich MA). Probes were synthesized via in vitro transcription with the Digoxigenin (DIG) RNA Labeling Kit using Sp6 polymerase for the antisense probes (digested with SmaI) or T7 polymerase for sense probes (digested with SbfI) (Roche Life Science, Indianapolis IN).

**4.4.4 Chromogenic RNA *in situ* hydridization:** *In situ* hybridization was carried out on *Exaiptasia pallida* anemones following an adapted protocol from *Nematostella vectensis* (31,32) and *Cassiopea* sp. following suggestions from Leslie Babonis and Bailey Steinworth (Martindale Lab, Whitney Labs, University of Florida) completing between four to ten biological replicates per gene. Anemones were immobilized in 7% MgCl$_2$ in filtered seawater, then fixed in ice-cold 4% paraformaldehyde with 0.2% glutaraldehyde for 1 min 30 sec, followed by 4% paraformaldehyde in phosphate-buffered saline with 1% Tween (PTw) at pH 8.8 for 1 hr at 4 °C. Anemones were digested in proteinase K for 35 min (45 min and longer resulted in sloughing off sensory cells from epidermis) and pre-hybridized overnight at 63 °C with hybridization buffer including salmon sperm. The DIG-labeled RNA probes with a concentration of 1 ng/ul in hybridization buffer with salmon sperm were hybridized at 63 °C up to 2 days (minimum of overnight). Development took part with the enzymatic reaction of NBT-BCIP as substrate for the alkaline phosphatase conjugated anti-DIG antibody. Samples were developed until purple precipitate was visible as dependent on each probe, development stopped and samples were washed before mounting. Whole anemones were mounted in 80% glycerol solution on glass slides. Fixed and developed anemones were viewed under bright-field using a

Leica DM5500 B microscope. Images were taken using a Leica DFC310 FX C-Mount 0.70x camera attachment and white balance restored in Adobe Photoshop.

## 4.5 Results

**4.5.1 Identifiable anatomy in whole mount *Exaiptasia pallida* anemones:** Anatomy, tissues, and cell types of the anemones were classified to identify the types of cells gluRs were expressed within or around. Anemones varied in size from 0.5 to 1 mm in length with oral diameters measuring from 0.2 to 0.4 mm. Tissue and cell types were visible within *E. pallida* from 200x to 1000x magnification (Fig. 4.1). Orange-red colored carotenoids are present in tentacles and the body column of some anemones (Fig. 4.1F-G). Carotenoids, associated with coral bleaching (loss of Symbiodiniaceae), are known for antioxidant responses as oxidative stress resistance within cnidarians were present in some of the anemones (26,33,34). Symbiodiniaceae, algal-dinoflagellates that form an endosymbiosis with cnidarians (7), have diameters of approximately 10 um and clearly located within tentacles and some tissues within the body column (Fig. 4.1B and D) (35–37). Acontinia, mesenterial filaments packed with mastigophores and isorhizas were visible in the column (Fig. 4.1D).

Tentacles have a variety of cell types, which were differentiated at 1000x (Fig. 4.1). Morphology was used to idententify sensory cells within tentacles of *E. pallida* (Fig. 4.1). Sensory cells that an apical cilium or a ciliary cone provide a point of contact to the external environment, similar to nematocytes, are closely associated with nematocytes

and spirocytes that run along a nerve plexus (20). The general term for cnidarian nematocytes and spirocytes is cnidocyte (38,39), which provides the namesake for the phylum Cnidaria. A cnidocyte contains a single cnida, the organelle known as a nematocyst or spirocyst (in general cnidocyst) (26,27) that launches the microtubule attached to the stylet. Once nematocysts are discharged out of the cell, the long microtubules are visible outside of the cell. There are two main groups of nematocytes: mastigophores that are within long oblong shaped cells distinguished by a prominent central shaft (Fig. 4.1B) (26) and basitrich isorhizas that have a less noticeable central shaft (25,27,40). In some images, tubules from mastigophores can be seen outside, as cnidae were ejected (Fig. 4.1C). The smaller defensive cells are spirocytes containing spirocysts, which lack a physical trigger hair or stereocilia. Spirocytes are distinguished by thin-walled spirocysts that have a visible accordion-pleated spiral tubule (Fig. 4.1B and D; (24). To identify each of these cell types within the microscopy images allows gluRs localization and association to the system of sensory and signaling cells.

**4.5.2 Localization of gluR genes in whole mount *Exaiptasia pallida*:** Expression of nine gluRs (*E. pallida* iGluR genes) (Table 4.1) was localized in whole mount *E. pallida* anemones via chromogenic *in situ* hybridization. Between concentrations of 1 ng/ul and 8 ng/ul, RNA probe concentration of 1 ng/ul provided the best resolution for *in situ* hybridization in the experimental pilot. Sense probe controls had rare false positive occurrence and presented little background noise across replicates (Fig. 4.2 and 4.3). For each gene, at least four biological replicates for antisense probes and three for sense probes were completed to demonstrate reproducibility (Table 4.3).

Expression of gluR was confinedto the epidermal tissue layers (Fig. 4.6). Patterns of gluR expression differed between tentacles and column (Table 4.3, Fig. 4.2). There are several patterns that present more strongly than others. Localization occurred mostly within tentacles and adjacent to sensory cells. Expression of gluRs .1, .2, 4, 8, 10, 15, 21 and 22 displayed tentacles with more pigment relative to the body (Fig. 4.2). At increased magnification, gluR .1, .2, 8, 15, and 21 expression was concentrated at tips of tentacles (Fig. 4.3). Punctate patterns within tentacles occurred for gluR4 (Fig. 4.4), gluR10 (Fig. 4.3E), and gluR22 (Fig. 4.3I). The overall expression of gluR12 appeared less concentrated at 1000x magnification relative to the other gluR genes and was recurrent throughout the column of the polyp (Fig. 4.3F and 4.9). There was no gluR expression around the 10 um Symbiodiniacea located within the gastrodermal layer (Fig. 4.3 and 4.4) (7,35). Localization of gluR expression was not detected within the internal gastrodermal tissues of the column or tentacles. The expression of *E. pallida* gluRs was not localized around carotenoids within the gastroderm (Fig. 4.1F and G), but gluR4 did express around the acontium where mastigophores are located (Fig. 4.7C). Considering the pattern of expression within the anemone column, some gluRs have evenly distributed probe localization across the entire body to the column of the polyps (Table 4.3, Fig. 4.6 and 4.10).

Polyp column staining was less prominent within chromogenic ISH of gluR mRNA expression. Expression within the column of the polyp (Fig. 4.2) occurs more prominently in gluRs 10, 12, and 22 where localized points of expression are scattered

throughout tissue (Fig. 4.2, Table 4.3). Gene expression of gluR8 was quite sparse in the polyp column relative to the punctate pattern of gluR12 throughout tentacles in addition to the column (Fig. 4.8 and 4.9).

**4.5.3 RNA localization of gluRs in relation to sensory cells:** Expression of gluRs was confined to the epidermal cell layer of tentacles, which is the tissue layer that contains sensory cells i.e., nematocysts and spirocyts (Fig. 4.3 & 4.6). Along tentacles, gluR.1 was evenly dispersed and expression localized to sensory related cells, mastigophores (Fig. 4.4), while expression of gluR4 has sparsely punctate expression (Fig. 4.5). The homolog gluR4 was also expressed in tissues around the pedal disc of the anemone, the mastigophore-filled acontium, and potentially a dissociated neural cell (Fig. 4.7). Expression of gluR4 was also confined to the tips of mastigophores (Fig. 4.5). In contrast to mastigophores, spirocytes tend to be more abundant in tentacles than in the column (24), which may contribute to the pattern of gluR expression isolated to the tentacles. The high concentration in the tentacles for gluRs .1, .2, 15, 21, and 22 implicates gluR localization to spirocytes (Fig. 4.3). On the contrary, gluRs 4, 8, 10 and 12 present patterns evenly spaced, with punctate expression (Fig. 4.3). Relatively lower number of positive cells presenting gluR expression  may account for less density of nematocytes versus spirocytes within the tentacles.

The punctate patterns of expression across the body column may also indicate gluR expression follow a path along the nerve net throughout the anemone (13). The gluRs that are expressed in this pattern, including gluR4, opens the possibility to gluR

expression within the neuroepiderm. The neuroepiderm is where the nerve cells are located below the most outer cells of the epidermal tissue layer (41). Expression points of gluR12 are evenly spaced along the epiderm of the polyp column (Fig. 4.9), which is consistent with the nerve network of anemones. False positives or non-specific background staining appeared to occur more commonly, albeit still to a minor extent, in gluR sense probes with corresponding antisense-localization to the column (Fig. 4.2F) and in those that presented staining along the polyp column (Fig. 4.2H-I, Table 4.3). Any staining from sense probes was used to compare with antisense whole mounts staining to account for background or non-specific binding.

## 4.6 Discussion

The expression of *E. pallida* mRNA occurred within the epidermal tissue layers, localizing to the tentacles and to a more limited extent to the column (Fig. 4.1-2), which is consistent with the hypothesis that iGluR variants can functional in different roles (16,42–46). Expression of gluRs on tentacles and sensory cells was hypothesized from localization of sensory-associated gene expression through cnidarian development (13,31,47–50) and presence of iGluRs from genomic evidence in anthozoan species (51,52). Signals occuring via synapses within sensory cells are found to perpetuate signals along the nerve plexus within cnidarians (20,23,24). We inferred gluRs were likely functioning in or alongside specific sensory cells, potentially within nematocytes and cnidocytes (25,27,29,41) from characterizational evidence (Ch. 2) on the plausibility of glutamate and iGluRs in cnidocyte defense (27). Resulting expression of gluRs around sensory cells, including nematocytes and spirocytes, followed the hypothesized

localization as deduced from findings of iGluR function and within developing

anthozoans related to *E. pallida*.

