Exploring reproductive signaling using nonconventional aquatic models: aging and reproductive function in killifish and sea anemones

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Aquatic animals possess surprising similarities to humans in reproductive signaling that are simplified or elaborated for life underwater, making them useful for studying reproductive control. In this thesis, killifish and sea anemones are used as models for reproductive function and aging. The aging model *Nothobranchius* (killifish) was used to investigate circadian rhythms and clock gene expression in reproductive tissues, and effects of the anti-aging compound resveratrol (RSV) were examined to elucidate the role of clock genes in fertility. Weak connections were found between gonadal clock gene expression and age, and RSV altered expression over time and shifted rhythmic patterns in old fish, suggesting that it may modulate circadian rhythms with age. Additionally, to understand the physiological mechanisms behind coral spawning for conservation and elucidate hormonal evolution, functions of the cnidarian leucine-rich repeat-containing G protein-coupled receptor LGRA2 were investigated in the model sea anemone *Aiptasia pallida*. LGRA2 was expressed near gonadal regions of sexually developed sea anemones, activated by gonadotropins, and modulated by estrogen, indicating that it is involved in regulating reproduction similarly to its mammalian homologs luteinizing hormone receptor and follicle

stimulating hormone receptor. These projects together provide a One Health view of reproductive signaling and aging.

Key Words: reproductive endocrinology, circadian rhythms, aging, gonadotropin signaling, coral reproduction
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Introduction

In sexually reproducing animals, reproductive success is dependent upon timing of multiple processes from gametogenesis to copulation. Yet, many holes exist in our current understanding of how sexual reproduction is controlled, why this control changes as we age, and how much similarity exists within these mechanisms among different animal taxa. This thesis has two aims: to address concerns regarding aging and fertility in humans and other animals using killifish as models, and to expand on established knowledge in mammalian reproductive endocrinology by examining how reproduction has evolved from cnidarians. Understanding the effects of a dietary supplement on circadian gene expression in the gonads of aging killifish could have implications for human reproductive health and fertility as well as breeding of both pets and zoo animals. Additionally, exploring the function of a gonadotropin-like hormone receptor in a cnidarian model species will enrich our understanding of the evolution of our own reproductive signaling systems, with additional applications to coral reef conservation and cnidarian husbandry in the laboratory. Furthermore, the use of aquatic model systems allows a look into the potential ecological impacts of disturbances such as endocrine disruptors or sex steroid runoff from agricultural areas, changes in environmental spawning cues with rising ocean temperatures, and circadian rhythm disruption due to light pollution from cities with busy waterways. Thus, these investigations provide a One Health outlook on reproductive signaling from which human, animal, and environmental health can each benefit.

Part 1: Examining reproductive aging in an aquatic model organism

Clock Genes, Circadian Rhythms, and Fertility

Circadian rhythms consist of daily fluctuations in an organism's behavior and physiological state which allow it to adjust for changes in temperature and sunlight as the earth

rotates, optimizing fitness. In most vertebrates, these cellular oscillations are synchronized by a central clock in the suprachiasmatic nucleus of the brain, located in the hypothalamus above the optic chiasm. This core clock converts light inputs into entrainment cues for modulating the expression of clock genes, many of which are intrinsically rhythmic. Entrainment, or "resetting of the clock", begins when the transcription factors BMAL1 and CLOCK dimerize and bind to E-box promoter regions to activate the circadian oscillation of genes that play roles in various cellular processes. The CLOCK protein is a histone acetyltransferase which relaxes condensed chromatin by acetylating histones, thereby allowing transcription. Included in the genes activated by the CLOCK-BMAL complex are Period (*Per*) and Cryptochrome (*Cry*) genes, which negatively feedback on their own transcription by inducing the degradation of BMAL1 and CLOCK[1]. Aiding in the regulation of this cycle are sirtuins, especially SIRT1, which is a histone deacetylase that interacts with the CLOCK-BMAL complex to re-condense affected chromatin, helping to maintain circadian amplitude[2]. In addition to the brain, SIRT1 and clock genes are also expressed in other tissues throughout the body, including the gonads[1]. However, many studies demonstrate that SIRT1 activity is lost with age, resulting in dampened circadian rhythms and thus providing a possible explanation for age-related decline in fertility[3]. This decline has vast impacts on society, as modern western women are waiting longer to have children and fertility treatments become more common place[4]. Similar problems can be observed in aging zoo animals that face reproductive challenges upon breeding[5]. Therefore, understanding the roles of SIRT1 and clock gene rhythmicity and amplitude in reproduction will have implications for human health as well as animal conservation and breeding.

Nothobranchius as a Model for Aging and Effects of Resveratrol on Fertility

Nothobranchius (killifish), is a genus of teleost fish native to northern Africa that includes *Nothobranchius furzeri* (turquoise killifish) in brackish ponds and *Nothobranchius*

guentheri (redtail notho) in rivers and freshwater marshes. Killifish are closely related to zebrafish, but because of the intermittent nature of their habitats, their lifespans are relatively accelerated, living their entire lives over a few months of rainy season before dying off to leave only desiccation-resistant eggs[6]. As a result, the average lifespan of N. guentheri is 43 weeks, and the average lifespan of N. furzeri is 12 weeks, compared to 3.5 years for zebrafish[7,8]. This makes them ideal models for aging studies, as they reach sexual maturity faster than zebrafish and can produce more generations in less time.

Resveratrol (RSV) is a polyphenol found in grapes and red wine that has been touted as an anti-aging supplement in numerous species including humans[9]. It has also been shown to increase the lifespan of *N. furzeri*, in addition to prolonging fertility and decreasing the likelihood of chromosome nondisjunction in offspring in aging female mice, resulting in healthier progeny[10,11]. While relatively little is known about the mechanisms of RSV action on fertility, it is known that RSV activates sirtuins, potentially making up for their natural decline with age and allowing for preserved circadian rhythmicity in the gonads[3]. SIRT1 and clock genes have been found to be expressed in the gonads of teleosts, suggesting that they could also play a role in the preservation of killifish fertility[12]. Elucidating the mechanisms of this action is paramount to further developing fertility drugs and recommendations for those hoping to conceive. We are thus using these animals as a model for reproductive aging and will explore the effects of RSV treatments on circadian gene expression in the gonads throughout life and resulting changes in fertility.

Part 2: Exploring mechanisms of coral reef reproductive capacity for the improvement of global health

Mammalian Reproductive Signaling—HPG Axis

Superimposed on this circadian rhythmicity, the hypothalamus-pituitary-gonad (HPG) axis also regulates mammalian reproductive timing. Gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile fashion from specialized GnRH neurons in the hypothalamus. Activation by GnRH on its cognate receptor stimulates gonadotrope cells in the anterior pituitary to secrete gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the bloodstream, allowing them to travel to the gonads. In males, LH binds luteinizing hormone receptor (LHR) in Leydig cells to stimulate steroidogenesis, and FSH binds follicle stimulating hormone receptor (FSHR) in Sertoli cells to support sperm production. Testosterone then completes a negative feedback loop involving both the hypothalamus and pituitary to stop further secretion of GnRH and gonadotropins. In females, FSH binds its receptor in granulosa cells, where it stimulates follicular development and estrogen production. Estrogen in turn modulates both FSHR and LHR expression at certain points in the reproductive cycle. LH binds LHR in multiple cell types within the ovary to maintain the luteal phase, in addition to stimulating production of enzymes involved in sex steroid hormone production. Estrogen acts in a negative feedback loop on the hypothalamus and pituitary for the majority of the cycle, but just before ovulation it switches to positive feedback, resulting in an LH surge and subsequent ovulation. During pregnancy, specialized gonadotropins such as human chorionic gonadotropin (hCG) also bind to LHR to maintain the corpus luteum and stop further ovulation. Although the importance of gonadotropins in reproductive signaling has been well-characterized in mammals, it is still relatively poorly understood in other animals such as invertebrates, especially those lacking central nervous systems. Understanding the mechanisms of reproductive signaling in simpler organisms such as these could help shed light on the complexities involved in mammalian neuroendocrine signaling.

