PHEROMONE MODULATION OF LOCOMOTOR RHYTHMICITY IN ADULT FEMALE SEA LAMPREY

By

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ABSTRACT

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All animals are partially controlled by a number of circadian rhythms. These are physical, behavioral, or genetic changes that occur on an approximate 24 hour time cycle and allow the organism to coordinate its internal and external environments. These rhythms are endogenous, but can be affected by external, environmental cues. Sea lamprey offer a unique model to understand locomotor rhythmicity, as throughout their life cycle they switch from a nocturnal rhythm in locomotor activity to arrhythmic multiple times. Adult female sea lamprey respond behaviorally to mature male sex pheromones that guide them to appropriate spawning grounds and induce spawning behaviors. In this dissertation, the overall hypothesis that locomotor rhythmicity in adult female sea lamprey is modulated by sex pheromone compounds is tested. In Chapter 1, field tests confirm that pre-ovulated and ovulated female locomotor activity is affected during exposure to spermiated male washings (SMW), and that in ovulated females SMW can alter the locomotor pattern. In Chapter 2, effects of individual SMW compounds are investigated using controlled laboratory studies, and results show that SMW as well as two components of SMW, 3-keto petromyzonol sulfate (3kPZS) and petromyzonol sulfate (PZS), have differential effects on pre-ovulated and ovulated female locomotor activity. Chapter 3 demonstrates that there are effects of these sex pheromones on the GABAergic system in the brain and pineal complex related to neural circuits that guide locomotor activity production. Chapter 4 investigates the endogenous circadian system, providing evidence that sex pheromone compounds alter the molecular framework responsible for circadian rhythms in the proposed

master circadian clock, the pineal gland, as well as peripheral clocks in the brain. Studies here provide a rare example of an exogenous stimuli on circadian locomotor production and add to the knowledge of sex pheromone influences on behavior, circadian timekeeping mechanisms, and locomotor activity and rhythm production in this species.

To my parents, David and Marjorie Walaszczyk, who introduced me to the magic of science and reading at a young age, and who have been there for me through thick and thin. Their unconditional love, support, and guidance have shaped me into the person I am today.

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CHAPTER 1

EFFECTS OF SEX PHEROMONES AND SEXUAL MATURATION ON LOCOMOTOR ACTIVITY IN FEMALE SEA LAMPREY (PETROMYZON MARINUS)

Walaszczyk E. J., Johnson N. S., Steibel J. P., Li W. 2013. Effects of sex pheromones and sexual maturation on locomotor activity in female sea lamprey (*Petromyzon marinus*). Journal of Biological Rhythms 28(3):217-226. (doi:10.1177/0748730413488994).

ABSTRACT

Synchronization of male and female locomotor rhythmicity can play a vital role in ensuring reproductive success. Several physiological and environmental factors alter these locomotor rhythms. As sea lamprey, *Petromyzon marinus*, progress through their life cycle, their locomotor activity rhythm changes multiple times. The goal of this study was to elucidate the activity patterns of adult female sea lamprey during the sexual maturation process, and discern the interactions of these patterns with exposure to male pheromones. During these stages, pre-ovulated and ovulated adult females are exposed to sex pheromone compounds, which are released by spermiated males and attract ovulated females to the nest for spawning. We monitored locomotor behavior of adult females in a natural stream with a passive integrated tag responder system as they matured and were exposed to a sex pheromone treatment (spermiated male washings) or a control (pre-spermiated male washings). Our results show that dependent on the hour of day, male sex pheromone compounds reduce total activity ($p < 0.05$) and cause increases in activity during several daytime hours in pre-ovulated and ovulated females. These results are one of the first examples of how sex pheromones modulate a locomotor rhythm in a vertebrate, and suggest that the interaction between maturity stage and sex pheromone exposure contributes to the differential locomotor rhythms found in adult female sea lamprey. This phenomenon may contribute to the reproductive synchrony of mature adults, thus increasing reproductive success in this species.

INTRODUCTION

Synchronization of internal and external environments by coordinating the proper timing of physiological and behavioral cycles is vital to enhance the survival and reproductive fitness of an organism. Diel locomotor rhythmicity is common in nature and can be influenced by several internal and external environmental stimuli, which may act simultaneously or one may dominate and mask the effects of the others to produce a final locomotor rhythm (Aschoff 1960). During seasonal reproduction, coordination of locomotor rhythmicity between males and females within a species becomes increasingly important to ensure reproductive success. The phenomena of locomotor activity alterations as a response to pheromones has been documented in insects (Shorey and Gaston, 1965; Baker and Cardé, 1979; Liang and Schal, 1990), and while in fish pheromones have been shown to produce activity responses (LaBerge and Hara, 2003), effects on overall locomotor rhythmicity are less well described.

The sea lamprey, *Petromyzon marinus*, has a life cycle consisting of several behaviorally different stages, offering a unique opportunity to study the factors that alter locomotor rhythmicity. Sea lamprey ammocoetes (larvae) are nocturnal, emerging at night to filter feed (Hardisty and Potter, 1971a). Ammocoetes metamorphose into a behaviorally arrhythmic juvenile stage, during which they parasitize large fish (Hardisty and Potter, 1971b). After feeding in open waters for a little longer than a year (Bergstedt and Seelye, 1995), immature (pre-ovulated and pre-spermiated) sea lamprey undergo an upstream spring migration through river and stream channels to appropriate spawning grounds, during which they are nocturnal (Manion and McLain, 1971) and guided by larval lamprey pheromone cues (Teeter, 1980; Wagner et al., 2009). Pre-spermiated male sea lamprey make this migration first, arriving at nests before females (Applegate, 1950). As they mature, they become arrhythmic, beginning to build nests

during the day and night (Applegate, 1950; Manion and Hanson, 1980). Another pheromone, 3-keto petromyzonol sulfate (3kPZS), is released by spermiated males and initiates searching behaviors, guiding ovulated female sea lamprey to nests (Li et al., 2002; Li et al., 2003; Siefkes et al., 2005; Johnson et al., 2009). Sea lamprey are semelparous and die shortly after spawning.

Until animals are sexually mature for reproduction, remaining nocturnal could be a benefit to escape predator pressure, as has previously been suggested (Semlitsch, 1987; Keitt et al., 2005). Additionally, timing of reproduction in this species is especially important because during the migration and spawning periods, sea lamprey do not eat and rely on the storage of nutrients acquired during the parasitic phase as energy for the maturation of gonads and the act of spawning (Beamish, 1979; Madenjian et al., 2003).

Our overarching hypothesis is that synchronized locomotor activity of ovulated female and spermiated male sea lamprey may be mediated by sexual pheromones, thus increasing the chances for successful reproduction. Specifically, we hypothesize that while sexual maturation and sex pheromone exposure will each have independent effects, it is their interaction that will have an effect on the overall locomotor rhythmicity. Therefore, we predict that pre-ovulated females will have a nocturnal rhythm, whereas ovulated females will increase their activity in the daytime hours, which likely will increase the chances for encountering behaviorally arrhythmic spermiated males. Furthermore, we speculate that females respond behaviorally to male sex pheromones, and exposure to these compounds acts as a signal to females that they have reached appropriate spawning grounds and mates, which will cause a decrease in locomotor activity of female sea lamprey to reserve energy for reproduction.

In this study, we aim to document the effects of sexual maturation and determine the direct effects of sex pheromone exposure on female locomotor activity and rhythms. Here, we

present observations on the diel locomotor activity of pre-ovulated and ovulated female sea lamprey in field conditions during exposure to a suite of pheromone compounds from washings of spermiated males or a control (pre-spermiated washings). Sexual maturation and sex pheromone exposure had independent effects on locomotor activity in adult female sea lamprey, but it was their interaction that disrupted the overall locomotor rhythms of the female sea lamprey, altering them from a nocturnal state when pre-ovulated to an arrhythmic state once reaching maturity.

METHODS

Animals

Sea lamprey were captured in mechanical traps operated by the United States Fish and Wildlife Service and Department of Fisheries and Oceans, Canada in the St. Marys River, which is an outflow of Lake Superior. Standard operating procedures for transporting, maintaining, handling, anesthetizing, and euthanizing sea lamprey were approved by the Institutional Committee on Animal Use and Care of Michigan State University. Males and females were separated based upon the protocol conducted by Vladykov (1949). Maturity stages were separated based upon the protocol established by Siefkes et al. (2003). Pre-ovulated females matured during the experiment. Ovulated females were classified as such if animals had a defined keel and eggs were expressed by manual pressure. Pre-spermiated and spermiated males were used for collecting washings and were separated based upon spermiated males having a dorsal ridge and expressing sperm using manual pressure.

Experimental Design

This study was conducted in the Little Ocqueoc River in Millersburg, MI between 15-Jul-

08 and 25-Jul-08. The Little Ocqueoc River is a tributary of the Ocqueoc River, which is above the sea lamprey barrier. A portion of the river with similar cross-sectional stream flow, shady conditions, slow-moving current, and depth was selected. The river was separated into two channels naturally with fallen trees and additional sand bags were added to ensure there was no cross flow. Temperature loggers (Hobo Temperature Data Logger, Onset, Bourne, MA, U.S.A.) were placed in the river near the cages to monitor the temperature throughout the duration of the experiment. Sea lamprey were exposed to natural lighting conditions. During this time, sunrise occurred at 0600 h and sunset at 2100 h, exposing the animal to approximately 15 hours of daylight, 9 hours of darkness, and two transitional twilight periods.

Two PVC cages 1 x 1 x 0.5 m lined with steel wire mesh were placed into each channel in a line parallel to stream flow (Figure 1.1A). Each cage had a hinged lid and contained a plexiglass rectangle covering the back half as an attachment surface as well as a refuge area from light created from a cement block placed on top of the plexiglass. Each cage was equipped with a circular passive integrated transponder (PIT) antenna connected to a multiplexer equipped with an internal system to record signals from PIT tags (Oregon RFID, Portland, OR, U.S.A.). The PIT antenna was placed in a line situated 0.25 m back from the front of each cage and 0.25 m below the top of the cage and tuned to ensure the sea lamprey would not rest in a position that would continually set off the system (Figure 1.1B). Each time a sea lamprey passed near the antenna, the time and uniquely identifiable tag number were recorded and used as a measure of locomotor activity. Due to the antenna placement, this system tracked a relative measure of activity, as some movements of each animal were inevitably missed. All of the animals within each cage, however, did show activity and provided large enough sample size numbers that we are confident that these behaviors are representative of this species. This measurement system

allowed us to infer activity patterns through comparisons of activity between maturity states, between treatment groups, and the overall rhythms of activity in each group.

Twenty pre-ovulated female sea lamprey equipped with a PIT tag placed within the gill slit openings were placed into each cage. A double-headed peristaltic, battery powered pump (Master-Flex 7553-70, Cole-Parmer, Vernon Hills, IL, U.S.A.) was used to pump either spermiated male washings (SMW) or pre-spermiated male washings (PSMW) into either side of the channel. Washings were collected by placing 20 spermiated or pre-spermiated males into a tank of 200 L of aerated water with no flow for 36 hours. Water samples were collected in one gallon containers and frozen at temperatures below -20°C until experimentation. Concentrations of natural 3kPZS in SMW and PSMW were quantified using ultrahigh performance liquid chromatography-tandem mass spectrometry according to the protocol described in Xi et al. (2011). The SMW contained 2.33 mg 3kPZS/L and the PSMW had no 3kPZS, therefore allowing it to be used as a control. Each pump was set at a constant rate of 30 ml/min. For cages 1 and 2, spermiated male washings were thawed and diluted with river water to create a final concentration of 10^{-12} M 3kPZS once mixed with stream water within the channel. The amount that the SMW were diluted was calibrated for every 24 hours and dependent on the stream flow discharge, which was measured with a Marsh-McBirney flow meter (Frederick, MA, U.S.A.) once a day. An equivalent volume of pre-spermiated male washings was added into the other channel as a control for cages 3 and 4.

Pre-ovulated females were placed into each cage at the start of the experiment and checked for maturity every two days. 1 mL blood samples were collected from a selected group of half of the animals in each cage via the caudal vein every third night of the experiment starting 15-Jul-08 between 0000 h and 0400 h for potential future experiments. The order cages were

selected for blood drawing was rotated every night blood was drawn. Cages were checked twice a day and dead sea lamprey were removed. The experiment continued until all of the sea lamprey expired.

Data Analysis

A generalized linear mixed model with a Poisson distribution and a log-link function was fit to the count data of movements. Fixed effects included were: treatment (spermiated male washings versus pre-spermiated male washings), maturity stage (pre-ovulated versus ovulated), hour of the day, and the interactions of treatment by hour, maturity by hour, and maturity by treatment by hour. Fixed effects of blood drawing, blood within treatment, and cage within treatment were also added into the model to account for any background effects that may be present. Random effects included in the model were animal ID, day, animal ID by day, and hour by day, to properly account for correlations due to the double repeated measure sampling process. ProcGlimmix of SAS (Littell et al., 2006) was used to fit the model. Our goal was to test the interactions of hour by maturity, hour by treatment, and the triple interaction of hour by maturity by treatment. We first compared levels of maturity (Figure 1.2) at each hour by treatment and subsequently compared treatment at each combination of hour and maturity (Figure 1.3). To clearly show the interaction of treatment and sexual maturation, we replotted the data from Figure 1.3 for each maturity stage treated with pheromone for comparison (Figure 1.4). We performed these comparisons based on the least square means of the linear predictor and back-transformed point estimates to the original count scale (Poisson variable) for our presentation and interpretation of results.

RESULTS

After transfer to the tanks or the cages, sea lamprey demonstrated transient bursts of

activity, but settled down into normal behavior after a few hours. When not moving, lamprey attached themselves to the sheets of plexiglass provided in each cage. Temperatures ranged between 15 and 19°C during the duration of the experiment, however, remained relatively stable near 17°C.

Response to Sexual Maturation

Maturity stage affected locomotor activity, which was dependent on the hour of the day ($F_{23,7748} = 8.67$, $p < 0.0001$). When pre-ovulated and ovulated females were exposed to the pre-spermiated male washings control, both had a nocturnal locomotor rhythm, however, ovulated animals showed a clear reduction in activity during several hours of the dark period (0200 h-0500 h, and 2100 h-2300 h) as well as an increase in activity during multiple hours (1200 h-1300 h) of the midday period (Figure 1.2). The peaks of locomotor activity in the diel profile also differed between the two maturity stages, with pre-ovulated females peaking in locomotion two hours after sunset (2300 h) and ovulated females peaking three to four hours after sunset (0000 h-0100 h).

Response to Sex Pheromones

Locomotor activity was affected by exposure to spermiated male washings, which was dependent on hour ($F_{23,7748} = 8.37$, $p < 0.0001$) in pre-ovulated as well as ovulated females (Figure 1.3). Pre-ovulated females were nocturnal whether they were exposed to the pre-spermiated male washings control or spermiated male washings, and the peak of activities did not change (Figure 1.3A). Exposure to spermiated male washings, however, caused a decrease in locomotion during all hours of the night (0000 h-0500 h and 2100-2300 h) as well as several hours of the day (0600 h-1300 h). Additionally, activity was increased in these animals during

several hours of the daytime period (1600 h and 1800 h-1900 h).

Ovulated females showed not only a change in activity levels, but also a change in rhythm when exposed to the spermiated male washings (Figure 1.3B). The females exposed to the control were nocturnal, with a peak at 0-0100 hours, however, treated females showed no clear rhythmic pattern or peak in diel locomotor activity. These treated females had a reduction in activity during several hours of the night (0000 h-0300 h, 0500 h, 1300 h, and 2200 h-2300 h) as well as an increase in activity one hour before and after sunset (2000 h-2100), which contributed to the overall loss of rhythm.

Interaction of Maturity and Sex Pheromones

The three-way interaction of maturity stage, sex pheromone exposure, and hour altered the locomotor rhythmicity ($F_{24,7748} = 5.24$, $p < 0.0001$). Pre-ovulated and ovulated female sea lamprey exposed to spermiated male washings had differential locomotor rhythms (Figure 1.4). Pre-ovulated females had a strong nocturnal pattern, peaking two hours after sunset (2300 h), while ovulated females had no clear locomotor rhythm or peak (hour had no significant effect on locomotor activity in ovulated females exposed to treatment, $F_{7,88} = 0.76$, $p = 0.6249$). Additionally, locomotor activity was reduced in ovulated females during several hours of the night (0000 h- 0300 h, 0500 h, 2200 h-2300 h) and was increased during several hours of the day (0700 h, 1000 h-1300 h, 1500 h, 1800 h, and 2000 h), demonstrating the alteration of the locomotor rhythm between these two life stages.