Expression of gluRs throughout tentacles is consistent with spirocysts using

chemical triggers rather than requiring physical contact to discharge (24,25). We predict

the rheostat determining where gluRs are expressed within anemone tentacles is related to

chemosensation and physical triggering of spirocysts and nematocysts via these

cnidocytes or neuro-synapse associated sensory cells. If gluRs require close proximal

physical contact, then there is the increased likelihood of association with nematocytes

that require cilia to act as as trigger. An alternative hypothesis is that these genes function

in nerve net signaling. There is a pattern of expression that is similar to the path of the

nerve net along the column of a polyp (Fig. 4.7A). As genes gluR4, gluR8, and gluR12

were chromogenically located throughout the entirety of the polyp (Fig. 4.8 and 4.9), the

localization of expression throughout the polyp is consistent with a connection of gluRs

to synaptic transmission within the nervous system.

It should also be considered that not all gluR genes within the genome have been

localized (Ch. 2). Possibly these genes that have not been cloned from transcripts have

wider localization within the nerve net. However, subfamily or class did not appear to be

a determinant in localization of gluRs, which was considered to as a possibility for

expression patterns from correlations between iGluR lineage and specific functions atune

with cell type. The majority of gluRs (aside from gluR21 GluN_cnid) belong to the

subfamily of Epsilon iGluRs (Ch.2). While chromogenic ISH provides a reproducible

protocol and foundation for localizing gluR RNA expression, it is necessary to pursue three-dimensional imaging using fluorescent *in situ* hybridization to more closely identify associated cells and overlap between gluR expression. Genes that were not uniformly expressed throughout an anemone potentially have a ligand response specific to different cells and tissues. The same concept can be proposed for all gluR genes with differential expression patterns and putative functions. Functional studies to determine the ligands that activate these gluRs are now needed. This will help the field separate which of these iGluRs are involved in primary responses to an external stimuli such as cnidocyte stinging cells, perpetuating neural signals within nerve net, or additional roles in the cnidarian nervous system.

### 4.7 Conclusion

*Exaiptasia pallida* gluR genes were localized to express adjacent to or within cells responsible for detecting and responding to chemical-specific environment cues, including stinging cells located within tentacles, but also punctate throughout the column of the polyps. Localization of gluRs within tentacles suggests iGluRs associate to spirocytes, while those expressed at less density and within the body column are predicted to have association to mastigophore nematocytes. As the expression of gluR genes is closely tied to these sensory cells that contain or are nearby glutamate-triggered mechanisms, iGluRs have a putative function in the discharge of nematocysts and spirocysts for hunting and protective mechanisms, along with neural signaling for the entire polyp. Close association of gluRs occurring along sensory cells supports their chemosensory nature within cnidarians. To further confirm the associated responses of

cnidarian iGluRs we propose to inject *Xenopus* oocytes with *E. pallida* gluR mRNA for functional experiments to determine ligand specificity.

## 4.8 Acknowledgements

Table 4.1 Coding sequence primers for *Exaiptasia pallida* gluR cloning.

| Primer | Accession | Gene | CDS size | TM °F | Sequence 5' – 3' |
|---|---|---|---|---|---|
| iGluR_CDS F | KXJ14767 | gluR(.1,.2) | 2.7kb | 60 | ATG GCG TTG GGA GTG TGT TTT ATT CC |
| iGluR_CDS R | | | | 62 | TCA GAG GTT GCT TTC GGG CGC |
| iGluR6_CDS F | KXJ18695 | gluR21 | 2.3kb | 54 | ATG CTA GAA AAA GCC CTC GAG |
| iGluR6_CDS R | | | | 54 | TTA GTG CAT TTT TCT TCC ATT CCT ATA GA |
| iGluR7_CDS F | KXJ13713 | gluR4 | 2.2kb | 53 | ATG ACT CAA AGA CCA CTG ATT ACA |
| iGluR7_CDS R | | | | 53 | TCA TGT TCT TGA GTC GTT ATT GTT TT |
| iGluR8_CDS F | KXJ29258 | gluR8 | 1.1kb | 55 | ATG GAT TTT ACA GCA CAT GGT TTG T |
| iGluR8_CDS R | | | | 55 | TCA AAA CGA TTT TTG ATG ACT CCA ACA |
| iGluR12_CDS F | KXJ29332 | gluR12 | 800bp | 59 | AAA GCA GAC ATG ATT GTG AGT GAT TTA GTC G |
| iGluR12_CDS R | | | | 62 | CAC GCT CCC CAT TTC GTA ACT TGC T |
| iGluR13_CDS F | KXJ29282 | gluR10 | 1.5kb | 57 | ATG TCA TCC ATT GAC CGT GTA CAG |
| iGluR13_CDS R | | | | 56 | TTA TTC GGA AGA TTT TTC TCT TGC GAT CCG |
| iGluR14_CDS F | KXJ20158 | gluR22 | 670bp | 59 | ATG TAC ACG TTT ATG GAA ACG TAT AAC GT |
| iGluR14_CDS R | | | | 58 | TTA GCT GTA AGT AAA AGC TGT TTC TGT AGC |
| iGluR15_CDS F | KXJ08909 | gluR15 | 1.6kb | 57 | ATG CAT CAT GAC ATG TGG AGG ATA ATG |
| iGluR15_CDS R | | | | 56 | TTA GGA AGC AAC GTG GAA GAA ACG AAC |

Table 4.2 *Exaiptasia pallida* gluR primers with pGEHME overhangs for directional assembly

| Primer | Gene | Sequence 5' – 3' |
|---|---|---|
| pGEMHE fwd | pGEMHE | GAA TTC TCT AGA GCA AGC TTG ATC TGG TTA C |
| pGEMHE rev | | GGA TCC CCG GGG AAT TGA TCT G |
| iGluR fwd | KXJ14767 | GAT CAA TTC CCC GGG GAT CCa tgg cgt tgg gag tgt gtt tta ttc |
| iGluR rev | | CCA GAT CAA GCT TGC TCT AGA GAA TTC tta gag gtt tga ggg tcg gcc |
| iGluR6 fwd | KXJ18695 | GAT CAA TTC CCC GGG GAT Cca tgc tag aaa aag ccc tcg ag |
| iGluR6 rev | | AAG CTT GCT CTA GAG AAT TCt tag tgc att ttt ctt cca ttc cta tag ata t |
| iGluR7 fwd | KXJ13713 | GAT CAA TTC CCC GGG GAT CCa tga ctc aaa gac cac tga tta cat tag |
| iGluR7 rev | | CAA GCT TGC TCT AGA GAA TTC tca tgt tct tga gtc gtt att gtt ttt aga g |
| iGluR8 fwd | KXJ29258 | ATT CCC CGG GGA TCC atg gat ttt aca gca cat ggt ttg t |
| iGluR8 rev | | TCT AGA GAA TTC tca aaa cga ttt ttg atg act cca aca tgc |
| iGluR12 fwd | KXJ29332 | TCC CCG GGG ATC Ccg gcc ccc aca cca ccg gtc att |
| iGluR12 rev | | TTG CTC TAG AGA ATT Ctc acg ctc ccc att tcg taa ctt g |
| iGluR13 fwd | KXJ29282 | AAT TCC CCG GGG ATC Cat gtc atc cat tga ccg tgt aca g |
| iGluR13 rev | | TAG AGA ATT Ctt att cgg aag att ttt ctc ttg cga tcc g |
| iGluR14 fwd | KXJ20158 | CAG ATC AAT TCC CCG GGG ATC Cat gaa tct cgt tca atc tta cgt ctt tgt a |
| iGluR14 rev | | AAG CTT GCT CTA GAG AAT TCc taa ttt gtg gct tgg gct cg |
| iGluR15 fwd | KXJ08909 | TCC CCG GGG ATC Cat gca tca tga cat gtg gag gat aat g |
| iGluR15 fwd | | TGC TCT AGA GAA TTC tta gga agc aac gtg gaa gaa acg aac |

