AN ABSTRACT OF THE DISSERTATION OF

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Title: Environmental Variables and Gene Expression Profiles Associated with Cueing Reproductive Events in Cnidarians

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Coral reproduction is vital to the persistence of coral reefs. Decades of ecological studies have correlated environmental variables, such as temperature and light, to the timing of reproduction in anthozoan cnidarians, including corals and sea anemones. However, elevated temperatures associated with climate change impair reproductive success and threaten the resilience of coral reefs globally. Empirically evaluating the effect of the key environmental variables temperature and light on the timing of reproduction, at ecological and molecular levels, will further our understanding of the impacts of a changing environment on the future of coral reproductive success. At the transcriptomic level, the signaling pathways and mechanisms involved in transducing environmental cues into molecular signals and coordinating reproductive events is poorly understood. The few studies examining molecular events associated with reproduction have focused on spawning corals: no study has examined these events in a brooding coral.

Cnidarians are both evolutionarily and ecologically significant. As early-diverging basal metazoans, they occupy a key position as the sister-taxon to bilaterians, and are the foundational species of coral reef ecosystems. Therefore, hypothesis-generating studies aiming to characterize both novel and homologous genes involved in coordinating reproductive events can provide a basis for detailing the genetic and physiological mechanisms governing reproduction in ancestral animal systems.

In this work, Chapters 2 and 3 investigate the impacts of elevated temperature on reproductive timing in the coral *Pocillopora damicornis* and integrate ecological and genomic methods to describe environmental and physiological components of reproductive timing in a brooding coral. My findings reveal that there is plasticity in the timing of reproduction at both an ecological and transcriptomic level, as elevated temperature results in earlier larval release and a disruption of transcriptomic profiles associated with the timing of reproduction. Chapter 4 explores the potential of the sea anemone developmental model, *Nematostella vectensis* as a reproductive model for corals and examines gene expression patterns associated with light + temperature-induced spawning. I describe smaller transcriptomic changes during reproduction, compared to brooding and spawning corals, and suggest that reproductive priming and post-translational regulation leads to modest transcriptional change. Overall, this work describes the impacts of elevated temperature on reproductive timing in a brooding coral and provides a detailed comparison of the transcriptomic mechanisms associated with light + temperature induced reproduction both in a brooding coral in nature and a laboratory-maintained sea anemone.

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APPROVED:

Major Professor, Zoology

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Camerron M. Crowder, Author

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1. INTRODUCTION

Reproduction is a central component of the life history strategies of all organisms including the Metazoa. The bioregulatory hormones, receptors, and signaling molecules involved in reproductive pathways are highly characterized in vertebrates but not well understood in early diverging Metazoa. Corals and sea anemones within the class Anthozoa, phylum Cnidaria represent early-diverging metazoans that branched from other Metazoa prior to the protosome-deuterostome split (Park *et al.* 2012). In addition to their ancestral evolutionary position, corals are the foundational species of coral reefs, ecologically important biogenic structures that provide habitat to a diversity of marine species and sustenance to human populations globally (Laurans *et al.* 2013; Pandolfi *et al.* 2011).

Coral reefs are in decline due to a variety of threats that act at both local and global scales. Changes in climate, such as increased sea-surface temperatures, in addition to other anthropogenic stressors, are causing rapid losses of coral reef habitats (Donner 2009; Foden *et al.* 2013; Hoegh-Guldberg *et al.* 2007). Currently, we are experiencing the 3rd and longest recorded global bleaching event in history, with approximately 93% of the Great Barrier Reef affected by bleaching (loss of photosynthetic endosymbiotic algae) and facing mortality due to increase sea-surface temperatures (Globalcoralbleaching.org 2015, ARC Centre of Excellence for Coral Reef Studies 2015)). In addition to impacting biodiversity, these stressors are impairing reproductive success. Corals suffer reduced fecundity and hindered reproductive development in response to increased ocean temperatures (Hoegh-Guldberg 1999; Szmant & Gassman 1990). Several studies have revealed that temperatures exceeding tolerance thresholds reduce the number of spawning

events, polyp fecundity, and gamete quality (Howells *et al.* 2013; McClanahan *et al.* 2009; Michalek-Wagner & Willis 2001). While many studies have examined the ecology and diversity of coral reproduction (Harrison 2011; Harrison & Wallace 1990b), less effort has been devoted to identifying the bioregulatory components that orchestrate reproduction and reproductive timing. Furthermore, little is known about the impacts of environmental variables, such as temperature, on coral reproductive physiology and improving our understanding is important for developing predictions for coral reef health in an era of climate change.

In this dissertation, I explore the effects of both temperature and light on transcriptional changes associated with the timing of reproduction in anthozoans. Through a field-based experiment conducted over an entire lunar month, the effects of temperature and lunar periodicity on reproductive timing and global gene expression profiles were investigated in the model coral *Pocillopora damicornis*. Using a lab-based time-course experiment during light + temperature-induced spawning, temporal changes in gene expression profiles, as well as spatial expression patterns on a selection of differentially expressed genes, were examined in the model sea anemone *Nematostella vectensis*. Together these two approaches provide information as to how light and temperature, individually and synergistically, impact the timing of reproduction and elicit changes in gene expression to orchestrate reproductive events on a cellular level in anthozoans.

The overall aims of this dissertation were to:

1) Evaluate the impacts of elevated temperature and lunar day on the timing of reproduction in a brooding coral.
2) Examine and compare global gene expression profiles during a reproductive cycle in both spawning and brooding anthozoans.
3) Identify gene expression pathways and networks correlated with sensing environmental variables and cueing reproduction in two model anthozoans.

1.1 Coral reproductive ecology

The ecology of coral reproduction has been studied for decades. To date, over 400 species have been categorized as either: hermaphroditic broadcast spawners, gonochoric broadcast spawners, hermaphroditic brooders, or gonochoric brooders, or mixed (both brooders and spawners) (Harrison 2011). The majority of these species, approximately 86%, are spawners, releasing eggs and sperm into the water column. Spawning typically occurs on an annual basis, often within a few hours on a single night (Brady *et al.* 2009; Harrison 2011). The most spectacular examples of spawning are on the Great Barrier Reef and off of western Australia, where multi-species mass spawning occurs on a large scale across reefs within in single night per year (Harrison *et al.* 1984).

Approximately 14% of examined coral species are brooders, where fertilization from sperm is internal and offspring are brooded. Planula larvae are released during a process known as planulation, which typically occurs multiple times annually, often on a monthly basis. The timing of planulation has a direct influence on larval survival, dispersal, and recruitment (Fan *et al.* 2002; Harrison & Wallace 1990b). Reproductive events optimally timed to environmental variables such as temperature and light can

affect larval survival particularly in larvae containing endosymbiotic dinoflagellates (Edmunds *et al.* 2005; Fan *et al.* 2006; Schnitzler *et al.* 2012; Yakovleva *et al.* 2009).

Although little is known about the mechanisms controlling reproduction, multiple environmental variables have been correlated to the timing of reproduction in corals and sea anemones. These variables include temperature, solar irradiance, and tidal cycles associated with seasonality, nocturnal illumination associated with lunar periodicity, and diel light-dark cycles (Babcock *et al.* 1986; Brady *et al.* 2009; Goodbody-Gringley 2010; Goodbody-Gringley & de Putron 2009; Harrison & Wallace 1990b; Jokiel & Guinther 1978; McGuire 1998; Villanueva *et al.* 2011). Although these variables are interrelated, it is unknown if one variable in particular acts as the proximate signaler for gamete and/or larval release. Furthermore, the impacts of changing environmental variables, such as increased sea-surface temperature associated with global climate change on the timing of reproduction have not been empirically evaluated.

1.2 Bioregulatory molecules associated with coral reproduction

Studies have described evidence of cellular signaling events related to reproductive timing in spawning corals. Gene expression profiles of two light-sensing cryptochromes (cry1 and cry2), belonging to an ancient family of blue-light sensing photoreceptors, were characterized in the reef-building coral *Acropora millepora,* with cry2 showing a peak in expression during the full moon (Levy *et al.* 2007; Vize 2009). Because spawning is cued by lunar and daily changes in light intensity and spectral quality, it has been suggested that cryptochromes are involved in this process (Hoadley *et al.* 2011; Levy *et al.* 2007; Reitzel *et al.* 2013b). Similarly, red-photosensitivity has been

documented in coral larvae and three coral opsins (acropsin 1-3), homologous to human rhodopsins, have been characterized in the coral *Acropora palmata,* suggesting that light detection associated with behaviors in corals may be the result of a rhodopsin-based visual system (Mason *et al.* 2012; Mason & Cohen 2012).

In addition, calcium has been described as a mediator of light responsivity in corals, and empirical evidence has shown that changes in [Ca^{2+}], brought on by shifts from day to night, control the time of spawning (Hilton *et al.* 2012). Similarly, recent reports in *A. millepora* corals showed that the lunar cycle influences gene expression, with higher expression occurring during the full moon, and that nocturnal illumination is an important factor in spawning, with light regime changes resulting in a desynchronization of gamete release (Kaniewska *et al.* 2015). Furthermore, this work also suggests that melanopsin-like proteins, neuropeptides and G protein-coupled receptors signaling pathways, acting via calcium, mechanistically coordinate coral spawning at the cellular level (Kaniewska *et al.* 2015; Zoccola & Tambutté 2015).

Little is known about the reproductive physiology of invertebrates, especially cnidarians. As a starting point, it is useful to investigate if homologous genes and bioregulatory molecules of well-studied vertebrate systems are present in cnidarians. Vertebrate systems, such as humans, involve the strict coordination of compartmentalized organs to release neuropeptides, gonadotropins (follicle-stimulating hormone (FHS), lutenizing hormone (LH)) and sex steroids (estrogens, testosterones, and progesterones) to time reproductive events (Figure 1.1). Although it remains unknown if cnidarians synthesize sex steroids *de novo*, homologs to vertebrate sex steroids have been detected

in anthozoan tissues and in some cases correlated with the timing of spawning (Figure 1.1) (Rougée *et al.* 2015; Tarrant *et al.* 2009; Twan *et al.* 2003; Twan *et al.* 2006).

In vertebrates, the neuropeoptide, gonadotropin-releasing hormone (GnRH), begins the cascade of events associated with the hypothalamic-pituitary-gonadal axis. An immunoreactive form of GnRH was detected in the coral *Euphyllia ancora* and induced the release of the vertebrate gonadotropin lutenizing hormone (LH) in a teleost pituitary cell (Twan *et al.* 2006). Estradiol, one of the sex steroids released by the gonads and the major estrogen involved in regulating gametogenesis and reproduction in vertebrates, was detected in coral eggs and surrounding seawater in Australia during a mass spawning event on the Great Barrier Reef (Atkinson & Atkinson 1992). Furthermore, estrone and estradiol were detected in the scleractinian coral *Montipora verrucosa* (now *M. capitata*) (Tarrant *et al.* 1999). In addition, testosterone, progesterone, and estradiol were detected in the tissues of the soft coral *Sinularia polydactyla* during mass spawning and were shown to vary in their concentrations on a monthly basis (Slattery *et al.* 1999). The scleractinian coral, *E. ancora*, displayed peak levels of estradiol, glucuronided (water soluble) estradiol, and testosterone concentrations just prior to spawning (Twan *et al.* 2006). Testosterone, estradiol, and progesterone were also detected in the anemone *Aiptasia diaphana*, with testosterone peaking in concentration prior to the appearance of gametes and estradiol steadily rising and peaking during oocyte maturation (Armoza-Zvuloni *et al.* 2014). All of these studies used similar methods of hormone detection including radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). However, there have been no high-throughout structural analyses of these sex steroids to

date, therefore it is still unknown if the hormones detected in anthozoans are identical to sex steroids in vertebrate systems.

Enzyme activity associated with vertebrate steroidogenesis pathways has been detected in anthozoans. The coral *M. capitata* demonstrates 17-β hydroxysteroid dehydrogenase (HSD) and 5α-reductase activity (Tarrant *et al.* 2003) and aromatase activity was detected in *E. ancora* (Twan *et al.* 2006). Although, the activity of aromatase or aromatase-like enzymes has been described, homologs to the aromatase gene, known as cyp19, have not been identified in the genome of the sea anemone *Nematostella vectensis* (Goldstone 2008; Tarrant *et al.* 2009).

1.3 Pocillopora damicornis and *Nematostella vectensis* as cnidarian reproductive models

P. damicornis has been established as a model coral for investigating reproductive timing, in part due to the frequency of its planulation. Multiple studies investigating the lunar periodicity of planulation in brooding corals have focused on *P. damicornis,* which generally planulates on a monthly basis (Fan *et al.* 2002; Fan *et al.* 2006; Jokiel & Guinther 1978; Richmond & Jokiel 1984; Tanner 1996; Villanueva *et al.* 2008). Collectively, these observations show variability in planulation patterns, with *P. damicornis* releasing planulae at every lunar phase during monthly lunar reproductive cycles, with some consistencies within geographical locations. This variation in lunar timing of reproduction has led to questions regarding the impacts of temperature on the lunar timing of planula release. Empirical evaluation of the impact of temperature on the

lunar timing of planulation is needed to improve our understanding of the environmental variables that control reproductive timing in corals.

N. vectensis is an established model system for developmental (Helm *et al.* 2013; Tulin *et al.* 2013), neurological (Matus *et al.* 2007) and steroid metabolism studies (Tarrant *et al.* 2009) in cnidarians, in part due to the availability of a published genome (Putnam *et al.* 2007). Although *N. vectensis* easily spawns in the laboratory (Stefanik *et al.* 2013), to date, no study has investigated gene expression patterns and signaling pathways associated with reproduction in this species. The cellular techniques and protocols already established including *in-situ* hybridization (Martindale *et al.* 2004; Matus *et al.* 2007) and microinjection of morpholino gene knockdowns (Layden *et al.* 2013) sets the stage for rigorous functional analysis of reproductive processes in this cnidarian model.

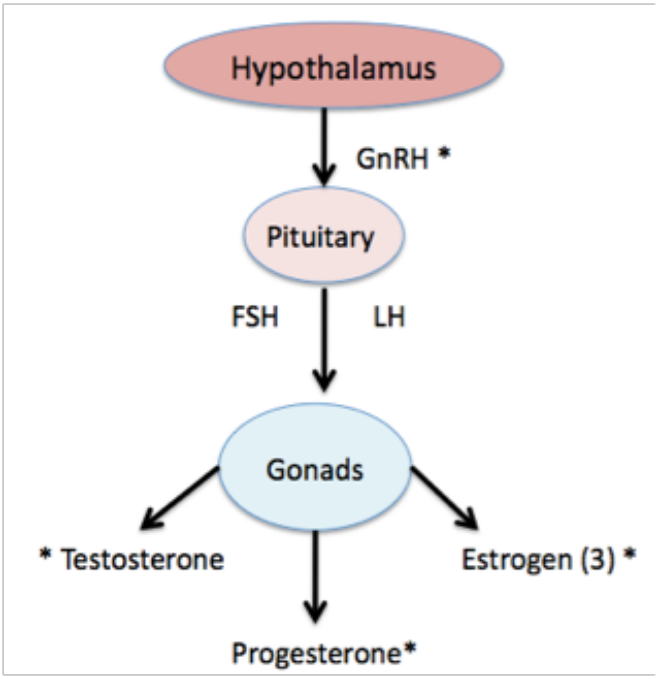

Figure 1.1 Depiction of the vertebrate hypothalamic-pituitary-gonadal axis. Circles represent organs in the axis, hormones emerging individual organs are denoted with arrows, and asterisks mark hormones that have been detected in cnidarian tissues.

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2. ELEVATED TEMPERATURE ALTERS THE LUNAR PERIODICITY OF PLANULATION IN THE BROODING CORAL *POCILLOPORA DAMICORNIS*

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2.1 SUMMARY

Reproductive timing in corals is associated with environmental variables including temperature, lunar periodicity, and seasonality. Although it is clear that these variables are interrelated, it remains unknown if one variable in particular acts as the proximate signaler for gamete and or larval release. Furthermore, in an era of global warming, the degree to which increases in ocean temperatures will disrupt normal reproductive patterns in corals remains unknown. *Pocillopora damicornis,* a brooding coral widely distributed in the Indo-Pacific, has been the subject of multiple reproductive ecology studies that show correlations between temperature, lunar periodicity, and reproductive timing although to date, no study has empirically measured changes in reproductive timing associated with increased seawater temperature. In this study, the effect of increased seawater temperature on the timing of planula release was examined during the lunar cycles of March and June 2012. To examine these effects, twelve brooding corals were removed from Hobihu reef in Nanwan Bay, southern Taiwan and placed in 23 and 28°C controlled temperature treatment tanks. For both seasons, the timing of planulation was found to be plastic, with elevated temperature resulting in significantly earlier peaks of planula release compared to the low temperature treatment. This suggests that temperature alone can influence the timing of larval release in *P. damicornis* in Nanwan Bay. Therefore, it is expected that continuing increases in ocean temperature will result in earlier timing of reproductive events in corals, leading to variations in reproductive success and recruitment patterns.

2.2 INTRODUCTION

Reproductive timing is a critical factor in coral reproductive success and has been correlated to multiple environmental variables including those associated with seasonality such as temperature, solar irradiance, tidal cycles, nocturnal illumination associated with lunar periodicity, and light-dark cycles corresponding with diel fluctuations (Babcock *et al.* 1986; Brady *et al.* 2009; Goodbody-Gringley 2010; Goodbody-Gringley & de Putron 2009; Harrison & Wallace 1990a; Jokiel & Guinther 1978; McGuire 1998; Villanueva *et al.* 2011). Previous studies have effectively demonstrated correlations between environmental variables and coral reproduction, however, examining the direct causality of individual variables on the timing of reproduction is critical to understanding the mechanisms controlling reproductive timing. Determining which variables are vital for the coordination of reproductive events will reveal information underlying coral reproductive function. Corals are both the bio-engineers and foundational primary producers of coral reefs ecosystems and therefore, understanding how environmental variables affect timing of reproduction is essential for predicting future impacts of climate change on coral reef ecosystem stability.

Temperature has been shown to be a critical variable affecting coral reproductive success. Corals of all life stages are negatively affected by increasing sea-surface temperature attributed to global climate change (Donner 2009; Hoegh-Guldberg *et al.* 2007; Randall & Szmant 2009). While significant effort has been dedicated to describing the effects of temperature on the physiology and ecology of corals, less attention has focused on the effects of elevated temperature on coral reproductive timing and success (Hoegh-Guldberg *et al.* 2007; Mayfield *et al.* 2013a). Recent studies have revealed that temperatures exceeding tolerance thresholds reduce polyp fecundity, gametic quality

(McClanahan *et al.* 2009; Michalek-Wagner & Willis 2001) and the number of reproductive events in corals (Howells *et al.* 2013). While it is evident that elevated temperatures can impair reproductive processes, predictable seasonal fluctuations in temperature might be a key component controlling the timing of reproduction. Multiple field studies have observed correlations between temperature and reproductive timing (De Putron & Ryland 2009; Nozawa & Harrison 2007). This correlation could demonstrate that some corals are displaying reproductive plasticity or alterations in the timing of release to adapt to changes, which could be an important mechanism for survival with rising ocean temperatures associated with climate change. Understanding how temperature affects the timing of reproduction will provide information as to how continued increases in sea-surface temperature may further alter reproductive processes and patterns in corals.

Approximately 15% of coral species brood internally fertilized larvae (planulae) that are released during a process known as planulation. Planulation typically occurs multiple times annually and in some cases on a monthly basis (Fan *et al.* 2002; Harrison 2011). Planulae are often buoyant and have the capacity for wide dispersal, but are also able to quickly settle upon release leading to fast rates of colonization (Harrison & Wallace 1990a; Villanueva *et al.* 2008). The timing of planulation has a direct influence on larval survival, dispersal, and recruitment (Fan *et al.* 2002; Harrison & Wallace 1990a). Reproductive events optimally timed to environmental variables such as temperature and light, can affect larval survival particularly in larvae containing symbiotic dinoflagellates (Edmunds *et al.* 2005; Fan *et al.* 2006; Schnitzler *et al.* 2012; Yakovleva *et al.* 2009).

The widely studied and ubiquitously distributed brooding coral *P. damicornis*, has been the subject of multiple studies investigating reproduction, particularly reproductive synchrony associated with lunar periodicity (Fan *et al.* 2002; Jokiel *et al.* 1985; Richmond & Jokiel 1984; Tanner 1996; Villanueva *et al.* 2008). Collectively, these observations show variability in planulation patterns. *P. damicornis* has been shown to release planulae at every lunar phase during monthly lunar reproductive cycles with some consistencies observed within similar geographical locations. This diversity in the timing of planulation between different geographical regions may be the result of phenotypic plasticity that increases fitness (Via *et al.* 1995). However, to date, little is known about phenotypic plasticity of coral reproductive timing, and specifically how this plasticity may be driven by environmental variables, such as rising ocean temperatures.

In the context of the ongoing debate as to the effect of individual environmental variables on the timing of planulation, it is timely to ask if changes in temperature can directly alter planulation patterns in *P. damicornis*. The purpose of this study was to determine the effect of increased seawater temperature on the timing of planulation within a single lunar cycle and between lunar cycles of different seasons. To investigate this, *P. damicornis* colonies were monitored in temperature-controlled tanks exposed to natural lunar cycles with seawater temperature set to either 23°C (low) or 28°C (high). For both seasons, the timing of planulation was found to be plastic, with elevated temperature treatments resulting in significantly earlier peaks of planula release. This suggests that temperature overrides lunar periodicity to affect the timing of larval release in *P. damicornis* in Nanwan Bay.

2.3 METHODS

2.3.1 Coral Collection

Twelve adult colonies of *P. damicornis* were randomly collected with permission from the Kenting National Park, Taiwan from a 10-meter area at Houbihu reef (21°56.799'N, 120°44.968'E) in Nanwan Bay, Taiwan in lunar March and June 2012. Colonies ranged in size from 7-15 cm in diameter and were removed from reefs 4-5 meters in depth. Within an hour after collection, colonies were transported back to The National Museum of Marine Biology and Aquarium (NMMBA) in Checheng, Taiwan.

2.3.2 Experimental Design

Six 150-liter tank mesocosms situated within a larger (30 ton) aquarium system as described by Mayfield et al. (2013) were used in this study. Three of the tanks were maintained at 23°C (mean winter ocean temperature) and three were maintained at 28°C (mean summer ocean temperature). Two coral colonies were randomly selected and assigned to each tank. Tanks were covered with shade cloth and exposed to natural outdoor sunlight and received a constant supply of sand-filtered seawater. Corals were allowed to acclimate to the experimental temperature conditions for one week prior to monitoring of planulation.

Monitoring of planulation began on lunar day 1 (new moon), March 22, 2012 (lunar March) and July 19, 2012 (lunar June). Every evening during the lunar cycle, corals from each tank were individually placed into flow-through containers surrounded by 100 μm mesh plankton netting. Flotation devices were placed inside the netting to prevent nets from touching coral colonies. Each morning, nets were removed and the

total number of planulae inside the netting was counted. Tank temperatures were recorded every 10 minutes using a HOBO temperature logger throughout lunar March and June 2012 (Figure 2.1).

2.3.3 Data Analysis

The percent of the total number of planulae released each day was calculated as the total number of planulae released per individual colony per day (mean ± standard deviation, n = 6 colonies per temperature treatment) divided by the total number of planulae released by each colony during the monthly reproductive cycle. Raleigh's test (Zar 1999) was used to test the null hypothesis that planula release occurred uniformly throughout the lunar cycle. If the null hypothesis was rejected, then the mean lunar day (MLD) and angular deviation of planulation were calculated using circular statistics (Zar 1999) to determine lunar periodicity of planulation.

A one-way nested ANOVA was performed to compare MLD, angular deviation, and individual coral colony size, with tank nested as a factor within temperature treatments. A mixed effect one-way ANOVA for completely random design (CRD) with subsamples was completed to compare differences in the total number of planulae released between temperature treatments. Statistical analyses were completed using the statistical package R (R Foundation for Statistical Computing Vienna, Austria).

2.4 RESULTS

Raleigh's test for uniform distribution indicated that planula release did not occur uniformly throughout the lunar cycle for either temperature treatment over both lunar

March and June ($p < 0.001$ for all tests) (Table 2.1). Differences were observed in the timing of planulation, measured as the percentage of planulae released per day with corals at 28°C releasing planulae earlier in the lunar cycle than those at 23°C for both lunar March and June (Figure 2.2). In lunar March, peak percentage of release occurred on lunar day 8 for the 28°C treatment, compared to lunar day 19 for the 23°C treatment. In lunar June, peak percentage of release was observed as two smaller peaks on lunar day 6 and 10 for the 28°C treatment, compared to a more significant peak at lunar day 12 for the 23°C treatment.

MLD of planulation closely resembled patterns in percentage of planulae released whereas angular deviation, a measure of dispersion, was only significant in lunar March and not lunar June where there was a larger spread in days of release (Table 2.1). In lunar March, the average MLD and angular deviation of release were 17.5 ± 1.1 and 23.7 ± 5.4 for the 23°C treatment and 8.4 ± 0.7 and 16.1 ± 1.9 for the 28°C treatment, respectively. In lunar June, the average MLD and angular deviation of release were 12.5 ± 1.0 and 23.8 ± 8.6 for the 23°C treatment and 7.7 ± 1.7 and 26.5 ± 10.4 for the 28°C treatment, respectively. One-way nested ANOVAs showed significant differences in MLD ($p < 0.001$) and angular deviation ($p = 0.004$) between temperature treatments in lunar March and significant differences in MLD ($p = 0.003$), but not angular deviation between temperature treatments in lunar June (Table 2.2).