Blood collection was not significant in affecting movement ($F_{1,7748} = 1.21$, $p = 0.271$) nor was there a significant blood within treatment interaction ($F_{1,7748} = 0.8$, $p = 0.773$). While cage within treatment was significant ($F_{2,7748} = 5.6$, $p = 0.0037$) and these cage differences were

incorporated into the model, this additional effect did not change the results and conclusions of the effects of maturity, treatment, and hour nor their interactions.

DISCUSSION

The results presented here document that the locomotor rhythm of the adult female sea lamprey is modulated by the interaction of sexual maturation and exposure to the male sex pheromone. We have shown that each factor has an independent effect on the locomotor activity, however, it is the interaction of the two factors that produces the switch in rhythm from a nocturnal state shown in pre-ovulated females to what is representative of an arrhythmic state with the addition of daytime activity exhibited in ovulated females. Conducting research in the quasi-natural environment required that activity be subsampled with PIT antennas instead of being monitored by cameras in a lab. The overall low activity recorded was a result of the subsampling. As each sea lamprey within each cage did show activity in our experiment, we are confident that the subsampled data, although not containing all activity, correctly characterized the locomotor activity of female sea lamprey. It is not uncommon for fishes of different maturation stages to have differential locomotor phases in the field (Emery, 1973; Johnson and Muller, 1978; Helfman, 1978; 1981; Helfman et al., 1982; Magnan and Fitzgerald, 1984; Reebs et al., 1995), however, to our knowledge this is the first demonstration of sex pheromones altering a locomotor rhythm in a vertebrate species.

For many decades, changes in the locomotor rhythms across the sea lamprey life cycle have been of interest. Several factors have been investigated to determine the cause of these changes. The corneal degradation that occurs with maturation was initially hypothesized to cause a loss of vision and the onset of daytime activity (Applegate, 1950; Manion and Hanson, 1980), however, this idea was put to rest when it was shown that the diel activity rhythm of sea lamprey

was not affected by blindness (Binder and McDonald, 2007). Later, it was discovered that the dominant photosensory organ involved in circadian locomotor control in lamprey species is the light-sensitive pineal gland (Tomotsu and Morita, 1986). The locomotor rhythm is lost in the river lamprey, *Lampetra japonica*, when the pineal gland is removed, implicating its role in rhythmicity control (Morita et al., 1992). The pineal gland can detect the day length photosensory changes (Morita and Dodt, 1971; Tamotsu and Morita 1986) across the spawning season and it was hypothesized that these alterations in the environment were responsible for the addition of daytime activity. However, in a study in which river lamprey were kept in cold water under natural lighting conditions, the onset of daytime activity in addition to sexual maturation was delayed (Sjoberg, 1977), suggesting that day length is not the key to controlling the locomotor rhythm. This is consistent with our presented results in which the locomotor rhythm was altered even though the day length remained consistent over each experimental period.

More recently, temperature has been implicated in the control of locomotor rhythmicity. Binder and McDonald (2007) demonstrated that pre-ovulated adult female sea lamprey exposed to low temperatures (7°C) or high temperatures (20°C) had a reduction in their nocturnal locomotor activity peak and an addition of activity during the daytime hours was observed at high temperatures. Additionally, they showed that rapid increases in temperature of 7°C or 8°C over a four hour period cause a brief increase in daytime activity. These results suggest that temperature is an important factor in modulating locomotor activity; however, sexual maturation was not examined, as only pre-ovulated females were used, and these results cannot be extrapolated to the change in locomotor rhythmicity demonstrated across adult life stages. Furthermore, in our study the temperature was consistent between a range of 15-19°C, with little change from the first to the final days of the experiment. The alterations in locomotor activity

and rhythms that we have demonstrated provide evidence that suggests other additional factors are contributing to these changes. It is possible that locomotor rhythms are only overt in an optimal temperature range. Sea lamprey migration upstream begins at approximately 10°C and the intensity of the migration varies with temperature (Applegate, 1950). Once within this thermal range, however, it is likely that sexual maturation and exposure to sex pheromones dominate the effects of temperature and, subsequently, are the determining factors of the locomotor rhythm. As a mostly nocturnal rhythm was still present in ovulated females exposed to a control, it is possible that sex pheromones are masking the effects of temperature and sexual maturation, contributing to the arrhythmic state that is displayed. Evidence has suggested in mammals that olfactory stimuli can alter circadian rhythms and that these two systems are linked functionally (Honrado and Mrosovsky, 1991; Fluxman and Haim, 1993; Haim and Rozenfeld, 1993; Goel and Lee, 1995). Therefore, an alternative explanation is that olfactory stimuli may influence circadian rhythms by modulating the activity of the circadian clock in the pineal gland through stimulation of structures that innervate this gland directly.

One possible rationale for the changes seen in locomotion as sea lamprey mature is that once sea lamprey reach spawning maturity, intraspecific communication becomes essential for the coordination of reproduction. The sex pheromone released by spermiated males may act as a signal to females that they are in appropriate spawning nest territory and to reduce energy expenditure on locomotion to promote energy for reproduction or gonadal development. In this species, only a finite amount of energy and, therefore, a narrow time range exists for reproduction due to the cessation of eating after the juvenile stage (Beamish, 1979; Madenjian et al., 2003). Furthermore, sea lamprey only spawn once in their life. Due to the limited amount of energy and time available for reproduction, the reproductive benefits that come from a reduction

in overall activity as well as an extension of activity to daytime hours outweigh the diurnal predation risks. Our results are consistent with previous studies in which adult fishes become arrhythmic during the spawning phase (see Reebs, 2002 for review).

Communication is also important to coordinate locomotor activities between mature male and female sea lamprey. Spermiated males are arrhythmic (Applegate, 1950), and as females mature, they may switch from a nocturnal to an arrhythmic state to optimally match the locomotor activity of the males. The sex pheromone cue may be the key to facilitate this synchronization of behavior, improving reproductive fitness through an increased chance of a spawning event to occur. This idea is consistent with previous studies in diverse fish species exhibiting that chemical signals participate in the synchronization between males and females by affecting both behavior and physiology (Partridge et al., 1976; Liley, 1982; Stacey and Sorenson, 1991; Sorenson, 1992; Olsen and Liley, 1993).

The idea that male sex pheromone compounds can alter female sea lamprey locomotor activity is not surprising. The male sex pheromone component 3kPZS alters behaviors in ovulated female sea lamprey (Siefkes et al., 2005, Johnson et al., 2009). Ovulated females exposed to 3kPZS show robust upstream movements, and when this compound was applied to a stream at a concentration greater than that of spermiated male washings, females were drawn to the source of 3kPZS (Johnson et al., 2009). Additionally, exposing ovulated females to spermiated male washings, which consists of a suite of pheromone compounds, retained them on the nests (Johnson et al., 2009). 3kPZS has also been shown to have effects on differential systems in male and female sea lamprey. In male sea lamprey, it has been shown that 3kPZS has a greater effect on the endocrine system (Chung-Davidson et al., 2013; Siefkes et al., 2005), whereas in contrast, it has a greater effect on the locomotor system of females (Li et al., 2002;

Johnson et al., 2009). In our study, the measured change between the treatment and control was the presence of 3kPZS. Due to this and previous evidence, we hypothesize that this sex pheromone may be the main component of spermiating male pheromone compounds affecting locomotor rhythmicity; however, further studies using ovulated females are needed to confirm this.

Overall, this study has provided evidence that sexual maturation and exposure to sex pheromones are contributing factors to locomotor activity rhythms in adult female sea lamprey. Pheromones have been implicated in locomotor rhythm changes in insects and changes in amounts of activity in other fish species, but this is one of the first examples of the effects of sex pheromones on the locomotor rhythm in a vertebrate species. Moreover, we have demonstrated that these key factors have independent effects that cause reductions in activity and an extension of activity into the daytime, but that it is their interaction that produces a shift in the locomotor rhythm. Future work should determine if the pheromones are having direct effects on the circadian clock system within the pineal gland and other circadian or locomotor control portions of the nervous system, or are exerting masking effects, or both.

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APPENDIX

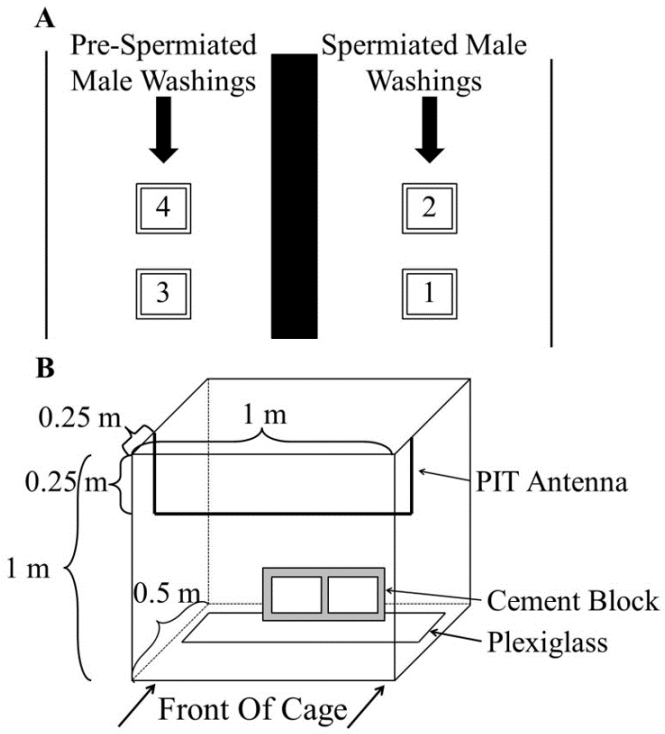

Figure 1.1: Experimental Stream Channel Design. (A) Cages 1 and 2 exposed to spermiated male washings. Cages 3 and 4 exposed to pre-spermiated male washings. Channels separated by line of sandbags. 20 pre-ovulated females placed in each cage and exposed to treatment for duration of experiment. (B) Individual experimental cage. Plexiglass and cement block placed in back half of cage. Red line denotes PIT antenna placed in top, front portion of cage. Blue arrows represent stream current direction.

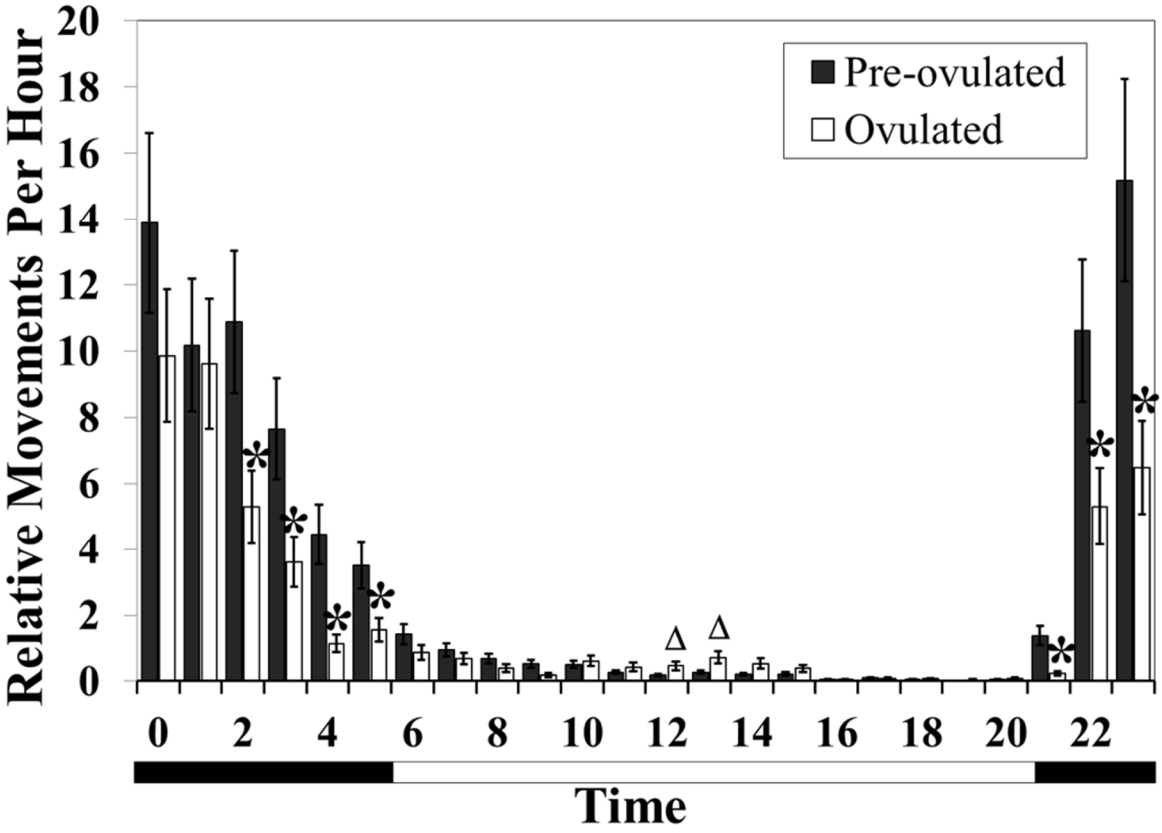

Figure 1.2: Effects of Maturity on Locomotor Activity. Asterisks denote that activity is significantly lower in ovulated females ($p<0.05$). The triangle represents the hour activity is significantly higher in ovulated females ($p<0.05$). Vertical bars denote one standard error. The bar below the graph is split into black for nighttime and white for daytime.

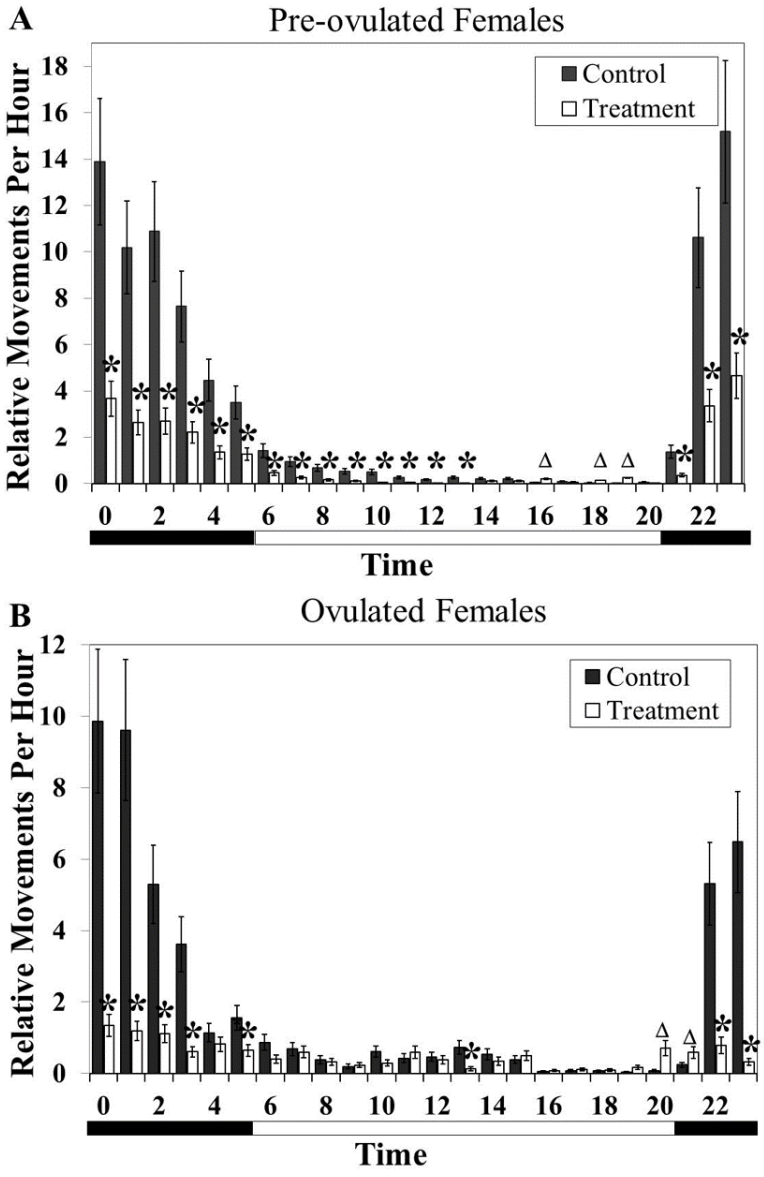

Figure 1.3: Effects of Sex Pheromones on Locomotor Activity and Rhythmicity in Adult Females. (A) Pre-ovulated female activity is altered in animals exposed to treatment. (B) Ovulated female rhythmicity and activity is altered in treated animals. Asterisks represent hours activity is significantly lower in treated females ($p<0.05$). Triangles denote hours activity significantly higher in treated females ($p<0.05$) Vertical bars denote one standard error. The bar below the graph is split into black for nighttime and white for daytime.