The Phylum Cnidaria as an Ancestral Model for Gonadotropin Signaling

The phylum Cnidaria, which encompasses corals, sea anemones, and jellyfish, provides habitat in the form of coral reefs for many species around the world. However, reef populations are severely threatened as ocean temperatures rise and seas become more acidic due to increased CO2 absorption. In recent years, there have been many major die offs and bleaching events, notably destroying a large portion of the Great Barrier Reef[13]. An improved understanding of the underlying mechanisms of coral reproduction is paramount for ensuring retention and reintroduction of these animals for global ecosystem health.

While the ecology of cnidarian spawning has been studied for many years, very little remains known about the physiological and cellular level of control they exert over these processes. Cnidarians demonstrate an astonishing variety in modes of reproduction. Some species are simultaneous hermaphrodites, some are gonochoristic, and some have even been found to be trioecious, containing males, females, and hermaphrodites together[14]. They can undergo both asexual reproduction (via budding, binary fission, or pedal laceration), and sexual reproduction in the form of brooding or broadcast spawning which can be synchronized among large populations. These spawning events are infrequent in certain coral species, with some only spawning once a year when the water temperature and lunar cycle line up perfectly. Gonadal development and spawning have been shown to be related to body size, food availability, and light cues in multiple species[15-17]. In the laboratory, spawning can be difficult to induce, although it can be achieved through months of light entrainment at proper temperatures and nutritional availability[18]. Understanding the molecular controls for timing of sexual development and spawning in Cnidarians is necessary for further laboratory experiments using corals or sea anemones, in addition to having applications for coral reef conservation as rising ocean temperatures interfere with ideal spawning conditions in the wild.

Aiptasia pallida is a small, fast-growing sea anemone that is easy to maintain in laboratory settings, and is therefore commonly used in symbiosis studies[19]. A receptor by the name of LGRA2 (leucine-rich repeat-containing G protein-coupled receptor A2) has been isolated in *A. pallida* that shows homology to mammalian LHR and FSHR (Heller, Goodall, Chappell, unpublished observations). Cnidarians also express circadian genes and clock genes such as *Clock* and *Cry*, which could potentially play a role in reproductive regulation similar to that in fish and humans[20]. While they have also been found to secrete both estradiol and GnRH, they lack a central nervous system, and as of yet no gonadotropins have been isolated[14,21]. This raises questions about the function of ancestral gonadotropin-like hormone receptors and the evolution of neuroendocrine signaling in reproduction. Illuminating the function of *LGRA2* in this ancestral invertebrate will help broaden our understanding of mammalian reproductive signaling and glycoprotein hormone function, resulting in widespread applications in human health, the breeding of other animals, and conservation.

GPHs and LGRs

LH and FSH are glycoprotein hormones (GPHs), which bind to leucine-rich repeat-containing G protein-coupled receptors (LGRs). GPHs make up a broad category of hormones also containing thyroid-stimulating hormone (TSH) and hCG, each containing a common alpha subunit and a unique beta subunit. When GPHs bind their LGRs, a downstream increase in cAMP intracellular signaling is induced[22]. In humans, eight separate LGRs have been isolated and grouped into three subfamilies. Subfamily A includes FSHR, LHR, and TSHR (or LGRs1-3)[23,24]. Subfamily B includes LGR4 (shown to have various functions in development), LGR5 (implicated in stem cell signaling and the Wnt pathway), and LGR6 (a suggested tumor suppressor), although each of these receptors remains an orphan without a known ligand[25-27]. Subfamily C is comprised of LGR7, which binds relaxin to help regulate parturition, and LGR8, which binds

insulin-like 3 (INSL3), whose loss has been implicated in cryptorchidism[28,29]. GPHs and LGRs most closely matching the A subfamily (LGRAs) have also been isolated in *Drosophila melanogaster* and *Caenorhabditis elegans*, and GPH alpha and beta subunit homologs have been found in the larvae and adult central nervous system of the mollusc *Aplysia californica,* suggesting that glycoprotein hormone signaling had already evolved by the emergence of bilaterians[22,24,30]. Additionally, cysteine-knot hormones partially resembling GPHs and corresponding LGRs have been found in sponges and comb jellies, and an elaborated LGR was isolated in cnidarians corresponding to GPH[31]. As more and more ancestral organisms are discovered to have genes for GPHs and LGRs, questions remain about the level of conservation in their function. Understanding the expression patterns and actions of LGRA2 in Cnidarians will help begin to answer some of these questions by shedding light on the evolutionary history of LGRs, GPHs, and the HPG axis. If *LGRA2* is found to be expressed similarly and act similarly to mammalian LH and FSH receptors, then that will provide evidence that this system began to evolve much earlier than previously thought, while if it is found to have distinct expression patterns or mechanisms of action, then that may indicate that LGRs diverged in their function over the course of evolution.

Experimental Questions and Results

Broad research question 1: What is the involvement of clock gene expression in reproductive aging?

To answer this question, quantitative PCR tests were carried out to investigate expression patterns of clock genes in the gonads and brains of aging *N. guentheri* and *N. furzeri* to look for temporal changes on a circadian scale as well as across lifespan. The pharmacological effects of RSV on these expression patterns were also assessed to investigate a possible method of manipulating circadian rhythms in the gonads. The results of these tests currently suggest a lack of correlation between RSV treatment, clock gene expression, and age, although more replicates are needed to be confident in these conclusions.

Is the expression of circadian rhythm genes altered with age in the reproductive system?

Expression of clock genes in ovaries of untreated control animals appeared to undergo changes with age, although significance could not be determined due to limited sample size (Fig 1). *Bmal1a* steadily increased from 9 to 16 weeks in *N. guentheri* ovaries before dropping down again at 22 weeks (roughly "middle-aged" for the species). *Bmal1a* expression slightly increased in *N. furzeri* from 13 to 14 weeks (very old for the species). *Cry1aa* expression showed a peak at 13 weeks in *N. guentheri* and began to decline slightly again from there, and it showed a slight increase from 13 to 14 weeks in *N. furzeri*. *Clocka* expression showed a marked increase at 22 weeks in *N. guentheri,* while it slightly decreased between 13 and 14 weeks in *N. furzeri.* These results suggest that clock gene expression in the gonads may be altered with age, possibly due to declined SIRT1 activity.

What role does RSV play in modulating overall expression of bmal1aa, clocka, and Cry1aa in the gonads with age?