Additionally, our results indicate that there were substantially more planulae released in lunar March (21,559 at 23°C and 8,724 at 28°C) compared to lunar June (1,077 at 23°C and 762 at 28°C) (Table 2.1). Significant differences were not found for

colony size or total number of planulae released between temperature treatments for either lunar March or June.

2.5 DISCUSSION

2.5.1 Influence of Seasonality

In this study we observed a substantial difference in the total number of planulae released between lunar March and June 2012. This observed difference in the total number of planule released is likely due to seasonality. This hypothesis is consistent with previous findings showing that there were differences in planulae abundance between seasons in Pocilloporid corals in the Northwestern Philippines with dry seasons (March-May) having higher numbers of planulae released then wet seasons in (June-October) (Villanueva *et al.* 2008). Differences in total number of planulae released in our study could also be attributed to colony size, since corals used in lunar March were, on average, 1 cm larger than those used in lunar June (Table 2.1). However, differences in planulation abundances are not expected to affect our results because timing of planulation was examined individually for each month.

2.5.2 Timing of Planulation

Lunar periodicity has been correlated with the timing of reproduction for multiple coral species (Harrison & Wallace 1990a; Harrison 2011) especially *P. damicornis*, where the timing of planulation is consistently linked to lunar phases (Fan *et al.* 2002; Richmond & Jokiel 1984; Tanner 1996; Villanueva *et al.* 2008). While lunar periodicity likely does play a role in the timing of reproduction, in some cases with good

predictability, other environmental factors may be able to disrupt these cycles. In our study we directly tested the influence of low (23°C) and high (28°C) temperatures on reproductive timing, and show that changes in temperature have the capacity to significantly alter the timing of planulation. Clear differences were observed in the timing of planulation, shown as the percentage of planulae released each day (Figure 2.2), and temperature was found to have a significant impact on the MLD of planulation for both lunar March and June (Table 2.1 & 2.2). The observed variations in lunar day of release between temperature treatments suggests that elevated temperature overrides other cues, such as lunar periodicity, to drive timing of release.

Early release in the high temperature treatment suggests that temperature has a Q_{10} effect (the change in rate of biological processes with an increase in temperature) on the reproductive physiology of the adult coral and/or the developing planulae, resulting in an acceleration of release (Schmidt-Nielsen 1997). This hypothesis is supported by a previous study on a broadcast spawning coral, *Echniopora lamellosa*, in Taiwan showing that reproductive processes, such as gametogenesis and spawning are plastic and can be accelerated by increasing seawater temperature (Fan & Dai 1999). Another study on Caribbean corals, within the genus *Madracis*, found that maturation of gametes was positively correlated with increases in seawater temperature, indicating that observed changes in timing could be attributed to internal cues associated with gametogenesis (Vermeij 2004).

Alternatively, shifts in the timing of planulation could be a result of negative changes in planula physiology. This hypothesis is supported by multiple studies that have observed decreases in larval survival with elevated temperature. Larvae of the Hawaiian

coral *Fungia scutaria* exposed to 27, 29, and 31°C showed gradual decreases in survivorship with animals incubated at 31°C having the highest rates of mortality (Schnitzler *et al.* 2012). Another study conducted on *P. damicornis* larvae in Taiwan found 28°C to be the thermal threshold for maximum respiration in planulae, with higher temperatures leading to reduced respiration and likely metabolic depression (Edmunds *et al.* 2011). Additionally, a 5-fold decrease in survival was observed with a 1.5°C increase in temperature in *Acropora palmata* embryos and larvae from the Caribbean (Randall & Szmant 2009). Although success of planulae after release was not assessed in this study, it has been shown that early-released planulae have lower settlement rates than those released later within a single reproductive cycle (Fan *et al.* 2002).

2.5.3 Reproductive Plasticity

Our results reveal that temperature can act as a driver for plasticity in reproductive timing. Reproductive plasticity can enhance individual success in harsh or fluctuating environments (Via *et al.* 1995). While our study did not examine reproductive plasticity specifically in an adaptive context, we postulate that the phenotypic plasticity observed in this study may suggest capacity for an adaptive response to elevated temperatures in corals. This ability to shift reproductive timing in high temperature environments may indicate that climate change induced increases in ocean temperature may not be detrimental to reproduction, but rather simply alters its timing. Our findings also indicate that such shifts in timing can occur relatively quickly, as reproductive plasticity was observed over a single reproductive cycle. This is similar to findings observed in *E. lamellosa*, that show early spawning, when transplanted from colder

northern to warmer southern Taiwan (Fan *et al.* 2002). Understanding reproductive plasticity in corals is important because plasticity may give corals the flexibility they need to be successful in a changing climate. However, many questions remain about how reproductive plasticity will influence the fate of corals, and their ecosystems, in the long term.

2.6 CONCLUSIONS

Our results provide empirical evidence that a 5°C increase in temperature, accelerates the timing of planula release in *P. damicornis* in Nanwan Bay, Taiwan. This reveals that there is plasticity in the timing of reproduction and these changes can occur rapidly, within a single lunar reproductive cycle. These results highlight the reality that increases in ocean temperature have the capacity to disrupt patterns of planulation in corals. Depending on how shifts in the timing of planulation correlate with other environmental variables and conditions, these alterations could lead to increased planulae mortality and decreased recruitment success, or may be indicative of the potential for adaptation to warming ocean temperatures. Understanding the affect of temperature on reproductive timing in *P. damicornis* provides information that can be used to predict patterns in reproductive success and colonization in a future of rapidly changing ocean climate conditions.

Table 2.1 Mean lunar day of planulation metrics.
Results of Raleigh's test for uniform distribution of planula release by *Pocillopora damicornis* colonies during March and June lunar cycles. Mean lunar day (MLD) ± standard deviation, angular deviation of release ± standard deviation, range of Raleigh's test statistic (z), *p* value for Raleigh's test (p), total number of planulae released (n) by all colonies, number (n) of colonies releasing planulae, and average colony diameter (cm) ± standard deviation.

	Lunar March		Lunar June	
	23°C	28°C	23°C	28°C
MLD	17.5 ± 1.1	8.4 ± 0.7	12.5 ± 1.0	7.74 ± 1.7
Angular Deviation	23.7 ± 5.4	16.1 ± 1.9	23.8 ± 8.6	26.5 ± 10.4
z	386-7261	2133-4638	93-208	11-291
p	< 0.001	< 0.001	< 0.001	< 0.001
n (Planulae)	21,559	8,724	1,077	762
Colony Size (cm)	10.4 ± 1.3	11.1 ± 1.8	9.7 ± 1.1	9.8 ± 1.4

Table 2.2 Planulation ANOVA for both lunar March and June. One-way nested ANOVA reporting mean lunar day (MLD), angular deviation, individual coral colony size, and number of planulae released during lunar March and June.

	Lunar March					Lunar June				
Mean Lunar Day	DF	SS	MS	F	P	DF	SS	MS	F	P
Temperature	1	251.42	251.42	553.52	3.87E-07	1	58.36	58.36	55.6	3.00E-04
Temperature:factor(Tank)	4	5.75	1.44	3.16	0.1	4	13.26	3.31	3.16	0.10
Residuals	6	2.73	0.45			6	6.30	1.05		
Angular Deviation										
Temperature	1	170.69	170.69	20.18	4.14E-03	1	21.90	21.93	0.68	0.44
Temperature:factor(Tank)	4	115.41	28.85	3.41	0.09	4	817.90	204.48	6.38	0.02
Residuals	6	50.75	8.46			6	192.40	32.07		
Colony Size										
Temperature	1	1.31	1.31	0.37	0.56	1	0.04	0.04	0.04	0.86
Temperature:factor(Tank)	4	3.96	0.99	0.28	0.88	4	9.35	2.34	2.05	0.21
Residuals	6	21.05	3.51			6	6.86	1.14		
Planulae										
Temperature	1	13,728	13,728	5.20	0.08	1	8,269	8,269	0.72	0.44
Temperature:factor(Tank)	4	10,555	26,388			4	45,892	11,473		
Residuals	6	26,936	44,894			6	43,150	7,192		

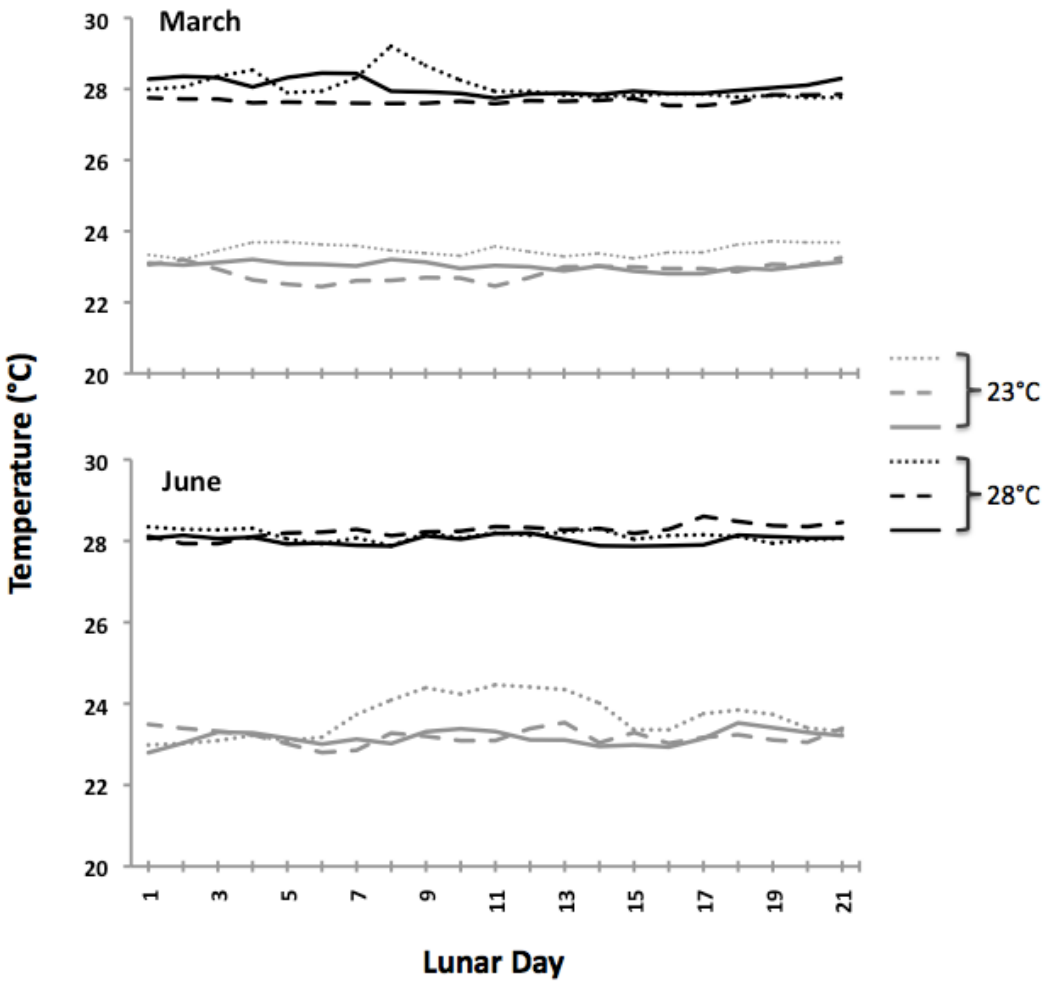

Figure 2.1 Experimental tank temperatures for lunar March and June. Average daily tank temperatures for both 23 and 28°C temperature treatments in lunar March and June 2012. Measurements were taken every 10 minutes and averaged for each lunar day.

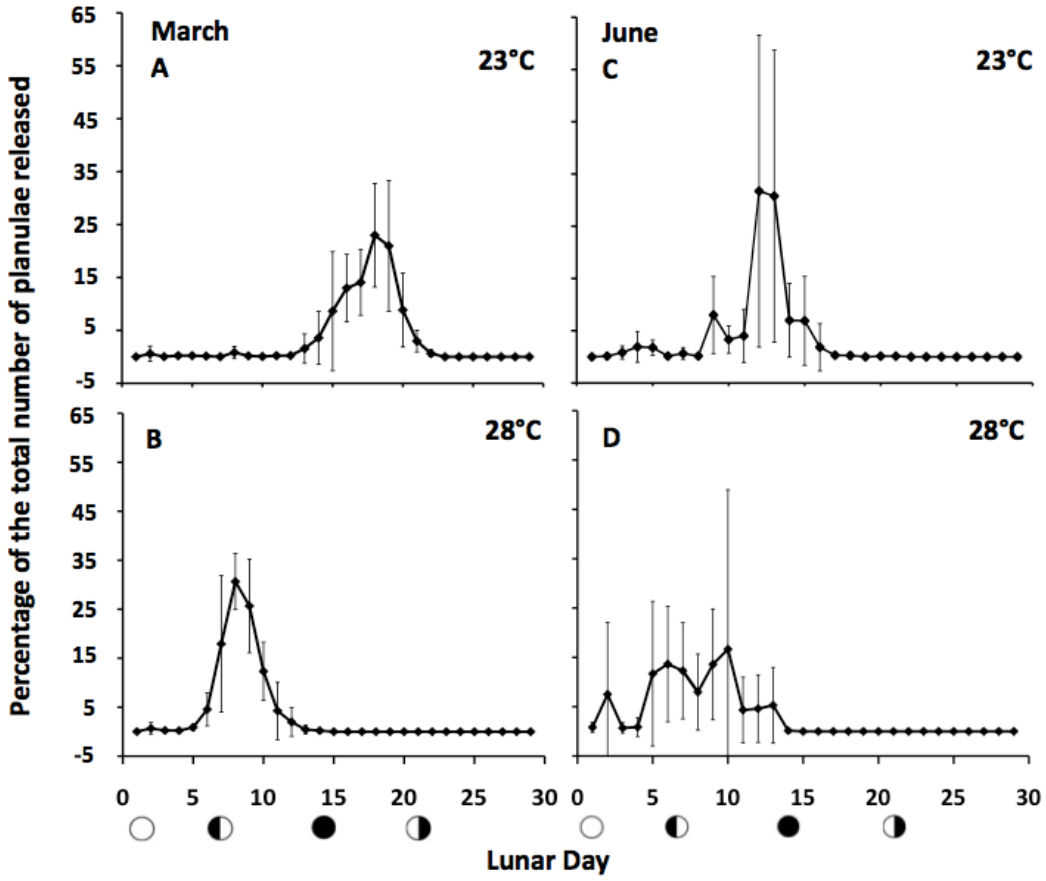

Figure 2.2 Percentage of total planulae released in lunar March and June. Percent of P. damicornis planulae released each day during lunar March (A and B) and Lunar June (C and D) 2012 reproductive cycles for colonies incubated at 23 (A and C) and 28°C (B and D). Points represent means of six colonies ± SE. Moon symbols represent lunar phases (new, 1st quarter, full, and last quarter).

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3. IMPACTS OF TEMPERATURE AND LUNAR DAY ON GENE EXPRESSION PROFILES DURING A MONTHLY REPRODUCTIVE CYCLE IN THE BROODING CORAL POCILLOPORA DAMICORNIS

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3.1 SUMMARY

Reproductive timing in brooding corals has been correlated to temperature and lunar irradiance, but the mechanisms by which corals transduce these environmental variables into molecular signals is unknown. To gain insight into these processes, global gene expression profiles in the coral *Pocillopora damicornis* were examined (via RNA-Seq) across lunar phases and between temperature treatments, during a monthly planulation cycle. The interaction of temperature and lunar day together had the largest influence on gene expression. Mean timing of planulation, which occurred at lunar day 7.4 for 28°C and 12.5 23°C-treated animals, was associated with an up-regulation of transcripts in individual temperature treatments. Individual transcriptomic profiles at the time of planulation were compared between temperature treatments. Comparisons of temperature-independent profiles showed a complete disruption of expression patterns associated with planulation. Gene functions, inferred from homologous matches to online databases, suggest complex neuropeptide signaling, with calcium as a central mediator, acting through tyrosine kinase and G protein-coupled receptor pathways. This work contributes to our understanding of coral reproductive physiology and the impacts of environmental variables on coral reproductive pathways.

3.2 INTRODUCTION

Corals are the foundational species of coral reefs and are in mass decline due to a variety of threats that act at both local and global scales. Changes in climate, such as increased sea-surface temperatures, in addition to other anthropogenic stressors are increasing in frequency and are causing rapid losses of coral reef habitats

(Donner 2009; Foden *et al.* 2013; Hoegh-Guldberg *et al.* 2007; Manzello 2015). In addition to impacting ecosystem structure and biodiversity, these stressors are impairing reproductive success. Corals suffer reduced fecundity and hindered reproductive development in response to elevated ocean temperatures (Hoegh-Guldberg 1999; Szmant & Gassman 1990). Temperatures exceeding tolerance thresholds reduce the number of spawning events, polyp fecundity, and quality of gametes (Howells *et al.* 2013; McClanahan *et al.* 2009; Michalek-Wagner & Willis 2001).

The timing of reproduction, shown to be synchronized with lunar and diel-light cycles (Fan *et al.* 2002) has been documented to shift, in some cases as much as five days, when exposed to prolonged elevated temperature (Crowder *et al.* 2014; Paxton *et al.* 2015). Although these changes in reproductive phenotypes are observed, the impact that temperature has on coral reproductive physiology is not well understood. Gaining insight into how elevated temperature affects intracellular reproductive regulatory physiology will help predict the future of coral reproductive success in a warming ocean.

Two general forms of sexual reproduction occur in corals, spawning and brooding. The majority of corals (approximately 86%) are spawners, which synchronously release gametes into the water column, often once annually, for external fertilization and development (Harrison 2011). The remaining 14% are brooders that release internally fertilized larvae (planulae), generally multiple times annually, in a process known as planulation (Harrison 2011). Although little is known about the internal mechanisms controlling reproduction, multiple environmental variables have been correlated to the timing of reproductive events. These include temperature, solar irradiance, and tidal cycles associated with seasonality, nocturnal illumination associated

with lunar periodicity, and diel light:dark cycles (Babcock *et al.* 1986; Brady *et al.* 2009; Goodbody-Gringley 2010; Goodbody-Gringley & de Putron 2009; Harrison & Wallace 1990b; Jokiel & Guinther 1978; McGuire 1998; Villanueva *et al.* 2011).

The few studies investigating the transduction of these environmental variables into molecular signals have been conducted on spawning corals. Gene expression profiles of two light-sensing cryptochromes (cry1 and cry2), belonging to an ancestral family of blue-light sensing photoreceptors, were described in the reef-building coral *Acropora millepora*, with cry2 showing a peak in expression during the full moon (Levy *et al.* 2007; Vize 2009). Because spawning is cued by lunar and daily changes in light intensity and spectral quality, it has been suggested that cryptochromes are involved in this process (Hoadley *et al.* 2011; Levy *et al.* 2007; Reitzel *et al.* 2013). In addition, calcium has been described as a mediator of light responsivity in corals, and empirical evidence has shown that changes in $[Ca^{2+}]$, brought on by shifts from day to night, control the time of spawning (Hilton *et al.* 2012). Similarly, recent reports in *A. millepora* corals showed that the lunar cycle influences gene expression, with higher expression occurring during the full moon, and that nocturnal illumination is an important factor in spawning, with light regime changes resulting in a desynchronization of gamete release (Kaniewska *et al.* 2015). Furthermore, this work also suggests that melanopsin-like proteins, neuropeptides and G protein-coupled receptors signaling pathways, acting via calcium, mechanistically coordinate coral spawning at the cellular level (Kaniewska *et al.* 2015; Zoccola & Tambutté 2015). Comparisons of the transcriptomic response associated with planulation in a brooding coral that exhibits lunar periodicity of planula release, will provide a

greater understanding of the transduction of environmental variables into molecular signals cueing reproductive events in corals.

Comparative approaches in coral reproductive physiology have looked to the well-characterized vertebrate systems for examples of types of bioregulatory molecules cueing reproductive processes. Using immunological methods, vertebrate sex steroids have been detected within tissues of both spawning and brooding corals (Armoza-Zvuloni *et al.* 2012; Rougée *et al.* 2015; Slattery *et al.* 1997; Tarrant *et al.* 1999; Twan *et al.* 2003; Twan *et al.* 2006). In the spawning coral *Euphyllia ancora*, concentrations of 17β-estradiol (E2) and testosterone (T), in free and glucuronided forms, were detected throughout the year, with E2, glucuronided E2, and T peaking in concentration just prior to spawning (Twan *et al.* 2006). Similarly, estrogens, both (E2) and estrone (E1), T, and progesterone were detected over two lunar reproductive cycles in tissues of the brooding coral *Pocillopora damicornis,* however significant fluctuations in hormonal concentrations were not observed (Rougée *et al.* 2015). Currently, there is little genomic evidence to support the hypothesis that vertebrate-type steroids act as bioregulatory molecules in corals and this reveals a need for unbiased hypothesis-generating approaches to identify the genes and pathways coordinating reproduction in corals.

The purpose of this study was to examine the transcriptomic events associated with lunar day in a brooding coral during a monthly planulation cycle, and to investigate if changes in temperature impact reproductive physiology at the molecular level. In order to identify how elevated temperature alters the reproductive physiology of planulation, the molecular events coordinating planulation need to be understood. Investigating how temperature and lunar day alter gene expression individually and synergistically across a

monthly reproductive cycle will provide information on the effects of these variables on the molecular mechanisms and signaling cascades associated with reproduction. We examined the effects of lunar day and temperature on *P. damicornis*, a widely distributed coral that is a model for studies examining reproductive timing in corals due to the predictability and multi-annual occurrence of its reproductive events (Fan *et al.* 2002; Fan *et al.* 2006; Jokiel & Guinther 1978; Richmond & Jokiel 1984; Tanner 1996; Villanueva *et al.* 2008). Colonies of *P. damicornis* were exposed to natural lunar cycles with seawater temperature set to either 23°C (low) or 28°C (high) for one lunar month. Differences in global gene expression profiles on different lunar days were compared between 23 and 28°C treatments using RNA-Seq analysis. This study provides information as to how these environmental variables affect molecular events underpinning reproductive timing in *P. damicornis* and provides information that can be used to predict patterns in reproductive success in the future of a rapidly changing ocean.

3.3 METHODS

3.3.1 Coral collection and experimental design

Twenty-four adult *P. damicornis* colonies were collected in June 2012 from a 10-meter area at Hobihu reef (21°56.799'N, 120°44.968'E) in Nanwan Bay, Taiwan with permission from the Kenting National Park. Colonies ranged in diameter from 7 to 15 cm and were removed from reefs 4 to 5 meters in depth. Within an hour after collection, colonies were transported back to The National Museum of Marine Biology and Aquarium (NMMBA) in Checheng, Taiwan.

Six 150-liter tank mesocosms, previously described by Mayfield *et al.* (2013b), were used in this study. Three of the tanks were maintained at a low temperature (23°C) and three of the tanks were kept at a high temperature (28°C) as described by Crowder et al. (2014). These temperatures were chosen to represent the thermal range that corals in Hobihu Bay experience on a monthly basis, where mean monthly temperature fluctuates from 23.5 to 28°C (Fan & Dai 1995). Four coral colonies were arbitrarily assigned to each of the six individual tanks. Corals were allowed to acclimate from ambient to the experimental temperature conditions for one week prior to lunar day 1 (July 19, 2012, lunar June). Water temperatures were recorded every 10 minutes using a HOBO temperature logger during the lunar cycle.

3.3.2 Sample collection and RNA extraction

Individual coral colonies were flash frozen in liquid nitrogen immediately upon removal from tanks in the morning on lunar day 1 (new moon), 7 (1st quarter moon), 14 (full moon), and 21 (last quarter moon). Tissue was removed using an airbrush apparatus and stored in RNA*later*® Stabilization Solution (Qiagen, CA, USA). Total RNA was extracted and purified from coral tissue using a combination of the TRIzol® RNA isolation (Life Technologies, CA, USA) and the RNeasy Mini Kit (Qiagen, CA, USA) protocols. Extracted RNA was DNase-treated using a TURBO DNA-Free Kit (Ambion, CA, USA) to remove genomic DNA contamination. RNA quantity and quality was assessed using the NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, MA, USA) and gel electrophoresis.

3.3.3 Reference transcriptome assembly and annotation

A reference transcriptome for *P. damicornis* was assembled using raw reads deposited on National Center for Biotechnology Information (NCBI). Details of the generation and sequencing of these raw reads are described by Vidal-Dupiol *et al.* (2013). Raw reads were filtered to eliminate low quality reads (> 20 positions with Phred scores < 20) and any reads containing adaptor sequences or homopolymer repeats (e.g., poly-A tails) were removed using custom Perl scripts (https://github.com/Eli-Meyer). Filtered reads were assembled using default settings in Trinity v2.0.2 (RNASeq_r2012-03017), a de Bruijn graph-based assembler that compiles transcripts in component groups (isogroups), which represent the collection of transcripts originating from a single gene (Grabherr *et al.* 2011). Transcriptome annotation was completed based on sequence comparisons to online databases using BLAST+ available from the NCBI (Package version 2.2.29) (Altschul *et al.* 1990) as described by (Kitchen *et al.* 2015). Each transcript was assigned a gene name through a comparison to UniProt protein sequence databases (SwissProt and TREMBL) using BLASTx (E value cutoff of 10^{-5}) (Consortium 2011). Gene names were based on best matches, excluding uninformative information (e.g., unknown, uncharacterized, or hypothetical). Gene Ontology (GO) terms, downloaded from the Gene Ontology website (Ashburner *et al.* 2000), were assigned based on GO-UniProt associations of best match and categorization of GO terms (biological, cellular, and molecular was completed using CateoGOrizer software (Hu *et al.* 2008). KEGG Orthology (KO) IDs were obtained by mapping to the Kyoto Encyclopedia of Genes and Genomes (KEGG) using the KAAS BBH method (Moriya *et*

al. 2007) to obtain pathway information and KEGG enrichment analysis was performed using the R package clusterProfiler (Yu *et al.* 2012).