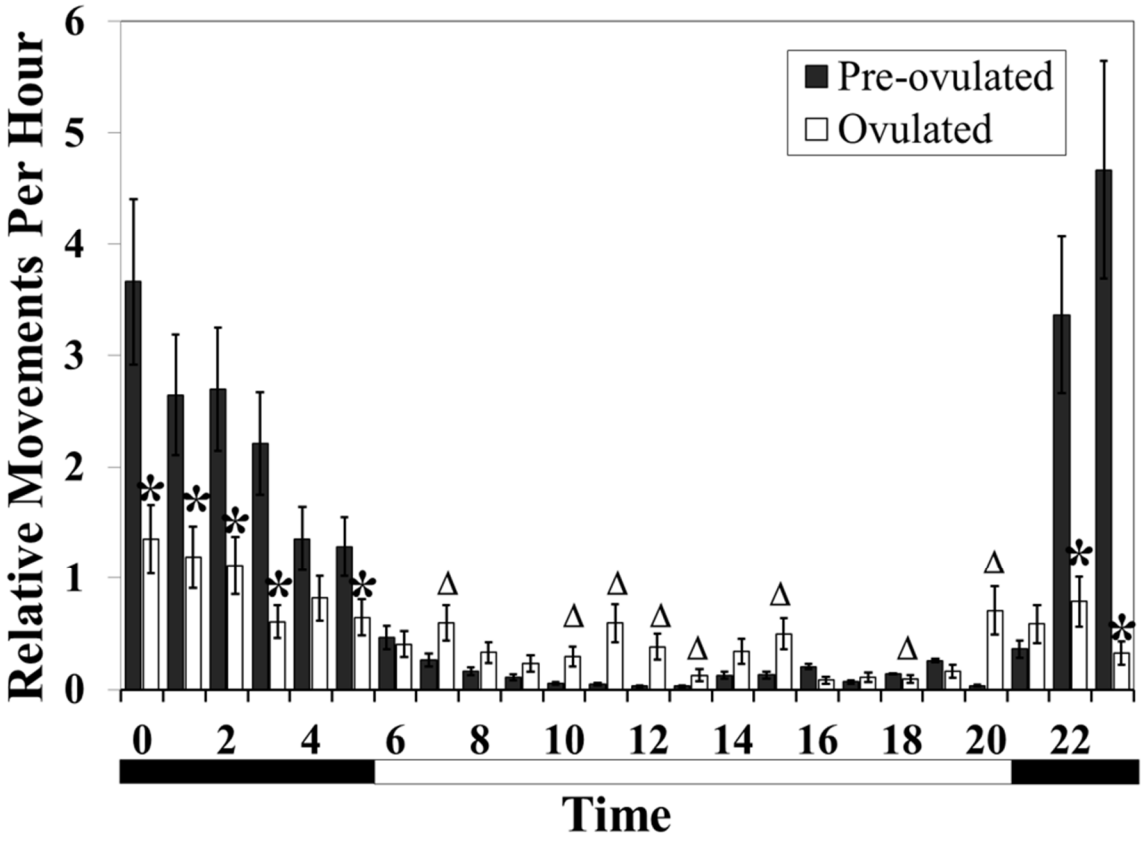

Figure 1.4: Effect of Sex Pheromones on Rhythmicity in Adult Females. Pre-ovulated females are nocturnal and ovulated females are arrhythmic when exposed to sex pheromones in sea lamprey. Asterisks denote that activity is significantly lower in ovulated females ($p<0.05$). The triangle represents the hour activity is significantly higher in ovulated females ($p<0.05$). Vertical bars denote one standard error. The bar below the graph is split into black for nighttime and white for daytime. Data presented in this figure are re-plotted from those of figure 1.3.

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CHAPTER 2

DIFFERENTIAL EFFECTS OF SEX PHEROMONE COMPOUNDS ON ADULT FEMALE SEA LAMPREY (PETROMYZON MARINUS)

ABSTRACT

Synchronization of male and female locomotor activity plays a critical role in ensuring reproductive success, especially in semelparous species. The goal of this study was to elucidate the effects of individual chemical signals, or pheromones, on the locomotor activity in the sea lamprey (*Petromyzon marinus*). In their native habitat, adult pre-ovulated females (POF) and ovulated females (OF) are exposed to sex pheromone compounds that are released from spermiated males and attract females to nests during their migration and spawning periods. In this study, locomotor activity of individual POF and OF was measured hourly in controlled laboratory conditions using an automated video-tracking system. Differences in the activity between a baseline day (no treatment exposure) and a treatment day (sex pheromone compound or control exposure) were examined for daytime and nighttime periods. Results showed that different pheromone compound treatments affected both POF and OF sea lamprey ($p < 0.05$), but in different ways. Spermiated male washings (SMW) and one of its main components, 3-keto petromyzonol sulfate (3kPZS), decreased activity of POF during the nighttime. SMW also reduced activity in POF during the daytime. In contrast, SMW increased activity of OF during the daytime, and an additional compound found in SMW, petromyzonol sulfate (PZS), decreased the activity during the nighttime. In addition, we examined factors that allowed us to infer the overall locomotor patterns. SMW increased the maximum hourly activity during the daytime, decreased the maximum hourly activity during the nighttime, and reduced the percentage of nocturnal activity in OF. Our findings suggest that adult females have evolved to respond to different male compounds in regards to their locomotor activity before and after final maturation. This is a rare example of how a species-wide chemosensory stimuli can not only affect the amounts of activity, but also the overall locomotor pattern in a vertebrate species.

INTRODUCTION

For animals to survive, it is vital for them to be synchronized with their external environments by coordinating the timing of behavioral and physiological cycles. This is especially true for those animals that undergo seasonal reproduction. Diel rhythms are used as a timekeeping system that allows an animal to anticipate and prepare for changes in the physical environment. Diel locomotor rhythmicity is prevalent in nature and can be influenced by several exogenous and endogenous factors. Studies of activity rhythms traditionally focus on the environmental effects of photoperiod or temperature, less commonly looking into other sensory modalities, such as olfaction. The sea lamprey, *Petromyzon marinus*, of the Laurentian Great Lakes has a life cycle consisting of several stages in which changes in locomotor activity patterns occur concurrently with changes in exposure to conspecific signals in the form of pheromones that can modulate behavior and provide information to the receivers, such as sex or reproductive status (Beamish, 1980; Albone, 1984; Johnston, 2000; Meckley et al., 2012). This allows for a rare opportunity to investigate the effects of an exogenous olfactory cue on locomotor activity and patterns.

Previous studies have documented locomotor patterns in particular life stages of the sea lamprey. Lamprey larvae live for several years in river and sediment beds, and although they spend most of their time burrowed, they are primarily nocturnal when active (Hardisty and Potter, 1971a; Potter, 1980; Almeida et al., 2005). Larvae metamorphose into a juvenile parasitic form and migrate downstream to lakes, during which they have been observed to be behaviorally arrhythmic and feed on larger fish (Hardisty and Potter, 1971b). After feeding in open waters for a little over a year (Bergstedt and Seelye, 1995), immature (pre-spermiated male and pre-ovulated female) lamprey undertake a spring migration through rivers and streams to appropriate

spawning grounds, guided by larval olfactory cues (Sorensen et al., 2003; Sorensen and Vrieze, 2003; Sorensen et al., 2005; Wagner et al., 2009). Petromyzonol sulfate (PZS or PS) is one of the metabolites released by the larvae that act as a potent odorant (Teeter, 1980, Venkatachalam, 2005; Fine and Sorenson, 2010). During this upstream migration, the immature lamprey exhibit a nocturnal locomotor activity pattern, being active at night and seeking refuge during the day (Manion and McLain, 1971; Kelso and Gardner, 2000; Vrieze et al., 2001). Binder and McDonald, 2007). Males make this migration prior to the females, and as they mature become arrhythmic, showing nest building activity during both the day and night (Applegate, 1950; Manion and Hanson, 1980). Due to the fact that sea lamprey are semelparous and die shortly after spawning (Applegate, 1950), and that during migration and spawning periods sea lamprey do not eat and rely on nutrients stored during their parasitic phase for gonad maturation and spawning behaviors, the timing of reproduction in this species is especially crucial (Beamish, 1979; Madenjian et al., 2003).

In this species, mature (spermiated) males release an array of sex pheromones that influence the activity of mature (ovulated) females. This includes the pheromone 3-keto petromyzonol sulfate (3kPZS), which guides ovulated females upstream over long distances to nests (Li et al., 2002; Siefkes et al., 2005; Johnson et al., 2006; Johnson et al., 2009; Johnson et al., 2012a). Washings collected from spermiated males (SMW), which include 3kPZS and several other active compounds, have been shown to have similar effects as 3kPZS and also induce additional spawning behaviors (Siefkes et al., 2005; Johnson et al., 2006; Johnson et al., 2009; Johnson et al., 2014). In a preceding study (Dissertation Chapter 1; Walaszczyk et al., 2013), we identified the effects of SMW and maturity stage on locomotor activity by showing that SMW reduced total activity in pre-ovulated and ovulated females during the normally active

nighttime hours. Additionally, ovulated females, which are overall less active than pre-ovulated females, showed an increase in daytime activity and a disruption of their nocturnal activity, which led to overall arrhythmic locomotion (Dissertation Chapter 1; Walaszczyk et al., 2013).

What remains unclear from the preceding study are the specific chemical compounds in SMW that contribute to the interaction between maturation and locomotor pattern changes. We hypothesize that pre-ovulated and ovulated females have evolved to respond differently to specific signal components identified in SMW, which will be reflected in their locomotor responses. We speculate that this is due to a difference in life history, as pre-ovulated females must be guided long distances to appropriate spawning grounds, whereas ovulated females must coordinate their reproductive activity with spermiated males at the spawning grounds. To test this hypothesis, we measured diel locomotor activity as well as changes in locomotor pattern characteristics in the form of nocturnal activity percentages and the maximum hours of activity during the daytime and nighttime periods of adult female sea lamprey in controlled laboratory conditions during exposure to compounds found within SMW.

METHODS

Animals

Standard operating procedures for transporting, handling, maintaining, and euthanizing sea lamprey were approved by the Institutional Committee on Animal Use and Care of Michigan State University (AUF#02/13-041-00). Sea lamprey were captured from late April through mid-July of 2013 and 2014 in standard mechanical traps operated by the U.S. Fish and Wildlife Service and Department of Fisheries and Oceans, Canada, from several tributaries of Lakes Huron and Michigan. Sea lamprey were transported to the laboratory of the University Research Containment Facility of Michigan State University each year after collection.

Females and males were separated based upon the protocol described by Vladykov (1949) and held in chilled water (6-8°C) in large flow through tanks provided with air stones. All sea lamprey were held in laboratory conditions for less than 30 days prior to experimental trial acclimation. Maturity stages were separated by utilizing the protocol established by Siefkes et al. (2003). Ovulated females were classified as such if a defined keel was present and eggs were expressed by manual pressure. Spermiated males ($N = 20$) were used for washings collection and were identified as such based on the presence of a dorsal ridge and sperm expression when manual pressure was applied.

Experimental Condition and Treatments

Experiments were conducted at the University Research Containment Facility of Michigan State University, East Lansing, MI, U.S.A. Pre-ovulated (weight: Mean ± SE = 264.5 ± 7.1 g, $N = 24$; length: Mean ± SE = 50.5 ± 0.5 cm, $N = 24$)) and ovulated (weight: Mean ± SE = 229.9 ± 9.8 g, $N = 38$; length: Mean ± SE = 46.1 ± 0.6 cm, $N = 38$) females were acclimated together (4 or 8 females) for 6 days prior to each experiment in a large circular, aerated flow through tank (120.1 cm diameter; 50.2 cm water height). Animals were held individually in experimental tanks, which were identical to the acclimation tank, for the duration of trials. A PVC pipe (10 cm diameter, 35.5 cm long) at the bottom of the tank provided a refuge from the light. Tanks were adjusted to a constant temperature of 15°C and equipped with an aquarium air stone (25.4 cm, Penn-Plax, Hauppauge, NY, U.S.A.). Temperature was checked at least twice daily using a digital thermometer (Extech, South Burlington, VT, U.S.A.). Temperature loggers (Hobo Temperature Data Logger; Onset, Bourne, MA, U.S.A.) were placed in each tank during a subset of trials and recorded the temperature every 30 minutes to ensure the temperature remained constant. Each tank was held under a 14:10 h light-dark cycle (lights on 0600 h, lights

off 2000 h) using light timers and exposed to approximately 500 lux on average across the surface of the water of 6500K light during daytime hours and < 1 lux dim red light (740 nm) during nighttime hours. Modified trolling motors were used (Minn Kota Model Endura C2 Transom-Mount; Johnson's Outdoor Inc., Racine, WI, U.S.A.) to imitate slow moving stream conditions (Mean ± SE = 0.04 ± 0.01 m/sec). Flow rates were determined using a portable flow meter (Model 2000; Hach Marsh-McBirney, Loveland, CO, U.S.A). Trolling motors were turned on for 24 hours every 3 days in the acclimation tank and remained on for the experiment duration.

Pre-ovulated female trials were conducted between 3-June-13 and 26-June-13, whereas ovulated female trials were conducted between 18-July-13 and 27-August-13 or 14-July-14 and 3-Sep-14. These dates are representative of the sea lamprey adult life cycle and, therefore, when each maturity stage was available for experimentation. On the morning of the start of each experiment, female lamprey were placed individually in one of four randomly selected experimental tanks at 0800 h and allowed to acclimate for 4 hours. At 1200 h, the trial began and 24 hours of activity (baseline period) were recorded using the video-tracking program EthoVision XT with the Multiple Arena Module (Version 9; Noldus Information Technology, Asheville, NC, U.S.A.) coupled to four CCD cameras (Model EQ-610; EverFocus, Taipei, Taiwan). The system ran on a Dell Precision Intel based computer with digitizing board (Euresys H264, San Juan Capistrano, CA, U.S.A.) to enable simultaneous live tracking and digital video recording with the EthoVision software. After 24 hours, a treatment was introduced to each tank (either a final tank concentration of 10^{-10} M 3kPZS (Bridge Organics, Vicksburg, MI, U.S.A.), 10^{-10} M PZS (Bridge Organics, Vicksburg, MI, U.S.A.), SMW with a concentration 10^{-10} M 3kPZS, or an equivalent amount of methanol (MeOH, vehicle; Sigma-Aldrich, St. Louis, MO,

U.S.A.)). The treatment selected for each tank was randomized prior to each trial. SMW used during all experiments were collected by placing 20 spermiated males into a tank of 200 L of aerated water with no flow for 36 hours. 1 L water samples were collected and frozen at -20°C until used. Concentrations of natural 3kPZS in SMW were quantified using ultra-high-performance liquid chromatography-tandem mass spectrometry according to the protocol described by Li et al. (2011). At the start of the treatment day, a dose of the desired compound(s) was added to bring concentrations up to the appropriate levels. Solutions based on each tank's volume and flow velocity were made and pumped into each tank via reef dosing pumps (Model The Sentry; Innovative Aquatics, Cumming, GA, U.S.A.) to account for outflow and keep the treatment in the tank held at a constant concentration throughout the trial duration. An additional 24 hours of activity was then recorded and deemed the treatment period. At the end of each experiment, females were euthanized with 0.5% MS222 (Tricaine methanesulfonate; Sigma-Aldrich) and tissues were collected for future experiments.

Locomotor Activity Recording and Data Analysis

Using EthoVision XT 9, we tracked movement defined as the seconds the animal was active per hour in one hour bins across the 48 hour trial. Hour times represent the start of the one hour time bin. To account for individual variation and small sample sizes, differences between the movement time of the baseline control day and treatment day were used for all analyses. To determine if pheromone treatments influenced the diel pattern of locomotor activity, movement time data was separated by hour and determined to be either daytime (0600-2000 h) or nighttime (2000-0600 h). To account for unanticipated, artificial bursts of activity when the lights turned on and off, hours 0600 and 2000 were not included in the analyses. Occasionally an ovulated female, those of which are more fragile due to the fact that they are physically degrading as they

approach the final act of spawning, died during the trial. If this occurred, the trial was stopped and data was not included in the analysis. All statistical analyses were performed in SAS version 9.4 (SAS Institute Inc., 2013, Cary, NC, U.S.A.).