Table 4.3 Summary of *Exaiptasia pallida* gluR localization patterns of gene expression and representation across biological replicates

| *Exaiptasia pallida* gene | Location | Pattern | Represented / Total replicates | Sense probe overall staining | Represented / Total replicates |
|---|---|---|---|---|---|
| gluR.1 | tentacles | continuous | 8/10 | Negative | 5/6 |
| gluR.2 | tentacles | continuous | 7/8 | Negative | 4/4 |
| gluR4 | tentacles pedal disc acontium | punctate | 6/6 | Negative | 4/5 |
| gluR8 | tentacles column | punctate sparse | 4/4 | Negative | 2/3 |
| gluR10 | tentacles column | punctate | 4/4 | Negative | 4/4 |
| gluR12 | tentacles column | punctate throughout | 4/5 | Negative | 3/5 |
| gluR15 | tentacles | continuous, some point expression | 4/4 | Negative | 4/4 |
| gluR21 | tentacles | concentrated at tips | 4/5 | Negative | 3/4 |
| gluR22 | tentacles column | punctate | 3/4 | Negative | 4/4 |
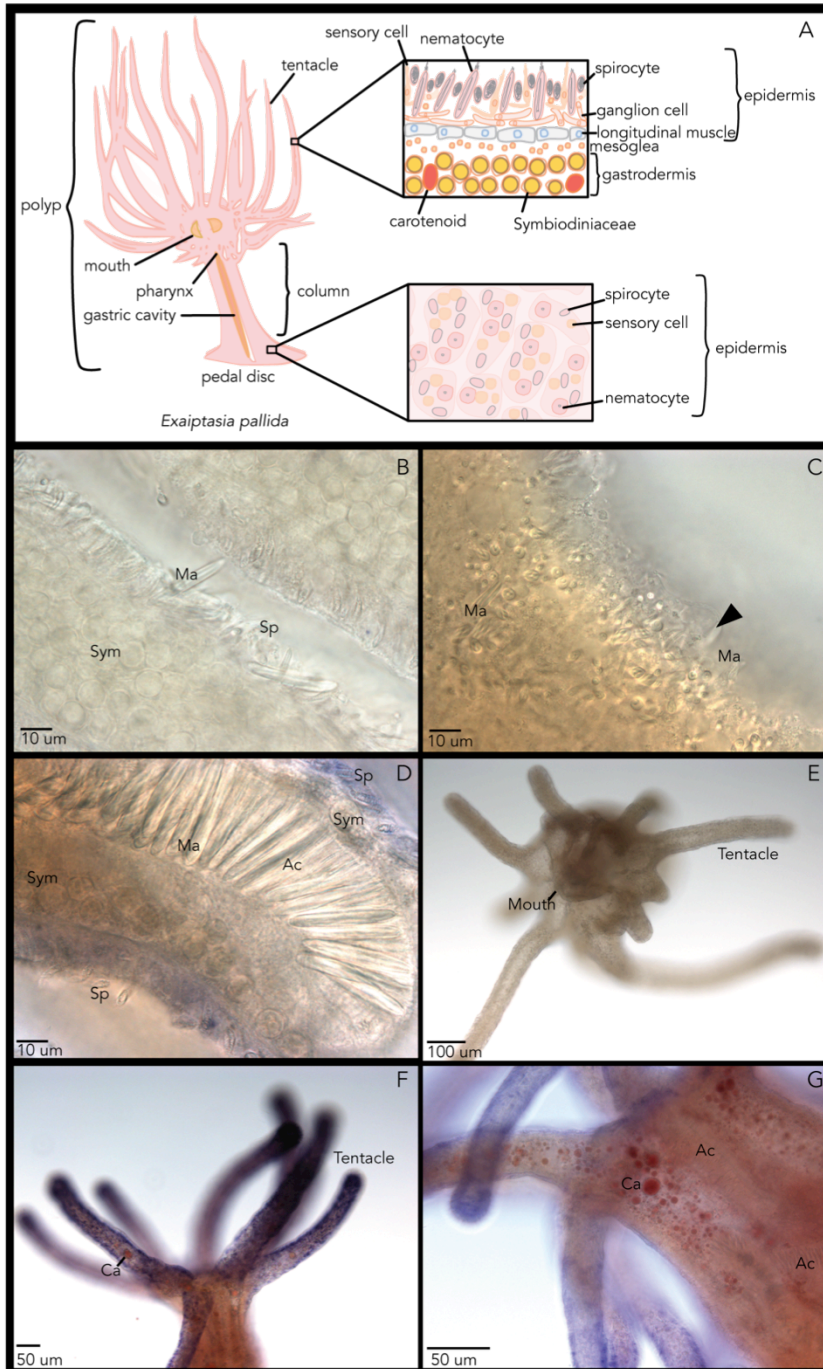
Figure 4.1 Cell differentation and anatomy of *Exaiptasia pallida* anemones.
A. Schematic of an anemone with anatomy labeled. Insets of tissue layers and cell differentiation. B. Tentacle filled with Symbiodiniaceae and lined with cnidae: nematocytes and spirocytes containing cnidocysts (nematocysts and spirocysts, respectively). C. Arrow pointing to launched tubule coil from a p-mastigophore on tentacle surface. D. Acontium packed with mastigophores.Visible spirocysts and mastigophores. E. Polyp from top view. F. gluR22 