3.3.4 Preparation of cDNA for Illumina sequencing

One μg of intact RNA from each sample was heat-fragmented at 95°C for 5 minutes. For samples with degraded RNA (below approximately 400 bp) no further heat fragmentation with required. Two samples containing less than 1 μg of total RNA were excluded from the analysis. Complementary DNA (cDNA) libraries were synthesized following a 3' tag-based RNA-Seq protocol described by (Meyer *et al.* 2011). Individual cDNA libraries were labeled with sequence-specific barcode adaptors and final cDNA samples ranging in size from 250-350 bp were excised from a 2% agarose gel.

3.3.5 Sequencing, processing and mapping

cDNA libraries were pooled and sequenced in a single lane on an Illumina HiSeq 2000 at the Center for Genomics and Research and Biocomputing (CGRB) at Oregon State University. Single-end sequencing of 22 cDNA libraries produced reads 50 bp in length. Sequences were de-multiplexed based on their sample-specific barcode adaptors. Raw sequences were filtered and trimmed as described above. High quality reads were mapped to the reference transcriptome using the software package SHRiMP (version 2.2.2) (David *et al.* 2011; Rumble *et al.* 2009). Alignments were further filtered using custom Perl scripts (https://github.com/Eli-Meyer) to exclude short, weak, or ambiguous alignments (reads showing equally strong alignments to more than one gene).

3.3.6 Identification of differentially expressed genes and profiling analysis

All calculations and statistical tests were conducted using R (version 3.2.1). Statistical comparisons of RNA-Seq count data typically use negative binomial models that account for the over-dispersed characteristics. However, currently available software implementing this approach does not model random factors as required for 'repeated measures' analysis. To balance these concerns, we transformed the counts data using a variance stabilized procedure (vsd) in the R module DESeq (version 1.14.0) (Anders & Huber 2010, 2012) designed to transform count data from RNA-Seq into weighted expression values suitable for linear modeling. The R package lme4 (Bates *et al.* 2014) was then used to determine differential expression of the normalized count data with lunar day, temperature, and the interaction of lunar day and temperature using a linear mixed-effects model. Lme4 was chosen for this analysis because it allowed for a mixed model design with lunar day and temperature as fixed factors and tank as a random factor nested within temperature. Raw *p* values were adjusted for multiple test corrections and false discovery rate using the Benjamini Hochberg procedure and a transcript was considered differentially expressed if the resulting adjusted *p* value was < 0.05.

The software program Short Time-series Expression Miner (STEM) was used to examine temporal patterns in gene expression and group transcripts into profiles based on patterns of expression (Ernst & Bar-Joseph 2006). Normalized expression data for biological replicates was averaged and fold changes, relative to lunar day 1, were calculated. Log fold-differences in expression levels were calculated as the difference between the mean expression of each transcript across all lunar day time points.

3.4 Results

3.4.1 *De novo* transcriptome assembly, mapping and differential expression

De novo assembly of the reference transcriptome produced a total of 144,922 transcripts, 23,634 of which passed mapping thresholds (mapped reads ≥ 24) and were tested for differential expression (Table 3.1). Lunar day and the interaction of temperature x lunar day had the most significant impacts on gene expression. Temperature alone did not significantly affect gene expression. Lunar day alone significantly affected the expression of 1,175 transcripts (adjusted p-value < 0.05), and the interaction of temperature x lunar day had the largest impact on expression, with 2,831 transcripts showing significant differences (adjusted p-value < 0.05). A heat map depiction of overall patterns in gene expression revealed that the expression patterns of replicate samples from lunar day 1 were more similar to each other than to samples from other days, for both lunar day alone (Figure 3.1) and the interaction of temperature x lunar day (Figure 3.2). Replicates largely grouped by day for later time points for lunar day-alone (Figure 3.1). However, replicate samples for the later time points in the temperature x lunar day interaction showed no patterning and were interspersed (Figure 3.2). Distribution of Gene Ontology (GO) terms associated with transcripts differentially expressed with lunar day revealed a variety of terms for biological, molecular and cellular categories, with multiple terms for some transcripts (Figure 3.3). Distribution of GO terms for transcripts differentially expressed with the temperature x lunar day interaction also showed a variety of terms for biological, molecular and cellular categories, with multiple terms for some transcripts (Figure 3.4). KEGG IDs were obtained for 3,368 (14%) of transcripts tested for differential expression and KEGG enrichment analysis,

performed on groupings of differentially expressed genes, revealed the enrichment of 3 KEGG pathways with lunar day and 47 in the temperature x lunar day interaction (Table 3.2).

Genes previously reported to be associated with elevated temperature stress were examined in the temperature x lunar day interaction (Barshis *et al.* 2013; Meyer *et al.* 2011; Weis 2008). Multiple differentially expressed genes associated with high temperature stress, were up-regulated at 28°C and down-regulated at 23°C, compared to lunar day 1, for lunar day 7, 14, and 21 due to the interaction of temperature x lunar day (Figure 3.5, Table 3.3).

3.4.2 Temporal patterns in expression

STEM was used to identify groups of transcripts showing similar temporal patterns of expression. Transcripts differentially expressed according to lunar day and the interaction of temperature x lunar day were examined separately for the two temperature treatments. The top five STEM profiles, based on transcript abundance, were the same for lunar day alone but occurred in a different order of abundance between the two temperatures (Figure 3.6A).

The most abundant profile for both temperatures was the same, showing an increase in expression from day 1 to a peak at day 14, followed by a decrease on day 21. For the temperature x lunar day interaction, the top profile for both temperatures was the same as for the lunar day alone groupings (Figure 3.6B). However from here, the patterns diverged between lunar day alone and temperature x lunar day groupings (comparing Figures 3.6A and B) and between temperature treatments within the

temperature x lunar day groupings (comparing within Figure 3.6B). For the 23°C treatment, the other four profiles (after the top profile) showed a decrease in expression at lunar day 14. In the 28°C treatment, all time points showed an increase in expression compared to lunar day 1, with lunar day 7 and 14 exhibiting the highest levels of expression.

Enrichment analysis of KEGG pathways ($p < 0.05$) (Table 3.2) in the temperature x lunar day interaction included estrogen, GnRH, and oxytocin signaling pathways. Examination of these three individual KEGG pathways revealed multiple genes shared in these pathways, as well as with the MAPK and calcium signaling pathways (Figure 3.7, Table 3.4). There were temporal differences in gene expression levels between temperature treatments. Only some of these genes were up-regulated on lunar day 7 (grey boxes), compared to lunar day 1, for the 28°C treatment (Figure 3.7). A subset of genes in the oxytocin pathway, that result in mammalian uterine muscle contraction, showed differences in expression levels at lunar day 7, compared to lunar day 1, between the 23 and 28°C temperature treatments (Figure 3.8). Based on KEGG pathway analysis associated with the interaction of temperature x lunar day and predicted to be involved with planulation a model was developed (Table 3.5, Figure 3.9). We propose that G protein-coupled receptor signaling, similar to signaling pathways involving the neuropeptides oxytocin and GnRH, intiate downstream signaling involing inositol triphosphate (IP_3), calcium (Ca^{2+}), and cyclic adenosine monophosphate (cAMP) resulting in cellular processes such as lipid metabolism, cell differentiation and muscle contraction associated with planulation (Table 3.5, Figure 3.9).

3.4.3 Impacts of temperature on expression profiles associated with planulation

In another study, on *P. damicornis* collected from the same reef and subjected to the same temperature treatments in the same tanks as the corals in this study, the mean lunar day of planulation was 12.5 for 23°C and 7.7 for 28°C treatments (Crowder *et al.* 2014). Using these data as an estimation of planulation for the corals sampled in this study, expression profiles of differentially expressed genes up-regulated at the time of planulation were grouped together (Figure 3.10) using STEM analysis. Four profiles showing stable expression or up-regulation from lunar day 7 to 14 (a total of 491 transcripts) were identified in the 23°C planulation group and four profiles showing peak expression at lunar day 7 (a total of 147 transcripts) were identified in the 28°C planulation group (Figure 3.10B, C).

To examine the impacts of temperature on expression of transcripts associated with planulation, expression profiles of individual planulation groups were compared between temperature treatments. The 491 transcripts in the 23°C planulation group with increased expression from lunar day 7 to 14 (Figure 3.11A) showed a clear breakdown in this expression pattern at elevated temperature (Figure 3.11B). Similarly, the 147 transcripts with increased expression at lunar day 7 in the 28°C planulation group (Figure 3.11C) displayed no such pattern at 23°C (Figure 3.11D).

3.4.4 Genes associated with planulation

Homologous gene matches from online databases were identified for 29% and 32% of transcripts associated with planulation in the 23°C (up-regulated from lunar day 7

to 14) (Table 3.6) and the 28°C treatment group (up-regulated at lunar day 7) (Table 3.7), respectively. Seventeen transcripts were shared between the two temperature-associated planulation groups, however none of these had homologous matches to publically available databases and therefore no information exists on the possible function of these transcripts. Genes in the 23°C planulation group included those involved in translation, transcription, cell cycle, apoptosis, development, motor activity, calcium signaling, protein transport and lipid metabolism (Table 3.6). Individual gene descriptions in the 28°C planulation group included genes associated with tyrosine-kinase activity, MAPK activity, cell differentiation, heat stress and G protein-coupled receptor activity (Table 3.7). Together these homologous matches represent a subset of transcripts that support the model proposed (Figure 3.9).

3.5 DISCUSSION

To examine the transcriptomic mechanisms involved in sensing environmental variables and coordinating reproductive events in a brooding coral, we investigated the impact of lunar day and temperature on global gene expression profiles. Our analysis revealed that (1) effects of the temperature treatments in our experiment are dependent on lunar day and (2) that both lunar irradiance and temperature influence gene expression and possibly reproductive timing in *P. damicornis*. These findings are similar to reports in *A. millepora* corals where transcriptional changes, at the time of spawning, were correlated with levels of moonlight, associated with lunar phases (Kaniewska *et al.* 2015). This indicates that lunar irradiance has a significant influence on gene expression in both spawning and brooding corals, at reproductively critical time points.

The influence of these environmental variables on gene expression is consistent with previous ecological studies that have correlated both of these exogenous factors with the timing of planulation. Lunar periodicity and elevated temperature, associated with seasonality, have been correlated with the timing of planula release in *P. damicornis*, from multiple geographical locations (Crowder *et al.* 2014; Fan *et al.* 2002; Harriott 1983; Jokiel & Guinther 1978; Richmond & Jokiel 1984; Stoddart & Black 1985; Tanner 1996; Villanueva *et al.* 2008). Furthermore, these findings are consistent with reports in other brooding corals where variables associated with seasonality such as temperature, tidal cycles, diel-light cycles as well as lunar periodicity were documented as being environmental signalers of planula release (Goodbody-Gringley & de Putron 2009; McGuire 1998; Villanueva *et al.* 2008).

3.5.1 Impacts of elevated temperature and stress

Temperature did not impacted gene expression as a main effect, however it did have an impact on gene expression, in the context of lunar day. Although, we did not visually observe a bleaching response (loss of endosymbiotic algae), previously-described signatures of thermal stress such as an up-regulation of genes involved with apoptosis, calcium ion homeostasis, and cell differentiation were observed (Barshis *et al.* 2013; Meyer *et al.* 2011; Vidal-Dupiol *et al.* 2014; Weis 2008). This suggests that although the high temperature treatment in this study did not result in a phenotypic stress response, the prolonged elevated temperature had an effect at the transcriptional level. The lack of visible bleaching could be due to the fact that corals in Nanwan Bay regularly experience a range of temperatures daily, due to tidally-induced upwelling, with 23 and

28°C representing the high and low of this fluctuation (Lee 1999). Corals in Nanwan Bay are therefore acclimated to cope with thermal fluctuations, and our data suggest that through the consistent up-regulation of stress related genes, they are able to manage physiological stress. It is also possible that some of the earlier time points could have captured a stress response in the internally fertilized larvae not yet released, since larvae have been shown to have higher sensitivity to elevated temperature (Putnam *et al.* 2010).

3.5.2 Temporal patterns of gene expression

The down-regulation of transcripts in four out of the five most abundant temperature x lunar day profiles in the 23 compared to the 28°C treatment (Figure 3.6B) indicates dramatic shifts in profiles dependent on both temperature and lunar day. Higher levels of up-regulation in the 28°C treatment could be explained by the increase in metabolism that occurs with higher temperature. This is supported by the top GO biological terms identified for the temperature x lunar interaction (Figure 3.4C). Metabolism, nucleic acid metabolism and protein metabolism were three of the top five hits accounting for 36% of the transcripts that were differentially expressed. Development was second behind metabolism on the list. Increases in expression of developmental genes could accelerate development, thereby causing the observed earlier planula release at elevated temperatures (Figure 3.4A).

3.5.3 Expression profiles correlated with planulation

Expression profiles exhibiting an up-regulation at the time of planulation for the two temperature treatments shared just seventeen common transcripts, none of which

show homology to known genes (Figure 3.10C). This lack of overlap could indicate only partial capture of genes associated with planulation. An increase in sampling around lunar day 7 and lunar day 12 could reveal additional genes differentially expressed at the time of planulation. This lack of overlap could also indicate that temperature has a profound impact on transcriptomic regulation of planulation, resulting in differing sets of mechanisms controlling the process at different temperatures. Genes identified in the 28°C planulation group included those associated with tyrosine-kinase activity, MAPK activity, cell differentiation, and G protein-coupled receptor activity.

3.5.4 Endocrine pathways differentially expressed with temperature x lunar day

Enriched analysis of KEGG pathways in the temperature x lunar day interaction included estrogen, GnRH, and oxytocin signaling pathways. GnRH and estrogen are key hormones in the vertebrate hypothalamic-pituitary-gonadal axis responsible for coordinating reproduction (Ojeda *et al.* 1994) and oxytocin is a pleiotropic hormone with many actions including induction of uterine contraction in mammals during birth (Sanborn 2001). Genes common to the GnRH, estrogen receptor and oxytocin pathways were also shared in the MAPK and calcium signaling pathways (Figure 3.7, Table 3.4) and expression of these genes differed with lunar day in the 28°C temperature group. No homologous matches to the neuropeptides themselves or to the receptors for GnRH, estrogen, and oxytocin were found in the transcriptome, however peptides similar in structure to oxytocin have been reported in cnidarians (Grimmelikhuijzen *et al.* 2004). A network within the oxytocin pathway that results in muscle contraction was differentially expressed (Figure 3.8) and differed between temperature treatments, with all genes up-

regulated at lunar day 7 in the 23°C treatment. Since lunar day 7 is just before planulation, in the 23°C treatment, this could indicate a homologous mechanism for contraction between mammals during birthing and corals during planula release from the mesenteries during planulation. Although matches to only portions of these pathways were present in our transcriptome, multiple genes associated with neuroendocrine signaling are represented. These include the neurotransmitter receptors: gamma-aminobutyric acid (GABBR), dopamine (DRD5), and glutamate receptor ionotrophic (GRIN1) (Figure 3.7, Table 3.4), some of which have been described in corals, where neuropeptides are the dominant transmitters in the cnidarian nervous system (Grimmelikhuijzen & Hauser 2012).

3.5.5 Calcium acts as a central mediator

Multiple genes associated with calcium binding and signal transduction pathways involving calcium were up-regulated in both the 23 and 28°C planulation groups (Table 3.5 and 3.6). Calcium has previously been shown to mediate light responsiveness in spawning corals (Hilton *et al.* 2012) and genes associated with calcium binding were up-regulated at the time of spawning (Kaniewska *et al.* 2015) and during the day compared to the night in the spawning coral *A. millepora* (Bertucci *et al.* 2015). Together the differential expression of these calcium-related homologous in reproductive corals suggests that calcium plays a role in sensing moonlight and coordinating reproduction in both brooding and spawning corals.

3.5.6 No support for vertebrate-type steroidogenesis

Corals can take up estrogens from the environment, therefore it remains unclear if steroids detected in coral tissues are synthesized by the corals themselves or acquired from their diet or from surrounding seawater (Tarrant *et al.* 2001). There is little evidence to support the *de novo* synthesis of vertebrate-type sex steroids in corals, given that key steroidogenic pathways and receptors specific to vertebrate steroids are not present in cnidarian genomes examined to date (Goldstone 2008; Markov *et al.* 2009). Likewise, our analyses did not detect steroid pathway or steroid receptor homologous. For example, targeted searches of vertebrate steroidogenesis enzymes whose activity has been reported in coral tissues (aromatase (CYP19) and cytochrome P450-17 (CYP17)) were not detected in our *de novo* assembled transcriptome (Rougée *et al.* 2015; Twan *et al.* 2006). This leaves the possibility that corals may be able to synthesize vertebrate-like steroids from cholesterol using analogous enzymes with convergent functions and that these steroids bind other nuclear receptors or function through alternative pathways (Tarrant *et al.* 2009). Regardless, there is a need for thorough genomic investigations to further examine both the origin and role of these hormones in coral reproduction.

3.6 CONCLUSIONS

Multiple ecological studies have described the role of the lunar phase and temperature on reproductive timing in corals, and this study adds, to our knowledge, the first transcriptome-based study that investigates the role of these environmental variables on the timing of planulation. Not only does elevated temperature shift the timing of reproduction by almost a week (Crowder *et al.* 2014), it also disrupts patterns of expression associated with lunar timing of planulation. This indicates that elevated

temperatures impact coral reproductive physiology considerably at the molecular level. Numerous transcripts were associated with the interaction of temperature and lunar day together, indicating a synergistic role of these two environmental variables in coordinating planulation in brooding corals. Homologous matches of differentially expressed transcripts to online databases revealed a potential role for neuroendocrine signaling during reproduction, with calcium acting as a central mediator, similar to previous findings in a spawning coral (Kaniewska *et al.* 2015). This suggests that moonlight perception and reproduction in an ancestral metazoan is regulated by a network of neuropeptides acting through G protein-couple receptors, as opposed to more derived vertebrate-type sex steroid pathways.

Table 3.1 Transcriptome assembly, mapping and differential expression statistical values.

***De novo* Transcriptome Assembly**	**Sequences**
Number of reads after quality filtering	45,072,448
Total number of contigs	144,922
Total subcomponent isogroups	78,519
Average contig length	520
Maximum contig length	7,701
Minimum contig length	201
N50 of all contigs	643
Total number of transcripts tested for differential expression	23,634
Percentage of transcripts tested with UniProt matches	41%
Percentage of transcripts tested with Gene Ontology category matches	39%
Percentage of transcripts tested with KEGG pathway matches	14%
Mapping	
Average number of reads per sample	4,680,151
Average number removed during filtering	1,666,463
Average number of reads per sample after filtering	3,013,688
Differential Expression	
Number of transcripts passing thresholds and tested for differential expression	23,634
Number of transcripts differentially expressed with temperature ($p < 0.05$)	0
Number of transcripts differentially expressed with lunar day ($p < 0.05$)	1.175
Number of transcripts differentially expressed with the interaction of temperature x lunar day ($p < 0.05$)	2,831

Table 3.2 KEGG pathways enriched for with lunar day and temperature x lunar day.

	KO ID	KEGG Pathway Description	*p*-value	Count
Lunar Day	ko04142	Lysosome	2.5E-02	6
	ko03040	Spliceosome	3.1E-02	6
	ko04141	Protein processing in endoplasmic reticulum	4.5E-02	6
Temperature x lunar day	ko03010	Ribosome	1.0E-24	42
	ko04141	Protein processing in endoplasmic reticulum	9.2E-05	19
	ko05169	Epstein-Barr virus infection	1.8E-04	20
	ko05016	Huntington's disease	4.9E-04	18
	ko04144	Endocytosis	1.0E-03	19
	ko04915	Estrogen signaling pathway	1.0E-03	11
	ko04210	Apoptosis	1.5E-03	14
	ko04912	GnRH signaling pathway	2.8E-03	10
	ko03018	RNA degradation	3.0E-03	11
	ko03040	Spliceosome	3.0E-03	14
	ko05222	Small cell lung cancer	3.0E-03	10
	ko05200	Pathways in cancer	4.8E-03	25
	ko03050	Proteasome	6.2E-03	8
	ko04971	Gastric acid secretion	6.7E-03	8
	ko05010	Alzheimer's disease	1.1E-02	14
	ko04962	Vasopressin-regulated water reabsorption	1.8E-02	6
	ko04540	Gap junction	2.0E-02	8
	ko04010	MAPK signaling pathway	2.3E-02	16
	ko04261	Adrenergic signaling in cardiomyocytes	2.5E-02	10
	ko04918	Thyroid hormone synthesis	2.5E-02	7
	ko04145	Phagosome	2.8E-02	10
	ko04921	Oxytocin signaling pathway	2.8E-02	11
	ko04972	Pancreatic secretion	2.8E-02	8
	ko03410	Base excision repair	2.8E-02	6
	ko04330	Notch signaling pathway	2.8E-02	5
	ko04391	Hippo signaling pathway - fly	2.8E-02	7
	ko04510	Focal adhesion	2.8E-02	13
	ko04622	RIG-I-like receptor signaling pathway	2.8E-02	7
	ko04750	Inflammatory regulation of TRP channels	2.8E-02	8
	ko05146	Amoebiasis	2.8E-02	9
	ko04612	Antigen processing and presentation	2.9E-02	6
	ko04745	Phototransduction - fly	2.9E-02	4
	ko05203	Viral carcinogenesis	2.9E-02	12
	ko04974	Protein digestion and absorption	3.2E-02	7
	ko04015	Rap1 signaling pathway	3.5E-02	13
	ko04925	Aldosterone synthesis and secretion	3.6E-02	7
	ko05012	Parkinson's disease	3.6E-02	11
	ko05164	Influenza A	3.9E-02	11
	ko04512	ECM-receptor interaction	4.1E-02	7

Table 3.2 (Continued)

	KO ID	KEGG Pathway Description	*p*-value	Count
Temperature x lunar day	ko04924	Renin secretion	4.1E-02	6
	ko04151	PI3K-Akt signaling pathway	4.4E-02	17
	ko03420	Nucleotide excision repair	4.4E-02	6
	ko05145	Toxoplasmosis	4.5E-02	9
	ko04970	Salivary secretion	4.6E-02	7
	ko05110	Vibrio cholerae infection	4.6E-02	6
	ko05160	Hepatitis C	4.6E-02	8
	ko03022	Basal transcription factors	4.8E-02	5

Table 3.3 Selected genes associated with heat stress featured in Figure 3.5.

GO category	Gene	UniProt description
Apoptosis	Agrin	Agrin
Apoptosis	Eif2ak3	Eukaryotic translation initiation factor 2-alpha kinase 3
Apoptosis	Casp2	Caspase-2
Cell differentiation	Eda2r	Tumor necrosis factor receptor superfamily member 27
Calcium ion transport	Cacna2d1	Voltage-dependent calcium channel subunit alpha-2/delta-1
Calcium ion transport	cacnb4	Voltage-dependent L-type calcium channel subunit beta-4
Calcium ion transport	Megf6	Multiple epidermal growth factor-like domains protein 6
Chaperone	HSP70-17	Heat shock 70 kD protein 17

Table 3.4 Genes included in the GnRH, estrogen, oxytocin, MAPK, and calcium-signaling pathways featured in Figure 3.7.

Transcript	Gene	Gene Description
comp63809c0	ACTBG1	Actin beta/gamma 1
comp63126c2	ADCY5	Adenylate cyclase 5
comp63126c1	ADCY6	Adenylate cyclase 6
comp59345c0	CACNA2D1	Voltage-dependent calcium channel alpha-2/delta-1
comp40360c0	CACNB2	Voltage-gated-dependent calcium channel beta-2
comp55736c0	CALM	Calmodulin
comp60517c0	DRD5	Dopamine receptor D5
comp48844c0	FGFR3	Fibroblast growth factor receptor3
comp62413c0	FKBP45	FK506-binding protein 4/5
comp59489c0	FLNA	Filamin
comp62109c1	GABBR	Gamma-aminobutyric acid type B receptor
comp51849c0	GADD45	Growth arrest and DNA-damage-inducible protein
comp62391c0	GNAS	Guanine nucelotide-binding protein G subunit alpha
comp52730c0	GRIN1	Glutamate receptor ionotrophic
comp54036c1	HSPA1-8	Heat shock 70kDa protein 1/8
comp64424c0	htpG	Molecular chaperone HtpG
comp54319c0	IKBKG	Inhibitor of nuclear factor kappa-B kinase subunit gamma
comp60542c0	ITPR1	Inositol 1,4,5-triphosphate receptor type 1
comp58456c0	MAP2K6	Mitogen-activated protein kinase kinase 6
comp63261c2	MAP2K7	Mitogen-activated protein kinase kinase 7
comp55025c0	MAP3K13	Mitogen-activated protein kinase kinase kinase 13
comp61968c0	MAP3k4	Mitogen-activated protein kinase kinase kinase 4
comp59465c1	MYC	Myc proto-oncogene protein
comp56395c0	MYL6	Myosin light chain 6
comp41143c0	MYLK	Myosin light-chain kinase
comp54742c0	NFKB1	Nuclear factor NF-kappa-B p105 subunit
comp48165c1	PLCB	Phosphatidylinositol phospholipase C, beta
comp46665c0	PPIF	Peptidyl-prolyl isomerase F
comp61425c0	RASGRP3	RAS gunayl-releasing protein 3
comp60045c1	SLC25	Solute carrier family 25
comp48078c0	SOS	Son of sevenless
comp56417c0	SRF	Serum response factor

Table 3.5 Genes included in the proposed model of neuropeptide signaling featured in Figure 6. Multiple transcripts resulted in homologous matches to genes listed.