Effects of the pheromone compounds and maturity on movement were analyzed for each time period (daytime or nighttime) using repeated measures general linear models implemented within the PROC MIXED function of SAS. The fixed effects of treatment (MeOH control vs. SMW, vs. 3kPZS, and vs. PZS), maturity (pre-ovulated, POF vs. ovulated, OF), and their two-way interaction were fit to the model, with the random terms controlling for repeated measures within the individual number (ID) of each animal. Akaike (AIC) information criteria were used to select the best fitted model prior to examining the hypothesis tests. The autoregressive covariance structure was selected for the model, which has homogenous variances and correlations that decline exponentially with distance, due to the nature of the repeated data collected across time. Post-hoc comparisons were performed with the Dunnett test, which allows for comparisons between treatment groups and a control (reference) group, for each maturity stage (Zar, 1999) or with a Bonferroni test when comparing two treatment groups. To test that there were no tank effects for the pre-ovulated and ovulated female groups, we compared the baseline movement from each treatment group using the same model.

To investigate how pheromone compounds and maturity influenced the diel locomotor pattern, we examined changes in daily movement by calculating the differences in percent of nocturnal activity after exposure to each treatment. This was calculated by taking the amount of activity during the nighttime period, dividing it by the total amount of activity during the daytime and nighttime, and turning it into a percentage. We then calculated the differences of these percentages between the baseline and treatment days, comparing among different treatment

groups and maturity stages. In addition, we tested the effects of each of these factors on the maximum hours of movement during the daytime and nighttime. These were defined as the maximum movement during a one hour time period within the daytime or the nighttime. The corresponding maximum hour of activity differences were then calculated by taking the difference between the treatment period maximum hour of activity and the baseline period maximum hour of activity. We conducted two-way ANOVAs using PROC GLM of SAS to account for our unbalanced design for the fixed effects of treatment (MeOH control vs. SMW, vs. 3kPZS, and vs. PZS), maturity (pre-ovulated females, POF vs. ovulated females, OF), and their two-way interaction on nocturnal activity differences and each time period's maximum hour of activity. When significant ($\alpha < 0.05$), the Dunett post-hoc test was used to test for differences between treatment groups and the control for each maturity stage.

RESULTS

After transfer to the tanks, sea lamprey had transient bursts of activity, but settled down to normal behavior prior to the start of the trial. Temperatures remained constant during the experiments (temperature: Mean ± SE = 15.1 ± 0.01°C across the duration of testing of all tanks, $N = 7$ trials). There were no treatment effects during the first 24 hours of the experiment in which no treatment odor was administered, neither for the nighttime (pre-ovulated females: Figure 2.1, $F_{3,189} = 0.21$, $p = 0.89$; ovulated females: Figure 2.2, $F_{3,279} = 0.31$, $p = 0.82$) nor for the daytime (pre-ovulated females: Figure 2.1, $F_{3,109} = 0.77$, $p = 0.51$; ovulated females: Figure 2.2, $F_{3,82} = 1.17$, $p = 0.32$). These data were used as a baseline control day to make comparisons with the treatment day.

Nighttime Movement

Pre-ovulated and ovulated females both demonstrated a nocturnal locomotor pattern during the baseline day. There was a significant effect of the treatment by maturity interaction ($F_{3,143} = 3.53$, $p = 0.017$) on movement during the nighttime period (Figure 2.3B). During the nighttime, SMW reduced locomotor movement in pre-ovulated females compared to the methanol control (Dunnett post-hoc test: $t = -4.04$, $p = 0.00061$). Additionally, the pheromone compound 3kPZS produced a reduction in locomotor movement in this maturity stage (Dunnett post-hoc test: $t = -2.22$, $p = 0.028$), and had the same effect on movement as SMW (Bonferroni post hoc test: 3kPZS vs. SMW: $t = 1.79$, $p = 0.63$) There was no effect in pre-ovulated females exposed to PZS (Dunnett post-hoc test: $t = -1.41$, $p = 0.55$).

Interestingly, as opposed to pre-ovulated females, in ovulated females the pheromone compound PZS reduced the activity during the nighttime hours compared to the methanol control. (Figure 2.3B, Dunnett post-hoc test: $t = -3.11$, $p = 0.013$) There were no effects documented from exposure to 3kPZS (Dunnett post-hoc test: $t = -0.52$, $p = 0.99$) or SMW (Dunnett post-hoc test: $t = -0.68$, $p = 0.985$).

Daytime Movement

During the daytime hours, the treatment by maturity interaction ($F_{3,135} = 6.44$, $p = 0.00040$) affected movement (Figure 2.3A). While treatment did not affect daytime movement within the pre-ovulated females ($F_{3,144} = 2.22$, $p = 0.089$), treatment did affect ovulated female locomotor activity ($F_{3,128} = 5.41$, $p = 0.0015$). Locomotor movement increased during the daytime when ovulated females were exposed to SMW (Figure 2.3A, Dunnett post-hoc test: $t = 3.78$, $p = 0.00020$) compared to the methanol, ovulated female control. In contrast, 3kPZS (Dunnett post-hoc test: $t = 0.91$, $p = 0.93$) and PZS (Dunnett post-hoc test: $t = 0.28$, $p = 0.99$) had no effect during this time period.

Diel Movement

The treatment by maturity interaction affected the change in the percentage of nocturnal activity ($F_{3,52}$ = 3.97, p = 0.013). Further analysis revealed that in ovulated females, the percentage of nocturnal activity was reduced compared to the ovulated, methanol control (Figure 2.4A, Dunnett post-hoc test: t = -2.84, p = 0.03). Similarly, the daytime and nighttime maximum hours of activity were affected by the interaction between treatment and maturity stage ($F_{3,53}$ = 3.9, p = 0.013 and $F_{3,53}$ = 4.85, p = 0.0047, respectively). Ovulated females exposed to SMW showed an increase in movement during the daytime maximum hour of activity (Figure 2.4B, Dunnett post-hoc test: t = 3.95, p = 0.0016) and a reduction in movement during the nighttime maximum hour of activity (Figure 2.4C, Dunnett post-hoc test: t = -2.41, p = 0.019) compared to the ovulated, methanol control. There were no effects on the movement during the maximum hour of activity in pre-ovulated females for daytime (Dunnett post-hoc tests; 3kPZS: t = -0.57, p = 0.99; PZS: t = -0.13, p = 0.90; SMW: t = -1.31, p = 0.63) or nighttime (Dunnett post-hoc tests; 3kPZS: t = -1.4, p = 0.557; PZS: t = -1.3, p = 0.63; SMW: t = -2.26, p = 0.13). Other treatments had no effect on movement during the maximum hour of activity in ovulated females during the daytime (Dunnett post-hoc tests; 3kPZS: t = 2.26, p = 0.13; PZS: t = 1.79, p = 0.37) or nighttime (Dunnett post-hoc tests; 3kPZS: t = 0.21, p = 0.83; PZS: t = -2.35, p = 0.07).

DISCUSSION

We have identified two specific molecules released from spermiated male sea lamprey that have diverse effects on the locomotor activity of different stages of adult females. Our results show that the response to specific compounds of SMW is maturity dependent, which is reflected in the distinct responses during the nighttime of pre-ovulated females to 3kPZS and ovulated females to PZS. Additionally, we have confirmed our previous results (Dissertation

Chapter 1; Walaszczyk et al., 2013) that SMW has a differential effect on each maturity stage. While SMW reduces nighttime activity in pre-ovulated females, it does not have an effect on the percentage of nocturnal activity or the daytime movement maximum hour of activity, thus not affecting the overall diel pattern. Contrastingly, SMW contributes to the alteration of the diel pattern of locomotor activity in ovulated females, reducing the percentage of nocturnal activity and nighttime maximum hour of activity as well as increasing the daytime maximum hour of activity. This is consistent with our previous studies, in which ovulated females become arrhythmic in their locomotor activity when exposed to SMW in semi-natural, field conditions (Dissertation Chapter 1; Walaszczyk et al., 2013). Although these results are also congruent with previous literature in other species documenting that sex pheromones can affect locomotor activity amounts in insects (August, 1971; Rust and Bell, 1976; Hawkins, 1978; Pontes, 2014) as well as fish (Bjerselius et al., 1995; Amcoff et al., 2014), we have provided a rare example of how a specific, exogenous chemical cue can not only alter the amounts of activity, but also the overall pattern of locomotor activity in a vertebrate species.

In natural environments, adult female lamprey are exposed to a complex array of pheromone plumes, which change as females make their upstream migration and mature. The olfactory system of sea lamprey contains very sensitive and specific receptor mechanisms for both 3kPZS and PZS (Li et al., 1995; Li and Sorensen, 1997; Siefkes and Li, 2004). 3kPZS is released in high levels from spermiated males, whereas PZS in the environment comes from lamprey larvae (Haslewood, 1969; Sorensen et al., 2005) and from spermiated males (Brant et al., 2013). While PZS can also be derived from a larval population, the spawning males are often upstream of these populations. Therefore, when females get close to the nests, the influences of PZS are most likely from spermiated males. It is possible that 3kPZS is also released by sea

lamprey of other stages, such as larvae, although no report has documented this possibility yet. When making their upstream migration, pre-ovulated females are at a further distance from the spermiated males and exposed to more diluted pheromone concentrations than ovulated females. In this ethological context, it makes sense these pre-ovulated females would respond to 3kPZS, which is more highly concentrated than PZS in this environment. Ovulated females, however, being closer to the spawning grounds, would be exposed to more concentrated levels of PZS at the spawning grounds compared to downstream where it becomes diluted as the distance is increased away from the sources of the compound. We speculate that pre-ovulated and ovulated females have evolved responses to different chemical signals at different concentrations or in different ratios in regards to their biological locomotor activity and pattern. Our results provide evidence that PZS is a contributing factor to the documented ovulated female switch in activity from nocturnal to arrhythmic, which assists in coordinating the vital, limited timing of activity between mature males and females, increasing the chance for reproduction. PZS may contribute to a signal for ovulated females to stay or resample a specific area that represents spawning territory with appropriate mates, thus reducing the energy expenditure on locomotor activity to reserve energy for gonadal development and the act of reproduction.

We have additionally provided evidence that several compounds within SMW are interacting to modulate overall locomotor activity and patterns, as the reduction in both diurnal and nocturnal activity by SMW for pre-ovulated females and the increase in diurnal activity in ovulated females cannot be solely explained by 3kPZS or PZS. This is consistent with insect studies that have shown that most characterized pheromones are complex mixtures that only produce strong behavioral responses when all compounds are present or represented in relative proportions of different components (Linn et al., 1987). These results suggest that there are

additional effective compounds, and are concurrent with preceding studies on sea lamprey that document that more than one component of SMW is needed to trigger the entire array of mature female sexual behaviors (Siefkes et al., 2005; Johnson et al., 2009; Johnson et al., 2012a; Li et al., 2013). In these experiments, although 3kPZS elicits the same robust upstream movement to an odorant release point as SMW does, ovulated females show a preference for the full compound suite of SMW, spend more time in nests baited with SMW, and show more spawning behaviors (Johnson et al., 2012b). Potential candidates for additional, effective odor cues include alternative bile salt compounds released by spermiated males, such as 3 keto-1-ene PZS or 3,12-diketo-4,6-petromyzonene-24-sulfate (DKPES), which attract ovulated females (Li et al., 2013; Johnson et al., 2014; Brant, 2015).

Our results are supportive of previous field tests, in the fact that ovulated females exposed to SMW show increased activity during the day (Dissertation Chapter 1; Walaszczyk et al., 2013). In these previous studies, however, ovulated females exposed to SMW also had reduced activity during the nighttime hours, whereas the results presented here are not significant. One explanation is that previous studies were conducted in the field, which has different conditions compared to the laboratory setting. Behavior was also measured over several days as females matured and were exposed to a SMW or a control, whereas in the current study the duration of exposure is much shorter, allowing us to examine the short term, continuous, and high concentration effects of each compound. Longer treatment exposure may have revealed a gradual switch to an arrhythmic state. An additional explanation is that full effects are dependent on the mixture of compounds found within SMW. Each batch gathered from spermiated males has its own unique compound profile. To control for this, the SMW are standardized to an equal amount of 3kPZS, but not PZS. It is possible that the batch we used in the present study had a

lower concentration of PZS compared to that of previous studies and, therefore, did not produce a significant effect during the nighttime hours.

We have revealed the behavioral effects of individual pheromone compounds on locomotor activity, and while it has been demonstrated from a neurological standpoint how pheromones affect locomotion in the sea lamprey (Derjean et al., 2010), the mechanism in which pheromones interact with endogenous biological clocks that control rhythmicity remains to be elucidated. This could involve neuronal activation of the pineal complex, which is vital for maintaining the timing of locomotor movement (Morita et al., 1992; Binder and McDonald, 2007) in this species. This complex indirectly connects to the pheromone-sensitive olfactory bulb and projects to the dorsal mesencephalic tegmentum, which is an important motor region in this species (Yáñez, et al., 1993; Yáñez et al., 1999). Additionally, circadian locomotor activity could be regulated through influences of the molecular circadian clock transcriptional and translational feedback loops consisting of several clock genes found within vertebrates that are critical for adapting an organism's body to the external environment (Zhang and Kay, 2010). Future work should aim to investigate the roles of these systems in locomotor activity alterations via pheromone exposure.

This study has revealed two specific pheromone compounds released by spermiated male sea lamprey that produce different effects on locomotor activity and patterns in adult pre-ovulated and ovulated females. In addition to these two compounds, we have demonstrated that there are others that contribute to changes in locomotor activity yet to be revealed. These results provide a rare example of an olfactory stimulus that affects the interaction between the overall locomotor activity pattern and maturity state in a vertebrate species.

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APPENDIX

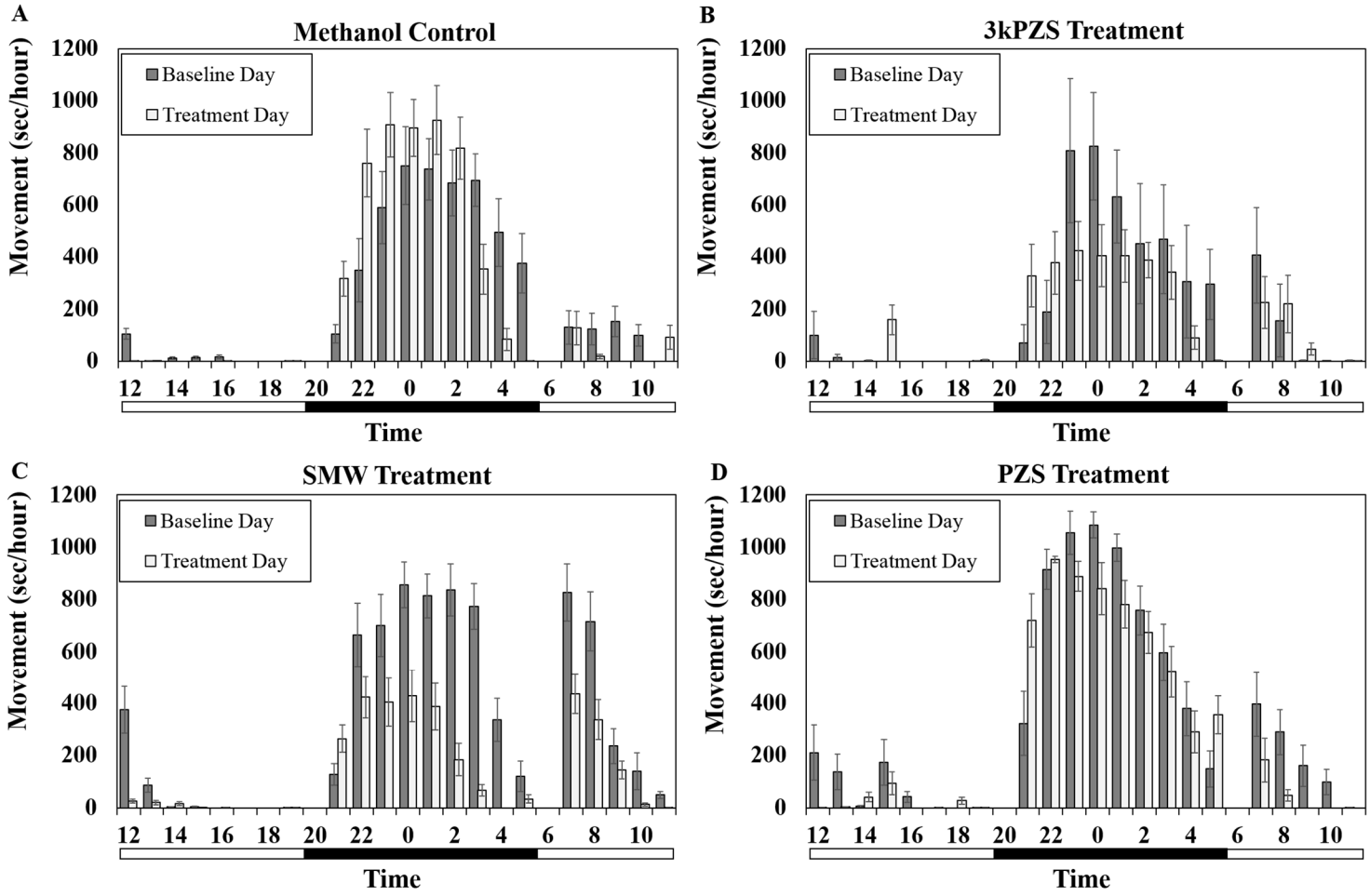

Figure 2.1: Effects of sex pheromone compounds on locomotor activity in pre-ovulated females. Activity is reduced after treatment of 3kPZS and SMW compared to the methanol control. Vertical bars denote 1 standard error. The bar below the graph is split into black for nighttime and white for daytime.