Antisense probe localization along the tentacles. Carotenoids present in anemone tentacles and body column. G. gluR.1 Antisense probe localization on tentacles and carotenoids within body and tentacles. ; Ac=acontium, Ca=carotenoid, Sym=Symbiodini-aceae, Ma=mastigophore, Sp=spirocyte, arrow=launched cnidae tubule
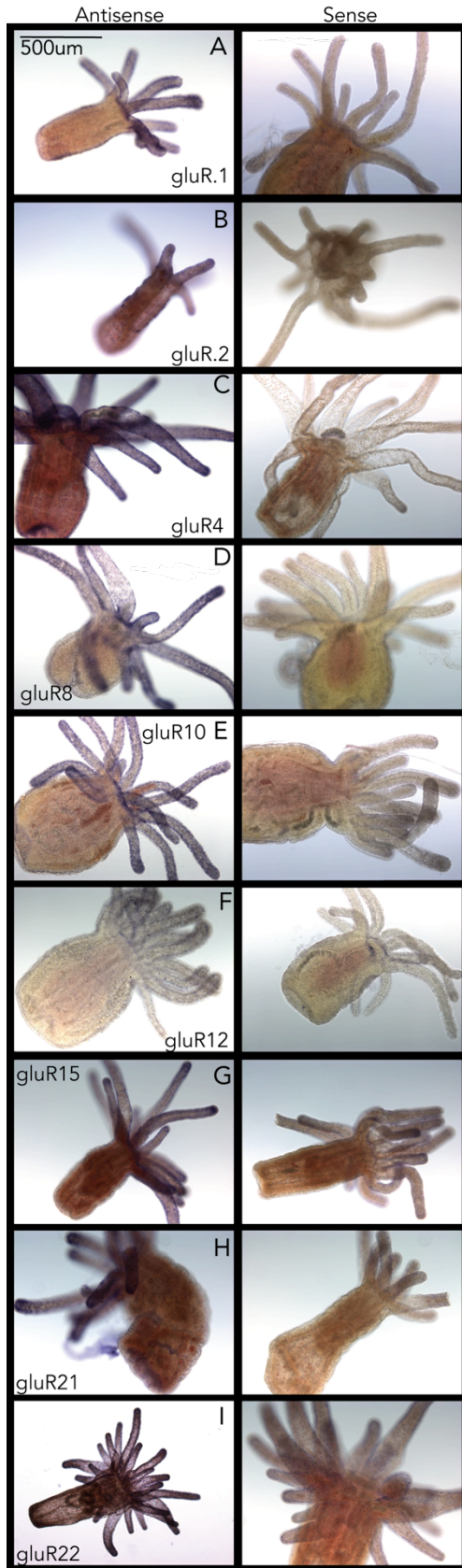
Figure 4.2 Whole mount *in situ* hybridization of *Exaiptasia pallida* anemones for gluR expression localization at 100X magnification under 70X camera magnification. Left antisense; right sense. Scale bar is 500 um. Representative images of A. gluR.1, B. gluR.2, C. gluR4, D. gluR8, E. gluR10, F. gluR12, G. gluR15, H. gluR21, I. gluR22
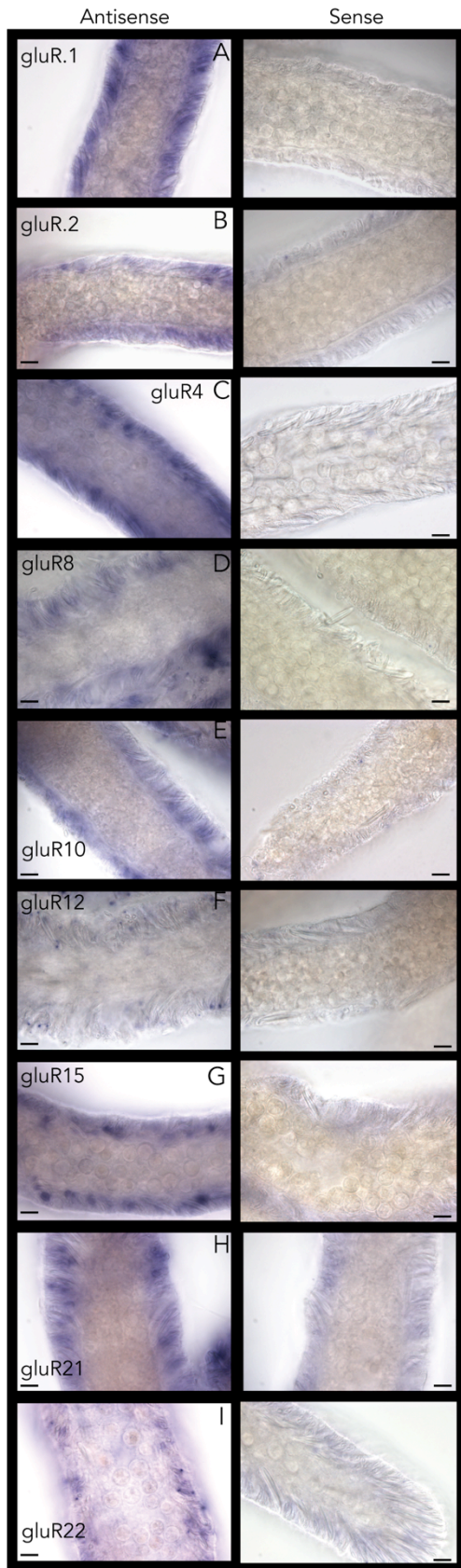
Figure 4.3 Tentacles of whole mount *in situ* hybridization of *Exaiptasia pallida* anemones for gluR expression localization at 1000X magnification under 70X camera magnification. Left antisense; right sense. The scale bar inset is 10 um, approximate size of Symbiodiniaceae. Representative images of A. gluR.1, B. gluR.2, C. gluR4, D. gluR8, E. gluR10, F. gluR12, G. gluR15, H. gluR21, I. gluR22
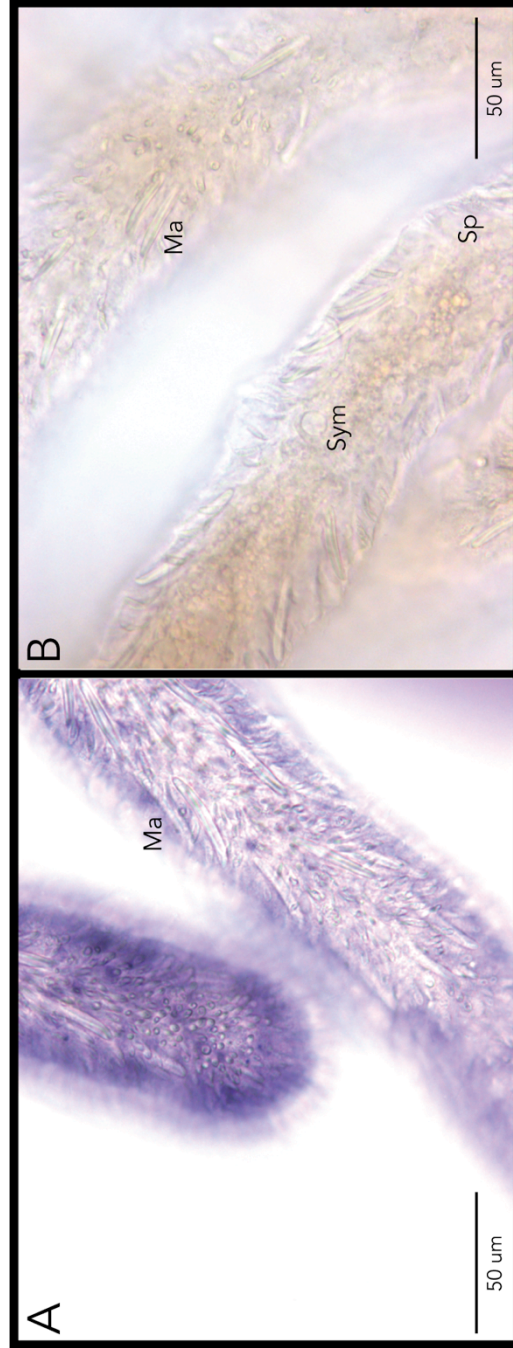
Figure 4.4 Expression of *Exaiptasia pallida* gluR.1 on tentacles at 400x magnification. Scale bar is 50 um. Representative images of A. Antisense probe  B. Sense probe
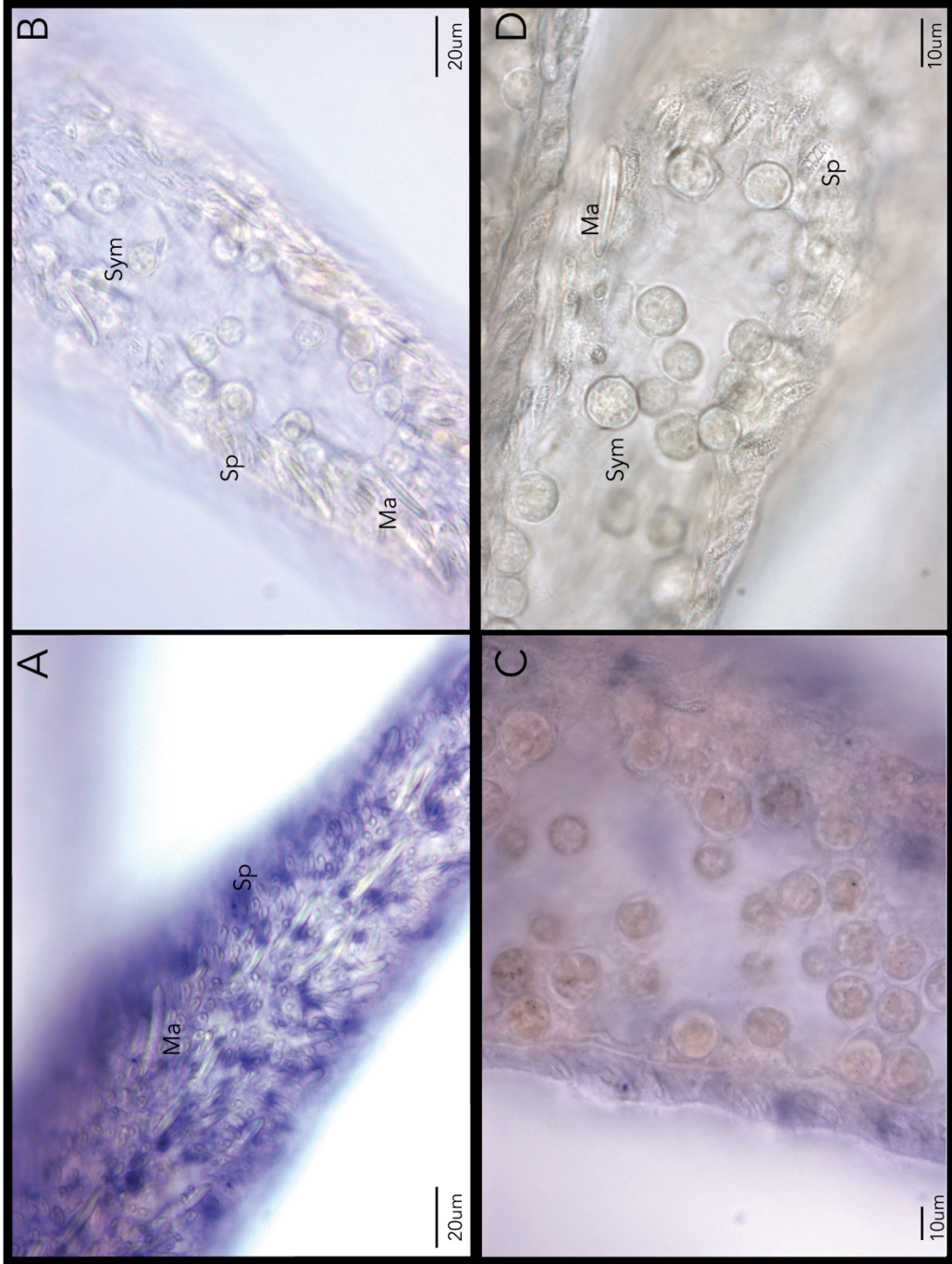