Gene	Gene Description
GPCR	G protein-coupled receptor
Gs	Guanine nucelotide-binding protein G subunit
PLC	Phosphatidylinositol phospholipase C
IP3R	Inositol 1,4,5-triphosphate receptor
ADCY	Adenylate cyclase
PKA	Protein kinase A
VGCC	Voltage-gated calcium channel
CALM	Calmodulin
MLCK	Myosin light-chain kinase
MLC	Myosin light chain
Actin	Actin

Table 3.6 Transcripts with UniProt matches in the 23°C planulation group.

Transcript	KO ID	UniProt ID	UniProt Gene Description
comp59320c0	K02888	Q7Z2W9	39S ribosomal protein L21
comp45827c0	N/A	Q96EL3	39S ribosomal protein L53
comp9716c0	K02949	Q9XSU4	40S ribosomal protein S11
comp28130c0	K02960	Q98TR7	40S ribosomal protein S16
comp64428c0	K02971	Q90YQ2	40S ribosomal protein S21
comp64439c0	K02989	P97461	40S ribosomal protein S5
comp28138c0	K02997	P29314	40S ribosomal protein S9
comp64448c0	K02943	Q6X9Z5	60S acidic ribosomal protein P2
comp9757c0	K02875	O46160	60S ribosomal protein L14
comp41999c0	K02877	P61314	60S ribosomal protein L15
comp9725c0	K02894	P62832	60S ribosomal protein L23
comp64409c0	K02908	P58374	60S ribosomal protein L30
comp42782c2	K02917	Q90YT3	60S ribosomal protein L35a
comp57714c0	K02920	Q4PM12	60S ribosomal protein L36
comp38901c0	K02924	P51424	60S ribosomal protein L39-1
comp61970c0	N/A	Q0V9K1	Transcription factor Maf
comp51883c0	K02730	P60901	Proteasome subunit alpha type-6
comp61961c0	N/A	Q54U49	Mediator of RNA polymerase II transcription subunit 12
comp45156c0	K15151	Q5R6P5	Mediator of RNA polymerase II transcription subunit 10
comp61604c0	N/A	P67829	Casein kinase I isoform alpha
comp55628c0	K15622	Q9QWV4	Myeloid leukemia factor 1
comp51929c0	N/A	P36872	Protein phosphatase PP2A 55 kDa regulatory subunit
comp25242c0	K08857	Q7ZZC8	Serine/threonine-protein kinase Nek9
comp57642c0	K12859	P83877	Thioredoxin-like protein 4A
comp59674c0	K16219	Q4KLE6	N-terminal Xaa-Pro-Lys N-methyltransferase 1-B
comp63915c2	K09209	O14901	Krueppel-like factor 11
comp44894c0	K06698	Q5RFD3	Proteasome activator complex subunit 3
comp54981c0	K08964	Q5ZLP2	Methylthioribulose-1-phosphate dehydratase
comp57505c0	K03936	P23709	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3
comp58034c0	K11187	P30044	Peroxiredoxin-5
comp26791c0	N/A	A5WUY6	O(6)-methylguanine-induced apoptosis 2
comp56832c0	K15614	Q62231	Homeobox protein SIX1
comp62078c0	N/A	Q7T3Q2	Cysteine-rich motor neuron 1 protein
comp63266c0	K10295	Q8BK06	F-box only protein 9
comp63808c0	K05125	Q62371	Discoidin domain-containing receptor 2
comp12539c0	K09084	Q96SQ7	Protein atonal homolog 8
comp59821c0	N/A	O70480	Vesicle-associated membrane protein 4
comp48363c0	N/A	P07087	Anti-lipopolysaccharide factor
comp58108c0	K11990	P54120	Protein AIG1
comp58988c0	K11251	P02264	Histone H2A
comp51551c0	K12236	Q12986	Transcriptional repressor NF-X1
comp47184c0	N/A	Q9DAJ5	Dynein light chain roadblock-type 2

Table 3.6 (Continued)

Transcript	KO ID	UniProt ID	UniProt Gene Description
comp61460c2	K07374	P41383	Tubulin alpha-2/alpha-4 chain
comp55813c0	K07375	P68372	Tubulin beta-4B chain
comp54733c0	N/A	O75506	Heat shock factor-binding protein 1
comp63659c0	K17495	P0C6B8	Sushi
comp55744c0	K12304	Q8K4Y7	Soluble calcium-activated nucleotidase 1
comp63901c2	N/A	O14830	Serine/threonine-protein phosphatase with EF-hands 2
comp60175c1	N/A	Q9JJ00	Phospholipid scramblase 1
comp60121c0	N/A	P42325	Neurocalcin homolog
comp57672c0	N/A	P54357	Myosin-2 essential light chain
comp56395c0	K12751	P54357	Myosin-2 essential light chain
comp12480c0	K02503	P62958	Histidine triad nucleotide-binding protein 1
comp55736c0	K02183	Q95NR9	Calmodulin
comp63436c2	N/A	P12815	Programmed cell death protein 6
comp62641c0	N/A	P26990	ADP-ribosylation factor 6
comp51958c0	K02868	Q90YV7	60S ribosomal protein L11
comp46665c0	K09565	P54985	Peptidyl-prolyl cis-trans isomerase
comp55920c1	K09481	Q5RB31	Protein transport protein Sec61 subunit beta
comp57155c0	K11979	Q8BU04	Putative E3 ubiquitin-protein ligase
comp27893c0	K09568	Q8LGG0	Peptidyl-prolyl cis-trans isomerase FKBP12
comp47727c0	K09500	Q6EE31	T-complex protein 1 subunit theta
comp62817c0	K07891	P51154	Ras-related protein Rab-22A
comp63233c0	N/A	Q5ZHW4	Ras-related protein Rab-5B
comp57935c0	N/A	Q9FGZ9	Ubiquitin-like protein 5
comp45900c0	K10457	Q6DFF6	Kelch-like protein 20
comp62365c0	N/A	Q71U00	S-phase kinase-associated protein 1
comp63657c0	N/A	Q8IUS5	Epoxide hydrolase 4
comp63285c1	N/A	Q9XTR8	Lipase ZK262.3
comp50872c0	K07897	Q3T0F5	Ras-related protein Rab-7a
comp64177c0	K13356	A1ZAI5	Putative fatty acyl-CoA reductase
comp53821c1	N/A	Q9BXW6	Oxysterol-binding protein-related protein 1
comp62808c0	K12186	Q6KAU4	Multivesicular body subunit 12B
comp56961c1	K00121	P79896	Alcohol dehydrogenase class-3
comp63332c1	N/A	P46434	Glutathione S-transferase
comp57524c0	K01581	P09057	Ornithine decarboxylase
comp62997c1	K01953	Q1LZA3	Asparagine synthetase
comp43256c0	K18342	Q7ZV00	OTU domain-containing protein 6B
comp62902c0	N/A	P11442	Clathrin heavy chain 1
comp55447c0	N/A	P98088	Mucin-5AC
comp51868c0	N/A	A5PJX7	Sodium- and chloride-dependent GABA transporter 2
comp62477c0	N/A	Q96CG8	Collagen triple helix repeat-containing protein 1
comp51685c0	N/A	Q7SXW4	ER membrane protein complex subunit 3
comp43844c0	N/A	P20702	Integrin alpha-X
comp60045c1	K05863	O49447	ATP carrier protein 3
comp75825c0	N/A	Q03114	Cyclin-dependent kinase 5

Table 3.6 (Continued)

Transcript	KO ID	UniProt ID	UniProt Gene Description
comp62477c0	N/A	Q96CG8	Collagen triple helix repeat-containing protein 1
comp50277c0	K08371	Q5E965	Cytochrome b561 domain-containing protein 2
comp14280c0	N/A	Q5RCS9	Ufm1-specific protease 2
comp60037c0	K04794	O76387	Probable peptidyl-tRNA hydrolase 2
comp43828c0	K18668	Q9C0D7	Probable ribonuclease ZC3H12C
comp9733c0	N/A	Q8N6F8	Williams-Beuren syndrome chromosomal region 27
comp51730c0	N/A	Q2KID7	Oligosaccharyltransferase complex subunit OSTC
comp57798c0	K02735	O73817	Proteasome subunit beta type-3
comp60952c1	K05619	P23606	Protein-glutamine gamma-glutamyltransferase K
comp62242c0	K00933	O15992	Arginine kinase
comp63165c0	N/A	B5DG42	Eukaryotic initiation factor 4A-III
comp63764c1	K12856	Q99PV0	Pre-mRNA-processing-splicing factor 8
comp52539c0	K12815	Q17R09	Pre-mRNA-splicing factor RNA helicase
comp56460c0	N/A	Q5RC80	RNA-binding protein 39
comp59688c0	N/A	Q06185	ATP synthase subunit e
comp58760c0	K10408	Q3V0Q1	Dynein heavy chain 12
comp44071c0	N/A	Q15058	Kinesin-like protein KIF14
comp54738c0	N/A	P79781	Ubiquitin-40S ribosomal protein S27a
comp48913c0	K10646	Q6Q0C0	E3 ubiquitin-protein ligase TRAF7
comp55303c0	K03627	Q5ZMC0	Endothelial differentiation-related factor 1
comp55605c3	K02136	P05631	ATP synthase subunit gamma
comp63332c0	K15690	Q4VGL6	Roquin-1
comp9754c0	K14648	Q503V9	Poly(U)-specific endoribonuclease-B
comp62284c0	K08955	O80983	ATP-dependent zinc metalloprotease FTSH 4
comp60670c0	N/A	Q96MM6	Heat shock 70 kDa protein 12B
comp45788c0	N/A	Q57997	Universal stress protein MJ0577
comp60860c0	K01587	P38024	Multifunctional protein ADE2
comp61816c1	N/A	O75486	Transcription initiation protein SPT3
comp58936c0	K09350	Q60554	Homeobox protein Nkx-6.1
comp52285c0	N/A	Q503Y7	Peptidyl-prolyl cis-trans isomerase
comp62889c1	N/A	Q6NVN0	Transcription factor Sox-2
comp27891c0	K11251	O93327	Core histone macro-H2A.1
comp51794c0	N/A	P86854	Perlucin-like protein
comp62902c0	N/A	P11442	Clathrin heavy chain 1
comp43973c0	K14443	P34743	Protein BTG1
comp64007c0	N/A	O00471	Exocyst complex component 5
comp50277c0	K08371	Q5E965	Cytochrome b561 domain-containing protein 2
comp63818c3	N/A	P18075	Bone morphogenetic protein 7
comp60517c0	K05840	P19020	D(3) dopamine receptor
comp57557c0	N/A	A7S3I2	BBSome-interacting protein 1
comp53159c0	K03037	Q9V3G7	26S proteasome non-ATPase regulatory subunit 6
comp9828c1	K02889	P46778	60S ribosomal protein L21

Table 3.7 Transcripts with UniProt matches in the 28°C planulation group.

Transcript	KO ID	UniProt ID	UniProt Gene Description
comp60165c1	N/A	A2AJ76	Hemicentin-2
comp63435c0	N/A	A2ASQ1	Agrin
comp45825c0	N/A	A2BIL7	Tyrosine-protein kinase BAZ1B
comp59479c0	N/A	A2VDK6	Wiskott-Aldrich syndrome protein family 2
comp27681c0	N/A	A7SGF0	Protein MEF2BNB homolog
comp86474c0	N/A	C8VTR6	Putative acyl-coenzyme A synthetase
comp47456c0	N/A	E9Q555	E3 ubiquitin-protein ligase RNF213
comp12378c0	N/A	F4JMJ1	Heat shock 70 kDa protein 17
comp61968c0	K04428	O08648	Mitogen-activated protein kinase kinase kinase 4
comp63685c0	K00654	O15270	Serine palmitoyltransferase 2
comp63820c1	N/A	O70475	UDP-glucose 6-dehydrogenase
comp49822c0	K07152	O75880	Protein SCO1 homolog
comp63333c0	N/A	O76484	Casein kinase II subunit alpha
comp62483c0	K08889	P00541	Tyrosine-protein kinase transforming protein Fps
comp62427c2	N/A	P15848	Arylsulfatase B
comp46211c0	N/A	P25386	Intracellular protein transport protein USO1
comp56369c0	K03124	P29054	Transcription initiation factor IIB
comp59272c0	N/A	P29074	Tyrosine-protein phosphatase non-receptor type 4
comp58989c0	K03141	P32780	General transcription factor IIH subunit 1
comp53489c0	K00297	P42898	Methylenetetrahydrofolate reductase
comp64505c0	K02150	P54611	V-type proton ATPase subunit E
comp60071c0	K13110	P55080	Microfibrillar-associated protein 1
comp63370c0	N/A	P69566	Ran-binding protein 9
comp61245c0	K08375	P83861	Pyroglutamylated RFamide peptide receptor
comp63459c1	N/A	P98110	E-selectin
comp47500c0	N/A	Q10836	Thyrotropin-releasing hormone-degrading enzyme
comp55644c0	K03174	Q13114	TNF receptor-associated factor 3
comp64043c0	N/A	Q26417	Homeobox protein OTX
comp71078c0	N/A	Q3UR85	Myelin regulatory factor
comp62132c0	N/A	Q3ZBA0	Tectonin beta-propeller repeat-containing protein 1
comp70495c0	N/A	Q499P8	UPF0420 protein C16orf58 homolog
comp47226c0	K15189	Q4V7W3	Protein HEXIM
comp54779c0	K01493	Q5M9G0	Deoxycytidylate deaminase
comp43594c0	K00771	Q5QQ57	Xylosyltransferase 1
comp60365c0	N/A	Q5VZ89	DENN domain-containing protein 4C
comp58115c0	N/A	Q5ZJ69	AT-rich interactive domain-containing protein 5B
comp30292c0	K08371	Q641Y1	Cytochrome b561 domain-containing protein 2
comp64082c1	K11665	Q6ZPV2	DNA helicase INO80
comp62806c1	N/A	Q7T308	E3 ubiquitin-protein ligase RNF168
comp48547c0	N/A	Q8BX35	Tumor necrosis factor receptor superfamily 27
comp64039c0	N/A	Q8JG38	Fibroblast growth factor receptor 2
comp46155c0	N/A	Q8MYF0	Mitochondrial genome maintenance protein

Table 3.7 (Continued)

Transcript	KO ID	UniProt ID	UniProt Gene Description
comp47901c0	N/A	Q9BYK8	Helicase with zinc finger domain 2
comp40763c0	K10848	Q9QYM7	DNA repair endonuclease XPF
comp59940c1	K08892	Q9V9J3	Tyrosine-protein kinase Src42A
comp78548c0	N/A	Q9VCA8	Ankyrin repeat and KH domain-containing protein
comp59211c0	N/A	Q9WTQ1	Serine/threonine-protein kinase D1

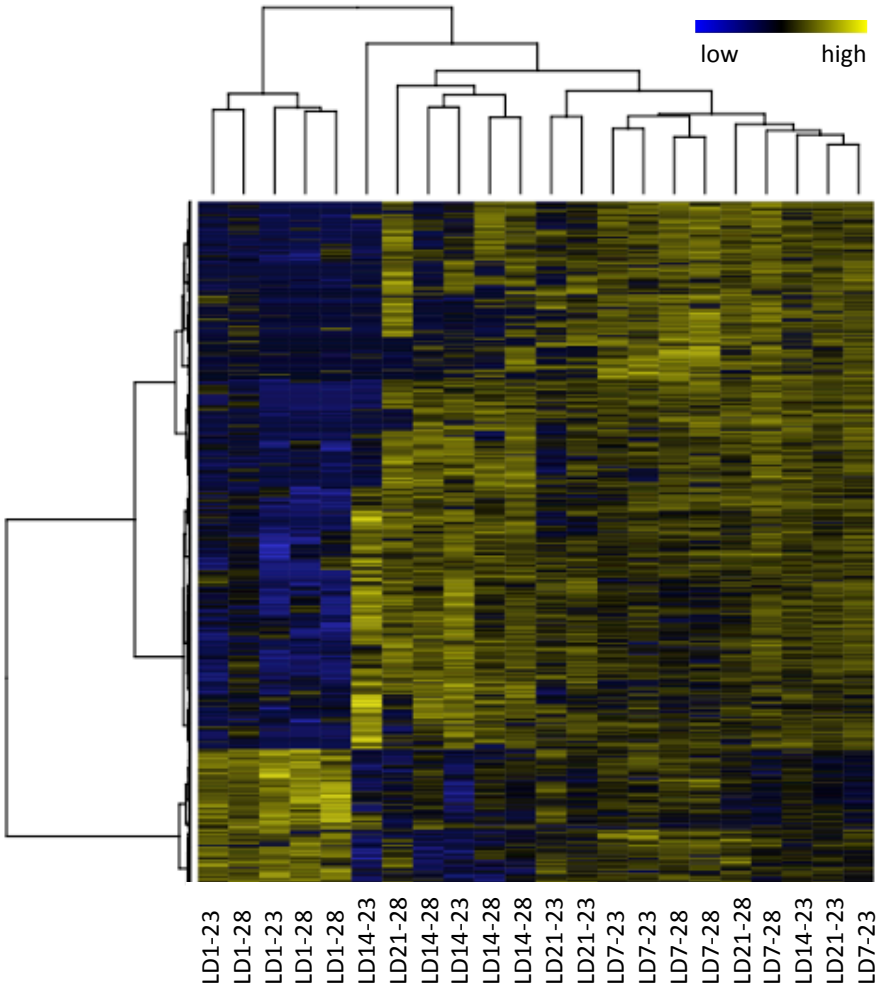

Figure 3.1 Heat map depicting the impact of lunar day on gene expression. Expression patterns for the 1,175 transcripts differentially expressed due to lunar day. Sample names, including biological replicates (samples with the same name), are provided on the x-axis (LD = lunar day, 28 = 28°C, 23 = 23°C) and rows represent individual transcripts.

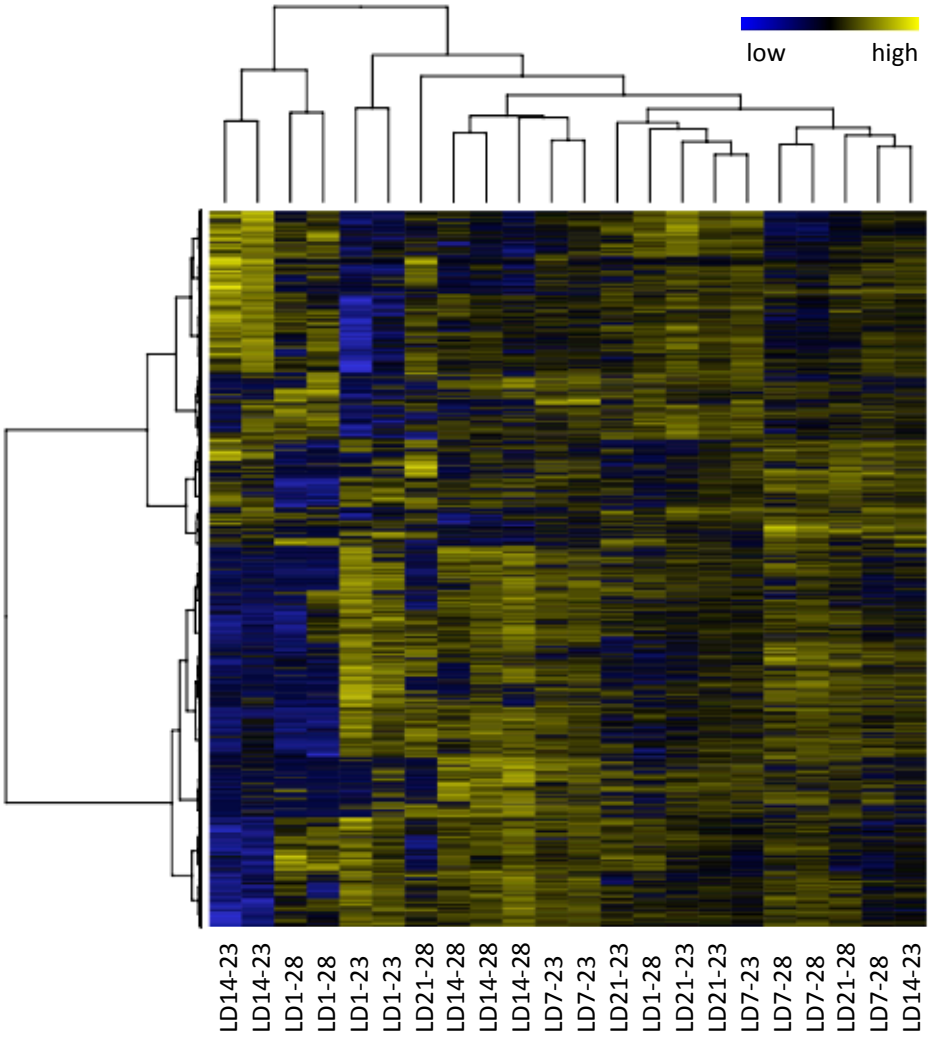

Figure 3.2 Heat map depicting the impact of the interaction of temperature x lunar day on gene expression. Expression patterns in for the 2,831 transcripts that were differentially expressed due to the interaction of temperature x lunar day. Sample names, including biological replicates (samples with identical names), are provided on the x-axis (LD = lunar day, 28 = 28°C, 23 = 23°C) and rows represent individual transcripts.

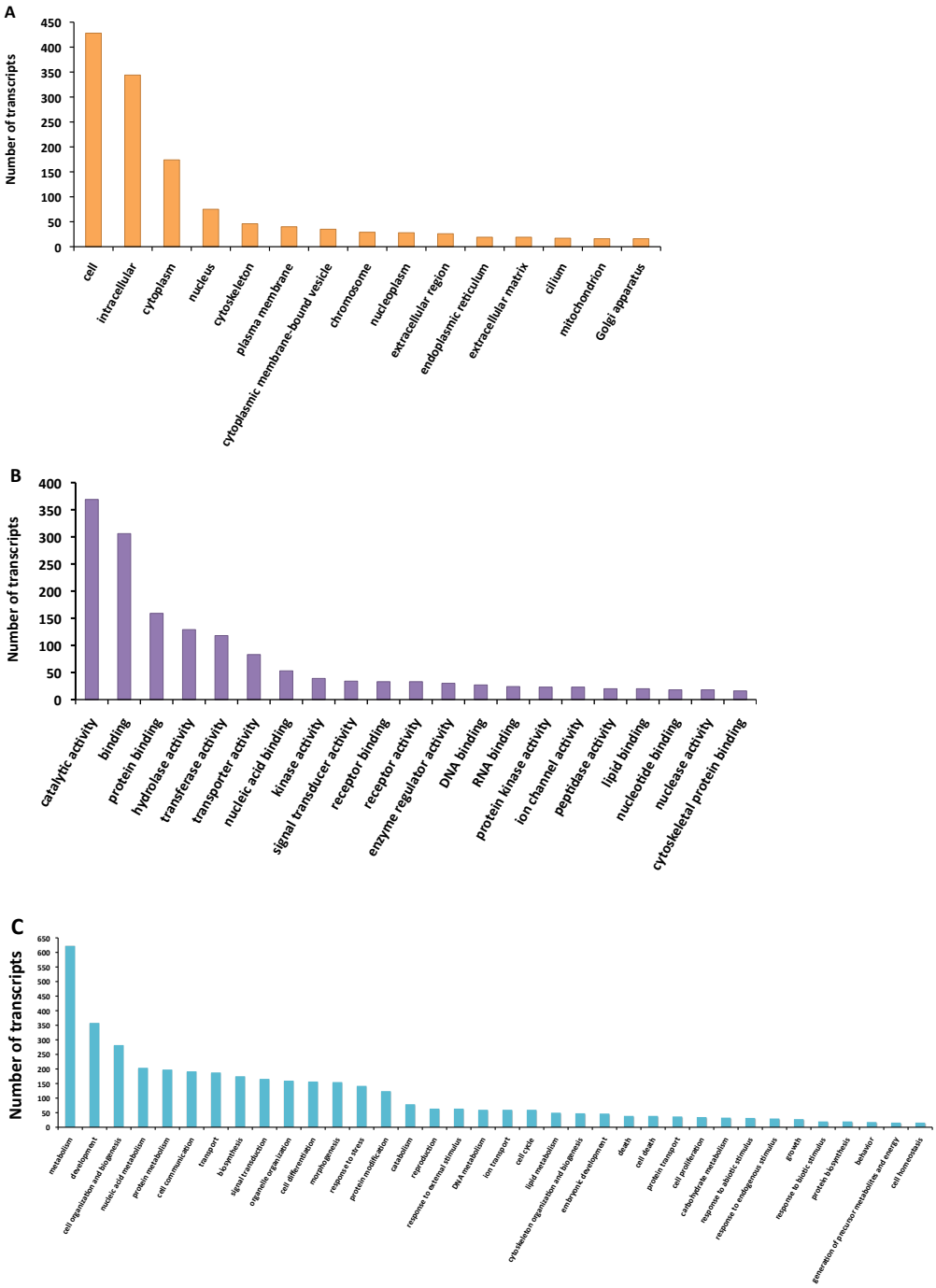

Figure 3.3 Distribution of GO terms for differentially expressed transcripts assoicated with lunar day. Distribution of (A) cellular (B) molecular and (C) biological GO categories.