There were not enough replicates to determine if there were significant differences in expression by RSV treatment or discrete age. However, generally, expression appeared to be dampened or remained constant in RSV-treated groups, while controls were more variable over time (Fig 1). When ovary expression values were binned by age, (into relatively young at 9-13 or 10-13 weeks of age and relatively old at 14-23 weeks of age), no significant difference was found between clock gene expression by age or treatment, although the effect of treatment on *Bmal1a* expression produced a p-value that was close to significant, at p=0.0652 (Fig 2). Due to lost samples, low RNA yields, and undetectable quantities in real-time PCR, there were not enough replicates from brain to be binned in to young and old categories in the same manner for statistical analyses, but expression patterns with age and treatment did not seem notably different from those in the ovaries, with little discernable relationship between treatment, age, and expression in any of the genes.

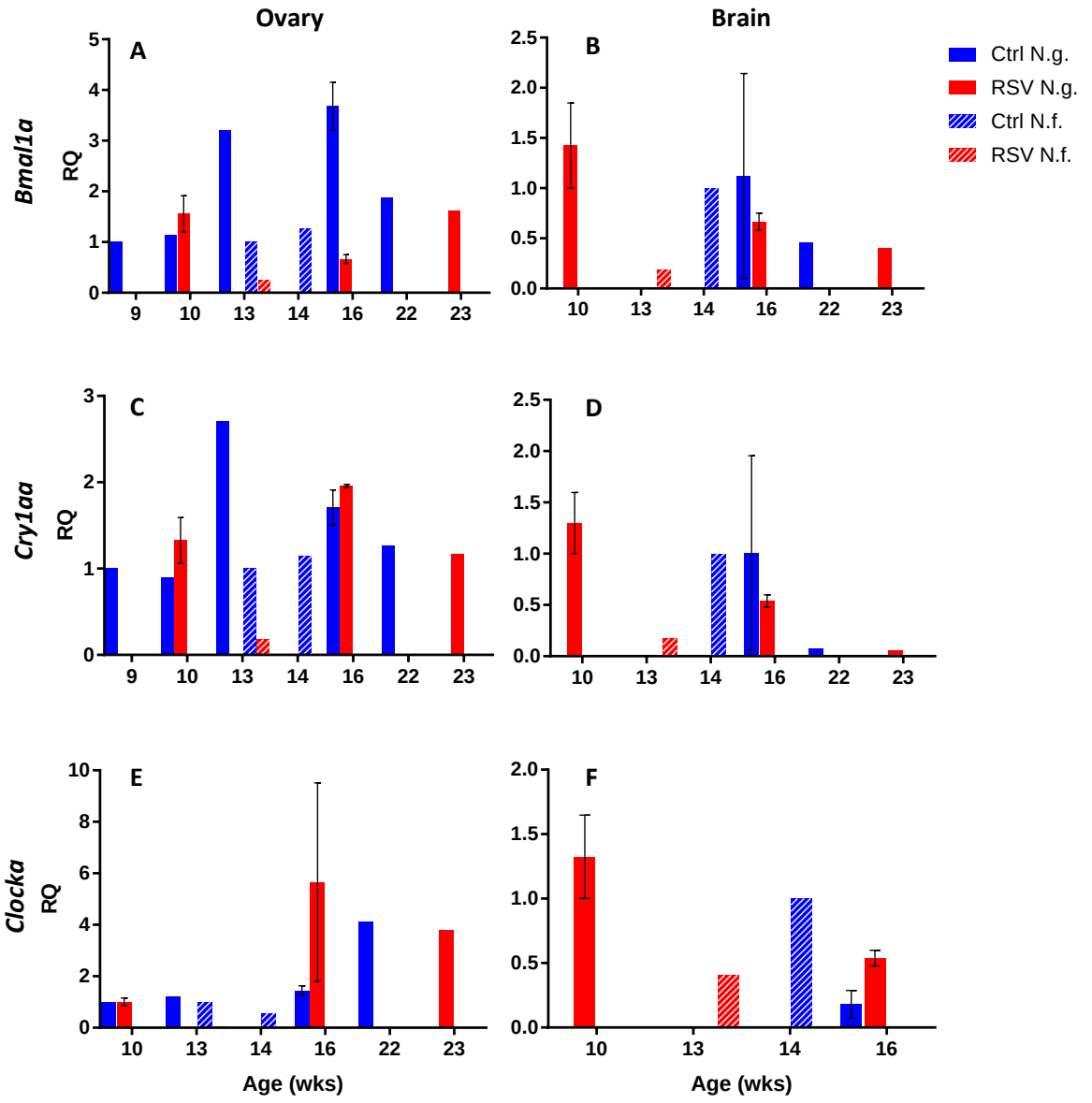

Figure 1. Relative expression of clock genes in ovaries (left) and brains (right) of *N. furzeri* and *N. guentheri* at different ages with and without RSV treatment. Left graphs represent expression in ovaries of *bmal1a*(A), *Cry1aa*(C), and *clocka*(E). Right graphs depict expression in brains of *bmal1a*(B), *Cry1aa*(D), and *clocka*(F). *N. furzeri* values are standardized separately from *N. guentheri* and are shown here on the same graph for conciseness, although actual expression in *N. furzeri* was much higher for all genes than in *N. guentheri*. All n values are equal to 1, except for Ovary-RSV N.g.-10wk (n=3), Ovary-Ctrl N.g.-16wk (n=2), Ovary-RSV N.g.-16wk (n=2), Brain-RSV N.g.-10wk (n=2), Brain-Ctrl N.g.-16wk (n=2), and Brain-RSV N.g.-16wk (n=2).