Figure 4.5 Pattern of *Exaiptasia pallida* gluR4 expression on tentacles. Representative images of A. Antisense 630x B. Antisense 630x C. Antisense 1000x D. Sense 1000x ; Ma=mastigophore, Sp=spirocyte, Sym=Symbiodiniaceae
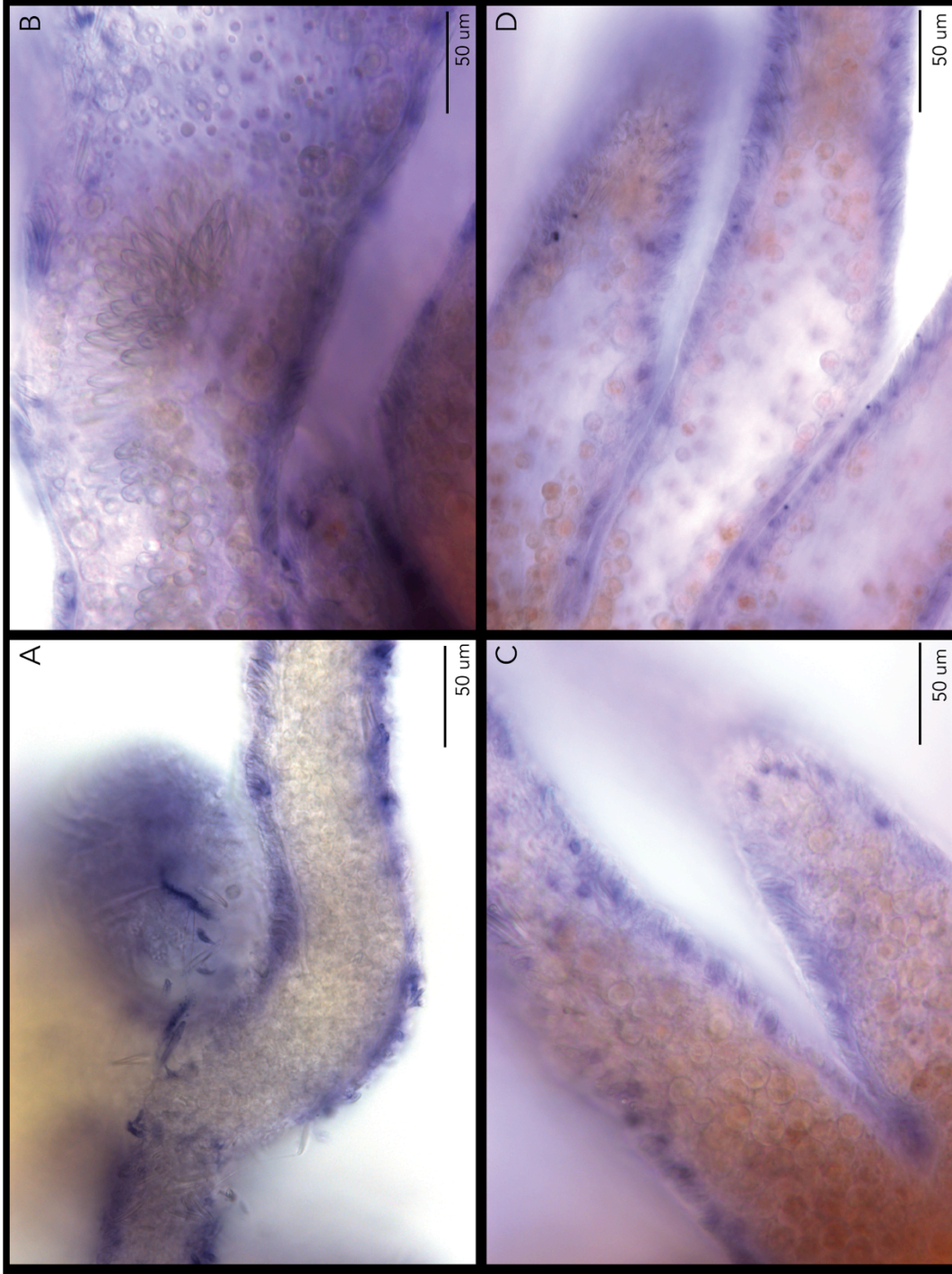
Figure 4.6 *Exaiptasia pallida* gluR expression occuring at epidermal tissue layers. Representative images of A. gluR.2 Antisense 400x B. gluR4 Antisense 630x C. gluR15 Antisense 630x D. gluR22 Antisense 630x
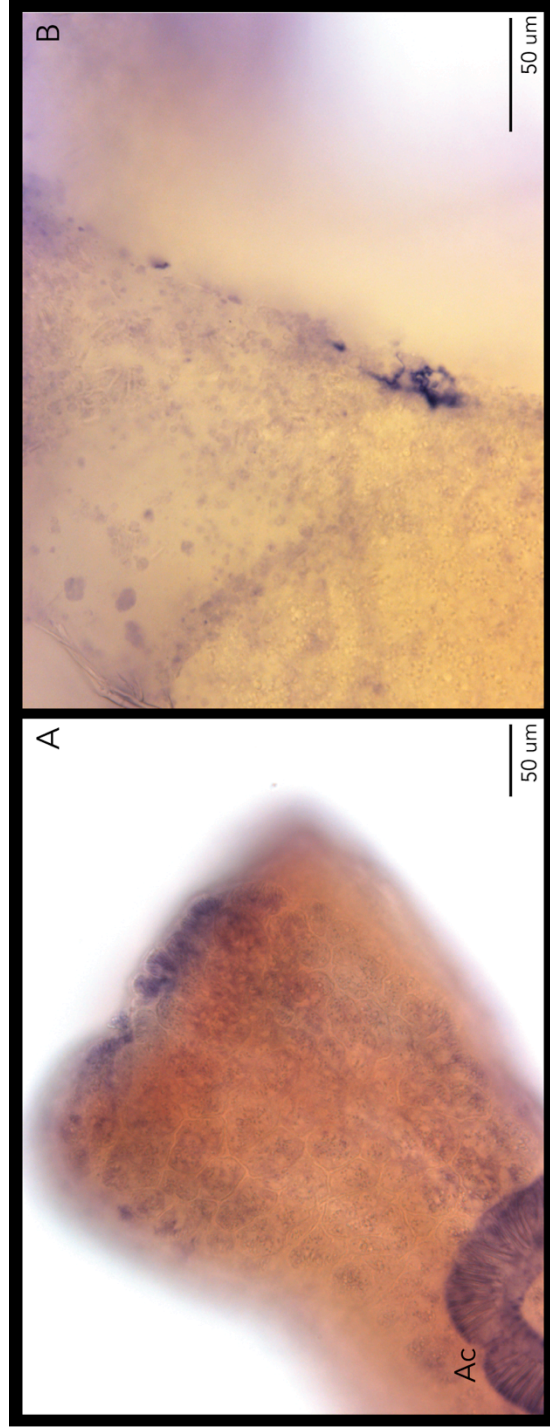
Figure 4.7 *Exaiptasia pallida* gluR4 expression occuring at epidermal tissue layers on polyp column. Representative images of A. Antisense 400x B. Antisense 630x; Ac=acontium
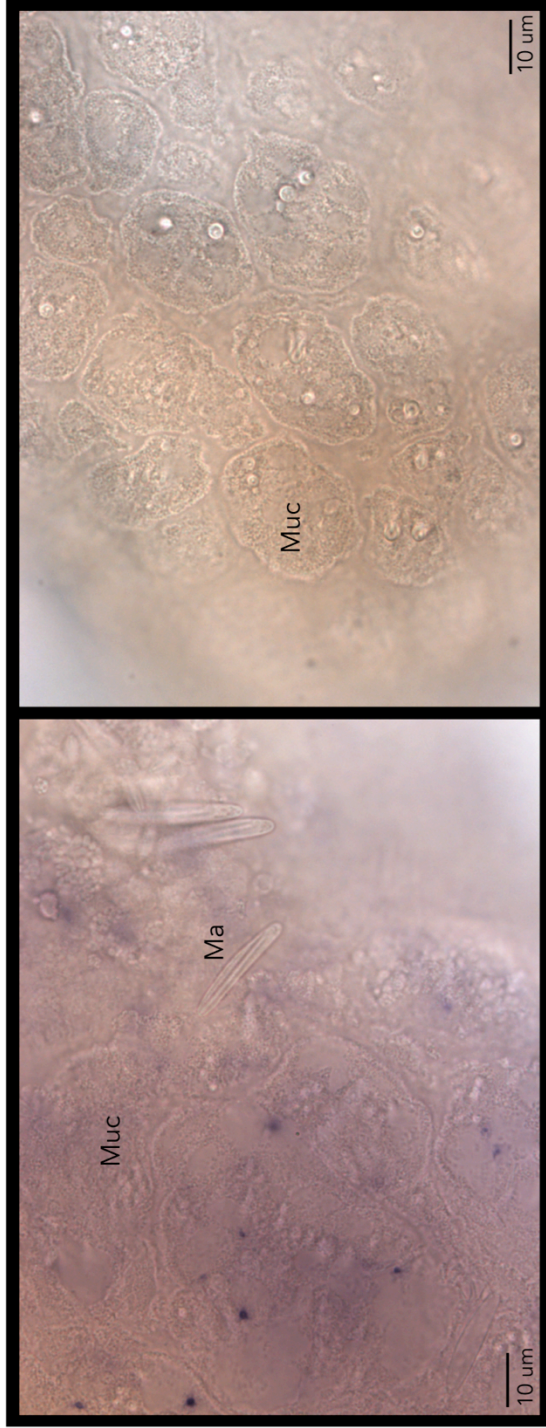
Figure 4.8 *Exaiptasia pallida* gluR8 expression patterns on polyp column at 1000x magnification. Representative images of A. Antisense B. Sense; Muc=mucocyte, Sp=spirocyte
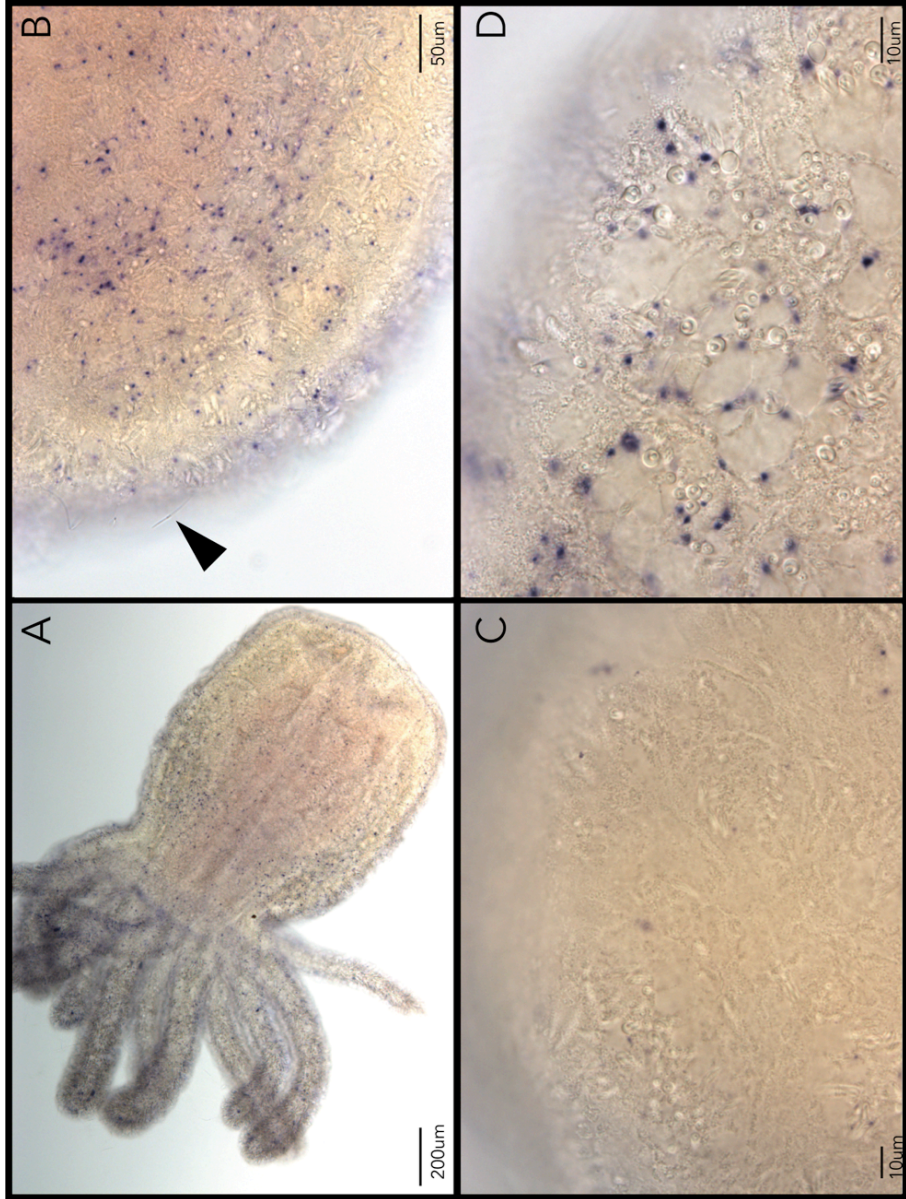
Figure 4.9 *Exaiptasia pallida* gluR12 expression patterns on polyp column. Representative images of A. gluR12 Antisense 100x B. gluR12 Antisense 400x, arrow marks launched cnidae tubule C. gluR12 Sense 1000x D. gluR12 Antisense 1000x ; Sp=spirocyte, Ma=mastigophore
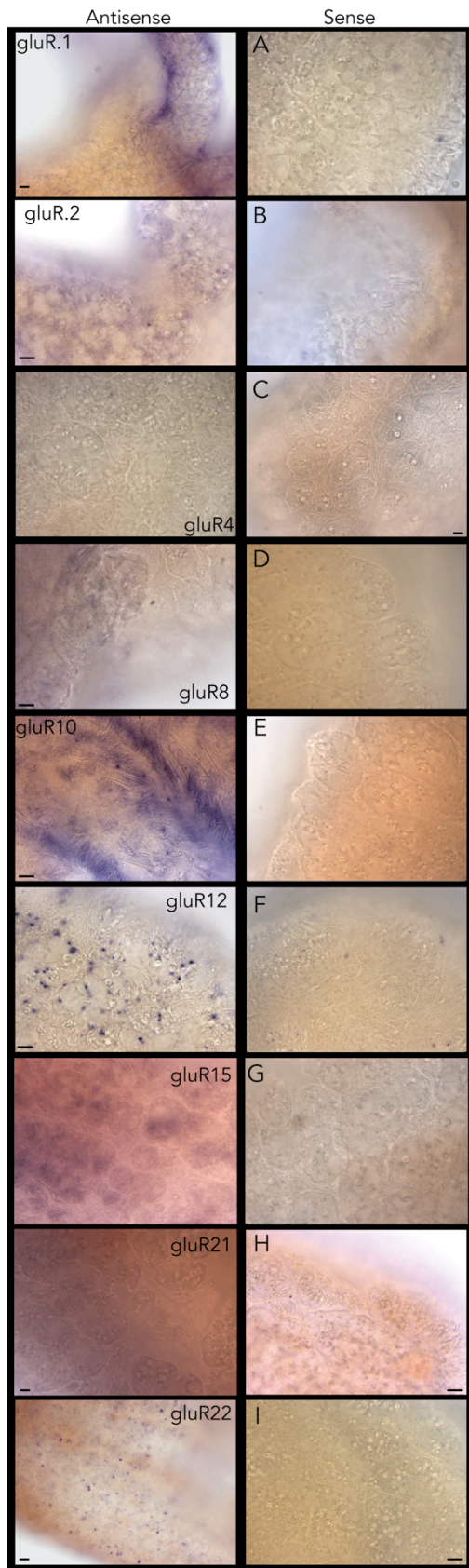
Figure 4.10 Expression of *Exaiptasia pallida* gluRs on polyp column of whole mount anemones. Viewed at 630x or 1000X magnification under 70X camera magnification. Left antisense; right sense. Scale bar set to 10um. Representative images of A. gluR.1, B. gluR.2, C. gluR4, D. gluR8, E. gluR10, F. gluR12, G. gluR15, H. gluR21, I. gluR22