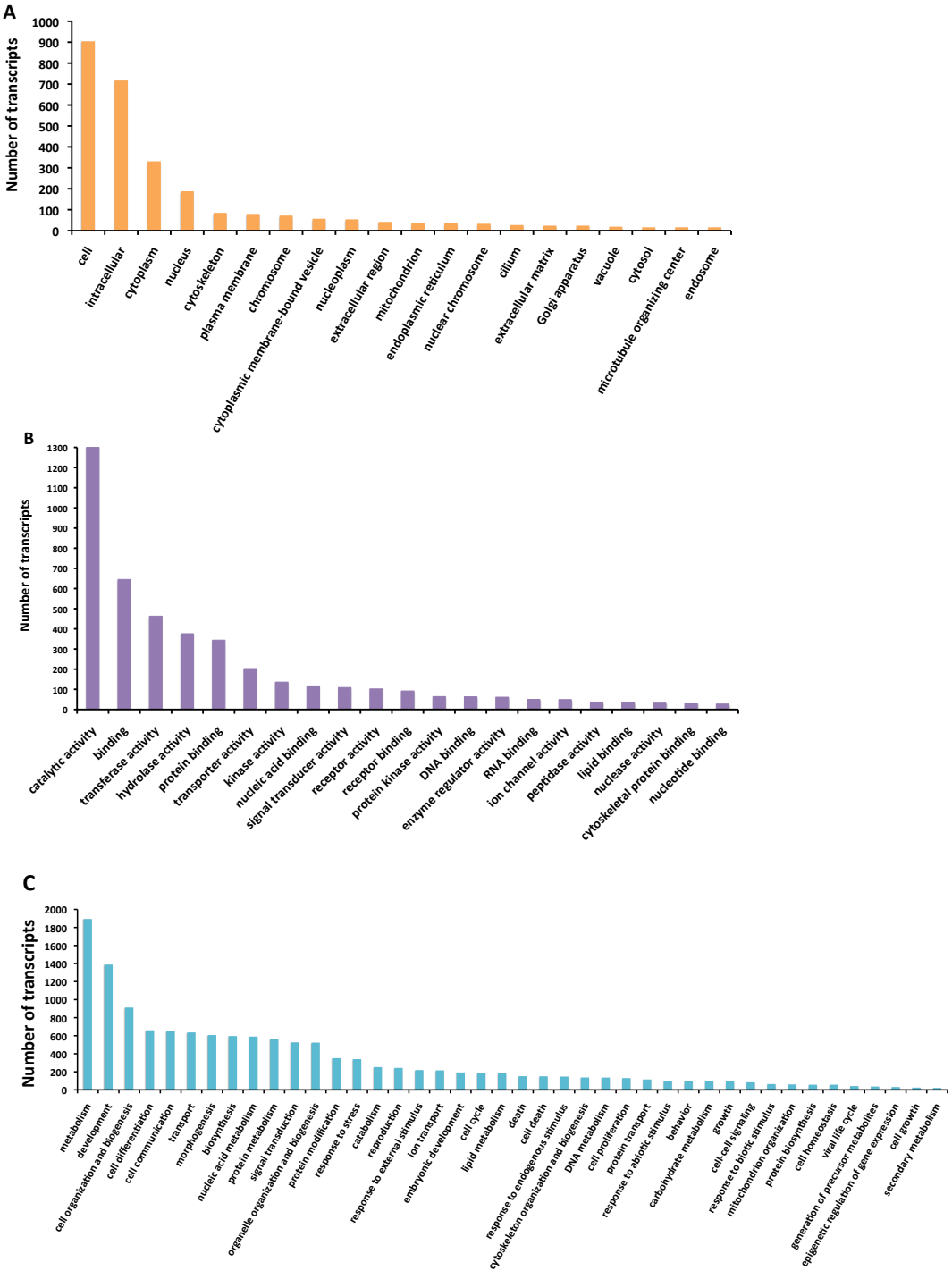

Figure 3.4. Distribution of GO terms for differentially expressed transcripts assoicated with the interaction of temperature x lunar day. Distribution of (A) cellular (B) molecular and (C) biological GO categories.

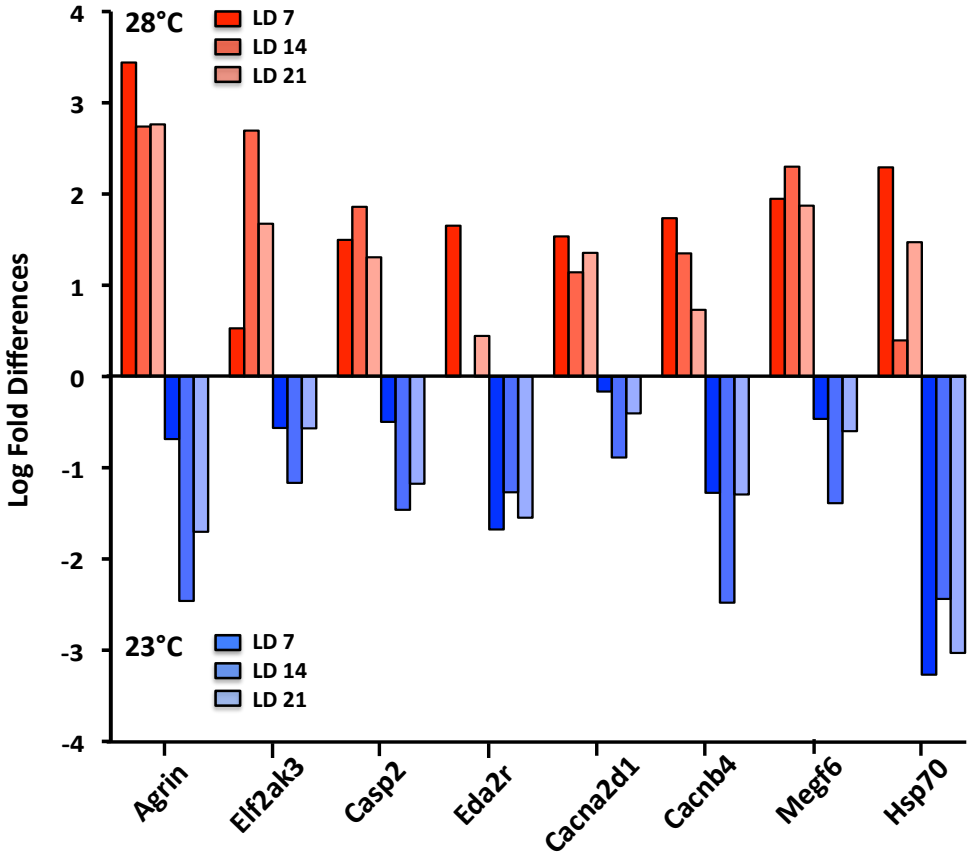

Figure 3.5 Expression levels of previously reported genes associated with heat stress in corals. Levels of expression across lunar days 7, 14, and 21 (compared to lunar day 1) in the 28°C treatment group (all up-regulated) are colored red. Levels of expression across lunar days 7, 14, and 21 in the 23°C treatment group (all down-regulated) are colored blue.

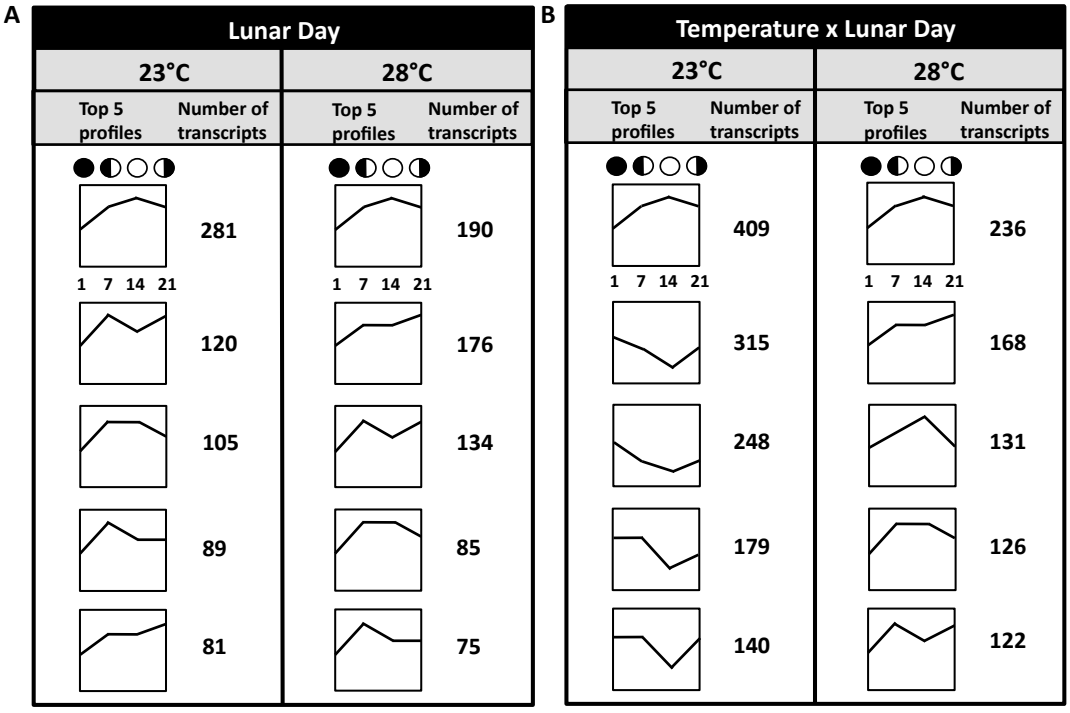

Figure 3.6 Abundant STEM profiles differed between lunar day and the interaction of temperature x lunar day. Grouping of the top five STEM expression profiles for transcripts differentially expressed with (A) lunar day and (B) the temperature x lunar day interaction. Top five profiles, based on the total number of transcripts, are listed for individual temperature treatments. Left panels: top five profiles for the 23°C treatment, right panels: top five profiles in the 28°C treatment. Lunar days and phases are provided to represent sampling time points: new moon (lunar day 1), first quarter moon (lunar day 7), full moon (lunar day 14), and last quarter moon (lunar day 21).

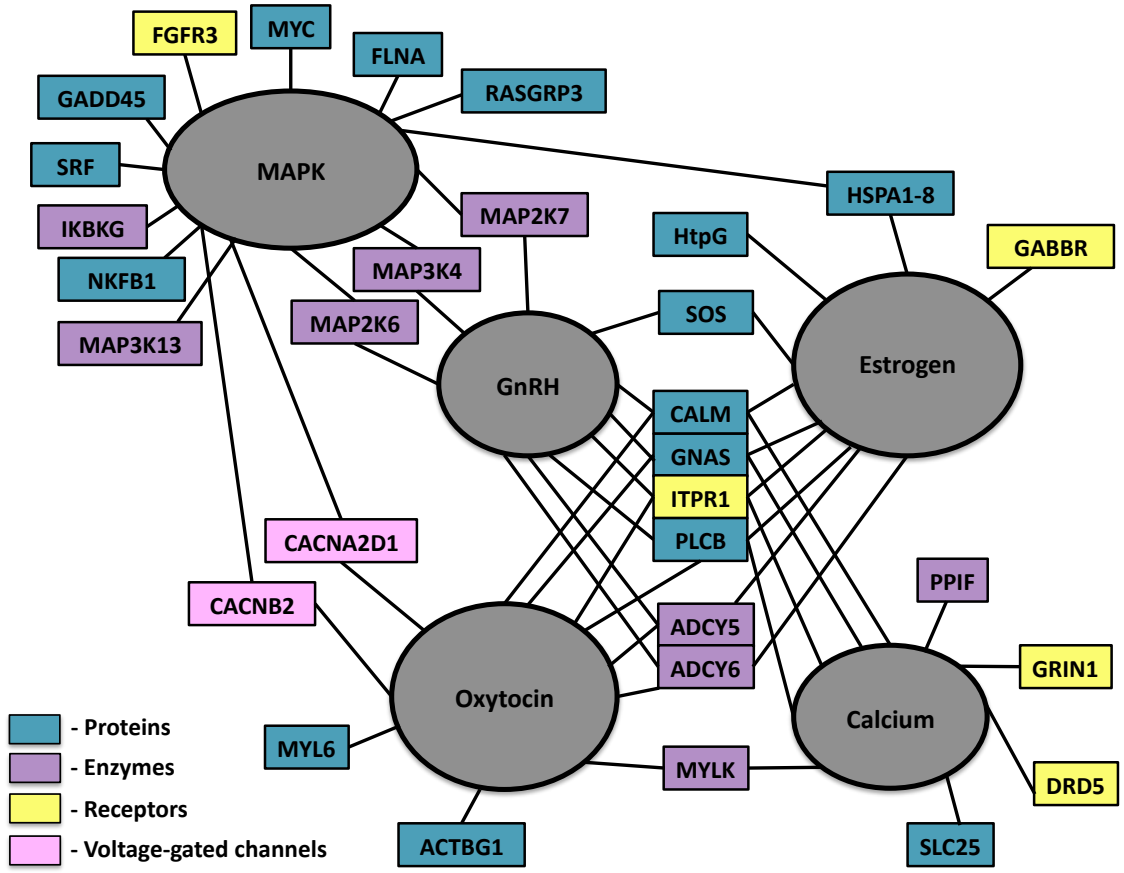

Figure 3.7 Network of selected KEGG signaling pathways depicts pleiotropic gene functions. Five KEGG pathways differentially expressed with the interaction of temperature x lunar day. Circles represent signaling pathways, rectangles represent genes, lines connect genes to individual pathways they are involved in, for a full list of genes see Table 3.4.

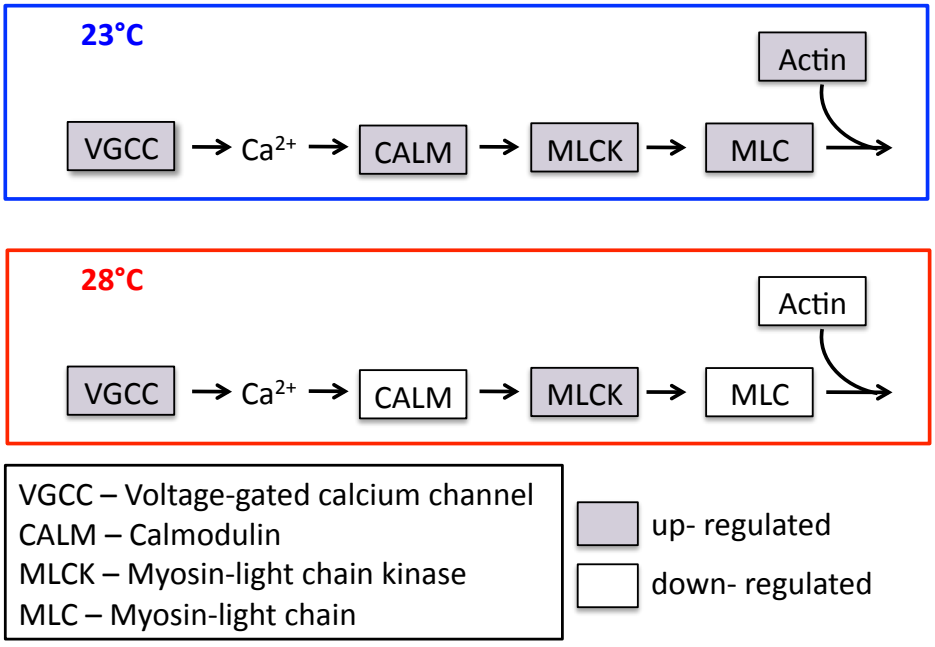

Figure 3.8 Expression levels of genes in the oxytocin signaling pathway, associated with contraction, differ with temperature. Selection of the oxytocin pathway that leads to contraction in mammals. Genes are represented by rectangles. Grey coloration represents up-regulation at lunar day 7.

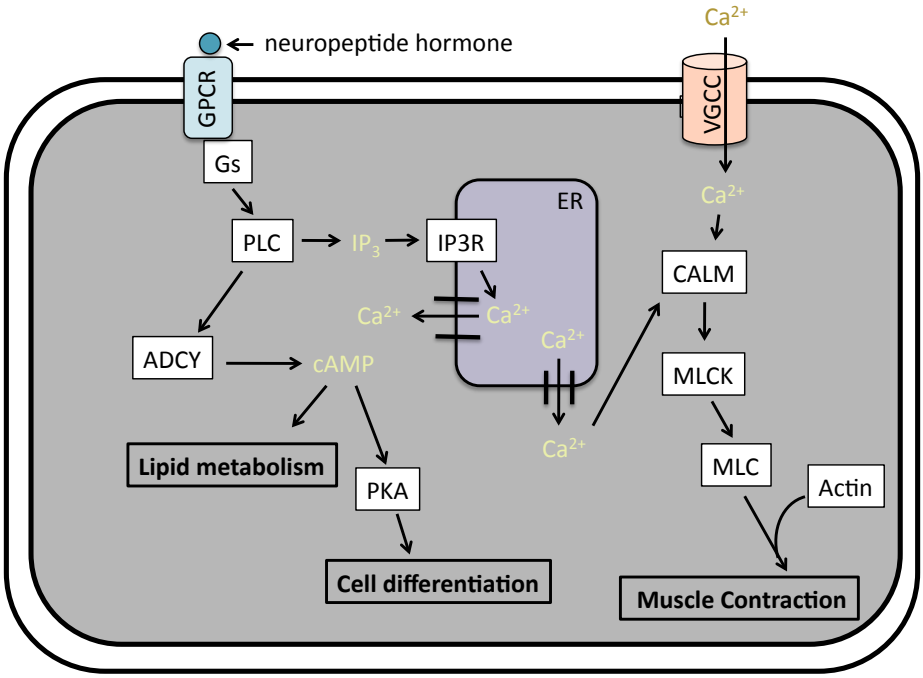

Figure 3.9. Proposed model of neuropeptide signaling associated with planulation. Model is based on KEGG pathways of differentially expressed genes with the interaction of temperature x lunar day. Neuropeptide hormones (such as oxytocin or GnRH-like) bind to g protein-coupled receptors and intiate downstream signaling involing inositol triphosphate (IP_3), calcium (Ca^{2+}), and cyclic adenosine monophosphate (cAMP) resulting in cellular processes such as lipid metabolism and cell differentiation. Calcium stores released from the endoplasmic reticulum (ER) and imported into the cell via voltage-gated calcium channels leads to muscle contraction during planulation (homologous to signaling events in the oxytocin signaling pathway that result in uterine contraction). Genes are identified as rectangles, the blue rounded-edged retangle represents a g protein-coupled receptor, pink cylinder represents voltage-gated calcium channel, signaling molecules are colored yellow, endoplasmic reticulum (ER) is colored purple, and downstream processes are described in bold, for a full list of genes included see Table #.

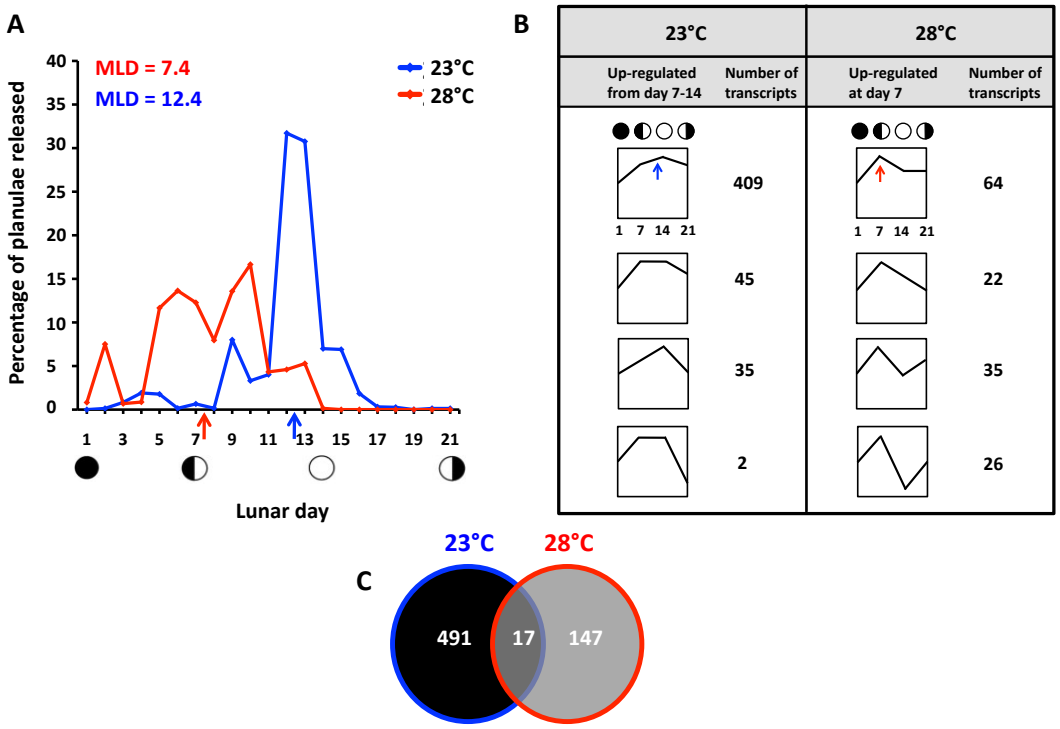

Figure 3.10 Expression profiles associated with the timing of planulation. (A) Percentage of the total number of planulae released in lunar June 2012, data taken from Crowder *et al.* (2014). (B) Grouping of transcripts based on STEM expression profiles associated with the timing of planulation (mean lunar day of planulation is indicated by arrows) for individual temperature treatments. Left panel: STEM profiles for the 23°C treatment that comprise the 491 transcripts associated with timing of planulation in the 23°C treatment. Right panel: STEM profiles that comprise the 147 transcripts associated with the timing of planulation in the 28°C treatment group. **C.** Venn diagram indicating the total number of transcripts associated with individual temperature treatments.

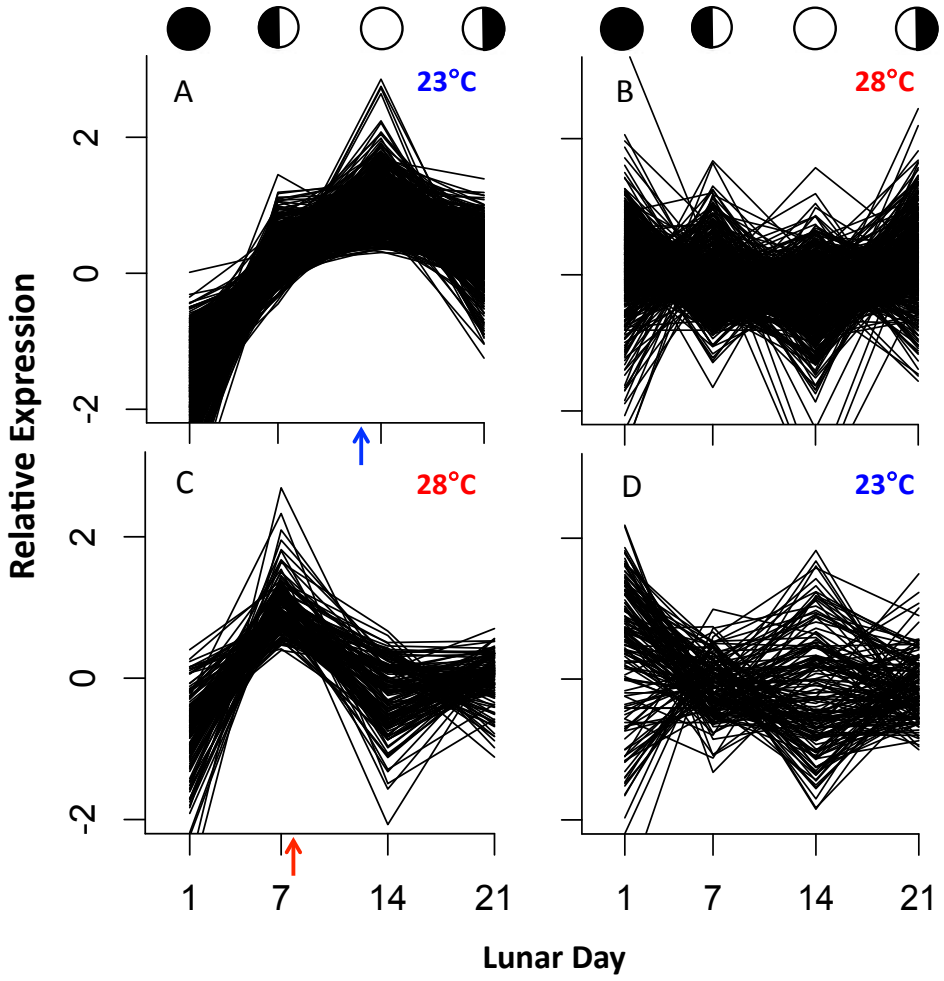

Figure 3.11 Disruptions of planulation profiles with temperature. Comparisons of relative mean expression profiles associated with planulation for individual treatment groups. (**A**) Expression patterns for the 491 transcripts up-regulated from lunar day 7 to 14 in the 23°C treatment and (**B**) the same transcripts at 28°C. Expression patterns for the 147 transcripts up-regulated at lunar day 7 in the 28°C treatment (**C**) and the same transcripts at 23°C (**D**). Arrows depict mean lunar day of planulation for individual temperature treatments reported by Crowder *et al* 2014.