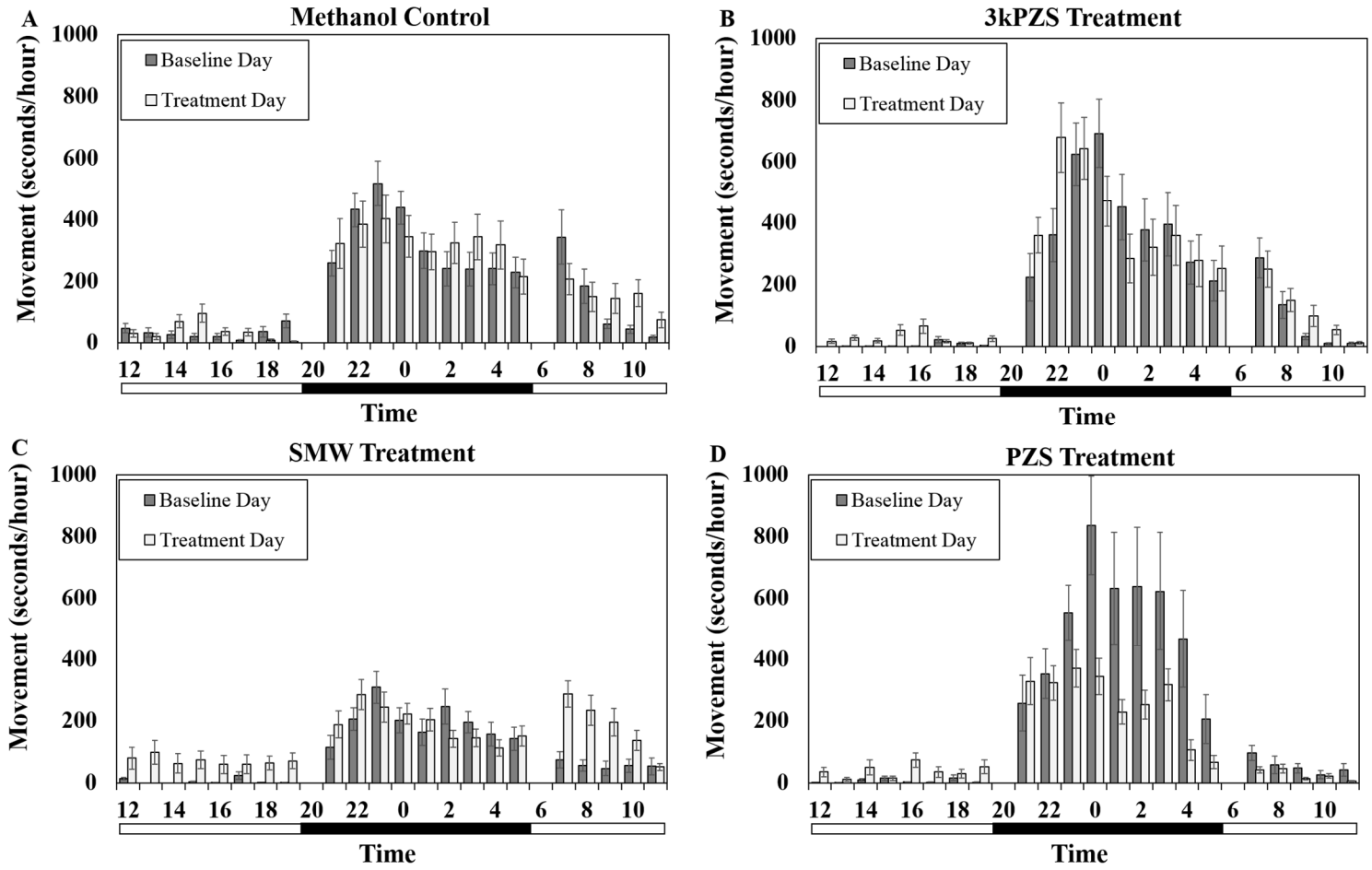

Figure 2.2: Effects of sex pheromone compounds on locomotor activity in ovulated females. Activity is reduced after treatment of PZS during the nighttime and increased after treatment of SMW during the daytime compared to the methanol control. Vertical bars denote 1 standard error. The bar below the graph is split into black for nighttime and white for daytime.

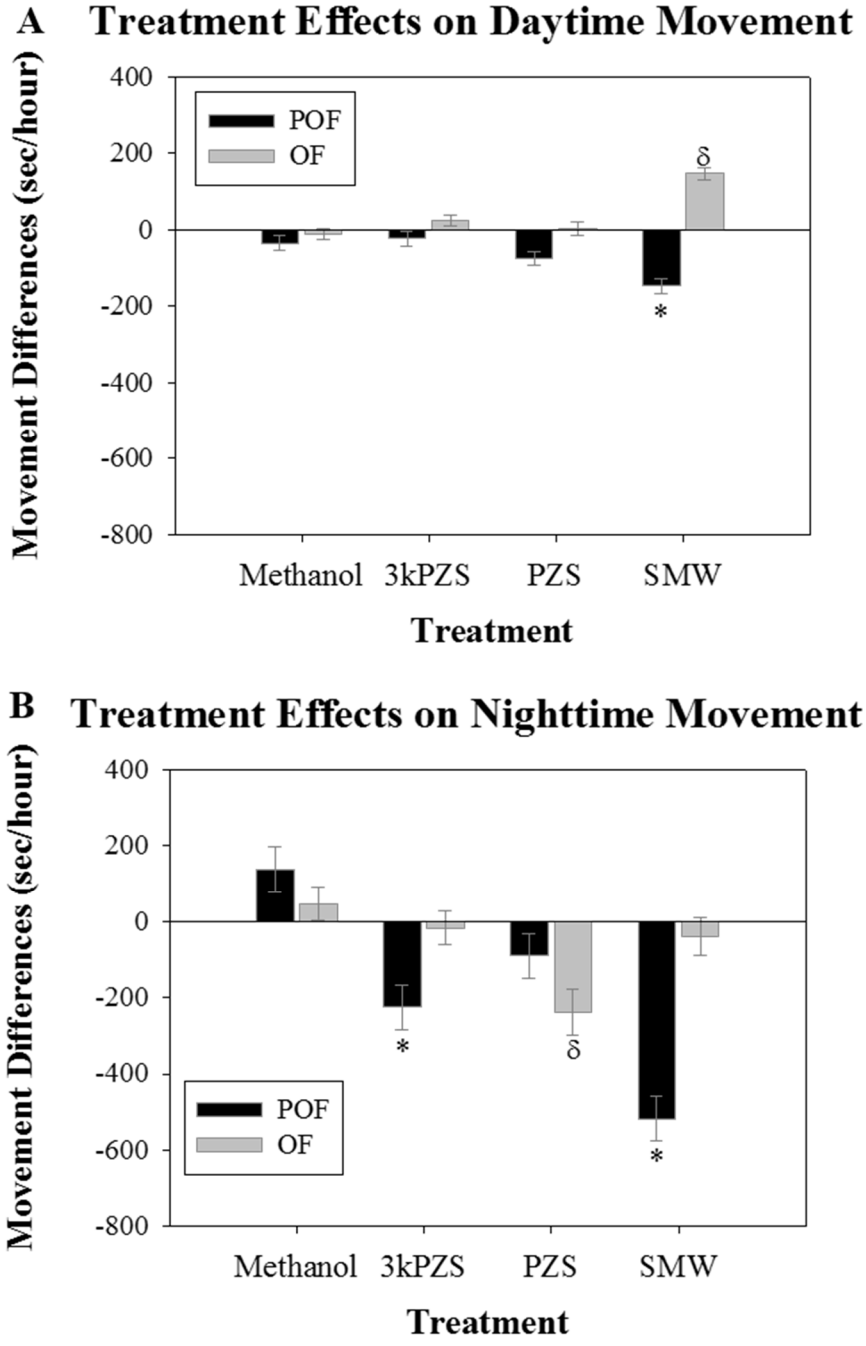

Figure 2.3: Effects of sex pheromone compounds on (A) daytime movement and (B) nighttime movement differences between the baseline and treatment days of adult female sea lamprey. POF denotes pre-ovulated females and OF denotes ovulated females. * represent groups that are significantly different than the pre-ovulated, methanol control. δ represent groups that are significantly different than the ovulated, methanol control. Vertical bars denote 1 standard error.

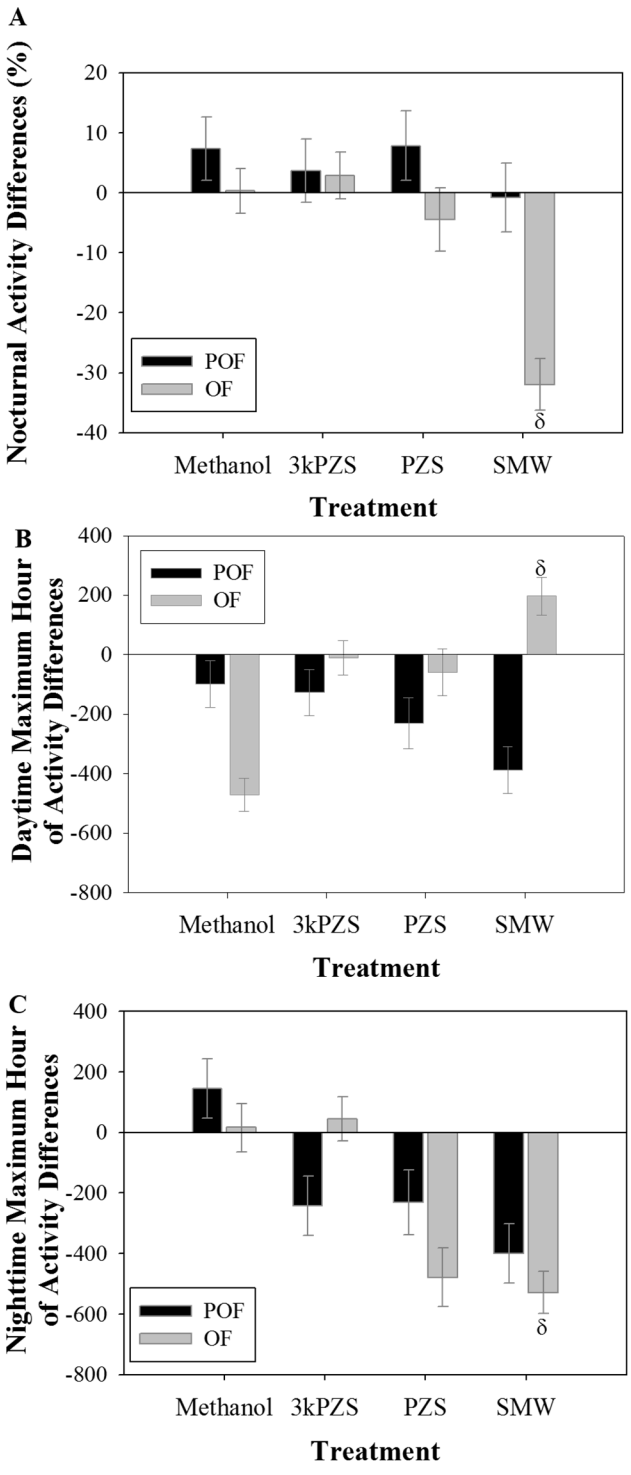

Figure 2.4: Effects of sex pheromone compounds on locomotor pattern characteristics of adult female sea lamprey. (A) The differences in percentage nocturnal activity between the baseline and treatment days. (B) The differences in movement during the daytime maximum hour of activity between the baseline and treatment days. (C) The differences in movement during the nighttime maximum hour of activity between the baseline and treatment days. POF denotes pre-

Figure 2.4 (cont'd)

ovulated females and OF denotes ovulated females. δ represents group that is significantly different than the ovulated, methanol control. Vertical bars denote 1 standard error. OF denotes ovulated females. δ represents group that is significantly different than the ovulated, methanol control. Vertical bars denote 1 standard error.

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CHAPTER 3

EFFECTS OF SEX PHEROMONES ON THE GABAERGIC SYSTEM IN THE BRAIN OF ADULT FEMALE SEA LAMPREY (PETROMYZON MARINUS)

ABSTRACT

Adult female sea lamprey (*Petromyzon* marinus) locomotor activity and patterns are affected by sex pheromone compounds released from mature males. In this species, gamma-aminobutyric acid (GABA) is a dominant inhibitory neurotransmitter in the brain and plays a role in the locomotion system. We hypothesize that GABA is involved in the neural circuits in the brain and pineal gland that modulate locomotor rhythmicity in response to sex pheromone compounds. In this species, the pineal gland is critical to maintaining locomotor activity rhythms. This structure receives inputs from the olfactory system and provides outputs to motor regions. To test this hypothesis, we examined the effects of washings from spermiated males (SMW) or individual sex pheromone compounds found within SMW on the GABAergic system of pre-ovulated females, including: (1) GABA concentrations in the forebrain and hindbrain using liquid chromatography-tandem mass spectrometry; (2) *Gabrb3*, *Gabra5*, and *Gabbr1* GABA receptor gene expression in the forebrain, the hindbrain, and the pineal complex using real-time quantitative PCR; and (3) $GABA_AR\beta3$ and $GABA_BR1$ GABA receptor protein expression in the forebrain, hindbrain, and pineal gland using immunocytochemistry techniques. GABA concentrations were increased in the forebrain in those females exposed to 3kPZS compared to the methanol control ($p < 0.05$). All sex pheromone treatments reduced *Gabrb3* and *Gabra5*$_5$ receptor expression in the forebrain and hindbrain, and *Gabbr1* expression was reduced in the forebrain ($p < 0.05$). Within the pineal complex, only SMW increased the transcript expression of the same receptors ($p < 0.05$). Exposure to all treatments examined affected the immunoreactivity of $GABA_A R\beta3$ and $GABA_BR1$ within several areas of the brain. Similar to the gene expression data, only SMW increased $GABA_AR\beta3$ immunoreactivity in the pineal gland. These results demonstrate that sex pheromone compounds affect the GABAergic system in pre-ovulated female sea lamprey.

INTRODUCTION

Circadian rhythms are physical, behavioral, or genetic changes that occur on an approximate 24 hour time scale and are used as a timekeeping system that allows an organism to participate and prepare for changes in the physical environment as well as control internal changes that take place in coordination with one another (Dunlap, 1999; Bell-Pedersen et al., 2005). The ability of an animal to synchronize its internal and external environments is critical to its well-being and survival. Circadian rhythms are endogenous, but can be influenced by external, environmental cues called zeitgebers (Aschoff et al., 1982; Wenderoth and Bock, 1999).