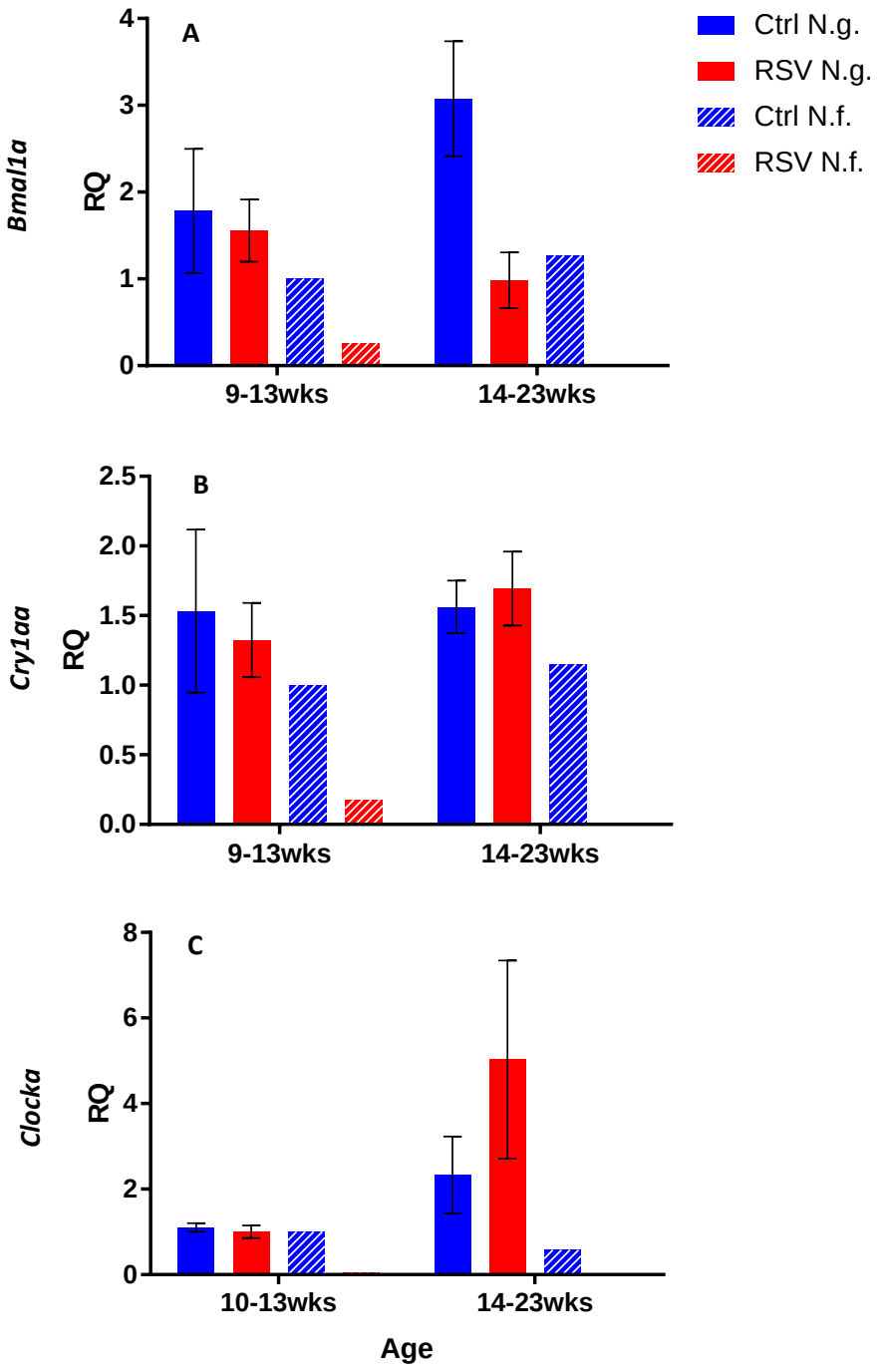

Figure 2. Mean expression of clock genes in *N. furzeri* and *N. guentheri* ovaries grouped by young and old fish with or without RSV treatment. *Bmal1a* (A), *Cry1aa* (B), and *clocka* (C) expression. N=3 for all *N. guentheri* bars except for Ctrl 10-13wks (for which n=2). N=1 for all *N. furzeri* bars.

Does RSV treatment alter circadian expression patterns of bmal1aa, clocka, and Cry1aa in aged gonads?

In ovaries of *N. guentheri* between 22 and 23 weeks of age which had been on RSV or control treatment for 12-16 weeks prior to sacrifice, RSV-treatment appeared to produce changes in circadian expression of all genes, although not enough replicates were performed to determine significance (Fig 3). RSV-treated ovaries showed a peak in *bmal1a* expression at 12hrs ZT, compared to nontreated ovaries which steadily increased, peaking closer to ZT20. In contrast, brain *bmal1a* exhibited a rhythm with peak levels at ZT16 in controls, while RSV treatment appeared to abrogate this rhythm. *Cry1aa* expression in RSV-treated ovaries showed a gradual decrease throughout the day, in contrast with controls which exhibited a peak towards ZT16-24. This dampening effect of RSV was also seen in RSV-treated brains compared to control brains, which showed a high peak in *Cry1aa* expression at 16hrs followed by a drop off and another peak at 24hrs. *Clocka* was not expressed in clear circadian patterns in either treated or non-treated ovaries. *Clocka* expression was not detectable in all but three brain samples, so those data were not included in figures. These results suggest that although clock gene expression varies with age and cyclicity is diminished in old fish, RSV does not modulate clock gene expression significantly.

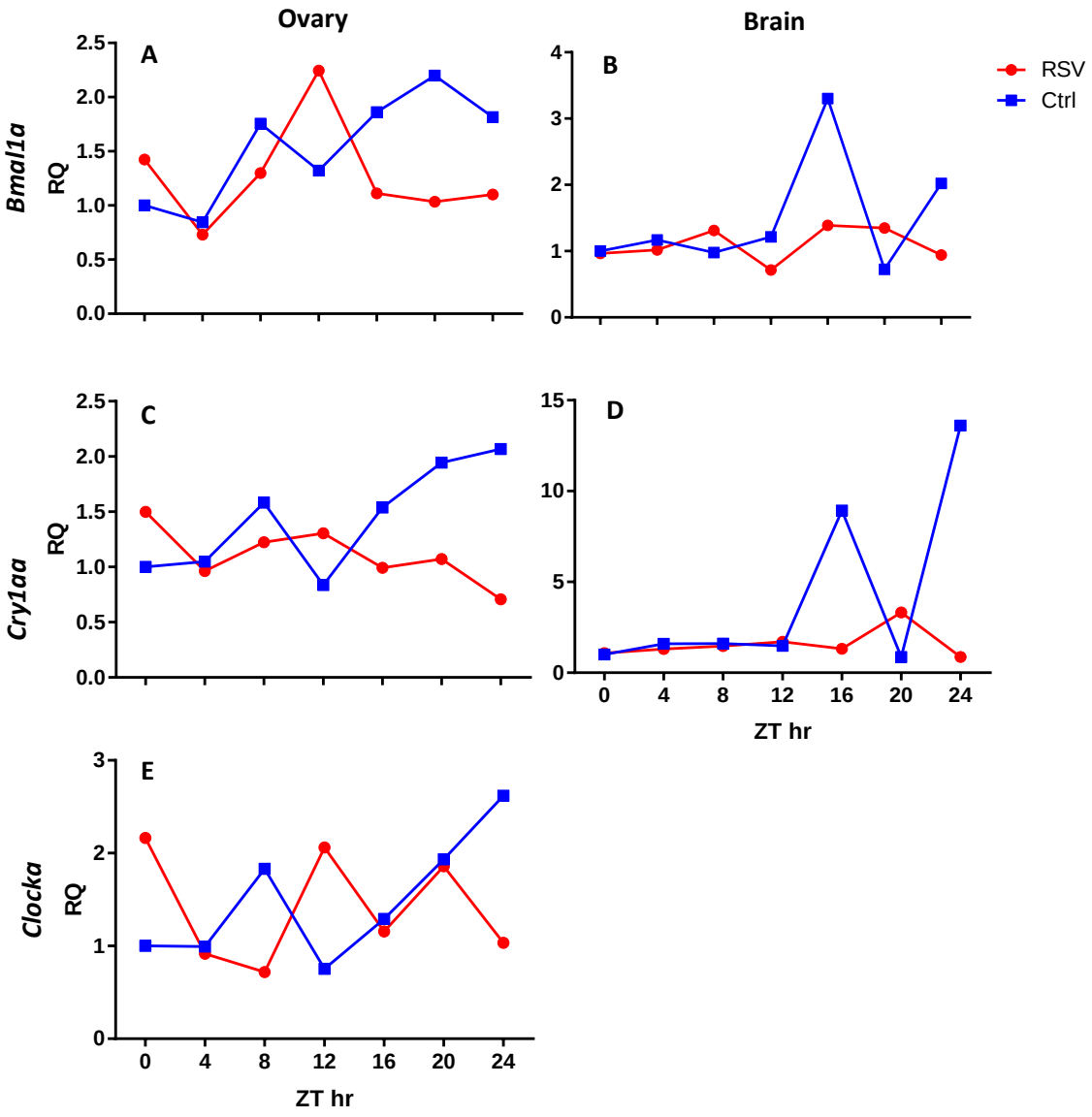

Figure 3. Expression of *bmal1a* (A, B), *Cry1aa* (C, D,) and *clocka* (E) in ovaries (left) and brains (right) over 24 hours. Expression of *clocka* in brains was too low to read in real-time PCR. N=1.