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4. CHARACTERIZATION OF GENE EXPRESSION PROFILES ASSOCIATED WITH LIGHT + TEMPERATURE-INDUCED SPAWNING IN THE MODEL CNIDARIAN *NEMATOSTELLA VECTENSIS*

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4.1 SUMMARY

The sea anemone *Nematostella vectensis*, is a model system for comparative studies exploring the evolution of developmental processes. The success of *N. vectensis* as a model is in part due to the ease of spawning, which can be predictably induced by an increase in temperature together with an extended period of light exposure. Although, these environmental parameters have been identified as triggers, little is known about the transduction events and molecular mechanisms that coordinate gametogenesis and spawning. Gene expression profiling using RNA-Seq analysis allows for an unbiased assessment of global gene expression through time. Here I examine temporal and spatial expression patterns of *N. vectensis* genes across time points prior to, during and after light + temperature-induced spawning.

There were 178 genes differentially expressed between time points, with peaks in expression occurring after the onset of light exposure and an hour before spawning. I observed an unexpectedly small difference at the transcriptomic level between spawning and non-spawning anemones, with 71 genes being differentially expressed. Genes up-regulated in spawning animals included major components of the *N. vectensis* oocyte proteome. This suggests that bi-weekly spawning, in laboratory-maintained *N. vectensis*, could lead to reproductive priming that allows for a rapid spawning response. *In situ* hybridization of differentially expressed genes in developing embryos and adult anemones revealed expression in early-developing and adult mesenteries, where gonadal development occurs. This examination of the transcriptomic regulation of spawning complements previous works detailing gene expression changes throughout development and expands our understanding of the physiological processes in this model cnidarian.

4.2 INTRODUCTION

Nematostella vectensis, an estuarine sea anemone, has been established as a model cnidarian for studies investigating the evolution of animal development. Cnidarians occupy a key phylogenetic position as early-diverging basal metazoans, sister group to the Bilateria, and as the first cnidarian with a sequenced genome, *N. vectensis* has provided key genomic information on the complexity and evolution of animal lineages (Putnam *et al.* 2007). Studies in *N. vectensis*, examining the origins of axial patterning (Ryan *et al.* 2007), process of regeneration (Bossert *et al.* 2013) and the development of the nervous system (Layden & Martindale 2014; Matus *et al.* 2007), have provided evidence of a high degree of conservation in gene repertoire, structure and complexity between cnidarians and bilaterians.

The success of *N. vectensis* as a model system for the study of development, is in part due to the ease of inducing frequent spawning events in the laboratory (Hand & Uhlinger 1992; Stefanik *et al.* 2013). In the majority of spawning cnidarians, reproduction is unpredictable and in most cases occurs once annually (Grawunder *et al.* 2015; Harrison 2011). Although spawning is crucial to the success of *N. vectensis* for developmental studies, surprisingly, no work has focused on the physiology of the reproductive process in this species.

Laboratory populations of *N. vectensis*, a gonochoric spawner, release eggs and sperm into the water column, as often as weekly, upon exposure to a combination of a dark-light cycle and increased temperature (Fritzenwanker 2002). Females become reproductively active at approximately 2-3 months of age by developing gonads in

mesenteries, that extend radially from the inner surface of the gastrodermis. Females release a gelatinous egg mass that containing hundreds of large eggs (~200 μm in diameter) (Darling 2005).

Little is known about the transduction of light and temperature cues into molecular signals that coordinate spawning. Previous studies in *N. vectensis* and the coral *A. millepora* have shown that blue-light sensing cryptochromes increase in expression when animals are exposed to blue- and full-light spectra (Levy *et al.* 2007; Reitzel *et al.* 2010). A recent transcriptomic study in spawning *A. millepora* suggested that the molecular mechanisms cueing spawning involve melanopsin-like proteins, neuropeptides, and G protein-coupled receptor signaling pathways (Kaniewska *et al.* 2015).

While many studies have examined the spatial and temporal patterns of candidate developmental genes throughout embryonic stages in *N. vectensis* (DuBuc *et al.* 2014; Kusserow *et al.* 2005; Magie *et al.* 2005; Marlow *et al.* 2009; Matus *et al.* 2007) less effort has been devoted to investigating gene expression associated with gametogenesis and spawning in developing embryos and adults. This is surprising given that *N. vectensis* is such an important cnidarian model system.

Recently studies in *N. vectensis* have shifted from candidate gene approaches to a global transcriptomic and proteomic examinations (Helm *et al.* 2013; Lotan *et al.* 2014; Tulin *et al.* 2013). Two studies by Helm *et al.* (2013) and Tulin *et al.* (2013) using RNA-Seq, have profiled all genes expressed during embryogenesis and developmental stages and two others by Lotan *et al.* (2014) and Levitan *et al.* (2015) have examined the entire proteome of a newly fertilized *N. vectensis* egg cell, providing a catalog of all oocyte proteins. These large datasets have made significant contributions to the field by

providing valuable resources for future hypothesis testing. In this study, we aim to add to these datasets by exploring global gene expression profiles associated with light + temperature-induced spawning and characterize the transcriptomic signatures of gametogenesis and spawning in *N. vectensis.* To accomplish this, we examined the temporal transcriptomic profiles of spawning and non-spawning adults prior to, during, and after light + temperature-induced spawning, followed by an investigation of spatial expression patterns, using whole mount *in situ* hybridization on a subset of differentially expressed genes.

4.3 METHODS

4.3.1 Animal husbandry and experimental design

Sixty-four genetically identical adult female *N. vectensis*, housed in individual wells of 6-well plates, were stored in an incubator at 16°C in the dark. Anemones were feed brine shrimp nauplii and received water changes weekly. Two-months prior to this study, on a bi-weekly basis, animals were fed chunks of oyster and relocated to a 24°C incubator and exposed to a 7-watt compact fluorescent light for 8 hours, to induce spawning, a procedure described in detail by Stefanik *et al.* (2013).

In June 2014, 32 of these 64 anemones were placed into a 24°C incubator and again exposed to the 8-hour light treatment (spawning treatment). Beginning at 8 am (time 0), four replicate anemones were sacrificed from both the spawn-inducing treatment (light + temperature) and control treatment (dark + 16°C), followed by continued sampling of replicate anemones at: 1, 2, 4, 6 hours post-lights on, pre-spawn (9 hours post-lights on), eggs visible in column (10 hours post-lights on), and post-spawn (11

hours post-lights on with eggs released) (Figure 1). Individual anemones were immediately placed into 1 ml of RNA*later*® Stabilization Solution (Qiagen, CA, US) and stored at 4°C for 24 hours. Following the 4°C incubation, RNA*later*® was removed and tissue was stored at -80°C until transport on dry ice to Oregon State University (OSU).

4.3.2 RNA extraction, cDNA library preparation, and sequencing

Total RNA was extracted from adult *N. vectensis* following a combination of the TRIzol® RNA isolation (Life Technologies, CA, US) and RNeasy Mini Kit (Qiagen, CA, US) extraction protocols. Whole anemones were homogenized in 500 μL of TRIzol® and the manufacturer's protocol was followed up to the chloroform extraction. The aqueous extraction phase was then combined with an equal volume of 100% ethanol and purified using the RNeasy Mini Kit. Total RNA was DNase-treated using a TURBO DNA-Free kit (Ambion, CA, US) to remove genomic DNA contamination. RNA was quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific, MA, US) and quality was determined with gel electrophoresis. Some samples were lost during extraction and purification procedures.

All samples were processed using a 3' tag based method described by Meyer *et al.* (2011). Heat fragmentation at 95°C for 5 minutes was completed on 1 μg of RNA in 10 μL of 10 mM Tris (pH 8.0). Three samples containing less than 1 μg of total RNA were excluded from the analysis. Preparation of cDNA libraries proceeded using the following methods. A list of all oligonucleotide primer sequences used in library preparations is provided (Table 4.1). First-strand cDNA was synthesized by incubating fragmented RNA at 65°C for 5 minutes with 1 μL of 10 μM 3ILL-20TV oligonucleotide

followed by incubation at 42°C for 1 hour with a master mix consisting of 1 μL water, 1 μL dNTP (10mM), 2 μL DTT (0.1 M), 4 μL 5x First-Strand Buffer, 1 μL ILL-4N-TS RNA oligonucleotide (10 μM), and 1 μL Super Script II Reverse Transcriptase (Life Technologies, CA, US)). Amplification of cDNA libraries was completed using a master mix consisting of 5 μL of first-strand cDNA, 67.5 μL water, 2.5 μL dNTP (10 mM), 20 μL 5X Phusion PCR Buffer, 2 μL 5ILL oligonucleotide (10 μM), 2 μL 3ILL-20TV oligonucleotide (10 μM), 1 μL Phusion Taq and a thermal profile with an initial incubation at 98°C for 5 seconds followed by 20 cycles of: 98°C for 10 s, 60°C for 30 s, 72°C for 1.5 min. PCR products were purified using an E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek, GA, US). Further amplification of cDNA libraries (4 cycles) was completed to incorporate sample specific-barcode adaptors for multiplexing. Products were then size-selected (200–350 bp fragments) and gel extracted. Samples were quantified using qPCR, pooled, and purified again using the E.Z.N.A. Cycle Pure Kit, prior to sequencing on an Illumina Hi-Seq at OSU's Center for Genomics and Bioinformatics (CGRB).

4.3.3 Transcriptomic processing, annotation and mapping

The *N. vectensis* reference transcriptome **"**Additional_file_1_reference.fasta" described by Helm *et al.* (2013) was downloaded from http://www.biomedcentral.com/content/supplementary/1471-2164-14-266-s1.zip. This transcriptome was modified from previously published genomic references (Putnam *et al.* 2007). In order to reduce mapping redundancy, cd-hit-est was run to cluster similar transcripts into a single cluster (Li & Godzik 2006). In order to obtain as much information possible, putative genes names and functional categories (Gene Ontology

(GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)) were assigned to individual transcripts. A detailed description of these annotation procedures is described in Kitchen *et al.* (2015).

A total of 55 cDNA libraries were pooled and distributed between two lanes that were sequenced on an Illumina HiSeq 2000 at the Center for Genomics and Research and Biocomputing (CGRB) at Oregon State University. Single-end sequencing of cDNA libraries produced 50 bp reads. An additional single-end 100 bp run was completed to further sequence a few samples whose total read count was < 1 million raw reads. Sequences were de-multiplexed based on their sample-specific barcode adaptors. Raw reads were filtered to eliminate low quality reads (> 20 positions with Phred scores < 20) and any reads containing adaptor sequences or homopolymer repeats (e.g., poly-A tails) were removed using custom Perl scripts (https://github.com/Eli-Meyer). High quality reads were mapped to the reference transcriptome using the software package SHRiMP with default settings (version 2.2.2) (David *et al.* 2011; Rumble *et al.* 2009).

4.3.4 Differential expression and profiling analysis

All calculations and statistical tests were conducted using R (version 3.2.1) (R Development Core Team, 2008). Statistical comparisons of RNA-Seq count data were completed using the R package DESeq2 (version 1.9), which uses a variance stabilizing procedure (vsd) to transform count data into weighted expression values suitable for linear modeling (Love 2014). Raw *p*-values were adjusted for multiple test corrections and false discovery rate using the Benjamini Hochberg procedure. A transcript was

considered differentially expressed if the resulting adjusted *p*-value was < 0.1, the default setting in DESeq2.

The software program Short Time-series Expression Miner (STEM) was used to examine temporal patterns in gene expression and group transcripts into profiles based on their expression patterns (Ernst, 2006). Normalized expression data for biological replicates exposed to the spawn inducing treatment were averaged and fold changes, relative to the 8 am (time point 0), were calculated as the difference between the mean expression of each transcript across all time points.

4.3.5 Whole-mount *in situ* hybridization

Twenty-three digoxigenin (DIG) labeled probes were synthesized for a subset of genes in the "light" and "spawn" profiles using Ambion's MEGAscript T7 Transcription Kit (ThermoFisher Scientific, MA, US). Genes of interest were amplified from previously constructed PGEM-T plasmids (Promega,WI, US) from an existing expressed sequence tag (EST) library (Martindale & Technau, unpublished data). Visualization of the spatial expression patterns of gene probes was completed using whole mount *in-situ* hybridization (WISH) procedures that have previously been described elsewhere (Martindale *et al.* 2004; Matus *et al.* 2007; Wolenski *et al.* 2013). Probe hybridizations were performed at 65°C for ~ 24 hours. Probe binding was detected using NBT/BCIP and developed at 4°C. Total development times varied with each gene probe. Developing embryos were collected at the following stages: blastula (24 hrs), gastrula (48 hrs), planula (72 hrs) and early-stage polyp (240 hrs). Both embryos and adults were fixed in 4% formaldehyde + 0.2% gluteraldehyde for 30 s prior to WISH.

4.4 RESULTS

4.4.1 Mapping, sequencing and differential expression

The reference transcriptome contained 25,212 individual genes, of which 12,164 passed a mapping threshold (mapped reads ≥ 48) and were tested for differential expression (Table 4.2). Seven samples with < 1 million filtered reads were excluded from the analysis. All three variables (time point, treatment, and the interaction of time point x treatment) had an impact on gene expression (adjusted p-value < 0.1).

Time point alone affected the expression of 178 transcripts, of which 78% had homologous matches to online databases (Table 4.3). Forty-seven of these transcripts were significantly differentially expressed at an adjusted p-value < 0.05 and a heat map depiction of these transcripts displays variable patterns in expression between biological replicates for time point, since not all replicates grouped together (Figure 4.2).

Homologous matches in UniProt databases for time point revealed multiple genes associated with cytoskeletal organization, motility, heat stress, reproduction, post-transcriptional regulation of gene expression and G protein-coupled nuclear receptor activity (Table 4.3). Top matches in the Gene Ontology (GO) databases listed were, in the biological category: metabolism, cell organization, development and cell differentiation; cellular category: cell, intracellular, cytoplasm, and cytoskeletal; and molecular category: binding, catalytic activity, protein binding and hydrolase activity (Figure 4.3). Lastly, KEGG pathway mapping revealed ribosome, metabolic, phagosome, vasopressin-regulated water reabsorption pathways and biosynthesis of secondary metabolites as the pathways with the most homologous matches (Table 4.4).

The effect of treatment impacted the expression of 71 transcripts, 85% of which had homologous matches to online databases (Table 4.5). A heat map depiction of expression patterns of differentially expressed (adjusted *p*-value < 0.05) genes showed clear differences (albeit with two outliers) between spawners and controls (Figure 4.4). Homologous matches in UniProt databases showed multiple genes associated with cytoskeletal organization, heat stress, sperm-associated proteins, and post-transcriptional regulation of gene expression (Table 4.5). Top matches in the GO databases listed were, in the biological category: metabolism, biosynthesis, nucleic acid metabolism and development; cellular category: cell, intracellular, cytoplasm, nucleus and cytoskeletal; and molecular category: binding, catalytic activity, protein binding, and nucleic acid binding (Figure 4.5). Lastly, KEGG pathway mapping revealed metabolic, PI3K-Akt signaling, estrogen signaling, and vasopressin-regulated water reabsorption as the pathways with the most matches (Table 4.6).

The interaction of both time point x treatment together impacted the expression of 2 transcripts (adjusted *p*-value < 0.1). Both of these had homologous matches to online databases (Table 4.7) and included genes associated with cell adhesion and actin binding. However, thirteen transcripts were in common in these two groups and therefore were differentially expressed with both time point alone and treatment alone (Table 4.8), but had adjusted *p*-values above the cut-off threshold for differential expression in the time point x treatment interaction term.

Genes differentially expressed with time point and treatment were compared to a recently published transcriptomic study in spawning on the day of spawning compared to non-spawning *A. millepora* corals, where a larger number of genes (184, $p < 0.05$) where

shown to be differentially expressed (Kaniewska *et al.* 2015). Six genes were differentially expressed in both studies (Table 4.9).

Several genes differentially expressed as a function of both time point alone and treatment alone had homologous matches to genes with reproductive functions or to proteins that were abundant in the *N. vectensis* oocyte proteome (Lotan *et al.* 2014). Genes associated with reproduction were: two genes involved with germ cell development and meiosis including meiosis-specific nuclear structural protein-1 and tudor domain-containing protein-1 were differentially expressed with time point (Table 4.3). Unexpectedly, since animals tested were female, four genes associated with spermatogenesis and sperm motility were differentially expressed: spermatogenesis-associated serine-rich protein-1, nuclear autoantigenic-sperm protein, Protein NDRG3 and sorbitol dehydrogenase, with the latter two being differentially expressed with treatment (Table 4.3 and 4.5). Multiple proteins described as components of the *N. vectensis* oocyte proteome were differentially expressed in time point and treatment: the most abundant oocyte protein vitellogenin, the cytoskeletal proteins actin and tubulin, elongation factor, peroxiredoxin-6, ferritin, as well ribosomal and transport protein (Lotan *et al.* 2014).

4.4.2 Temporal patterns in gene expression

The software program STEM was used to identify groups of transcripts showing similar temporal patterns of expression. Based on significance and abundance, the top profiles were the same for time point and treatment (Figure 4.6A and B). The top profile showed a peak in expression an hour after lights on and will be referred to as the “light”

profile. In this profile there were 25 and 6 genes differentially expressed with time point and treatment, respectively (Figure 4.6A and B). Homologous matches to genes in the light profile included those associated with cytoskeletal organization, lipid metabolism, ion and protein transport (Table 4.10).

The second most significant profile for both time point and treatment showed a peak in expression an hour after lights off and just prior to spawning and will be referred to as the "spawn" profile. In this profile there were 24 and 4 genes differentially expressed with time point and treatment respectively (Table 4.11). Homologous matches in the spawn profile revealed genes associated cytoskeletal organization, cell migration and post-transcriptional regulation.

4.4.3 Whole-mount *in situ* hybridization

Three genes each from the "light" and "spawn" profiles were selected as candidates for WISH in both developing embryos and adults, due to their up-regulation during development in a previous study by Helm *et al.* (2013) that characterized all genes differentially expressed during early-stage development. Homologous matches to the UniProt database of the three selected from the "spawn" profile were: male specific lethal-1, spermatogenesis-associated serine rich protein 2, and zinc finger matrin-type protein 2. Homologous matches of the three selected in the "light" profile were: pancreatic granule membrane major glycoprotein, ras-related protein Rab-5c, and peroxiredoxin-6.

Two of these genes (peroxiredoxin-6 (P6) and pancreatic secretory granule membrane glycoprotein (GP2)) showed clear expression patterns in early developmental

stages (Figure 4.7). Early up-regulation of GP2 was observed in the pharyngeal ring surrounding the blastophore and in developing mesenteries (Figure 4.7B). P6 was highly expressed in the mesenteries of early-stage polyps and in dissected adults, with dark staining observed in mesenterial endoderm compared to ectoderm (Figures 4.7 & 4.8).

4.5 DISCUSSION

4.5.1 Patterns in gene expression

Time point had the largest impact on gene expression (178 differentially expressed genes), followed by treatment (66 genes) and the interaction of time point x treatment (2 genes) (Tables 4.2). This level of differential expression is comparable to that found in the coral *A. millepora*, where 184 genes were reported as differentially expressed between animals at the time of spawning compared to two months earlier (Kaniewska *et al.* 2015). Homologous matches of differentially expressed genes in our study compared to the *A. millepora* study revealed an up regulation of six genes during spawning common to both datasets (Table 4.9). These genes included proteins involved in DNA binding, metabolic processes, and protein transport, but did not include light-sensing cryptochromes, melanopsin proteins, or G protein-coupled receptors that were suggested to be regulators of signal transduction in *A. millepora* during spawning. This scant similarity in differentially expressed genes could indicate significant differences in induction and or in spawning mechanisms between laboratory-maintained *N. vectensis* and corals collected from nature immediately before spawning.

Expression patterns of biological replicates did not strongly group as a function of time point (Figure 4.2) suggesting the presence of biological variation between anemones

sampled at the same time points. This was unexpected given that animals used in the study came from a single clonal lineage exposed to an identical treatment. This could indicate that the spawning treatment impacted individual animals differently at the transcriptomic level, transcriptomic responses occurred at slightly different time scales, or that other non-transcriptional mechanisms are involved in coordinating spawning.

Expression patterns of replicates grouped much more strongly with treatment (Figure 4.4). The number of transcripts differentially expressed with treatment was unexpectedly low, given that all treated animals and no controls spawned. This modest transcriptomic difference could indicate a sexual reproductive strategy that allows for high reproductive plasticity and rapid spawning. This is in marked contrast to most spawning corals that spawn once annually and commit to spawning months prior to gamete release (as evidenced by gonadal development) (Shikina *et al.* 2012; Szmant-Froelich *et al.* 1980; Vermeij 2004). This could indicate a strategy *N. vectensis* has developed for living in an estuarine habitat with highly variable fluctuations in temperature, salinity, and oxygen. Since all life stages exhibit high tolerance thresholds and low vulnerability to changes in temperature, salinity, and oxygen levels (Reitzel *et al.* 2013a) acute shifts in environmental variables could trigger a rapid largely non-transcriptional response to induce spawning.

Alternatively, the observed modest transcriptional response could be due to long-term acclimation to laboratory conditions. Samples used in this study had been entrained to a bi-weekly spawning schedule for an extended period of time. This artificial laboratory setting could have an impact on transcriptional patterns. A comparison to animals collected from nature could provide insight into the impact of bi-weekly

entrainment on transcriptional patterns. Lastly, the time points sampled in this study could have missed a large transcriptomic response associated with reproduction. A follow up study that compares non-spawning animals the week between spawning with those undergoing spawning has the potential of revealing a stronger transcriptomic response.

4.5.2 Mechanisms of induction

Elevated expression, prior to spawning, of genes involved in post-transcriptional modifications could reveal an alternative mechanism for inducing spawning in *N. vectensis.* Post-translational modifications, involving microRNA silencing, have been described in *N. vectensis* where microRNAs are suggested to play a regulatory role in developmental processes (Moran 2014). A comparison of the proteomes between spawning and control animals could reveal larger differences at the proteomic level, which would support this hypothesis. Furthermore, other post-transcriptional mechanisms or epigenetic factors, not yet described in cnidarians, could potentially play a role.

4.5.3 *In-situ* hybridization verifies gene expression patterns

In situ hybridization confirmed high levels of expression in both GP2 and P6, two genes involved with lipid transfer and metabolism. GP2, a glycoprotein involved in the anchoring of lipids to the cell membrane, showed expression early in development beginning with a ring around the blastophore in the oral ectoderm and highest levels of expression in developing mesenteries of early-stage polyps. This ring pattern of expression is similar to expression patterns observed in the *N. vectensis* homolog of dopamine beta hydroxylase, an enzyme that converts dopamine to norephinephrine

(Marlow *et al*. 2009). P6, a gene involved in lipid catabolism and present in the oocyte proteome (Lotan *et al.* 2014), showed high levels of expression in both early-stage polyp endoderm and adult mesenteries. Since gonadal development occurs in endodermal mesenteries, this supports a role of GP2 and P6 in reproductive signaling and or lipid transfer to developing oocytes, which are rich in lipids (Lotan *et al.* 2014). The early detection of gene expression in developing embryos could indicate pleotropic roles for these two genes in *N. vectensis*.

4.6 CONCLUSIONS

Differential gene expression across multiple time points prior to, during, and after spawning revealed a large up-regulation of genes coding for proteins that were expressed in *N. vectensis* oocytes as well as genes involved with cytoskeleton organization, nucleic acid binding, and post-transcriptional modifications. There was a lower than expected number of differentially expressed genes in spawning compared to non-spawning animals. This modest level of differential expression, coupled with the differential expression of genes potentially involved in post-transcriptional modifications could indicate post-transcriptional mechanisms at play, which would explain the rapidity and ease of spawning induction in this species of cnidarian. Further analyses are needed to investigate this hypothesis, but this type of reproductive control could be novel in the phylum Cnidaria and result from plasticity and flexibility in *N. vectensis*, a characteristic that may have contributed to its ease of culture in the laboratory and its success in marginal habitats.

Table 4.1 Oligonucleotide primers used during cDNA library preparation for Illumina sequencing. Oligonucleotide sequences © 2007-2012 Illumina, Inc.

Oligonucleotide	Sequence
3ILL-20TV	ACGTGTGCTCTTCCGATCTAATTTTTTTTTTTTTTTTTTTV
ILL-4N-TS	ACCGCATGCGGCTACACGACGCTCTTCCGATCTNNNNGGG
5ILL	CTACACGACGCTCTTCCGATCT

Table 4.2 Metrics and results for mapping and differential expression.

Mapping Results	Sequences
Average number of reads per sample	7,279,364
Average number removed during filtering	1,785,996
Average number of reads per sample after filtering	5,493,368
Percentage of reads lost to ambiguous mapping	22%

Differential Expression Results	Sequences
Number of transcripts in the reference transcriptome	25,212
Number of transcripts passing thresholds and tested for differential expression	12,164
Number of genes differentially expressed with time point	178
Number of genes differentially expressed with treatment	66
Number of genes differentially expressed with the interaction of time point x treatment	2

Table 4.3 Genes with UniProt matches differentially expressed with time point. Gene numbers represent gene identifiers listed in the Joint Genome Institute (JGI) website.