As sea lamprey, *Petromyzon marinus,* progress through their life cycle, their locomotor activity rhythm changes between nocturnal and arrhythmic multiple times (Kleerekoper et al., 1961; Sterba, 1962; Thomas, 1962; Manion and Smith, 1978; Moore and Mallatt, 1980). In this species, mature males release an array of sex pheromones that influences the activity of adult females. This includes the pheromone 3-keto petromyzonol sulfate (3kPZS), which guides ovulated (mature) females upstream over long distances to nests (Li et al., 2002; Siefkes et al., 2005; Johnson et al., 2006; Johnson et al., 2009; Johnson et al., 2012). Washings collected from spermiated males (SMW), which include 3kPZS and several other active compounds (Brant et al., 2013; Johnson et al., 2014; Brant, 2015), have been shown to have similar effects as 3kPZS, but also induce additional spawning behaviors (Siefkes et al., 2005; Johnson et al., 2006; Johnson et al., 2009; Johnson et al., 2014). In previous studies, we identified the effects of SMW and maturity stage on locomotor activity by showing that SMW reduced total activity in pre-ovulated (immature) and ovulated females during the normally active nighttime hours. Additionally, ovulated females, which are overall less active than pre-ovulated females, showed

an increase in daytime activity and a disruption of their nocturnal activity, which led to overall arrhythmic locomotion (Dissertation Chapter 1; Walaszczyk et al., 2013). Furthermore, we have demonstrated that different compounds found in SMW, 3kPZS and petromyzonol sulfate (PZS) can influence the locomotor activity of pre-ovulated and ovulated females, respectively (Dissertation Chapter 2). What effects these sex pheromones have on the internal mechanisms that modulate locomotor activity rhythms, however, remains unknown.

Gamma-aminobutyric acid (GABA) is one of the dominant neurotransmitters in the sea lamprey brain, and GABA is known to play a role in the regulation of locomotion in the sea lamprey (Nieuwenhuys and Nicholson, 1998; Menard et al., 2007). In the lamprey pineal complex, most projecting ganglion cells are GABAergic (Pombal et al., 1999; Menard et al., 2007). This complex, which comprises a dorsal pineal organ and a ventral parapineal organ (Hardisty, 1979), has been shown to be vital for maintaining circadian locomotor activity rhythms (Morita et al., 1992; Binder and McDonald 2007). The pineal complex receives inputs from the olfactory system (Polenova and Vesselkin, 1992), which is very well-developed and is highly sensitive to the male pheromone compounds (Li et al., 2002; Siefkes et al., 2005), and provides outputs to the motor region (Yáñez et al., 1993).

In addition, like in other vertebrates, the mesencephalic locomotor region in the brain of the lamprey plays a significant role in locomotor control, and projections from other brain regions in the diencephalon and telencephalon to this area have been identified as GABAergic (Menard et al., 2007; Derjean et al., 2010). Furthermore, experiments in semi-intact preparations of newly transformed and larval lamprey demonstrated that the GABA agonist Gabazine (antagonist of $GABA_A$ receptors) inhibited locomotion, whereas the GABA agonist muscimol (agonist of $GABA_A$ receptors) initiated locomotion (Menard et al., 2007). Given the vital roles of

the pineal complex and GABA for locomotion production and patterns in this species, we hypothesize that sex pheromone compounds affect the GABAergic system in the adult female sea lamprey brain and pineal gland. This study is the first to investigate GABA receptor protein expression and transcription levels of genes encoding for these proteins in a lamprey species as well as document pheromone effects on a neurotransmitter and its receptors in a vertebrate species.

The goal of this study was to elucidate the effects of sex pheromone compounds on GABA receptor expression and GABA concentrations in the brain and pineal complex of adult female sea lamprey. To do this, we investigated the effects of the entire suite of sex pheromones released from spermiated males into the water (SMW) as well as two specific sex pheromone compounds, 3-keto petromyzonol sulfate (3kPZS) and petromyzonol sulfate (PZS), on GABA concentrations in the forebrain and hindbrain using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Additionally, we measured transcriptional levels of genes that encode GABA receptors within the forebrain, hindbrain, and pineal gland of pre-ovulated adult female sea lamprey using real-time quantitative PCR (RT-QPCR) to understand the overall effects on these areas. Finally, we examined the effects of sex pheromone compounds on the protein expression of the same GABA receptors in the brain and pineal gland using immunocytochemistry (ICC) techniques to examine localized changes.

METHODS

Sea lamprey were captured in mechanical traps operated by the U.S. Fish and Wildlife Service and the Department of Fisheries and Oceans, Canada, in the St. Mary's River, which is an outflow of Lake Superior. Animals were transported to the University Research Containment Facility at Michigan State University, East Lansing, MI, U.S.A. The standard operating

procedures for transporting, maintaining, and handling were approved by the Institutional Committee on Animal Use and Care of Michigan State University (AUF#02/13-041-00). Males and females were separated based on the protocol conducted by Vladykov (1949). Spermiated males ($N = 20$) were identified based on the characteristic of a dorsal ridge and positive sperm expression when manual pressure was applied to the abdomen. These males were used to collect washings (SMW) treatments.

Experimental Conditions

Experiments were conducted at the University Research Containment Facility at Michigan State University as previously described (Dissertation Chapter 2). Briefly, adult pre-ovulated females were acclimated together for 6 days prior to each experiment in a large, circular flow through tank (120 cm diameter, 50 cm high) held at 15°C and equipped with an air stone (25.4 cm, Penn-Plax, Hauppauge, NY, U.S.A.). At the end of the acclimation period, females were randomly separated and placed into one of four experimental tanks that were identical to the acclimation tank size and environmental conditions. All tanks were exposed to a 14:10 h light-dark cycle (lights on: 0600 h, lights off: 2000 h). Tanks were exposed to approximately 500 lux (average across water surface of each tank) of 6500K light during the daytime hours and <1 lux of dim red light (740 nm) during the nighttime hours. A refuge from the light was provided at the bottom of each tank in the form of large PVC pipes (10 cm diameter, 35.5 cm long). Modified trolling motors (Minn Kota Model Endura C2 Transom-Mount; Johnson's Outdoor Inc., Racine, WI, U.S.A.) were placed in each tank to provide slow moving stream conditions (Mean ± SE = 0.04 ± 0.01 m/sec). These trolling motors were turned on every third and sixth day in the acclimation tank as well as kept on during the duration of experiments. Flow

rates were determined using a portable flow meter (Model 2000; Hach Marsh-McBirney, Loveland, CO, U.S.A.).

Experimental Design

Pre-ovulated female sea lamprey (N = 7 per group, N = 28 females per experiment) were randomly selected and transferred from the acclimation tank to experimental tanks at 0800 h the morning of each experiment. After a four hour acclimation period, each tank was randomly selected to receive one of four treatments (either a final tank concentration of 10^{-10} M 3kPZS (Bridge Organics, Vicksburg, MI, U.S.A.), 10^{-10} M PZS (Bridge Organics), SMW (with an equivalent amount of 10^{-10} M 3kPZS and average amount of methanol as other treatments), or an equivalent amount of methanol used as a vehicle control (MeOH, Sigma-Aldrich, St. Louis, MO, U.S.A.). SMW used for experiments were collected by placing 20 spermiated males into a no flow through tank of 200 L of aerated water for 36 hours. 1 L water samples were collected and stored at -20°C until use. Prior to each experimental year, natural 3kPZS concentrations in SMW were quantified using ultra-high-performance liquid chromatography-tandem mass spectrometry according to the protocol described by Li et al. (2011). To keep treatment levels constant, solutions were made based on each tank's volume and flow through velocity, and pumped into each tank during the duration of the experiment using reef dosing pumps (Model The Sentry; Innovative Aquatics, Cumming, GA, U.S.A.). Females were treated for 24 hours and then euthanized at 12:00 h the following day using 0.05% MS222 (Tricaine methanesulfonate; Sigma-Aldrich).

Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS) Analysis of GABA

28 pre-ovulated females were treated from 9-June-14 to 10-June-14. After euthanasia, forebrain and hindbrain samples were separated, removed, and snap frozen using liquid nitrogen,

and stored at -80°C. The pineal gland did not contain enough tissue for this type of analysis. Analyses for γ-Aminobutyric acid (GABA) followed the method developed by Gu et al. (2007) with minor modifications and were conducted in June 2014. GABA and GABA-d6 were purchased from Sigma-Aldrich. The transitions for additional compounds (internal standard) were optimized automatically and measurements were performed with Multiple Reaction Monitoring. The analysis method was validated and exceeded the minimum standards recommended in the Food and Drug Administration guidance. Briefly, each brain sample was weighed, and 1 mL of cold acetonitrile (1% formic acid) and 1 ng internal standard (GABA-d6) were added. Tissues were homogenized and incubated at -20°C for 15 minutes. The homogenized tissues were then centrifuged at 15,800 x g for 20 min. The supernatant was transferred to a new tube, freeze-dried overnight, and stored at -20ºC until analysis. Samples were reconstituted in 1 mL of water and placed in an autosampler for LC-MS/MS analysis. Treatment effects on the concentrations of GABA in the forebrain and hindbrain were analyzed by one-way ANOVA for each tissue followed by Bonferroni post-hoc tests if the ANOVA showed significant treatment differences ($p < 0.05$).

Real-Time Quantitative PCR (RTQ-PCR)

Samples for RTQ-PCR were collected from pre-ovulated females treated from 21-May-14 to 22-May-14. After euthanasia, forebrain, hindbrain, and pineal complex tissues were removed from the animal, separated, and immediately snap frozen using liquid nitrogen. Tissues were then transferred into -80°C until the time of processing. Frozen brains or pineal complexes were analyzed for transcripts of the genes encoding three GABA receptor subunits: $GABA_A$ receptor, subunit alpha-5 ($GABA_AR\alpha5$; gene= *Gabra5*), $GABA_A$ receptor, subunit beta-3 ($GABA_A R\beta3$; gene= *Gabrb3*), and $GABA_B$ receptor, 1 ($GABA_BR1$; gene= *Gabbr1*) using RTQ-

PCR during July 2014. Samples were also analyzed for the ribosomal subunit 60S, which served as our internal control and was confirmed to have no change in its expression level between treatments. Gene sequences were obtained from the sea lamprey genome as described previously (Smith et al., 2013). All primers and standards were designed using Primer Express software (Applied Biosystems, Foster City, CA, U.S.A.). The sequences for primers and standards (Sigma-Aldrich) for each mRNA are listed in Table 1.

RTQ-PCR was performed using the SYBR Green System (Applied Biosystems) and followed the procedure described by Chung-Davidson et al. (2008). Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.), treated with a TURBO DNA-free kit (Applied Biosystems), and then reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) and random hexamers (Promega, Madison, WI, U.S.A.). Synthetic oligos were used as standards and run on the sample plate. Reactions were analyzed on an ABI 7900 real-time PCR thermal cycler (MSU Genomics Technology Support Facility, MI, U.S.A.). RTQ-PCR data was analyzed by one-way ANOVA for each tissue followed by Bonferroni post-hoc tests if the ANOVA showed significant treatment differences ($p < 0.05$).

Immunocytochemistry (ICC)

Pre-ovulated females were treated for 24 hours from 26-May-15 to 27-May-15. The brain and pineal complex were dissected out and placed into 4% paraformaldehyde (in 0.1M phosphate buffer, pH 7.4) for two nights at 4°C and then transferred to 20% sucrose for cryoprotection. Tissues were then sectioned into 20 μm slices using a Leica CM1850 cryostat. Sections were mounted onto Superfrost Plus slides (Fisher Scientific, MA, U.S.A.) and immunostained for either $GABA_AR\beta3$ (1:300, AB5563: Millipore, Bellerica, MA, U.S.A.), $GABA_AR\alpha5$ (1:300,

AB9678: Millipore), or $GABA_BR1$ (1:300, AB2256: Millipore) proteins . Negative controls for immunocytochemistry (lacking primary antibody of interest) were performed simultaneously.

The general procedure for immunocytochemistry followed the protocol by Chung-Davidson et al. (2004). Briefly, sections were washed in Tris buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.2) 3 times (5 min each) in between each step. Sections were first reacted with 0.03% H_2O_2 (DAB substrate kit, Vector, Burlingame, CA, U.S.A) for 30 minutes to eliminate the endogenous peroxidase activity, incubated in the primary antibody (1:300 for all antibodies diluted in TBS with 0.05% Triton X-100) and normal goat serum (Vectastain ABC Elite Kit for rabbit IgG or Vectastain ABC Kit for guinea pig IgG, Vector) at 4°C for 48 hours, reacted with biotinylated secondary antibody (goat-anti-rabbit IgG, Vectastain ABC elite kit, or goat-anti-guinea pig IgG, Vectastain ABC kit, Vector) in TBS with 0.05% Triton- for 2 hours, and followed by ABC solution (Vectastain ABC Elite kit or Vectastain ABC kit, Vector) in 0.05% Triton X-100 for 2 hours. Sections were then reacted with 3,3′-diaminobenzidine and $NiCl_2$ (DAB substrate kit, Vector) for 13 minutes, counterstained with hematoxylin (Sigma-Aldrich) for 5 min, dehydrated through an ethanol series (70%, 95%, and 100%, 2 min each), clarified twice by xylene (5 min and 10 min), and finally covered with glass using DPX mounting media (Sigma-Aldrich). A Zeiss Axioskop II (Carl Zeiss, Thornwood, NY, U.S.A.) was used to visualize the stained sections and images were capture using a digital camera (Zeiss) and AxioVision Rel. 4.2 software (Carl Zeiss). Stained areas were identified according to Nieuwenhuys and Nicholson (1998).

RESULTS

Sex Pheromone Effects on GABA Levels in the Brain

In the forebrain, treatment affected the amounts of GABA (Figure 3.1, ANOVA, $F_{3,24}$ = 6.4, p = 0.002). Further analysis revealed that only females treated with the sex pheromone compound 3kPZS were affected and had an increase in GABA concentrations within the forebrain compared to the vehicle methanol control (Figure 3.1, Bonferonni test, 3kPZS vs. MeOH, p = 0.04). In the hindbrain, there were no differences between treatment groups in the levels of GABA (ANOVA, $F_{3,24}$ = 2.9, p = 0.06).

Sex Pheromone Effects on GABA Receptor Gene Expression

Sex pheromone compounds had the greatest effects on the expression of genes encoding for the $GABA_A$ receptor subunits examined. Within the pineal gland, treatment had an effect on the expression of *Gabra5* (ANOVA, $F_{3,24}$ = 4.9, p = 0.01) and *Gabrb3* (ANOVA, $F_{3,24}$ = 3.9, p = 0.02). The post hoc tests indicated that only the SMW treatment had an effect on the expression levels in the pineal gland, increasing them compared to the methanol control for both genes (Figure 3.2C, *Gabra5*: SMW vs. MeOH: p = 0.02; *Gabrb3*: SMW VS. MEOH: p = 0.03). In contrast, treatment exposure reduced expression of these genes in the forebrain (Figure 3.2A, *Gabra5*: ANOVA, $F_{3,24}$ = 11.04, p < 0.0001; *Gabrb3*: ANOVA, $F_{3,24}$ = 16.2, p < 0.0001), and the hindbrain (Figure 3.2B, *Gabra5*: ANOVA, $F_{3,24}$ = 6.6, p = 0.0017; *Gabrb3*: ANOVA, $F_{3,24}$ = 14.5, p = 0.01). Moreover, all three treatments (SMW, 3kPZS, and PZS) decreased the expression of *Gabra5* and *Gabrb3* in both the forebrain and the hindbrain (Bonferroni post hoc tests, p < 0.05 for each experimental treatment vs. MeOH).

In addition, within the forebrain, exposure to sex pheromone compounds also affected the transcript levels of *Gabbr1* (ANOVA, $F_{3,24}$ = 7.2, p = 0.001). Similarly to the other genes measured in the brain, all of the treatments examined reduced the expression levels of *Gabbr1* compared to the methanol control (Bonferroni post hoc test, p < 0.05) for all experimental

treatment comparisons with MeOH). This gene, however, was not affected by treatment in the pineal gland (*Gabbr1*: ANOVA, $F_{3,24} = 0.7$, $p = 0.6$) or the hindbrain (*Gabbr1*: ANOVA, $F_{3,24} = 0.4$, $p = 0.7$). There were no differences between treatment groups for our internal control, the ribosomal subunit 60S, in the forebrain (ANOVA, $F_{3,24} = 1.6$, $p = 0.2$), the hindbrain (ANOVA, $F_{3,24} = 2.4$, $p = 0.1$), or the pineal gland (ANOVA, $F_{3,24} = 0.9$, $p = 0.5$).

Sex Pheromone Effects on Receptor Protein Expression

Staining for $GABA_AR\beta3$ and $GABA_BR1$ proteins was seen across many areas of the brain. Most staining was diffuse across the neuropil areas and perikarya of neural cells, along with staining of some glial cells. Staining was more extensive in the hindbrain than in the forebrain. The antibody selected for $GABA_AR\alpha5$ did not produce positive staining in the tissues examined. Several areas of the brain and the pineal gland had differential staining between the vehicle control and all experimental treatment groups (Table 2, and Figure 3.3). Only females treated with SMW had changes in the expression of the $GABA_A R\beta3$ protein in the pineal gland, increasing expression compared to the other treatment groups (Figure 3.4).