Broad research question 2: What is the role of the LGRA2 gonadotropin-like hormone receptor in cnidarian reproductive development and spawning control?

To answer this question, receptor activation was studied first in a heterologous cell model and then *in vivo* in *A. pallida.* Receptor expression was also explored in animals at different stages of sexual development, examining both quantitative and spatial expression patterns. Finally, the effects of estrogen treatments on receptor expression in different *A. pallida* clones were studied to investigate steroid feedback and possible temporal or physiological variation.

Is the LGRA2 receptor stimulated by gonadotropins, activating similar intracellular second messenger pathways to mammalian LHR and FSHR?

Expression of *LGRA2* and peptide abundance were confirmed in stably transfected human embryonic kidney cells (HEK293) via immunohistochemistry and in situ hybridization (Fig 4). IHC confirmed the presence of this *A. pallida* receptor in transfected cell membranes, as would be expected for a putative G protein-coupled receptor. Stably transfected cell showed a significant increase in intracellular cAMP signaling when treated with 10µM mammalian LH (human) and/or FSH (bovine) compared to untransfected cells (Fig 5). Intracellular response to cAMP was also increased, evidenced by pCREB increases in gonadotropin-treated transfected cells compared to untransfected, indicating phosphorylation of cyclic AMP response element binding as a result of gonadotropin treatment and further supporting cAMP intracellular signaling as an action of LGRA2 (Fig 5).

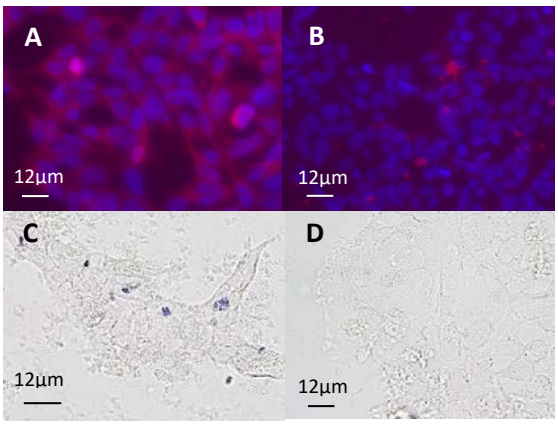

Figure 4. Stably transfected HEK293 cells stained for expression of *LGRA2* using anti-V5 primary (A) and negative control (B). Red indicates *LGRA2* expression in cell membranes around blue stained nuclei. Cells hybridized with *LGRA2* antisense probe (C), showing localized staining (purple dots) at locations of gene expression, and *LGRA2* sense probe (D), showing no staining.

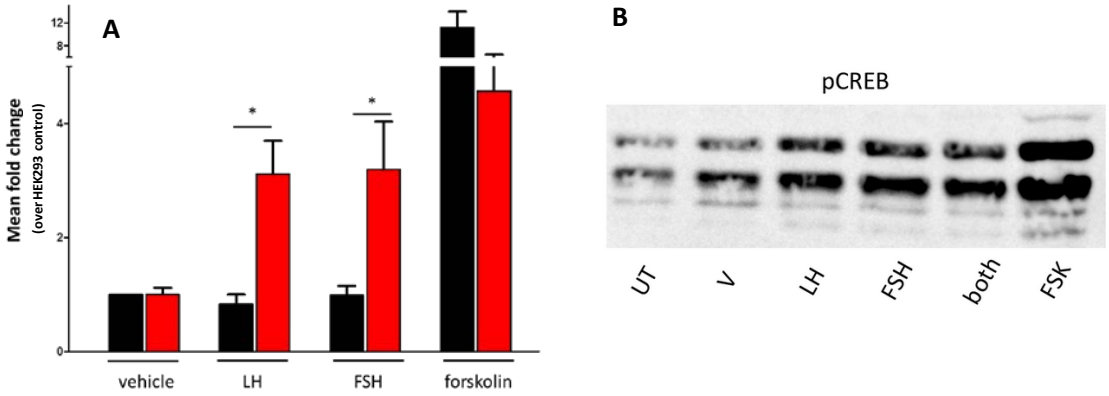

Figure 5. Stably transfected HEK293 cells expressing *LGRA2* (red bars) exhibit increases in cAMP 2nd messenger intracellular signaling when exposed to human gonadotropins LH and FSH in comparison to untransfected HEK293 cells (black bars) (A). N=3. Western blot showing pCREB in untransfected HEK293 cells (UT) and stably transfected cells following treatments of vehicle, gonadotropins, or forskolin (B).

Is LGRA2 expressed in sexually developed A. pallida, and if so, is it expressed in reproductive tissues?

Real-time PCR indicated that *LGRA2* was more highly expressed in an H2 clone which had been subjected to a simulated lunar light cycle to encourage sexual development than in a symbiotic MM2 and an aposymbiotic GMP clone, neither of which were subjected to the lunar light cycle and both of which were assumed to be sexually undeveloped (Fig 6). Upon hematoxylin and eosin staining, anemone populations under the lunar light cycle were confirmed to be reproductively developed by presence of mature oocytes (Fig 7). *In situ* hybridization of one of these anemones with antisense *LGRA2* probe showed positive staining in mesenteries near regions of gonadal development, compared to an absence of staining seen in sections hybridized with sense probe (Fig 7).

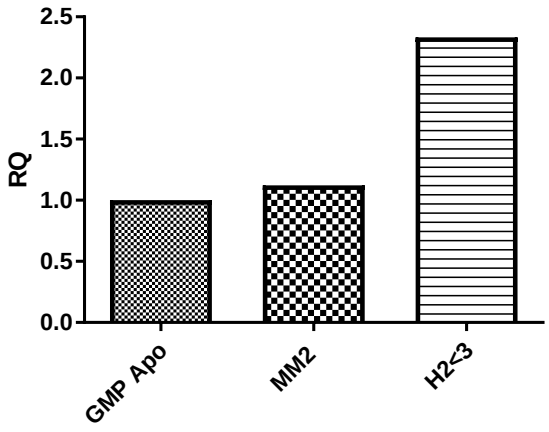

Figure 6. Relative expression of *LGRA2* in an aposymbiotic anemone assumed to be non-developed sexually (GMP Apo), a symbiotic anemone not under lunar light cycle conditions (MM2), and an anemone from a population of reproductively developed H2 clones housed under a simulated lunar light cycle (H2<3). N=1.

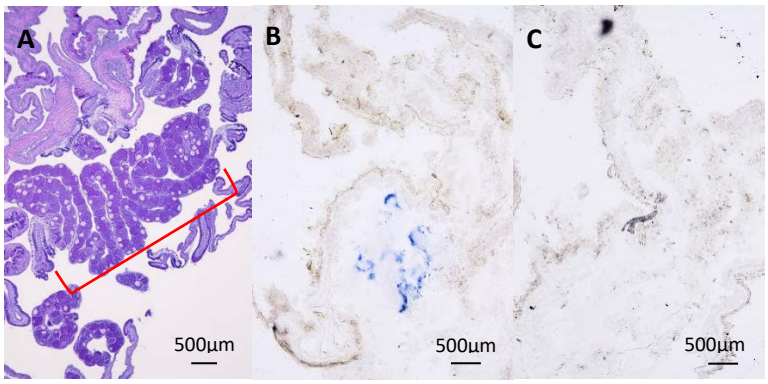

Figure 7. *LGRA2* expession in a reproductively developed H2 sea anemone clone. Hematoxylin and eosin stain showing presence of developed oocytes (red bracket) in mesenteries of anemone population housed under a simulated lunar light cycle (A). Slices of a reproductively developed H2 anemone hybridized with *LGRA2* antisense probe showing staining in mesenteries near regions of gonadal development (B), and sense probe showing no staining (C).