Gene ID	UniProt ID	UniProt Gene Description
175346	P42577	Soma ferritin
238419	Q8CBH5	Major facilitator superfamily domain-containing protein 6
245162	Q98TR7	40S ribosomal protein S16
195315	P63018	Heat shock cognate 71 kDa protein
240868	A4IF97	Myosin regulatory light chain 12B
168076	Q54271	Phosphonopyruvate decarboxylase
167582	Q05974	Ras-related protein Rab-1A
68375	Q3E811	Regulator of rDNA transcription protein 15
244746	Q9CZX5	PIN2/TERF1-interacting telomerase inhibitor 1
248175	A8MXE2	Putative UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase
241252	P51949	CDK-activating kinase assembly factor MAT1
236730	P25867	Ubiquitin-conjugating enzyme E2-17 kDa
229061	A2PYL8	Hexokinase-2
192806	P38117	Electron transfer flavoprotein subunit beta
236978	Q9D051	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial
229658	Q14692	Ribosome biogenesis protein BMS1 homolog
234225	Q5ZJF4	Peroxiredoxin-6
244590	Q2HJ86	Tubulin alpha-1D chain
158666	O16118	Guanine nucleotide-binding protein G(s) subunit alpha
113255	O73888	Hematopoietic prostaglandin D synthase
160811	P26352	Thymosin beta-12
85854	Q56K03	60S ribosomal protein L27a
247318	Q7T293	Protein SDE2 homolog
239031	Q06303	Aerolysin-4
245479	Q9NQX5	Neural proliferation differentiation and control protein 1
236044	P14272	Plasma kallikrein
230864	Q13310	Polyadenylate-binding protein 4
237519	P27659	60S ribosomal protein L3
177213	Q13231	Chitotriosidase-1
216404	A8KB59	Coiled-coil domain-containing protein 153
236679	O02414	Dynein light chain LC6, flagellar outer arm
190623	Q9JLR1	Protein transport protein Sec61 subunit alpha isoform 2
245966	A7SL20	Ribosomal RNA processing protein 36 homolog
86218	Q9UQ35	Serine/arginine repetitive matrix protein 2
167920	Q7ZV80	Survival of motor neuron-related-splicing factor 30
124712	P86179	L-rhamnose-binding lectin CSL3
91920	Q9QWW1	Homer protein homolog 2
242253	P25291	Pancreatic secretory granule membrane major glycoprotein GP2
90224	Q7ZW86	Peptidyl-prolyl cis-trans isomerase CWC27 homolog

Table 4.3 (Continued)

Gene ID	UniProt ID	UniProt Gene Description
243395	B0BM24	Protein FAM161A
189983	Q05975	Ras-related protein Rab-2
243751	Q1MSJ5	Centrosome and spindle pole-associated protein 1
167953	P63245	Guanine nucleotide-binding protein subunit beta-2-like 1
236543	O96952	Thioredoxin
238337	Q86XZ4	Spermatogenesis-associated serine-rich protein 2
173898	Q66I24	Argininosuccinate synthase
240939	Q5XFY4	B-cell CLL/lymphoma 7 protein family member A
179973	O76190	60S ribosomal protein L5
194342	Q8VEM8	Phosphate carrier protein, mitochondrial
177484	Q9GT45	40S ribosomal protein S26
66182	Q7ZVW9	Arginine and glutamate-rich protein 1-B
81224	Q6PCJ1	Dynactin subunit 1
158216	P51147	Ras-related protein Rab-5C
178746	Q9XSI3	60S ribosomal protein L10
169611	O93400	Actin, cytoplasmic 1
132332	Q9UJU2	Lymphoid enhancer-binding factor 1
244349	P41891	Protein gar2
175341	P42577	Soma ferritin
81851	Q0VFZ6	Coiled-coil domain-containing protein 173
59372	Q6CQE5	Protein TAR1
213913	Q8WWQ8	Stabilin-2
163923	Q6PGZ3	TRAF3-interacting protein 1
205229	P87498	Vitellogenin-1
245849	Q5T1M5	FK506-binding protein 15
161221	Q9NRZ9	Lymphoid-specific helicase
105818	Q9CQU1	Microfibrillar-associated protein 1
245689	Q9LQU4	Protein Plant cadmium resistance 2
167737	P11833	Tubulin beta chain
169502	P94598	Glutamate dehydrogenase
26812	Q9ULJ8	Neurabin-1
244319	Q6RUV5	Ras-related C3 botulinum toxin substrate 1
29518	P15870	Histone H1-delta
232813	P55112	Zinc metalloproteinase nas-4
244785	A1L2I9	Transmembrane protein 214-B
165664	P62263	40S ribosomal protein S14
237468	P46781	40S ribosomal protein S9
155941	P29693	Elongation factor 1-delta
243257	P20478	Glutamine synthetase 2 cytoplasmic
221552	Q9D565	WD repeat-containing protein 64
165407	Q96NC0	Zinc finger matrin-type protein 2
240276	A9JRX0	Male-specific lethal 1-like 1

Table 4.3 (Continued)

Gene ID	UniProt ID	UniProt Gene Description
215658	B3EWY8	Skeletal aspartic acid-rich protein 2
168739	Q642C0	DnaJ homolog subfamily C member 8
170702	P52565	Rho GDP-dissociation inhibitor 1
197216	Q6PBH5	Cytochrome c oxidase subunit NDUFA4
183698	Q14008	Cytoskeleton-associated protein 5
218444	Q86Y13	E3 ubiquitin-protein ligase DZIP3
191841	P24369	Peptidyl-prolyl cis-trans isomerase B
248018	O13035	Proactivator polypeptide
228736	Q0V8M0	Protein KRI1 homolog
245662	Q66HD3	Nuclear autoantigenic sperm protein
169263	Q9D7W4	Tetraspanin-17
78945	Q9UQB3	Catenin delta-2
239375	Q5TD94	Radial spoke head protein 4 homolog A
172695	Q5R1W1	Septin-7
245400	P98069	Bone morphogenetic protein 1 homolog
228922	Q6GMH0	Pre-mRNA-splicing factor 18
192745	Q96HN2	Putative adenosylhomocysteinase 3
180651	Q95029	Cathepsin L
170524	B8V7S0	CUB and peptidase domain-containing protein 1
28519	A1L224	Cyclic AMP-responsive element-binding protein 3-like protein 2
181774	Q10651	Beta-amyloid-like protein
239161	P46783	40S ribosomal protein S10
161077	Q803V3	Serine/threonine-protein phosphatase 2A subunit B subunit gamma
190019	Q26636	Cathepsin L
167144	P55201	Peregrin
102755	A0JNC0	Tropomodulin-1
236404	Q4GXG7	60S ribosomal protein L18
242566	P32969	60S ribosomal protein L9
164493	Q0VCR1	Protein RTF2 homolog
239786	P25291	Pancreatic secretory granule membrane major glycoprotein GP2
243925	Q7TSA6	Proline and serine-rich protein 3
130809	Q3UU96	Serine/threonine-protein kinase MRCK alpha
241341	P28648	CD63 antigen
164776	Q7L014	Probable ATP-dependent RNA helicase DDX46
85009	Q3TJ91	Lethal(2) giant larvae protein homolog 2
176892	A4IJ21	Meiosis-specific nuclear structural protein 1
116145	Q3SZ12	Probable ribosome biogenesis protein RLP24
189121	Q9UPN3	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5
245261	Q8ISP0	40S ribosomal protein S18
162224	P24049	60S ribosomal protein L17
226223	Q90YT0	60S ribosomal protein L37a
234970	Q6PBF0	60S ribosomal protein L8
188243	Q964E3	Actin, cytoplasmic
234207	P27604	Adenosylhomocysteinase

Table 4.3 (Continued)

Gene ID	UniProt ID	UniProt Gene Description
216486	A5PK74	Bifunctional lysine-specific demethylase and histidyl-hydroxylase
180912	A8XWX5	Chondroitin proteoglycan 2
197623	O13067	Condensin complex subunit 2
167011	Q9H1H9	Kinesin-like protein KIF13A
240796	Q9Y2U5	Mitogen-activated protein kinase kinase kinase 2
169568	P19804	Nucleoside diphosphate kinase B
226967	D3ZUQ0	RILP-like protein 1
246464	Q5ZI43	Snurportin-1
186331	P13607	Sodium/potassium-transporting ATPase subunit alpha
237552	P42577	Soma ferritin
241141	O18405	Surfeit locus protein 4 homolog
247520	O57594	Surfeit locus protein 6 homolog
245679	Q99MV1	Tudor domain-containing protein 1

Table 4.4 Selected KEGG pathways of genes differentially expressed with time point. KEGG pathway ID, pathway name, and the number of genes within the pathway with matches are listed.

KO ID	KEGG Pathway	Genes
ko03010	Ribosome	16
ko01100	Metabolic pathways	13
ko04145	Phagosome	7
ko04962	Vasopressin-regulated water reabsorption	6
ko01110	Biosynthesis of secondary metabolites	5
ko03040	Spliceosome	5
ko04024	cAMP signaling pathway	4
ko04261	Andrenergic signaling in cardiomyocytes	4
ko04540	Gap junction	4
ko04810	Regulation of actin cytoskeleton	4
ko04972	Pancreatic secretion	4
ko05205	Proteoglycans in cancer	4
ko00220	Arginine biosynthesis	3
ko00250	Alanine, aspartate and glutamate metabolism	3
ko04010	MAPK signaling pathway	3
ko04015	Rap1 signaling pathway	3
ko04141	Protein processing in endoplasmic reticulum	3
ko04142	Lysosome	3
ko04151	PI3K-Akt signaling pathway	3
ko04152	AMPK signaling pathway	3
ko04210	Apoptosis	3
ko04390	Hippo signaling pathway	3
ko04510	Focal adhesion	3
ko04520	Adherens junction	3
ko04724	Glutamatergic synapse	3
ko04728	Dopaminergic synapse	3
ko04915	Estrogen signaling pathway	3
ko04916	Melanogenesis	3
ko04918	Thyroid hormone synthesis	3
ko04922	Glucagon signaling pathway	3

Table 4.5 Genes with UniProt matches differentially expressed with treatment. Transcript numbers represent gene identifiers listed in the JGI website.

Gene ID	UniProt ID	UniProt Gene Description
158601	Q5REB8	Acyl-CoA synthetase short-chain family member 3
166341	Q8CG45	Aflatoxin B1 aldehyde reductase member 2
114134	B2G331	Analgesic polypeptide HC1
236455	Q5XGM3	Betaine-homocysteine S-methyltransferase 1
96382	Q9NRL2	Bromodomain adjacent to zinc finger domain protein 1A
166897	Q3T168	Calcyclin-binding protein
114346	P53568	CCAAT/enhancer-binding protein gamma
238912	B3EWZ3	Coadhesin
158054	Q8IYE0	Coiled-coil domain-containing protein 146
81851	Q0VFZ6	Coiled-coil domain-containing protein 173
237783	Q96HJ3	Coiled-coil domain-containing protein 34
246171	Q28IQ9	Cold-inducible RNA-binding protein
158749	A7RHL5	Costars family protein v1g158749
242787	P27925	Cyclic AMP-responsive element-binding protein 1
28519	A1L224	Cyclic AMP-responsive element-binding protein 3-like protein 2
239399	Q606D6	Dihydroxy-acid dehydratase
122680	P11387	DNA topoisomerase 1
236679	O02414	Dynein light chain LC6, flagellar outer arm
220102	Q7TPV2	E3 ubiquitin-protein ligase DZIP3
239505	E9Q555	E3 ubiquitin-protein ligase RNF213
240518	B8VIW9	Fibronectin type III domain-containing protein
160673	Q6DCZ7	Formin-binding protein 1
245724	P14314	Glucosidase 2 subunit beta
172093	Q05966	Glycine-rich RNA-binding protein 10
178640	P30946	Heat shock protein HSP 90-alpha
185184	Q196Z2	High mobility group protein
29518	P15870	Histone H1-delta
194445	P84245	Histone H3.3
208718	Q16543	Hsp90 co-chaperone Cdc37
172038	Q566I3	Hypoxia up-regulated protein 1
124712	P86179	L-rhamnose-binding lectin CSL3
161221	Q9NRZ9	Lymphoid-specific helicase
239233	Q174D3	Mediator of RNA polymerase II transcription subunit 19
177611	P54357	Myosin-2 essential light chain
245479	Q9NQX5	Neural proliferation differentiation and control protein 1
242253	P25291	Pancreatic secretory granule membrane major glycoprotein GP2
182009	Q8IVL5	Prolyl 3-hydroxylase 2
243395	B0BM24	Protein FAM161A
247562	Q9UGV2	Protein NDRG3
164493	Q0VCR1	Protein RTF2
248175	A8MXE2	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase

Table 4.5 (Continued)

Gene ID	UniProt ID	UniProt Gene Description
173264	Q5E9C0	Ras suppressor protein 1
160897	P55006	Retinol dehydrogenase 7
160402	Q569B7	RWD domain-containing protein 4
26678	P26686	Serine-arginine protein 55
198399	A1L1M4	Serine/threonine-protein phosphatase 4 regulatory subunit 2-B
215658	B3EWY8	Skeletal aspartic acid-rich protein 2
193535	Q58D31	Sorbitol dehydrogenase
240418	Q92543	Sorting nexin-19
163923	Q6PGZ3	TRAF3-interacting protein 1
241026	Q7YQM3	Transcriptional regulator ATRX
186200	P08537	Tubulin alpha chain
176499	P41383	Tubulin alpha-2/alpha-4 chain
245872	O46037	Vinculin
240820	Q8N2E2	Von Willebrand factor D and EGF domain-containing protein
120741	Q5ZLX5	Zinc finger Ran-binding domain-containing protein 2

Table 4.6 Selected KEGG pathways of genes differentially expressed with treatment. KEGG pathway ID, pathway name, and the number of genes within the pathway with matches are listed.

KO ID	KEGG Pathway	Genes
ko01100	Metabolic pathways	6
ko04151	PI3K-Akt signaling pathway	5
ko04915	Estrogen signaling pathway	4
ko04962	Vasopressin-regulated water reabsorption	3
ko04925	Aldosterone synthesis and secretion	3
ko04922	Glucagon signaling pathway	3
ko04916	Melanogenesis	3
ko04728	Dopaminergic synpase	3
ko04141	Protein processing in endoplasmic reticulum	3
ko04024	cAMP signaling pathway	3
ko04022	cGMP-PKG signaling pathway	3

Table 4.7 Genes differentially expressed with the interaction of time point x treatment. Transcript identifiers represent gene identifiers listed in the Joint Genome Institute (JGI) website.

Gene ID	UniProt ID	UniProt Gene Description
207295	Q8C6K9	Collagen alpha-6(VI) chain
81172	Q7JQD3	Gelsolin-like protein 1

Table 4.8 Shared Genes differentially expressed with both time point alone and treatment alone.

UniProt ID	UniProt Gene Description
A1L224	Cyclic AMP responsive element-binding protein 3
A8MXE2	UDP-GlcNAc:betaGal beta-1,3 N-acetylglucosaminyltransferase
B0BM24	Protein FAM161A
B3EWY8	Skeletal aspartic acid-rich protein 2
O02414	Dynein light chain LC6, flagellar outer arm
P15870	Histone H1-delta
P25291	Pancreatic secretory granule membrane major glycoprotein GP2
P86179	L-rhamnose-binding lectin CSL3
Q0VCR1	Protein RTF2
Q0VFZ6	Coiled-coil domain-containing protein 173
Q6PGZ3	TRAF3-interacting protein 1
Q9NQX5	Neural proliferation differentiation and control protein 1
Q9NRZ9	Lymphoid-specific helicase

Table 4.9 Differentially expressed genes shared with spawning *A. millepora* corals. Genes both up-regulated in spawning compared to non-spawning corals (Kaniewska *et al.* 2015), and differentially expressed with time point and treatment in this study.

UniProt ID	UniProt Gene Description
P15870	Histone H1-delta
Q6PBH5	Cytochrome c oxidase subunit NDUFA4
P94598	Glutamate dehydrogenase
Q9H1H9	Kinesin-like protein KIF13A
Q05975	Ras-related protein Rab-2
P55006	Retinol dehydrogenase 7

Table 4.10 Genes in the light profile. Grouping of genes identified with STEM that were up-regulated an hour after being subjected to the light + temperature-induced spawning treatment. Gene ID numbers represent genes listed in the JGI website.

Gene ID	UniProt ID	UniProt Gene Description
243395	B0BM24	Protein FAM161A
170524	B8V7S0	CUB and peptidase domain-containing protein 1
248018	O13035	Proactivator polypeptide
169611	O93400	Actin, cytoplasmic 1
167737	P11833	Tubulin beta chain
29518	P15870	Histone H1-delta
169568	P19804	Nucleoside diphosphate kinase B
242253	P25291	Pancreatic secretory granule membrane major glycoprotein
236730	P25867	Ubiquitin-conjugating enzyme E2-17 kDa
237519	P27659	60S ribosomal protein L3
175346	P42577	Soma ferritin
175341	P42577	Soma ferritin
237552	P42577	Soma ferritin
158216	P51147	Ras-related protein Rab-5C
124712	P86179	L-rhamnose-binding lectin CSL3
81851	Q0VFZ6	Coiled-coil domain-containing protein 173
177213	Q13231	Chitotriosidase-1
244590	Q2HJ86	Tubulin alpha-1D chain
85854	Q56K03	60S ribosomal protein L27a
234225	Q5ZJF4	Peroxiredoxin-6
163923	Q6PGZ3	TRAF3-interacting protein 1
190623	Q9JLR1	Protein transport protein Sec61 subunit alpha isoform 2
245689	Q9LQU4	Protein plant cadmium resistance 2
241430	NA	NA
241424	NA	NA
241092	NA	NA
214761	NA	NA
245535	NA	NA
240969	NA	NA

Table 4.11 Genes in the spawn profile. Grouping of genes identified with STEM for both time point and treatment that were up-regulated an hour after before spawning in the light + temperature-induced spawning treatment. Gene ID numbers represent genes listed in the JGI website.

Gene ID	UniProt ID	UniProt Gene Description
245966	A7SL20	Ribosomal RNA processing protein homolog
240276	A9JRX0	Male-specific lethal 1-like 1
243395	B0BM24	Protein FAM161A
29518	P15870	Histone H1-delta
242253	P25291	Pancreatic secretory granule membrane major glycoprotein GP2
176499	P41383	Tubulin alpha-2/alpha-4 chain
124712	P86179	L-rhamnose-binding lectin CSL3
81851	Q0VFZ6	Coiled-coil domain-containing protein
246171	Q28IQ9	Cold-inducible RNA-binding protein
168076	Q54271	Phosphonopyruvate decarboxylase
168739	Q642C0	DnaJ homolog subfamily C member
59372	Q6CQE5	Protein TAR1
163923	Q6PGZ3	TRAF3-interacting protein
247318	Q7T293	Protein SDE2 homolog
243925	Q7TSA6	Proline and serine-rich protein 3
167920	Q7ZV80	Survival of motor neuron-related-splicing factor
66182	Q7ZVW9	Arginine and glutamate-rich protein
90224	Q7ZW86	Peptidyl-prolyl cis-trans isomerase CWC27 homolog
238337	Q86XZ4	Spermatogenesis-associated serine-rich protein 2
165407	Q96NC0	Zinc finger matrin-type protein
105818	Q9CQU1	Microfibrillar-associated protein
244746	Q9CZX5	PIN2/TERF1-interacting telomerase inhibitor
248433	NA	NA
238402	NA	NA
238199	NA	NA
240262	NA	NA
242829	NA	NA
205031	NA	NA
239592	NA	NA
241430	NA	NA

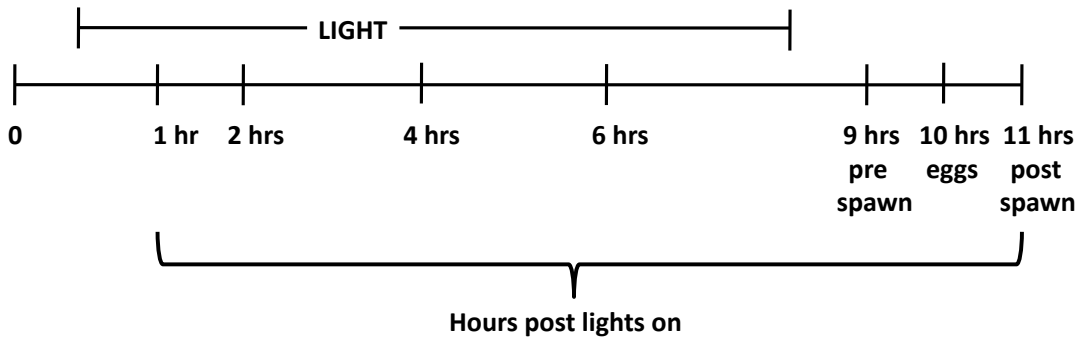

Figure 4.1 Experimental design and sampling time points during the spawning treatment. Depiction of sampling time points (indicated by hour marks) and length of light exposure for the spawning treatment. Control samples were collected at the same time points for animals kept in the dark at 16°C.

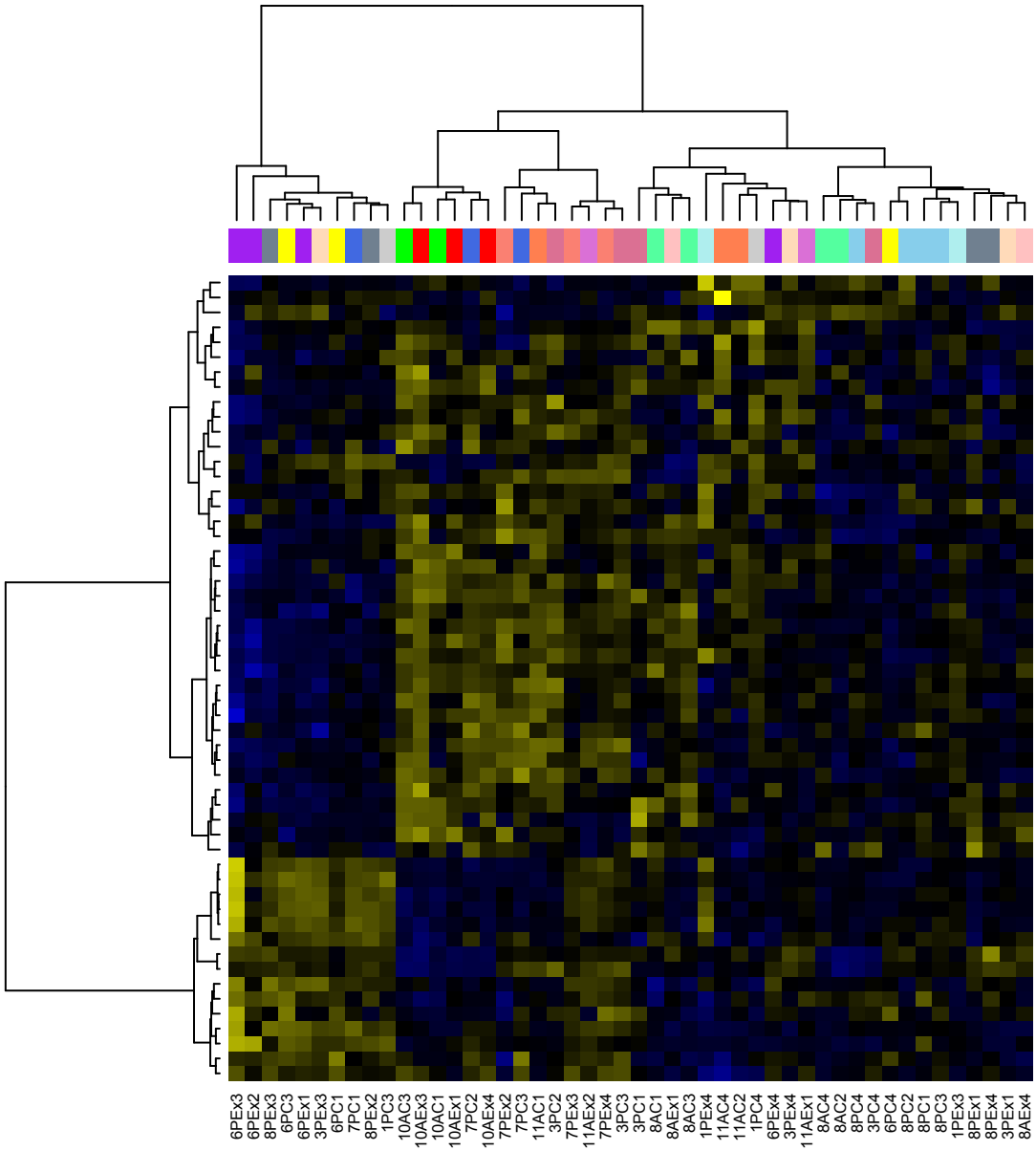

Figure 4.2 Heat map depiction of genes differentially expressed with time point. Patterns of differential expression ($p < 0.05$) associated with time point (x-axis represents biological samples (replicate samples have the same name), y-axis represents individual genes. Colors at the top represent individual treatment and time point groups.