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CHAPTER V. CONCLUSIONS AND SYNTHESIS

**5.1 Synthesis of findings**

This project began as an investigation into the involvement of chemosensory proteins in an innate immunity-focused perspective and grew to encompass the study of the evolution and explore putative functions of chemosensory ionotropic glutamate receptors (iGluRs) within Cnidaria, explicitly using the model cnidarian, sea anemone *Exaiptasia pallida* (Agassiz in Verrill, 1864). The results of the findings presented through Data Chapters II-IV, have led to the development of several hypotheses on the evolutionary roles of neural networks and chemical signaling.

Chemosensation, or chemical sensing, is a fundamental signaling pathway correlating innate immunity and neural signaling within the anemone, *E. pallida* through chemosensory iGluRs. Polyphyletic *E. pallida* iGluRs are predicted to act as sensory receptors and respond to immune and environmental stimuli. This dissertation explored the diversity of iGluRs within cnidarians, measures expression of *E. pallida* iGluRs in response to two environmental stimuli, and chromogenic localization of expression in cells and tissues within whole *E. pallida* anemones. Several methods were used to characterize the iGluR protein family within Cnidaria. Phylogenetic analyses were used in the approach to determine the extent of cnidarian diversity across metazoans. Further bioinformatic analysis in homolog conservation was used as a starting point for predicting putative functions from protein functional domains. The available genome and transcriptome of *E. pallida* was important for both bioinformatics analysis and within the

approach for gene expression. Bacterial challenges and diurnal cycling have implications of the environment in eliciting changes in expression of gluRs in *E. pallida.* Chromogenic localization of genes is consistent with the hypothesis that gluR expression correlates to sensory cells and the nervous system in the putative function of iGluRs within Cnidaria.

## 5.2 Chapter summaries

The range of iGluR diversity in available genomes along with complementary data on putative cnidarian functions has given rise to hypotheses on the cnidarian role of iGluR function. Overall characterizations are summarized in Table 5.1. There were 22 unique genes coding for *E. pallida* GluR (one set of isoforms, named gluR.1 and gluR.2) dispersed over four phylogenetic clades across subfamilies and classes of iGluRs (Ch. 2) with expansions in Cnidaria. Four of these genes from clades C2 and C4 had significant changes in expression, as a response to bacteria challenge, while two within C4 did not respond at all (Ch. 2). In addition to inference of GluN and GluE expression responses to bacteria, there was the underlying implication of time as a factor to investigate.

Two iGluRs differentiated in expression cycle when quantified via a diel zeitgeber, a 12 hr light: 12 hr dark cycle versus a 12 hr dark: 12 hr dark cycle sans light stimulus (Ch. 3). *Exaiptasia pallida* gluR.2 (isoform of GluR), a respondent to bacterial challenge, was calculated to have a 16-hr period, compared to an 8-hr period in LD cycle. *Exaiptasia pallida* gluR8, which had not responded to bacterial challenge, was calculated to have a 24-hr period within DD, referred to as a diel or circadian, compared to an 8-hr

132

period in LD. While, we cannot claim gluR8 expression has a circadian rhythm, gluR expression changes are consistent with changes in transcriptomic data of iGluRs from the symbiotic relationship with algal-dinoflagellates and in *Exaiptasia diaphana* (Rapp, 1829) (Ch. 3) (1). The expression changes in sea anemones from symbionts and diurnal fluctuations imply the metabolic outputs of Symbiodiniacea via primary productivity affect transcription of iGluRs (Fig. 5.1).

Expression of *E. pallida* gluR genes was localized mostly to *E. pallida* tentacles, where nematocysts and sensory cells reside, while some gluRs were expressed throughout the column of anemone polyps (Ch. 4). The expression of gluRs to only tentacles implies the sensory proteins align to spirocysts or nematocysts, which is consistent with the connections of glutamate to cnidocyte firing (2,3). *Exaiptasia pallida* gluRs that did not have an expression response to bacteria are potentially associated to internal nervous system signaling. It is possible that different *E. pallida* gluRs are prescribed for different functional roles from synapses to environmental receptors, dependent on their association with sensory cells.