Does gonadotropin treatment in vivo elicit similar activation of native LGRA2 in A. pallida?

Similarly to the transfected cells, cAMP increased in H2 anemone clones treated with 10µM mammalian LH and FSH as above (Fig 8). However, gonadotropin treatment in MM2 clones resulted in a decrease in cAMP, indicating either genetic or temporal variation between clones. Basal levels of cAMP were low in H2 clonal *A. pallia* in comparison to MM2. Yet, thirty minutes following treatment with LH, FSH, or both, H2 cAMP was elevated more than tenfold, while MM2 levels decreased by a factor of four. Reasons for this variation in responses remain unclear, although it could be due to adaptive differences between clones based on their geographical origin, as MM2s are a Floridian strain while H2s came from Hawaii. More replicates are needed to determine significance.

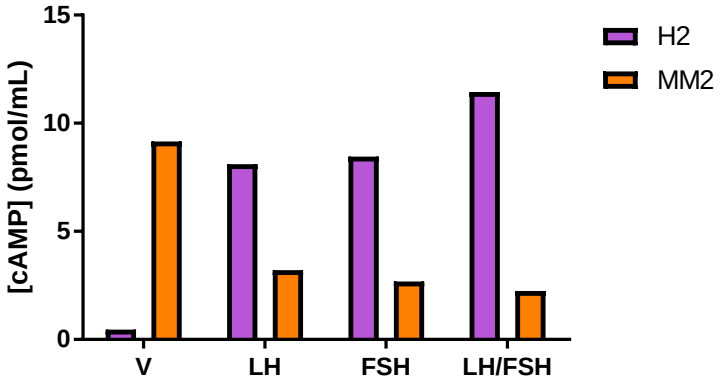

Figure 8. Cyclic AMP concentration in H2 and MM2 clones of *A. pallida* after treatment with vehicle or mammalian gonadotropins. N=1.

Does estradiol exposure modulate LGRA2 expression in A. pallida?

Although real-time PCR revealed no significant difference in *LGRA2* expression after treatment with estradiol for 24 or 72 hours compared to vehicle, H2s exhibited an increase in expression following 72hrs of exposure compared to 24hrs, while MM2s experienced a decrease (Fig 9). The increase in *LGRA2* expression in vehicle-treated MM2s at 72hrs and H2s at 24hrs may reflect normal temporal differences between the two clones. More replications will continue to shed light on possible variation between clones.

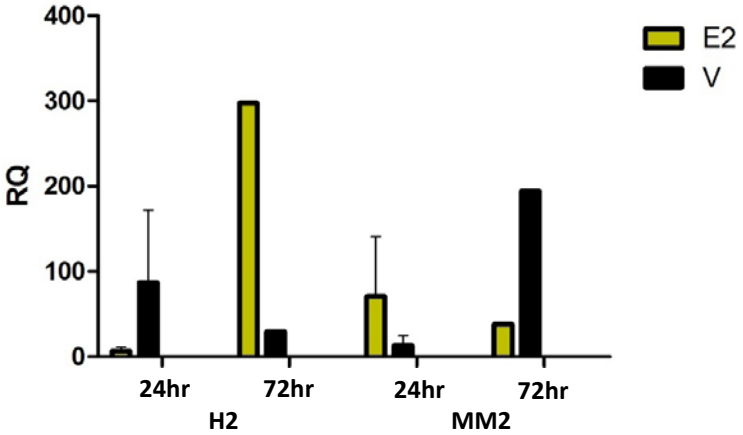

Figure 9. Relative expression of *LGRA2* in H2 and MM2 clones following treatment with estradiol or vehicle for 24 and 72 hours. For H2-E2-24hr, n=4, for H2-V-24hr, n=3, for H2-72hr (E2 and V), n=1, for MM2-24hr (E2 and V), n=2, and for MM2-72hr (E2 and V), n=1.

Discussion

Implications of Clock Gene Expression in Reproductive Aging

Data presented here do not strongly support RSV modulating clock gene expression in the gonads via SIRT1 as hypothesized. However, these data are preliminary, and more replicates are needed to determine significance and rule out the effects of compounding variables such as the length of time fish were on treatments. Slight differences were seen in overall clock gene expression with age, showing increases in non-treated ovaries over time. This could be correlated to a decline in SIRT1 histone deacetylase activity resulting in higher transcription of select clock genes. Expression of clock genes in RSV-treated ovaries did not appear to have a strong relationship to age, which could be a sign of preserved SIRT1 activity. Furthermore, it was observed in the 24-hour study that RSV treatment produced a shift in peaks in *bmal1a* and *Cry1aa* compared to vehicle. Repeating a 24-hour timecourse experiment with fish at earlier ages could help identify whether this disparity reflects a preservation of original rhythmicity compared to a degradation with age experienced by control animals. Investigating *Per* expression would help complete this picture as well by adding to the patterns seen in *Cry*, *BMAL*, and *CLOCK* expression. Additionally, it may be beneficial to revisit *clocka* primer design to obtain real-time expression data more consistently in order to get a more holistic view of expression patterns in the gonads over time.

The lack of strong evidence for RSV modulating clock gene expression in aging gonads does not rule it out from maintaining female fertility via other mechanisms. Next steps to determine possible correlations between clock gene expression include measures of spawning behavior, viable egg numbers, and health of progeny. If further evidence is found supporting a connection between circadian rhythms in reproductive tissue and fertility, then this may open

the door to the pursuit of pharmacological treatments that target clock gene expression or SIRT1 activity. The development of such treatments would have health benefits for humans, zoos, and breeders, each of whom may experience increased reproductive problems with age. Investigating the importance of clock gene rhythmicity on fertility could also provide vital information for how circadian cues should be maintained for those hoping to become pregnant, either by getting enough regular sleep at night in the case of humans or via careful husbandry protocols in zoos and animal facilities. Furthermore, the knowledge that proper circadian entrainment is paramount to fertility could inform conservation and policies concerning light pollution in areas with endangered species, including cities with busy waterways. Each of these applications make the continued study of circadian rhythms and their connection to reproductive function with age useful for human, animal, and environmental health.