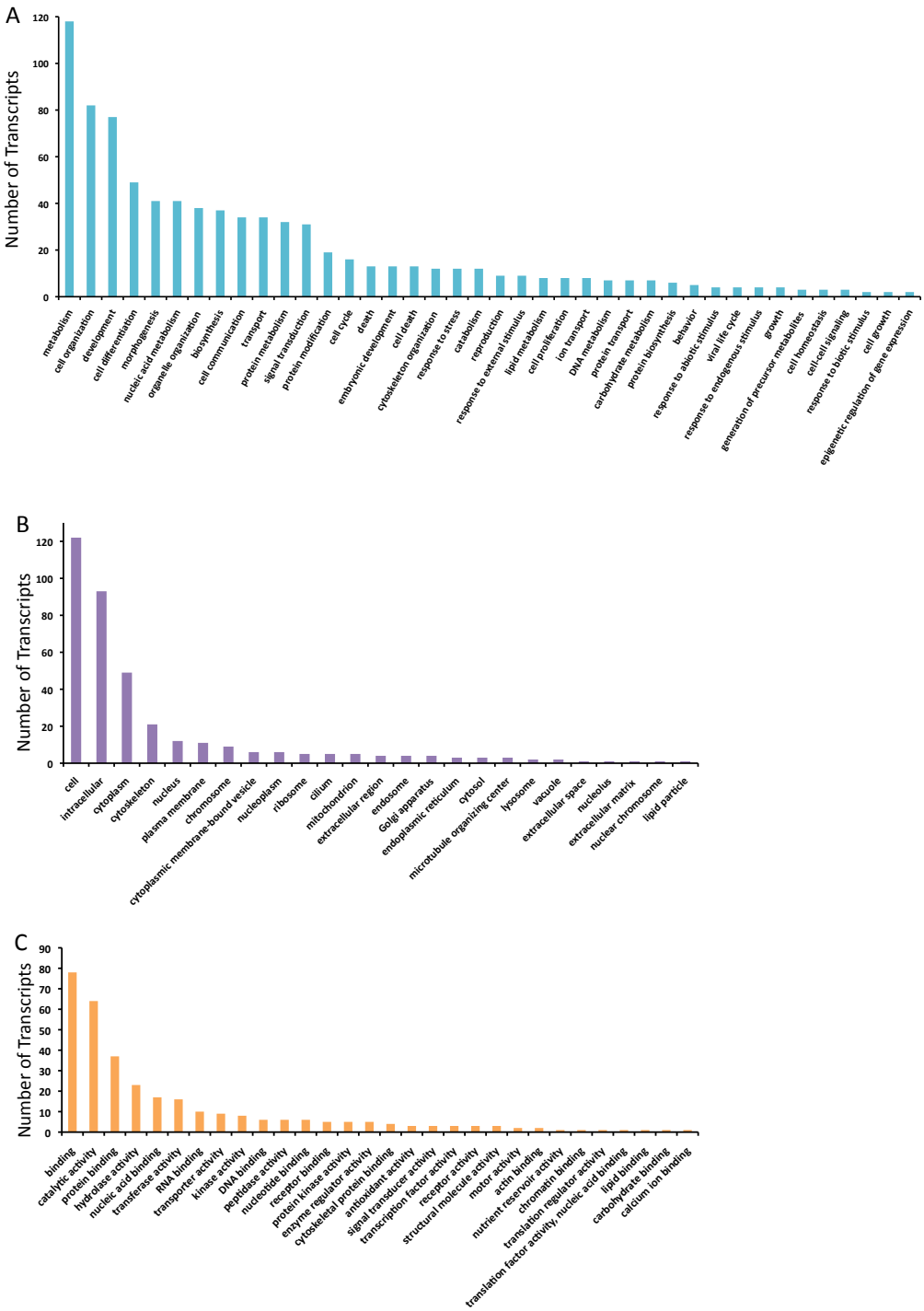

Figure 4.3 Distribution of GO terms for differentially expressed genes associated with time point. Number of genes (y-axis) associated with (A) biological (B) cellular and (C) molecular categories.

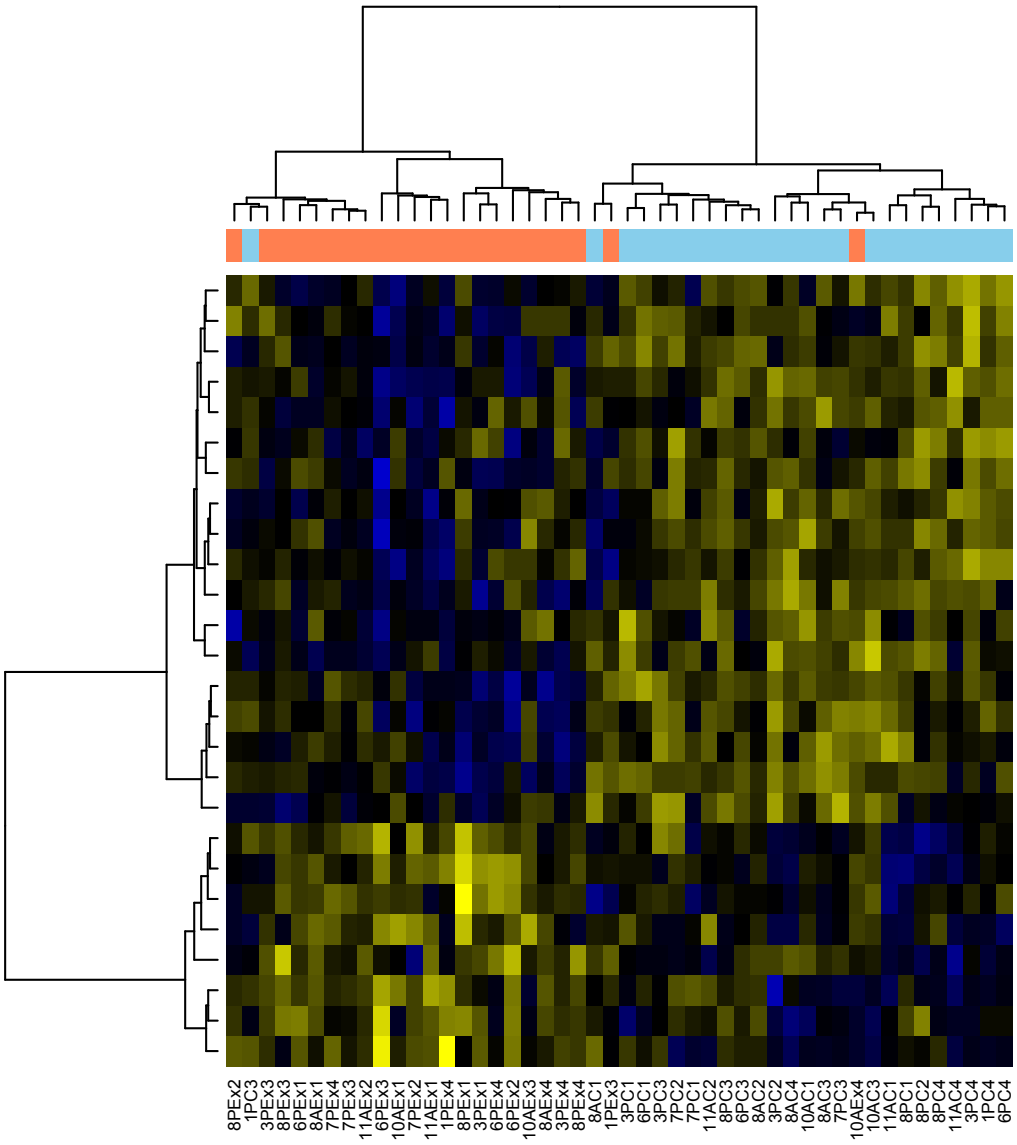

Figure 4.4 Heat map depiction of genes differentially expressed with treatment. Patterns of differential expression ($p < 0.05$) associated with treatment (x-axis represents biological samples (replicate samples have the same name), y-axis represents individual genes. Side colors represent red for spawning samples and blue for control samples.

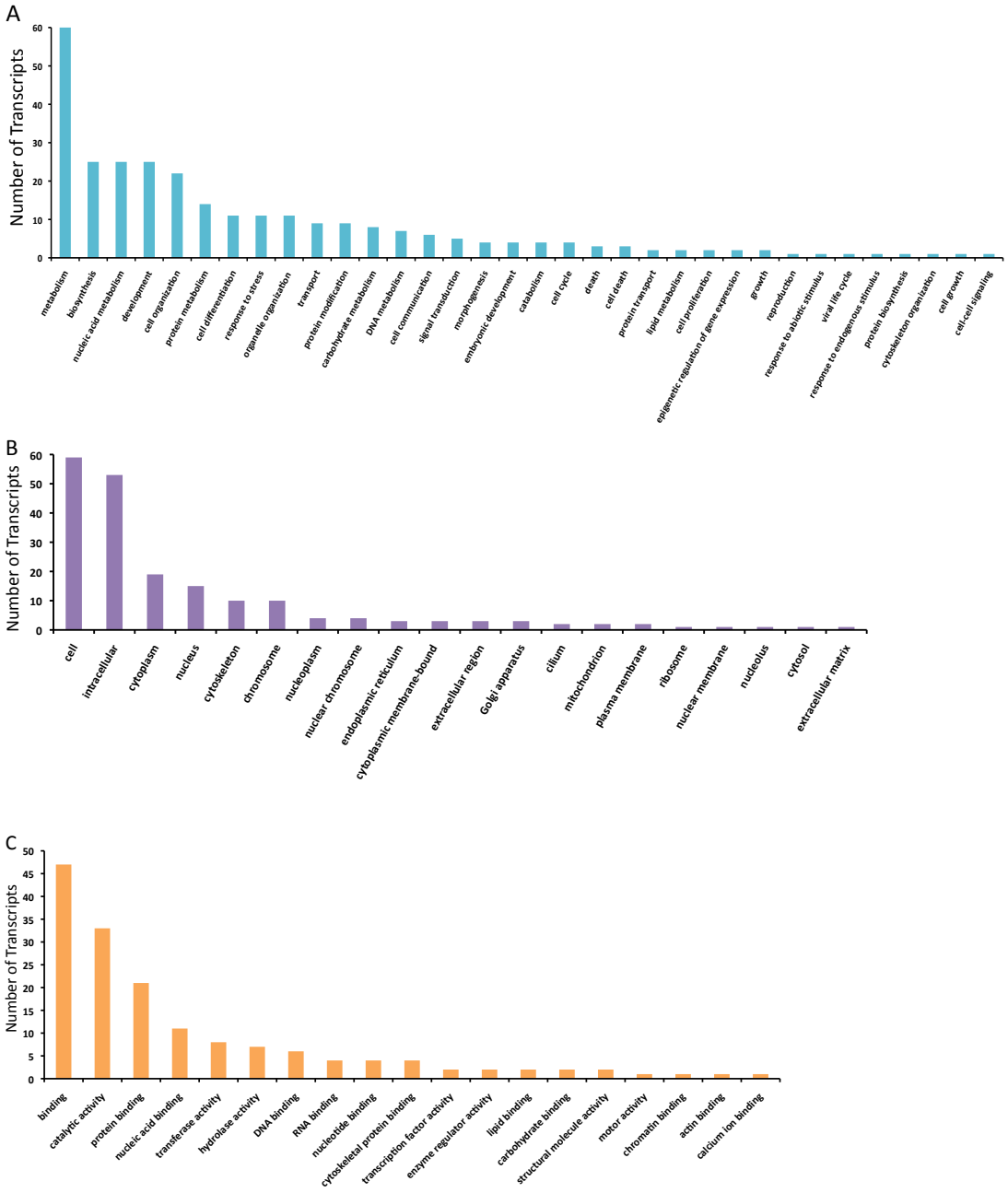

Figure 4.5 Distribution of GO terms for differentially expressed genes assoicated with treatment. Number of genes associated with (A) biological (B) cellular and (C) molecular GO categories.

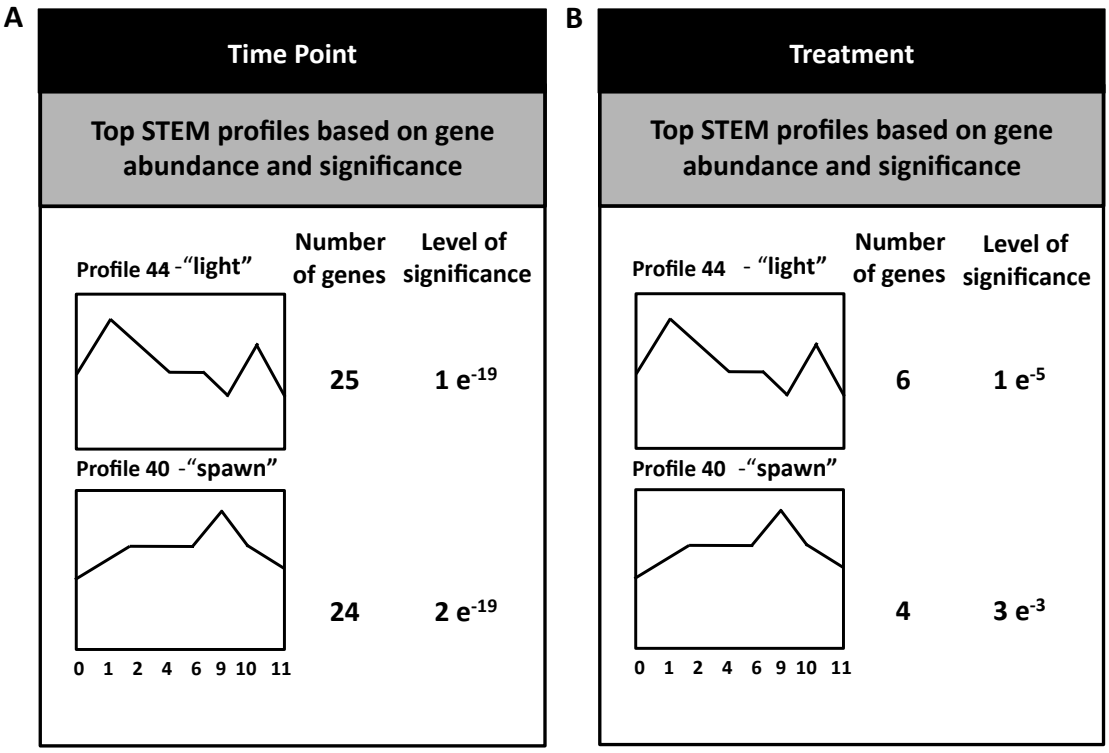

Figure 4.6 Significant and abundant STEM profiles associated with time point and treatment. STEM profiles indicating temporal patterns in expression. Profile selection was based on the level of significance and transcript abundance, y-axis: level of expression (normalized expression relative to time 0), x-axis: sampling time points (numbers represent hours post lights on), as described in Figure 1. Top profiles differentially expressed with (A) time point and (B) treatment were the same despite the profiles being comprised of different genes between the two treatments.

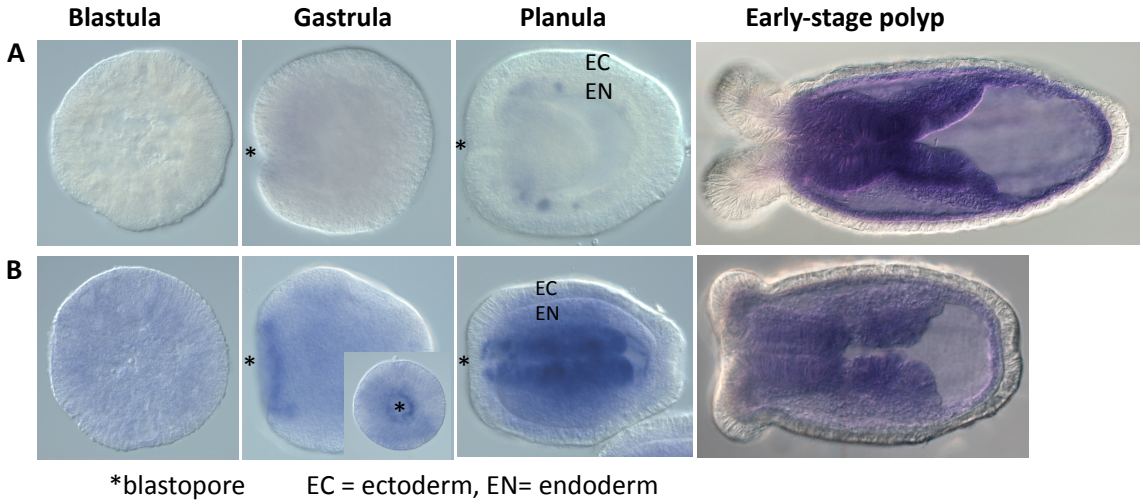

Figure 4.7 WISH showing expression of genes differentially expressed in the light profile throughout development. Staining (dark purple) of (A) peroxiredoxin-6 (P6) is present mainly in mesenteries of early-stage polyps and (B) pancreatic secretory granule membrane glycoprotein (GP2) is visible around the blastophore of the oral ectoderm and in the mesenteries of planula and early-stage polyps.

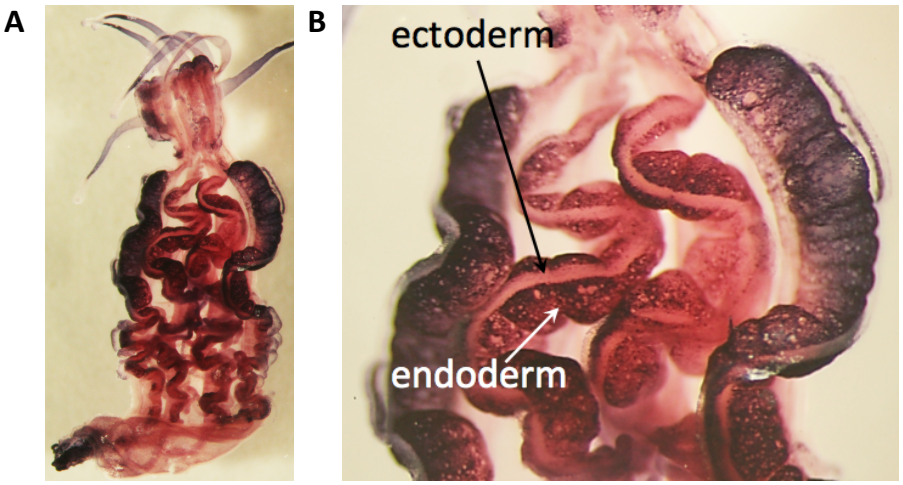

Figure 4.8 Peroxiredoxin-6 *in situs* of dissected adults.
Spatial expression patterns of a (A) peroxiredoxin-6 homolog in a dissected *N. vectensis* adult and (B) a close up of the mesenteries of same animal with mesenterial ectoderm and endoderm labeled. Dark purple indicates gene expression.

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5. CONCLUSIONS

This dissertation presents new information regarding anthozoan reproduction by examining: 1) the impacts of temperature on the timing of reproduction in a brooding coral at both an ecological and transcriptional level and 2) the combined influence of light + temperature on temporal and spatial gene expression profiles associated with the induction of spawning in a model sea anemone. These findings offer new insights into how increases in seawater temperature associated with climate change may affect patterns of coral reproduction and presents new hypotheses regarding the transduction of environmental variables into transcriptional changes in ancestral metazoans.

5.1 Influence of temperature on reproductive patterns in a brooding coral

In Chapter 2, the influence of low (23°C) and high (28°C) temperature on the timing of reproduction was empirically evaluated for a brooding coral and elevated temperature resulted in a significantly earlier peak in planulation timing in both lunar March and June (Figure 2.2). This finding revealed that there is plasticity in the timing of planulation and that these changes can occur rapidly, within a single lunar reproductive cycle. Depending on the timing of planulation, shifts in planulae release could lead to decreased survivorship of brooded planulae, since early released *P. damicornis* planulae have been shown to be of lesser quality (i.e., decreased size, protein content, and symbiodinium densities) than planulae released during peak planulation (Cumbo *et al.* 2012). Early-released planulae with fewer stored nutritional resources are likely limited in their dispersal distances, resulting in faster recruitment in order to acquire more nutrients for survival. Decreases in planula dispersal could in turn have impacts on coral

reef biodiversity and connectivity, leading to less localized genetic diversity and increased vulnerability to disease and other threats associated with changing climate. Additional studies that track the survival and dispersal distances of early-released planulae would confirm these predictions.

Chapter 3 presents the first transcriptome-based study to investigate the role of temperature and lunar day on global gene expression profiles during reproduction in a brooding coral and showed that elevated temperature disrupts patterns of expression associated with lunar timing of planulation (Figure 3.9 & 3.10). Together with Chapter 2, these findings reveal that prolonged elevated temperature exposure impacts coral physiology at a phenotypic and transcriptomic level. Comparisons of transcriptomic profiles at the time of planulation for individual temperature treatments revealed that 17 transcripts were shared between temperature independent planulation profiles (Figure 3.9). This modest number of shared transcripts could indicate the presence of different transcriptomic mechanisms involved in coordinating reproduction at high and low temperatures. Differing transcriptomic mechanisms at the time of planulation could reveal shifts in resources from planula development to regulation of genes associated with acclimation to increased thermal stress. This hypothesis is supported by an up-regulation of previously-described heat stress genes in corals at all lunar day time points in high compared to low temperature treatments (Figure 3.5). This shift in gene expression could lead to a change in resource allocation away from planula development, which in turn could lead to the release of prematurely developed planulae less suited for survival at higher temperatures. Alternatively, lunar day time points sampled in this study did not fully capture transcriptional changes associated with planulation resulting in only

a partial overlap between temperature independent expression profiles. Additional sampling of more time points centered around the timing of planulation for both individual temperature treatments could capture additional transcripts in common associated with planulation and determine whether alternative mechanisms occur with elevated temperature.

5.2 The combination of light and temperature cues associated with reproduction correspond to significant transcriptional changes

Significant transcriptional changes were observed with lunar day and the temperature x lunar day interaction during a monthly reproductive cycle in *P. damicornis* corals (Table 3.1) and during a light + temperature spawn-inducing treatment in *N. vectensis* anemones (Table 4.2). The strong interaction of temperature x lunar day in *P. damicornis* corals revealed that the influence of temperature is dependent on lunar day at the transcriptomic level, displaying the close interplay of these two variables in the molecular coordination of reproduction in brooding corals. These findings corroborate ecological data suggesting lunar irradiance, diel-light cycles and increased temperature having roles in cueing reproductive events in cnidarians. Furthermore, these studies describe a strong combinatorial effect of temperature and light on gene expression, and the synergistic role of these variables in inducing a transcriptomic response involved in cueing reproduction.

Levels of differential expression varied in these two datasets, with a greater than tenfold increase in genes differentially expressed with lunar day and the interaction of temperature x lunar day in *P. damicornis* compared to levels observed during spawning in *N. vectensis*. Differences at the transcriptome level in brooding *P. damicornis* corals

collected from the field compared to laboratory-maintained *N. vectensis* anemones suggests alternative internal mechanisms at play to coordinate reproductive processes in these two anthozoans. This does not support the use of *N. vectensis* as a model for reproductive studies in corals and reinforces the extreme plasticity and flexibility of reproductive strategies in anthozoans. Explorations of spawning frequency and transcriptomic changes associated with spawning in *N. vectensis* in their natural estuarine environments are lacking and are needed in order to better understand the findings in laboratory animals. Comparisons of gene expression profiles of spawning and non-spawning *N. vectensis* in the field would provide an important comparator for the modest changes observed in laboratory-reared cultures. Additional high-throughput investigations are needed to confirm and describe potential post-transcriptional processes, such as microRNA silencing or protein modifications associated with the induction of spawning in *N. vectensis*.

5.3 Potential mechanisms for signal transduction during reproduction include neuropeptide-like signaling rather than steroid hormones

In Chapter 3, multiple genes associated with neurotransmitter, G protein-coupled receptor, and calcium signaling pathways were differentially expressed with the interaction of temperature x lunar day (Table 3.2). This suggests that G protein-coupled receptors, commonly activated by light-sensitive compounds, neurotransmitters and peptide hormones, are involved in cell signaling events associated with sensing moonlight and cueing reproduction in corals (Figure 3.11). These findings are supported by previous reports where calcium was shown to mediate light responsiveness in spawning corals (Hilton *et al.* 2012) and genes associated with calcium binding were up-

regulated at the time of spawning (Kaniewska *et al.* 2015) and during the day compared to the night in the spawning coral *A. millepora* (Bertucci *et al.* 2015). A comparison of these two datasets and previously published reports in spawning corals suggests that differing mechanisms occur in corals undergoing reproduction in nature and the induction of spawning in laboratory-maintained *N. vectensis* anemones. Although, multiple studies have detected the presence of vertebrate sex steroid compounds in corals, no evidence to support vertebrate steroidogenesis pathways that lead to synthesis of vertebrate sex steroids (estrogens, progesterone, and testosterone) in *P. damicornis* or *N. vectensis* was found in these studies.

5.3 FUTURE STUDIES

The two RNA-Seq studies (Chapter 3 and 4) in this dissertation have led to two general hypotheses regarding mechanisms of signal transduction and reproductive physiology in cnidarians: 1) Ancestral neuropeptides, G protein-coupled receptors, and calcium signaling pathways are involved in reproductive events in brooding corals and 2) light + temperature-induced spawning in *N. vectensis* involves potentially novel reproductive post-transcriptional modifications. More work is needed to test these hypotheses and confirm the function of homologous genes in cnidarians. Furthermore, more work is needed to characterize and examine the function of unannotated genes that were significantly differentially expressed in both RNA-Seq datasets.

This work, in combination with reports in spawning corals (Bertucci *et al.* 2015; Hilton *et al.* 2012; Kaniewska *et al.* 2015), sets the stage for investigations into alternative bioregulators, other than vertebrate-type sex steroids, such as neurotransmitters and peptides as reproductive signalers in cnidarians. More emphasis on

the confirmation of biochemical assays with precise high-throughput analyses to identify the molecular structure of detected molecules is needed. Immunohistological, pharmacological, and high-throughput approaches to detect peptide hormones, such as oxytocin-like and GnRH-like neuropeptides could confirm the role of these neuropeptides in coral reproduction. Additionally, studies that block calcium channels or prevent the release of calcium from the endoplasmic reticulum, similar to work done in spawning corals, (Hilton *et al*. 2012) could provide support for calcium-mediated signaling in brooding corals.

The field of cnidarian reproductive biology is pushing forward in exciting new directions and the era of next-generation sequencing is offering new avenues for hypothesis generation and exploration. It's imperative that there is more overlap between coral reproductive ecology, endocrinology and genomic studies that all aim to interpret mechanisms of reproductive regulation. New studies that intertwine these three disciplines to evaluate ecologically themed issues in coral biology are necessary to predict the impacts of changing climate on coral reef regeneration and improve our understanding of the future persistence of coral reefs.

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