## 5.3 Exploring ligand specificity

Phylogenetic and structural analyses suggest identified *E. pallida* iGluRs maintain functional domains of chemosensory proteins (Ch. 2). Coupling bioinformatics with transcriptional data implies iGluRs have a putative function in the context of bacterial defense (Ch. 2). The GluR sequences from *E. pallida* span several clades, designating *E. pallida* iGluRs as polyphyletic. From our knowledge of classical iGluRs, we know this

family of receptor proteins diverges into different clades based on their specificity of synthesized ligands. Drawing this parallel, we can hypothesize that each of these clades of *E. pallida* iGluRs (C1-C4) has a specific ligand (or several ligands), which requires further investigation to confirm. While the ligand-binding domain may detect glutamate or glycine or a bacterial-specific ligand site, we do not know at this stage as to which specific chemical each GluR subunit responds. Ligand specificity for different iGluR subunits have been documented as GluN1, GluN3, and GluD binding glycine and D-serine, while GluA and GluK bind glutamate (4–6). I hypothesize GluR subunits classified in C1 putatively respond to NMDA and glycine, GluRs within C2 to glycine and D-serine, and GluRs within C3 and C4 to glycine and/or glutamate. *Exaiptaisa pallida* iGluRs within C2 and C4 cnidarian subclades are conjectured to demonstrate cnidarian-specific ligand binding associations within GluN and GluE subfamilies, which is consistent with species-specific ligand specificity within respective expansions. Ligand specificity within species expansions has been demonstrated in ctenophore binding specificity toward glycine (7).

A standard method to assess ligand specificity is through measuring ion channel activity via expressing ion channel proteins within *Xenopus* oocytes (8,9). *Xenopus* oocytes are standard to use as model cells for *in vivo* experimentation; the oocytes can be de-folliculated from the ovarian sacs and logistically important, are relatively large in size (8). A large cell size is ideal for studying ion channels as a two-electrode clamp can fitted on the cell to measure extracellular currents flowing through the membrane with a vibrating probe (9). This approach would use full-length *E. pallida* cDNA sequences of

iGluRs within a pGEHME vector (provided from samples preserved during Ch. 4) to be transcribed into mRNA *in vitro* or synthesized into cRNA, and injected into *Xenopus* oocytes for expression, ultimately isolating the receptors individually or in co-receptor pairs to measure ligand response. Through this experimental system the selected iGluR proteins can be expressed alone or in specified combinations. To measure activity of the iGluRs, oocytes expressing receptors of interest would be exposed to candidate ligands related to immunity and chemosensation for ligand-specific measurements of electrophysiological response. Specific ligands that will be targeted include various known agonists and antagonists i.e., AMPA, NMDA, kainate, L-glutamic acid, L-aspartic acid, glutamate, glycine, D-serine and DNQX (5,10) and immunological agents of *V. coralliilyticus, S. marcescens*, lipopolysaccharide, and peptidoglycan. Data from ligand specificity would allow us to confirm accuracy of the naming conventions used for iGluRs in applicability to invertebrates and more importantly, determine functional responses and the role of iGluRs within chemosensory pathways.

## 5.4 Putative functions of *Exaiptasia pallida* iGluRs

Initial exploration into gluR putative functions occurred through developing an immune challenge experiment using two bacteria, *Vibrio coralliilyticus* and *Serratia marcescens* (Ch. 2). It should be noted here the GluR with two isoforms, gluR.1 and gluR.2, responded to *S. marcescens*. In response to *V. coralliilyticus*, gluR.2 displayed the strongest transcriptional changes compared to the other gluR genes. Also from C4, *E. pallida* gluR4 and gluR8 did not have a significant response to the bacterial challenges. However, bacterial treatment did significantly impact C2 gluR21 and C3 gluR23. Across

C2-C4, there is a bacterial response, but this does not mean every *E. pallida* gluR gene will respond to this stimulus. Sampling over the course of several days demonstrated that the 24-hour time point is the critical to the affect of time.

I further explored the expression of the bacteria-responsive gluR.2 and non-responsive gluR8. Within diurnal experimentation via light cycling *E. pallida* gluR.2 and gluR8 expressed ultradian 8-hr periods under normal light:dark conditions. The period changed for both expression patterns of gluR genes when compared to anemones in constant darkness; implying *E. pallida* iGluRs have different expression cycles without a diurnal signal. Symbiosis tightly connects to diurnal cycles in cnidarians with endosymbiotic Symbiodiniaceae (11–13). Recent expression data from the facultative-symbiotic anemone *Exaiptasia diaphana* suggested iGluRs have different cycles dependent on the presence of symbionts (1). Transcriptional regulation of iGluR gene expression was consistent with altering from a symbiosis-associated 24-hr period to aposymbiotic-associated 12-hr period. While the pattern did not hold fast for every iGluR sequence, expression cycles consistently differed between symbiotic states for the anemones. Endosymbiotic Symbiodiniaceae rely on light for metabolism via primary productivity, which changes from day to night. When I interrupted the light cycle during the diurnal experiment (Ch. 3), I uncovered a potential endogenous period cycle for the *E. pallida* iGluRs. The rhythm would only be endogenous if it is circadian. Regardless, removing light changed iGluR transcription, which was consistent with iGluR transcription changes to Symbiodiniacea in *E. diaphana*.

Expression data is not complete for the entirety of all *E. pallida* iGluRs, because of financial and practical restrictions including the number of individual anemones required for each experiment and consequent amount of processing. In addition to the number of iGluRs, there is also the consideration of the 24-hr sample period versus a 48-hr period. Measuring expression over 48 hours would provide stronger support for the cycle of the biological rhythm (14), however, the aim of this experiment was to determine if there was a periodicity or factor of time in gluR expression. The most interesting finding for me within Chapter II was change in expression between light cycles and how this was consistent with the altering expression cycles of iGluRs from Symbiodiniacea (1). In order to demonstrate biological rhythms in *E. pallida*, a transcriptomics study sampling anemones at least every four hours over a 48-hr period with the experimental set up testing the factors of light as a diel factor or alternatively the presence and absence of Symbiodiniacea. In a transcriptomic study, the data could be explored to uncover iGluR genes and their expression cycles as I did with the *E. diaphana* data (1).

## 5.5 Chromogenic localization of gluRs within *Exaiptasia pallida*

To establish a chromogenic *in situ* hybridization protocol, I cloned and sequenced nine *E. pallida* iGluRs from cDNA. The *E. pallida* gluRs were inserted into a pGEMHE-containing vector, which was necessary for the initially planned functional experiments. Each of these cloned genes was used to create probes specific to each gene. *In situ* localization has not always been successful within *E. pallida*, but I was able to develop a protocol and process nine probes, both sense and anti-sense, for characteristically low-expressed receptors. *Exaiptasia pallida* gluRs were expressed for the most part within the

tentacles of the whole mount anemones. I hypothesize the location of the *E. pallida* gluRs overlaps closely to sensory cells and defensive nematocytes and spirocytes. Anemones have a denser, higher concentration of spirocytes relative to nematocysts within tentacles. The genes expressed at high concentrations, total coverage of tentacles, are predicted to be expressed within or adjacent to spirocytes, while those with greater spacing between expression points within or adjacent to nematocytes. This idea is purely conjecture based on the concentrations of spirocytes versus nematocytes in anemone tissues (3,15). However, the *E. pallida* iGluRs are expressed within the epidermal layer and not within the gastroderm. I originally hypothesized *E. pallida* iGluRs would be located within the gastroderm because of phagocytic cells and the initial connection of iGluRs to cnidarian innate immunity (16).

At this juncture, I hypothesize *E. pallida* iGluRs are more likely linked to neural signaling within the dispersed nervous system of the anemones. Anemones have variety of neural cells dispersed throughout a nerve net, that the anemone uses as communication across the polyp. Instead of having a CNS to process signals, anemones act purely on an immediate 'gut' reaction, quick signaling and identification of the biological or environmental elicitor that set off those signals via the nerve net. Perhaps in these early-diverged metazoans, the large diversity across clades is necessary for more specificity and nuanced specificity between ligands. It is possible the *E. pallida* iGluRs act as co-receptors and forming various combinations of subunits, therefore, even with only four clades and 22 genes, it is possible there are many more potential ligands or specificity to different signals.

To continue localizing gene expression of iGluRs, whole mount fluorescent *in situ* hybridization (FISH) can assess localization of iGluR gene expression (17,18). FISH can be used to determine where iGluR genes are transcribed through fluorescent markers that bind to iGluR-specific probes. Co-expression can be determined by overlapping iGluR FISH with multiple probes and specific markers for each probe, which would provide potential pairs to inject into *Xenopus* oocytes. Then the ability to use RFamide to outline the cnidarian nervous system (3,15,19) would allow overlap between labels to identify how closely *E. pallida* iGluRs associate to the nerve net.

**5.6 Ecological and applicable relevance of cnidarian iGluRs**

The primary goal of an organism is to maintain homeostasis, to stay within those set bounds for cellular operations. Changes within environment provoke behavioral changes or physiological responses, dependent on adaptations to environmental fluctuations. As anemones are sessile creatures, their responses are exclusively physiologically based and changing their internal environments. The most drastic physiological change cnidarians undergo is that disruption of symbiosis leading to coral bleaching, the event when polyp tissues either lose or eject Symbiodiniacea leaving bone-white ghostly coral frames. While unknown if these receptors are connected to bleaching, expression patterns of gluRs opens the possibility of influence by symbiosis. Their regulation changes in response to light and symbiotic state (Fig. 5.1). Alternating metabolic outputs, including extreme changes in pH and ion concentrations, may be the biochemical catalyst that impacts regulatory systems of *E. pallida* gluR expression within symbiotic anemones.