DISCUSSION

In this study, we present evidence that sex pheromones affect the GABAergic system within the adult female sea lamprey by demonstrating that the olfactory exposure to sex pheromone compounds affects brain GABA levels, $GABA_A$ receptor and $GABA_B$ receptor gene expression, and immunoreactivity of $GABA_A$ receptor and $GABA_B$ receptor proteins. Our results show that in the brain all three experimental groups reduced receptor gene expression. In contrast, in the pineal complex, only spermiated male washings increased levels compared the methanol control. This is consistent with our previous behavioral studies, in which SMW, 3-keto petromyzonol sulfate (3kPZS), and petromyzonol sulfate (PZS) have differential effects on the

locomotor activity of pre-ovulated and ovulated females in a controlled laboratory setting (Dissertation Chapter 2).

This study is the first to investigate GABA receptor protein expression and transcription levels of genes encoding for these proteins in a lamprey species as well as show how a pheromone can alter a neurotransmitter and its receptors in a vertebrate species. We have demonstrated that the genes and proteins of both receptor subtypes, $GABA_A$ and $GABA_B$ receptors, are present in the brain and the pineal gland. Our results show that SMW increases the *Gabrb3* expression and $GABA_AR\beta3$ immunostaining in the pineal gland, as shown in Figure 3.3. The pineal organ of lower vertebrates registers environmental photic conditions and transduces them into neural and neuroendocrine signals, which are relayed to other parts of the brain (Dodt and Meissl, 1982; Underwood, 1989). In the sea lamprey, the pineal gland has been shown to be critical for locomotor rhythmicity (Morita et al., 1992; Tomotsu and Morita, 1986), and we hypothesize that this organ is responsible for modulating the locomotor activity rhythm of adult female sea lamprey in response to sex pheromones (Dissertation Chapter 1; Walaszczyk et al., 2013). The results presented here provide additional evidence to support this hypothesis.

The pineal tract makes connections with a specific pathway within the central nervous system of sea lamprey that is dedicated to producing motor responses from olfactory inputs. This pathway includes relaying signals from the medial olfactory bulb, to the posterior tuberculum, to the mesencephalic locomotor region (MLR), and finally to the reticulospinal cells in the hindbrain (Derjean et al., 2010). These reticulospinal neurons activate inhibitory and excitatory interneurons along the spinal cord (Grillner et al., 1998), affecting locomotion. The pineal has connections with the aforementioned pathway via the posterior tuberculum, a ventral diencephalic structure that conveys inputs to the MLR, which is an area known to play a

significant role in the control of locomotion (Puzdrowski and Northcutt, 1989). The pineal gland also has indirect connections to the MLR via the thalamus (Yáñez et al., 1993). The male sex pheromone compounds may be affecting the timing of locomotor activity via influencing the pineal gland and its connections with the pathway transducing olfactory stimuli to motor responses.

In the sea lamprey, many of the descending projections to the MLR from several regions in the telencephalon and diencephalon are GABAergic. In mammals, the GABAergic system is involved in locomotor modulation. In these animals, the hypothalamic suprachiasmatic nucleus (SCN) controls locomotor rhythms. It has been estimated that nearly all neurons within the SCN contain GABA (van den Pol, 1992). Furthermore, GABA in the SCN appears to mediate phase-shifting effects from nonphotic stimuli as well as modulate phase-shifting effects of light on activity rhythms (Gillespie et al., 1997; Mintz et al., 2002). For example, in golden hamsters, the GABA agonist muscimol, which is highly selective for $GABA_A$ receptors (Enna and Karbon, 1986) can induce permanent shifts in the timing of the onset of activity (Smith et al., 1989). In addition, in the diurnal rodent *A. niloticus,* $GABA_A$ receptor activation in the SCN induces large phase delays of the circadian wheel running rhythm during the subjective day (Novak and Albers, 2004). These results suggest a part played by GABA and its receptors in supporting the regulation and generation of circadian locomotor rhythms.

Our data suggest pheromone exposure affects the fast acting mechanisms of the GABAergic system in the pineal complex. $GABA_A$ and $GABA_B$ receptors use different mechanisms to respond to GABA. $GABA_A$ receptors are fast-acting ligand-gated ion channels, whereas $GABA_B$ receptors have a slower response to GABA and are G-protein coupled receptors (Barnard et al., 1998; Bowery et al., 2002). $GABA_A$ and $GABA_B$ receptors are hypothesized to

have distinct and complementary roles in regulating neural activity and locomotor activity production. In the lamprey, it has been shown that $GABA_A$ and $GABA_B$ receptor subtypes coexist on spinal axons and that GABAergic transmission is of importance in the generation of locomotor activity (Alford and Grillner, 1991; Alford et al., 1991). In these studies, fictive locomotion in the spinal cord is severely disrupted by simultaneous antagonism of both $GABA_A$ and $GABA_B$ receptors, whereas disrupting either one alone does not have as great of an effect (Alford et al., 1991). This suggests that both of these receptors act together to modulate the locomotor activity. Our data have revealed that sex pheromone compounds affect the $GABA_A$ receptor subunits examined within the pineal gland. As the pineal gland is vital for maintaining a locomotor rhythm in the sea lamprey, we speculate that $GABA_A$ receptors may be playing role in the timing of locomotor activity in this species.

Within the brain, we have demonstrated that both types of GABA receptors are affected by sex pheromones at the protein level. One prominent example was seen in the hindbrain region near the trigeminal motor nucleus. Our results demonstrate protein expression increases in $GABA_{A\beta3}$ and $GABA_{B1}$ in this area after exposure to any of the sex pheromone compounds examined (see Figure 3.4 for representative examples). In the lamprey, the trigeminal nerve has both motor and sensory components, as in other vertebrate species (Northcutt, 1979). The cells located within the trigeminal descending tract have projections to the reticular nuclei and have been suggested to be the sensory relays to the reticulospinal neurons, which are involved in activating and coordinating the spinal locomotor circuits in the lamprey (Di Prisco et al., 2005). This is one area that is affected by sex pheromone compounds, which could contribute to the overall locomotor output.

Our results also show that the inhibitory neurotransmitter GABA increases in the forebrain of immature adult females after exposure to the sex pheromone compound 3kPZS. This is congruent with previous studies showing that 3kPZS affects serotonin, a neurotransmitter that plays a role in the regulation of locomotion of sea lamprey (Viana et al., 1992), in the forebrain and hindbrain of adult female sea lamprey (Chung-Davidson et al., 2015). We hypothesize that the change in GABA that is seen in this study is due to an increase of GABA in the mesencephalic locomotor region, which is involved in locomotor production (For review see Jordan, 1998). Interestingly, while this specific compound produced effects, the full array of sex pheromone compounds found in SMW had no effect compared to the vehicle control. In previous studies, we documented that SMW affects the locomotor activity and the activity rhythm in pre-ovulated and ovulated adult female sea lamprey (Dissertation Chapter 1; Walaszczyk et al., 2013). One possibility, therefore, is that the amount of GABA in the brain does not contribute directly to the overall timing of locomotor activity changes revealed in these previous studies and that changes occur at the receptor level in particular regions of the brain that contribute to locomotor activity. Another possibility is that there are changes in GABA levels within the pineal gland. This possibility needs to be confirmed by directly measuring GABA levels in this organ. In this study, we only examined one time point and the effects could be revealed at other times, especially given that adult female lamprey are nocturnal and the most dramatic effects on locomotor activity via sex pheromones occur during the nighttime hours. Future studies should focus on elucidating the effects of sex pheromone compounds on GABA and its corresponding receptor levels in the brain and pineal gland across multiple time points.

In conclusion, we have provided evidence that sex pheromone compounds affect the GABAergic system within the brain and the pineal gland of the adult female sea lamprey. While

all the treatments examined had effects on receptors in different regions of the brain, within the pineal gland only the full suite of compounds (SMW) increased the gene expression of the $GABA_A$ receptor subunits measured and on the corresponding protein immunoreactivity of $GABA_AR\beta3$ in the pineal gland. We speculate that these effects are related to the locomotor activity in this species, as our previous studies document that SMW, rather than individual compounds, affected the overall locomotor rhythm. It is possible that the pineal gland plays a role in modulating these activity rhythms via GABAergic connections to the motor system in adult female sea lamprey, and future studies are needed to confirm this idea.

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APPENDIX

Table 3.1: Sequences of designed primers and standards used for RT-QPCR. Primer sequences listed in the standard 5' to 3' direction.

Gene	5' Primer	3' Primer	Standard
Gabra5	TGGAAAAACGGCACCAACTACT	CGTGTAGTGCATCAGCTGGTACT	TGGAAAAACGGCACCAACTACTCGGTGGAAGTTGCTGAGGACGGCTCGCGCACCAACCAGTACCAGCTGATGCACTACACG
Gabrb3	GGAAAAACGGCACCAACTAC	CACGCGCGACCACTTGT	CCTCGCAGCTCAAGATCAAGATTCCGGATCTGAGCAACGTCAACGCCATCGACAAGTGGTCGCGCGTG
Gabbr1	CTCGCAGCTCAAGATCAAG	ATGTATCTGACGTTCCGGTTGTAG	CCTCGCAGCTCAAGATCAAGATTCCGGATCTGAGCAACGTCAACGCCATCGACAAGTGGTCGCGCGTG
60S	CGCATCCGCGCAATG	GTCGGGTATGTCCACGATCTG	CGCATCCGCGCAATGAAGACCATCCAGAGCAATCAGATCGTGGACATACCCGAC

Table 3.2: Summary of brain areas in which immunoreactive protein staining for $GABA_AR\beta3$ and $GABA_BR1$ receptors differed between sex pheromone treatment groups in the adult pre-ovulated female brain. Treatments were either washings (SMW with equivalent amount of 10^{-10} M 3kPZS), 10^{-10} M 3kPZS, 10^{-10} M PZS, or the vehicle control (MeOH) for 24 h. + represents positively stained cells and - represents lack of stained cells in each corresponding brain area. MeOH= methanol control; SMW= spermiated male washings.

	$GABA_AR\beta3$				**$GABA_B$ R1**			
Brain Region	MeOH	SMW	3kPZS	PZS	MeOH	SMW	3kPZS	PZS
Pineal	-	+	-	-	-	-	-	-
Habenular Region	-	-	-	-	+	-	+	+
Lateral Pallium	+	-	-	-	-	-	-	-
Medial Pallium	+	-	-	-	+	-	-	-
Hypothalamic Region	+	-	-	-	+	-	-	+
Trigeminal Motor Nucleus	-	+	+	+	-	+	+	+
Ependymal Cells Around 4th Ventricle	-	+	+	+	-	+	+	+

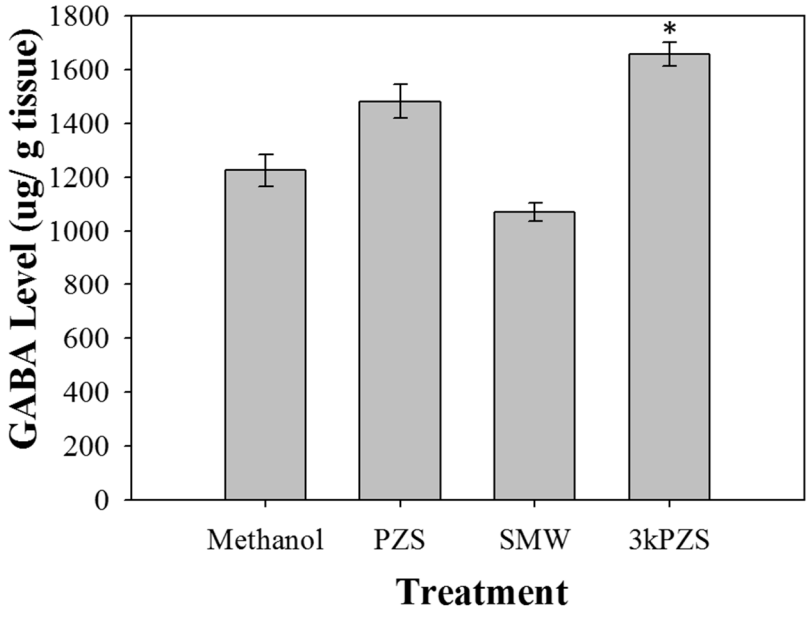

Figure 3.1: Effects of sex pheromone compounds on the inhibitory neurotransmitter GABA in the forebrain of pre-ovulated females after 24 h. 3kPZS significantly increases GABA levels compared to the methanol control. The asterisk represents the group that is significantly different than the, methanol control. Vertical bars denote 1 standard error.

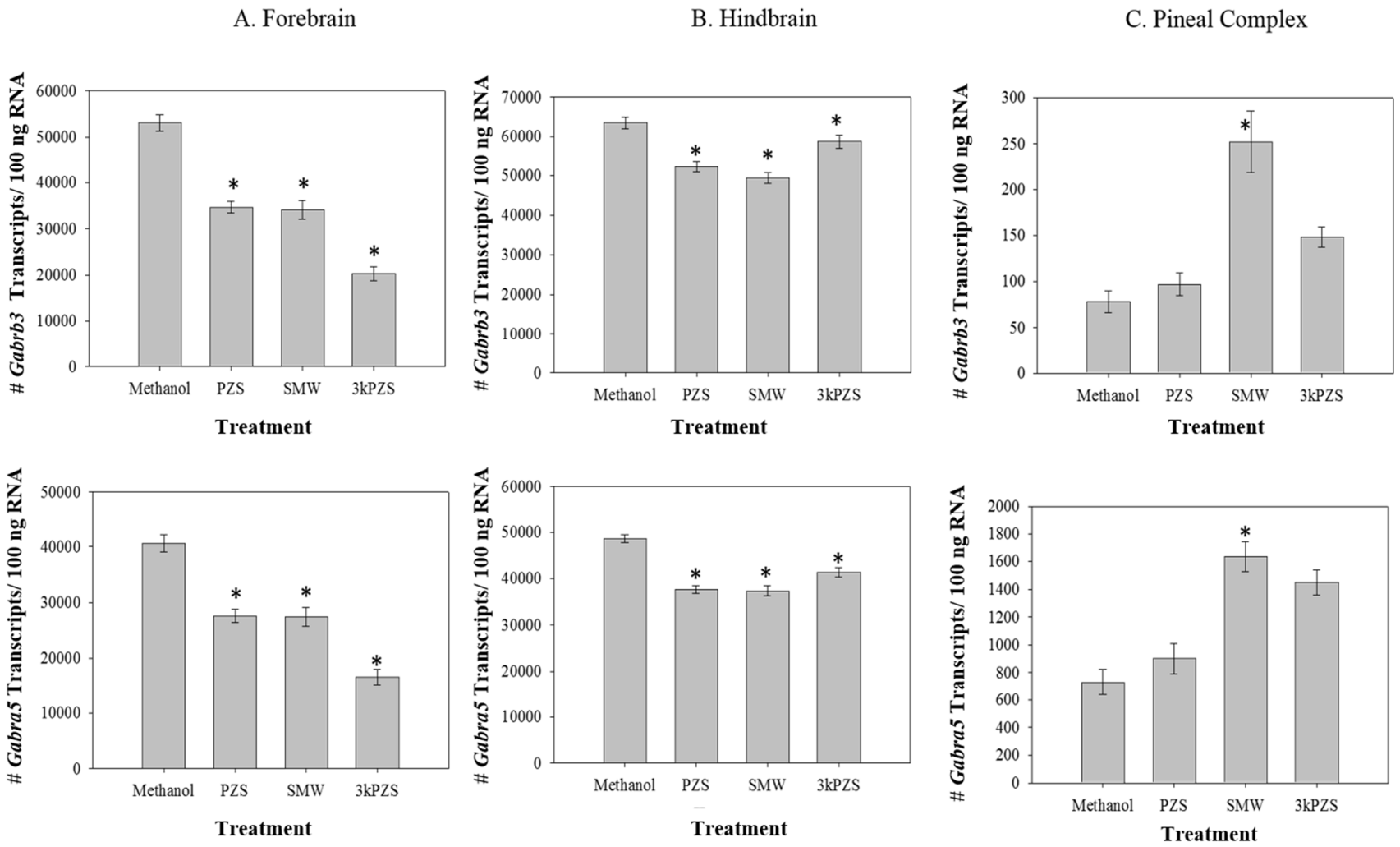

Figure 3.2: Effects on $GABA_A$ receptor subunit gene expression after 24 h exposure to sex pheromone compounds. (A) PZS, SMW, and 3kPZS reduce *Gabrb3* and *Gabra5* expression in the forebrain compared to the methanol control. (B) As in the forebrain, PZS, SMW, and 3kPZS reduce *Gabrb3* and *Gabra5* expression in the hindbrain compared to the methanol control. (C) Only the SMW

Figure 3.2 (cont'd)

treatment has an effect on *Gabrb3* and *Gabra5* expression in the pineal complex, increasing it compared to the methanol control. SMW= spermiated male washings. Asterisks denote that treatment is significantly different ($p < 0.05$) from methanol control. Vertical bars = one standard error.