Conserved Hormonal Pathways in Cnidarian and Mammalian Reproduction

Results presented in this thesis suggest that there is a level of conservation in the physiological mechanisms used by cnidarians to regulate spawning and those that make up the HPG axis in mammals. The presence of *LGRA2* expression near regions of gonadal development in *A. pallida,* along with evidence that prior "priming" of sexual development can change expression levels and that mammalian E2 can modulate these levels, gives evidence that the gene is linked to reproduction like its mammalian LGR homologs. Additionally, changes in second messenger cAMP downstream intracellular signaling upon treatment with mammalian gonadotropins show that LGRA2 is activated by homologous ligands in both heterologous *in vitro* models and *in vivo*. This supports the hypothesis that ancestral LGRAs have homologous functions to those seen in higher animals. It is important to note that other LGRAs such as *LGRA1*, which was also isolated by the Chappell lab and collaborators, could play a role in regulating reproduction that remains to be understood. In addition, the involvement of native

estrogens needs to be investigated to determine how much modulation occurs between cnidarian LGRs and sex steroids naturally. Furthermore, experiments carried out here examined immediate cellular responses to gonadotropin and estrogen treatment, but tests on the effects of these substances on spawning are needed to create a broader understanding of the physiological mechanisms controlling cnidarian reproduction.

These conclusions help begin to fill in the gaps in current understanding of cnidarian reproductive control, providing a physiological link between existing ecological and genomic knowledge. This helps us understand not only the evolution of our own signaling systems, but also the ways in which cnidarian reproduction is timed in nature. For example, knowledge of how sex steroids interact with LGRs and how this interaction affects sexual reproduction could provide a better understanding of how mass spawning events are synchronized within coral populations. Once these mechanisms are fully understood, they can inform future conservation efforts promoting coral reef spawning as ocean temperatures rise, as well as policies limiting runoff of sex steroids and endocrine disruptors from agricultural and urban areas. As a result, human as well as animal and environmental health will benefit.

Materials and Methods

Animals

Killifish

N. guentheri and *N. furzeri* embryos were kept on peat moss in a humid environment for several months until ready to hatch as deemed by the presence of eyes seen under a stereoscope. Hatching was performed by flooding the peat moss with reverse osmosis water at room temperature, incubating overnight, and then transferring the water and hatched fry to a shallow dish for raising. From this point, all fish were reared in a 14:10hr light:dark cycle. Fry were fed newly hatched artemia nauplii twice daily until around two weeks of age, at which point they were transferred into static tanks with sponge filters and brackish water (reverse osmosis water with sea salt added to 2000mS). Juveniles were transitioned to a diet of chopped frozen bloodworms at 4 weeks of age. Around 6 to 8 weeks of age, fish were moved to individual 2.8L tanks in an Aquaneering recirculating rack, with males separated from females to reduce aggression. For primer tests, *N. guentheri* and *N. furzeri* adult runts and fry that had been fed frozen bloodworms twice daily and died of natural causes were dissected into head and viscera and frozen for RNA extraction. For RSV tests, treatments were given to fish big enough to eat whole bloodworms (between 5 and 12 weeks of age) via food in the form of pellets prepared by soaking bloodworms in either 1.2mg/ml RSV dissolved in 95% water/5% ethanol or just 95% water/5% ethanol vehicle solution for two hours at room temperature, before adding 5% gelatin and storing at -20 degrees Celsius. The final concentration of RSV in food was 120ug/g, and fish ate approximately 25mg per day. Fish were euthanized with an overdose of buffered tricaine methylsulfonate (MS-222) at 500ppm, and brains and ovaries were dissected out and flash frozen before being stored at -80 degrees Celsius for RNA extraction. For aging of

clock gene expression experiments, five female *N. guentheri* were treated with RSV, and five were treated with vehicle, in addition to one female *N. furzeri* treated with RSV and two with vehicle. RSV-treated *N. guentheri* were sacrificed at ten and sixteen weeks of age, while control *N. guentheri* were sacrificed at nine, ten, thirteen, and sixteen weeks, all between 9:00am and 11:00am. The RSV-treated *N. furzeri* was sacrificed at thirteen weeks, and control *N. furzeri* were sacrificed at thirteen and fourteen weeks at the same time of day as well. To track clock gene expression over the course of a day, seven adult RSV-treated female *N. guentheri* and seven controls were sacrificed at 22-23 weeks of age, with one sacrifice of each treatment group every four hours. Upon sacrifice, one of the control fish was discovered to be a male that had not developed any color. To compensate for this, another same-age female that had been fed a diet of bloodworms without vehicle was sacrificed five days later.

Sea Anemones

H2 clones of *A. pallida* destined for *in situ* hybridization were housed individually in small glass jars containing filtered artificial sea water, loosely covered by saran wrap, and kept at 24 degrees Celsius under a simulated lunar light cycle as described by Grawunder for a period of four to six months to induce sexual development.[21] H2 and MM2 clones of *A. pallida* were also allowed to grow and produce pedal lacerates in either a 15cm cell culture dish or a 200ml plastic bottle cut in half with notches cut in the top edge for air flow that was covered by a clear plastic box lid. Both of these containers were kept under a 12:12hr light:dark cycle. Once pedal lacerates in these containers were developed and large enough to easily transport, they were moved to individual wells of six-well cell culture plates and allowed to acclimate and continue growing for cyclic AMP assays and gonadotropin exposure experiments. In these plates and jars containing anemones under the lunar light cycle, all further pedal lacerates were removed to allow for maximum growth of the original. All anemones were fed artemia nauplii every two to

three days, and sea water was changed a few hours after feeding. Sexual development of the population kept under the lunar light cycle was confirmed before *in situ* hybridization via hematoxylin and eosin stain on three different clones carried out by the OSU Veterinary Diagnostic Laboratory. Anemones were then anesthetized with 1:1 artificial sea water:0.37M magnesium chloride in the dark for 20 minutes and sacrificed by submerging them optimal cutting temperature medium in freezing molds with tentacles outstretched and as little water as possible and floating the molds in a bath of ethanol over dry ice briefly before freezing at -80 degrees Celsius. For cyclic AMP assays, anemones were treated with either 100nM of estradiol or ethanol vehicle for 24 or 72 hours and sacrificed by transferring to Eppendorf tubes, aspirating excess water, and freezing at -80 degrees Celsius. For quantification of *LGRA2* expression, anemones were treated with either 10µM of mammalian luteinizing hormone, follicle stimulating hormone, both, or DMSO vehicle for 20 minutes before being sacrificed similarly.

Anemone Sectioning

Anemones frozen in molds were floated in a bath of ethanol over dry ice for transport to a cryostat machine and kept there during cryosectioning of other specimens. 20 µm sections were cut and transferred to poly-L lysine coated slides which were stored over dry ice for the duration of sectioning. Slides were then thawed at room temperature before being fixed for 45 minutes in 4% paraformaldehyde in PBS for *in situ* hybridization.

Quantification of Clock Gene Expression in Killifish

Primers were designed for putative *N. furzeri* mRNA transcripts for elongation factor 1a (EF1a), *bmal1a*, *Cry1aa*, and *Clocka* identified via homology with *Danio rerio* sequences. Primers were then tested in *N. furzeri* and *N. guentheri* using standard PCR and gel electrophoresis.

Dissected tissues were homogenized in Trizol for RNA extraction. Samples were incubated with chloroform before spinning down and removing the top phase to new tubes. Samples were washed first in isopropanol and then in 70% ethanol before resuspending in Nanopure water. RNA was reprecipitated by incubating with a mixture of sodium acetate, glycogen, and 100% ethanol at -80 degrees Celsius overnight, before spinning down, rinsing in 70% ethanol, and resuspending in water. RNA was quantified using a Nanodrop Spectrophotometer and was then converted into cDNA using Thermo Fisher Scientific's High Capacity cDNA Reverse Transcription Kit. cDNAs were checked for quality by amplifying EF1a via standard PCR and performing electrophoresis using a 1% agarose gel. Expression of *bmal1a*, *Cry1aa*, and *Clocka* was then quantified by real-time PCR on an ABI OneStepPlus thermal cycler.