This dissertation has led me throughout various methods to characterize a large family of signaling receptors. From bioinformatics to predictive applications, I characterized iGluRs within the genome of *E. pallida* and addressed putative involvement of several receptors. However, this is only a start to the understanding of the importance of cnidarian iGluRs, which is possible through the established genome. Through characterizing the *E. pallida* gluR genes, I developed hypotheses for predicted functions. In order to completely characterize these receptors, functional work across the entire phylogeny of *E. pallida* iGluRs needs to be completed.

To illustrate the role of these receptors within an invertebrate is important in relation to our knowledge of how iGluR dysfunction can lead to neurological issues and degeneration within humans. Cnidarians do not exhibit the aging process in the apparent way humans do, so perhaps through this experimental model we can learn more about how to better sustain iGluRs within the environment of an aging brain. An anemone is constantly communicating with the outside world, there is barely a barrier to define where the organism begins and ends between the symbioses with Symbiodiniaceae to associated bacteria to the biogeochemical components of the ocean. We contemplate how receptors unremittingly exposed to a changing environment and internal cycling in a sessile organism continue to work efficiently. Through continued research, we can observe iGluR involvement in development and predict the role within a nerve net that utilizes conductor and environmental sensory cells. We recognize these receptors are essential for various signaling functions; it is possible iGluRs have a putative function to

maintain regulation and stability for homeostasis. From my dissertation research, I have opened several doors and explained how to continue exploration of these proteins and to learn how iGluRs function in cnidarians and their essential roles that required gene expansion.

## 5.7 Tables and Figures

Table 5.1 Overview of *Exaiptasia pallida* iGluR characterization and findings.

| gluR | Clade | Class/Subfamily | Immune Response | Biological Rhythm | Location |
|------|-------|-----------------|-----------------|-------------------|----------|
| .1 | C4 | GluE_cnid | bacteria: *Serratia* | | tentacles |
| .2 | C4 | GluE_cnid | bacteria: *Serratia* | LD 8-hr ; DD 16-hr | tentacles |
| 3 | C4 | GluE_cnid | | | |
| 4 | C4 | GluE_cnid | none | | tentacles, pedal disc, acontium |
| 5 | C4 | GluE_cnid | | | |
| 6 | C3 | GluE | | | |
| 7 | C3 | GluE | | | |
| 8 | C4 | GluE_cnid | none | LD 8-hr ; DD 24-hr | tentacles, column |
| 9 | C4 | GluE_cnid | | | |
| 10 | C4 | GluE_cnid | | | tentacles, column |
| 11 | C2 | GluN_cnid | | | |
| 12 | C4 | GluE_cnid | | | tentacles, column |
| 13 | C2 | GluN_cnid | | | |
| 14 | C2 | GluN_cnid | | | |
| 15 | C4 | GluE_cnid | | | tentacles |
| 16 | C2 | GluN_cnid | | | |
| 17 | C2 | GluN_cnid | | | |
| 18 | C2 | GluN_cnid | | | |
| 19 | C2 | GluN_cnid | | | |
| 20 | C3 | GluE | | | |
| 21 | C2 | GluN_cnid | bacteria treatments | | tentacles |
| 22 | C1 | GluN1 | | | tentacles, column |
| 23 | C3 | GluE | bacteria treatments | | |

Table 5.2 Summary of questions and hypotheses proposed in the disseration and concluding statements.

| QUESTION | PREDICTIONS | FINDING | CONCLUSION |
|---|---|---|---|
| 1a. What is the extent of cnidarian iGluR diversity? Within the genome of the sea anemone, *Exaiptasia pallida*? | N/A | N/A | There are 22 *Exaiptasia pallida* iGluR genes, one has two isoforms. These genes are polyphyletic, occuring across the evolution of iGluRs. |
| 1b. Are the protein structures of *E. pallida* iGluRs reflective of functional characteristics through conserved domains? | N/A. | Supported | Most GluRs have all or partially all distinctly iGluR conserved domains. Models of GluR with conserved domains mirror the functional structure of iGluRs. |
| 1c. What level are iGluRs involved during the response to microbial challenges? | If cnidarian gluRs respond to immune challenges presented by bacteria, expression will alter in response to either overwhelming signals or to set physiological responses in motion. | Supported/ Rejected | Of the six tested *E. pallida* gluR genes, four have significant transcriptional responses in gene expression to bacterial treatments. |
| 2a. Is there a daily expression cycle for cnidarian iGluRs? | **a.** If cnidarian gluRs follow physiological changes of glutamate amounts, then RNA transcription will decrease following glutamate synthesis. | Rejected | gluR.2 and gluR8 expression cycles do not follow changes in predicted glutamate levels. Rather, the expression changes with light cycle variation, i.e. light absence. |
| 2b. Do cnidarian iGluRs have a role in biological cycles? | **b.** If cnidarian gluRs follow a true circadian rhythm, there will be one peak and one trough within 24 hours of sampling. | Rejected | gluR8 had a 24-hr period when in constant darkness, but this did not hold true in the 12 hr light : 12 hr dark treatment. |
| 3. Where are iGluRs localized within *E. pallida*? | **Localization of gluRs is** likely to be within sensory tentacle cells and gastrodermal tissues that phagocytize pathogenic agents and within larvae expressed in the apical tuft. | Supported/ Rejected | gluRs are localized in epidermal tissues, particularly within tentacles around sensory cells. Putatively involved in neuro-nematocyst/spirocyst synapses. |
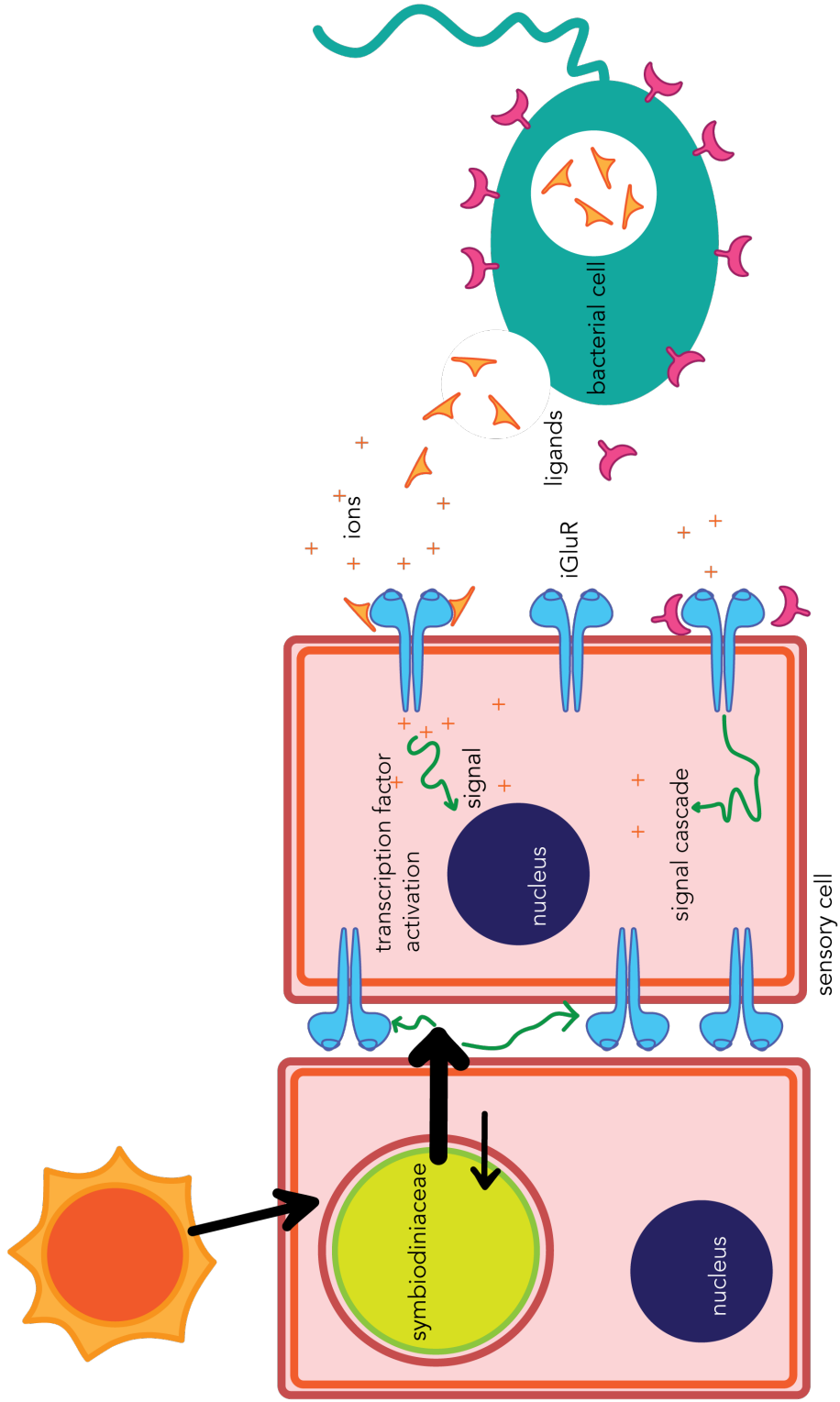
Figure 5.1 Graphic of hypothesis depicting bacterial and diurnal factors that affect *Exaiptasia pallida* iGluR expression. From transcriptional responses, expression of some gluR genes could be altered as a factor of bacterial challenge, time, and light stimulus.

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