Cell Transfection

Cell transfection, quantification of cyclic AMP in stably transfected cells, and a western blot for pCREB in stably transfected cells were performed previously. The gene for cnidarian *LGRA2* was cloned from an *Aiptasia pallida* library and inserted into a pcDNA3.1-V5 plasmid. Plasmid purification was performed via antibiotic resistance in competent *E. coli* bacteria. Human embryonic kidney cells (HEK293) were stably transfected with *LGRA2* via Lipofectamine, and expression was confirmed via immunohistochemistry using an anti-V5 antibody. A 10cm plate of cells was harvested in Trizol, and RNA was extracted as described above for probe synthesis. Additional 10cm plates of cells were passed 1:10 every three to four days before plating on glass coverslips in 6-well plates. Once confluent, cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 minutes before rinsing in PBS and storing in PBS at 4 degrees Celsius for later *in situ* hybridization.

Quantification of Cyclic AMP in Stably Transfected Cells

Cells were treated with a 10μM dose of either mammalian LH, FSH, both, or vehicle for 20 minutes before being harvested and processed as directed by an Arbor Assays High Sensitivity Direct Cyclic AMP Chemiluminescent Immunoassay Kit to quantify cAMP.

Western blot for pCREB in Stably Transfected Cells

Total protein concentrations from HEK293 cell lysates were determined via BCA assay kit (Pierce Biotechnologies, Rockford, IL). Plates were read using a SkanIt plate-reader (Thermo Fisher Scientific, Waltham, MA) at absorbance of 562 nm. Twenty-five micrograms of protein were separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Primary antibody for pCREB (Santa Cruz) was used to probe blots. Bound primary antibody was probed by goat anti-rabbit HRP and detected with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA). The blots were scanned on an ImageQuant LAS4000 (GE Healthcare Life Sciences, Marlborough, MS).

LGRA2 Probe Synthesis

HEK293 RNA was DNAse treated using an Ambion Turbo DNA-free kit before converting to cDNA using a SuperScript III kit. LGRA2 was amplified via standard PCR using primers designed previously in the Chappell lab and run on a 1% agarose gel to check for quality. PCR product was then used as template in further PCR reactions to incorporate a T7-tag on both sense and antisense probes, using a T7-tagged reverse primer for antisense and T7-tagged forward primer for sense. Product was run on a 1% agarose gel to check for quality before purifying with an EZNA Cycle Pure PCR purification kit. Purified product was quantified using a Nanodrop Spectrophotometer and sequenced with Sanger Sequencing by the OSU CGRB to check for T7 incorporation before running through a T7 RNA Polymerase Reaction to incorporate Dig-labeled nucleotides. Probes were then DNAse treated and reprecipitated using lithium chloride and

100% ethanol followed by a wash in 70% ethanol before being resuspended in DEPC-treated water. Probes were checked a final time on a gel before *in situ* hybridization on transfected cells to check for quality of binding.

In Situ Hybridization on Stably Transfected Cells

A piece of laboratory tape was placed sticky side down on the inside bottom and sides of a new six-well cell culture plate, and coverslips containing fixed transfected HEK293 cells were moved to these wells on top of the tape. Wells were then rinsed in a Triton X-100-PBS (PBST) solution, incubated in triethanolamine and acetic anhydride, rinsed again in PBST, and covered with hybridization buffer containing formamide, saline-sodium citrate (SSC), heparin, Denhardt's solution, Tween 20, and EDTA. Cells were left to incubate at 37 degrees Celsius for three hours while shaking before hybridization buffer was sucked off and replaced by new buffer containing either sense or antisense probe that had been denatured at 80 degrees Celsius for ten minutes. Hybridization cover slips were cut to cover the cell cover slips and placed on top before placing the six-well plate into a Tupperware lined with paper towels soaked in 5x SSC/50% formamide. The Tupperware was sealed and incubated at 60 degrees Celsius for two days. Hybridization coverslips were then removed and cells were rinsed and incubated in 0.2x SSC for two hours, rinsed again in SSC and washed in a buffer of Tris and sodium chloride before incubating in a blocking solution containing Triton X-100, sheep serum, Tris, and sodium chloride for one hour. Blocking solution was then replaced with fresh blocking solution containing anti-DIG antibody, and cells were left to incubate for 2 hours. Cells were then washed twice in Tris/sodium chloride buffer and a buffer containing Tris, Triton X-100, magnesium chloride, and sodium chloride. BM Purple AP was added in the dark and left for 40 minutes, and cells were checked for color development periodically by removing from the stain, rinsing in DEPC treated

water, and covering in phosphate buffered saline to view under a microscope. Once developed, cell coverslips were mounted on slides with gelatin and glycerol for imaging.

In Situ Hybridization on Frozen Anemone Sections

Fixed slides were washed in PBST twice and dehydrated in a series of methanol washes, washed again in PBST, and incubated in triethanolamine. Slides were then removed and placed in triethanolamine with acetic anhydride added, where they incubated for ten minutes before washing again in PBST and coating with hybridization buffer containing formamide, SSC, heparin, Tween 20, EDTA, t-RNA, and denatured salmon sperm DNA. Hybridization cover slips were added and slides were placed in sealed individual slide containers inside a Tupperware lined with paper towels soaked in 5x SSC/50% formamide, which was incubated at 37 degrees Celsius for 1 hour. Coverslips were then removed, and hybridization buffer was replaced with fresh buffer containing either sense or antisense probes that had been denatured at 80 degrees Celsius for 10 minutes prior. New hybridization cover slips were placed on the slides, and slides were placed back in their sealed containers inside the Tupperware in a hybridization chamber to incubate at 60 degrees Celsius overnight. The next morning, slides were incubated in 2x SSC/50% formamide at 55 degrees Celsius for five minutes before washing twice each in 2x SSC and 1x SSC at 37 degrees Celsius. They were then incubated in a pre-warmed solution containing RNAses A and T for thirty minutes at 37 degrees Celsius, rinsed twice in 0.1x SSC followed by Tris/sodium chloride buffer at room temperature, and incubated in blocking solution for one hour. Blocking solution was replaced with fresh solution containing anti-Dig antibody which was left to incubate for one hour and fifteen minutes before rinsing twice in Tris/sodium chloride buffer followed by buffer containing sodium chloride, magnesium chloride, Tris, and Triton X-100. Slides were then incubated in BM Purple AP overnight at 4 degrees Celsius to develop before being mounted in gelatin and glycerol and covered with a class cover slip for imaging.

Quantification of Cyclic AMP in A. pallida

Tubes containing anemones that had been exposed to estradiol or vehicle before sacrifice were spun down, and excess water was aspirated. Weights were then obtained and samples were homogenized in sample diluent provided in an Arbor Assays High Sensitivity Direct Cyclic AMP Chemiluminescent Immunoassay Kit. A cyclic AMP assay was then carried out following kit instructions.

Quantification of LGRA2 Expression

Frozen anemones that were treated with mammalian gonadotropins or vehicle prior to sacrifice were homogenized in Trizol, and RNA extraction, cDNA conversion, and real-time PCR were carried out as described for killifish. cDNA quality was checked by amplifying L10 as a housekeeping gene and running on a 1% agarose gel prior to real-time PCR.

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