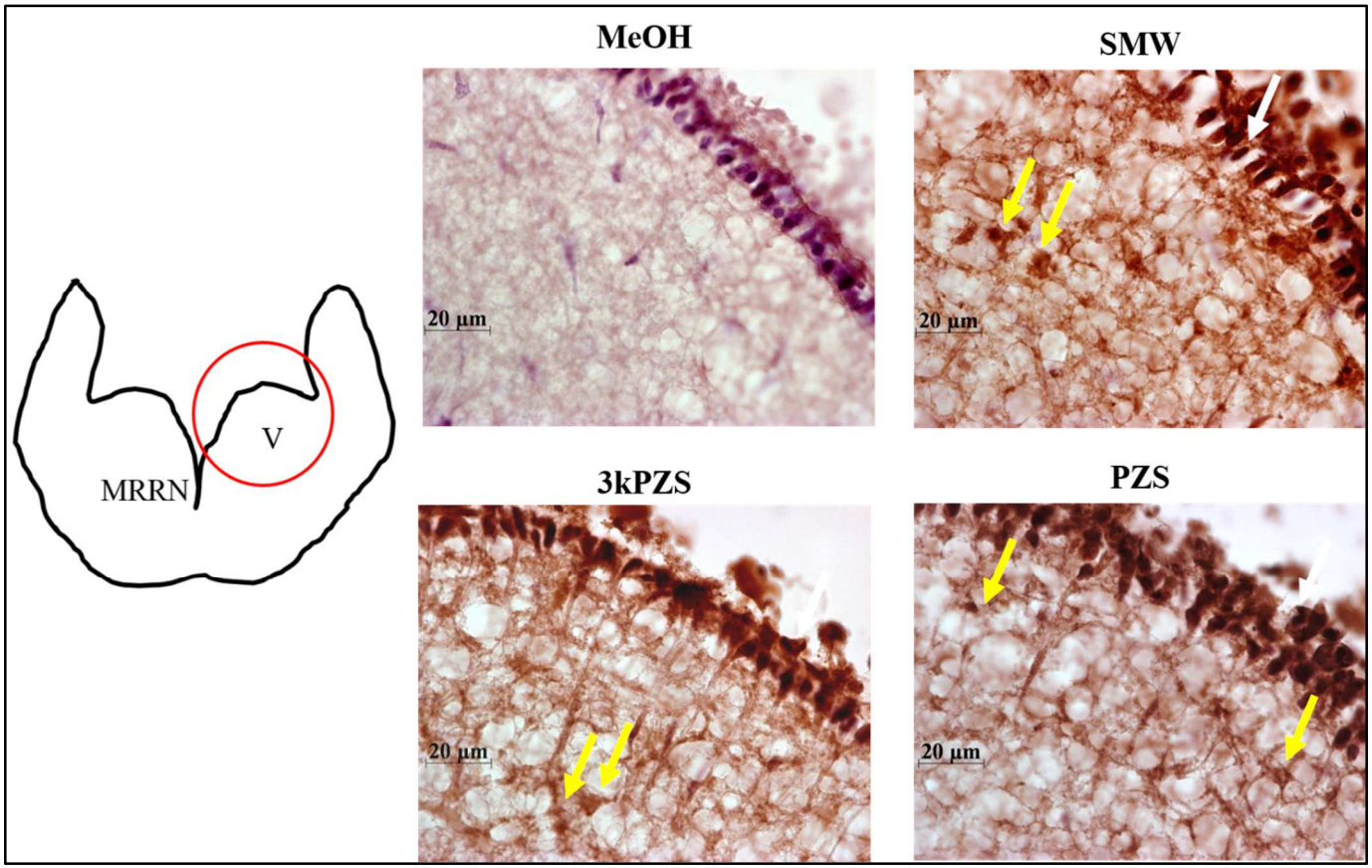

Figure 3.3: Representative example of increased immunoreactivity of $GABA_BR1$ protein in the hindbrain region near the trigeminal motor nucleus after a 24 h treatment of spermiated male washings (SMW with equivalent amount of 10^{-10} M 3kPZS), 3kPZS (10^{-10} M), or PZS (10^{-10} M). Schematic to left represents brain area seen in images. Yellow arrows represent examples of staining seen in trigeminal motor nucleus area. White arrows represent examples of staining seen in cells lining the 4th ventricle. V= area of trigeminal motor nucleus. MRRN= area of middle rhombencephalic reticular nucleus. MeOH= methanol control. Pictures taken at 100X.

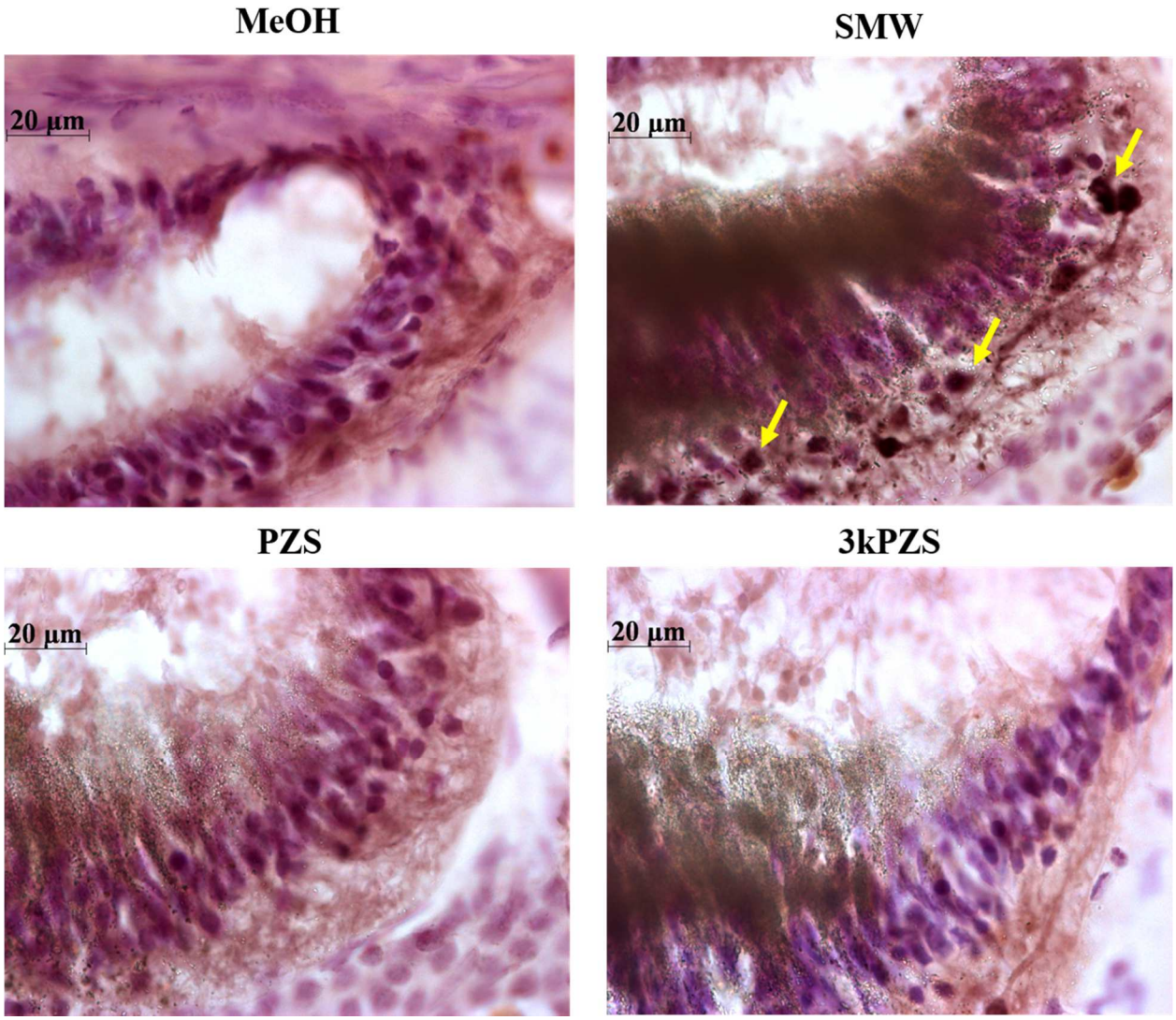

Figure 3.4: Representative example of increased immunoreactivity of $GABA_AR\beta3$ protein in the pineal gland after a 24 h treatment of spermiated male washings (SMW). Yellow arrows represent examples of immunoreactive cells within the pineal gland. Images taken at 100X. MeOH= Methanol Control.

SUPPLEMENTAL INFORMATION

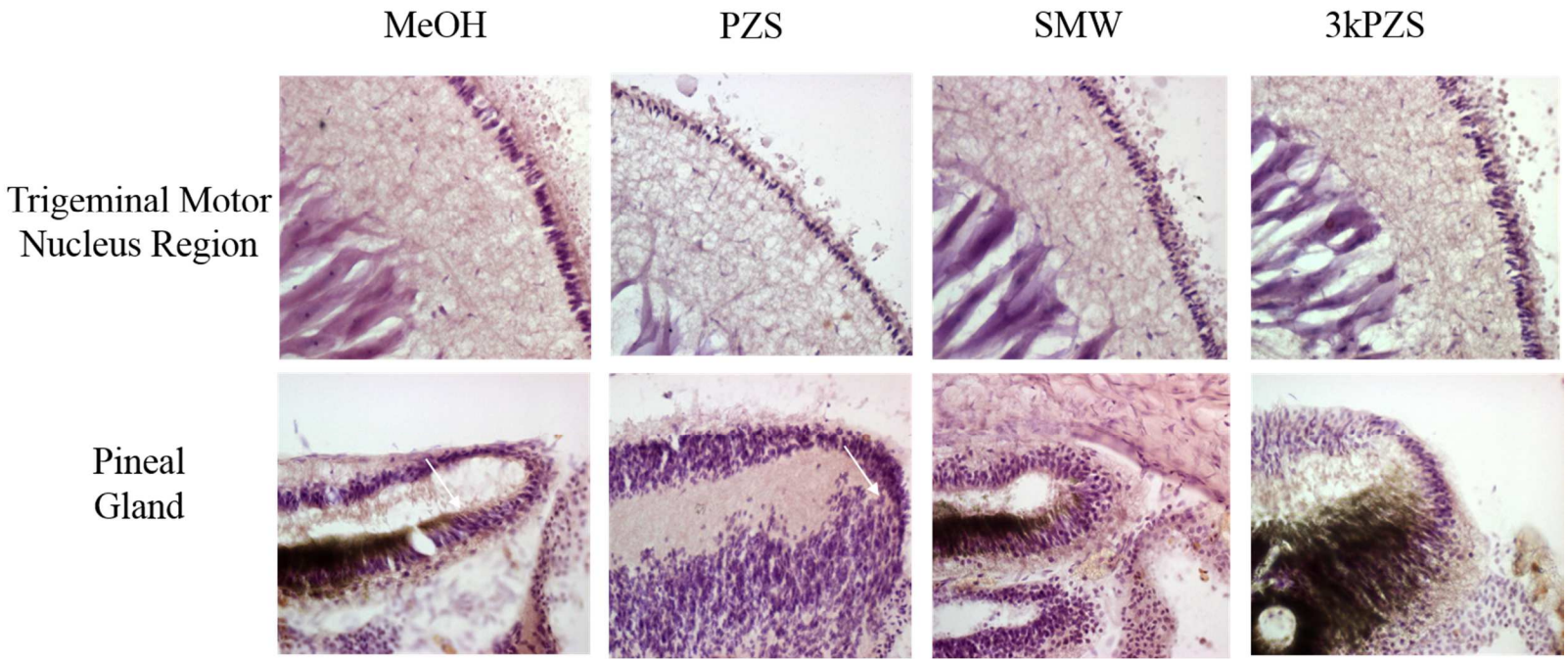

Figure S3.1: Representative normal goat serum controls for the hindbrain region near the trigeminal motor nucleus and the pineal gland of pre-ovulated females treated for 24 h with spermiated male washings (SMW containing equivalent amount of 10^{-10} M 3kPZS), 10^{-10} M 3kPZS, 10^{-10} M PZS, or the vehicle methanol control (MeOH) at 40x magnification. Slides treated with DAB and additional nickel. Cell were counterstained with hematoxylin (purple).

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CHAPTER 4

EFFECTS OF SEX PHEROMONES ON EXPRESSION OF CLOCK GENES IN THE BRAIN AND PINEAL GLAND OF ADULT FEMALE SEA LAMPREY (PETROMYZON MARINUS)

ABSTRACT

As adult female sea lamprey undergo sexual maturation (ovulation) and are exposed to sex pheromone compounds released by mature males, their activity pattern switches from nocturnal to arrhythmic. Because the pineal complex regulates locomotor rhythmicity and sexual maturation in lamprey, we speculate that this organ interacts with pheromone detection and motor regions of the brain to govern locomotion patterns. On a molecular level, the endogenous circadian clocks responsible for diel rhythm production are controlled via autoregulatory transcriptional-translational feedback loops that involve several clock genes (*Clock, Bmal1, Cry,* and *Per*) and their protein products. Here, we have provided the first demonstration of these genes and proteins in this species as well as examined the effects of sex pheromone compounds on the expression levels. To do this we measured clock gene mRNA expression levels using real-time quantitative PCR in pre-ovulated female forebrains, hindbrains, and pineal complexes after exposure to 24 h treatments of individual sex pheromone compounds or the multiple compounds found in spermiated male washings (SMW). We also examined these proteins in the brain and pineal complex using immunocytochemistry techniques after the same treatments. Our results showed that all sex pheromone treatments examined decrease the activity of all four clock gene expression levels within the forebrain ($p < 0.05$), whereas in the pineal complex only the full suite of compounds found in SMW increases only the gene expression of *Bmal1*. BMAL1 and CRY protein staining was also decreased in forebrains areas, and BMAL1 immunofluorescence was increased in the pineal complex. These results demonstrate a unique effect of a conspecific stimulus on the circadian clock system within a vertebrate species.

INTRODUCTION

Almost all organisms display circadian rhythms in various aspects of their behavioral, physiological, and biochemical processes (Dunlap, 1999; Bell-Pedersen et al., 2005). In animals, circadian rhythms have an approximate period of 24 hours and are controlled by biological clocks within the central nervous system as well as peripheral tissues (Brown and Schibler, 1999). The endogenous circadian system, or clock, acts as a timekeeping system that allows the organism to anticipate and prepare for changes in the physical environment and coordinate these changes with physiological and behavioral cycles (Meisel et al., 2003). This synchronization of internal and external environments is vital to the organism's well-being and survival, and can improve the fitness of organisms in constant as well as changing environments (Paranjpe and Sharma, 2005). Circadian clocks are endogenous and maintain rhythmic output in the absence of input signals, but can be reset by or entrained to external time cues (called zeitgebers; Aschoff et al., 1982; Wenderoth and Bock, 1999), such as light-dark (LD) cycles. The influence of these photic cues have been shown to act as one of the strongest zeitgebers for biological rhythms and have been studied extensively, but there are other non-photic cues that contribute to the entrainment of the circadian system and require further research.

On a molecular level, endogenous circadian clocks are controlled via autoregulatory transcriptional-translational feedback loops that involve several clock genes and their protein products, which have been well preserved through evolution (Vitaterna et al., 1994; Vatine et al., 2011). Although clock genes have been conserved from flies to humans, many vertebrates have evolved to have several homologs of each gene, and the functional roles of each gene can differ (Reppert and Weaver, 2001; Young and Kay, 2001; Okamura et al., 2002). Like in mammals, in fish the core loop consists of a positive complex formed by the CLOCK-BMAL1 (CLOCK:

Circadian locomotor output cycles kaput; BMAL1: Brain and muscle aryl hydrocarbon receptor nuclear translocator- like) heterodimer, which triggers the transcription of the negative elements of the clock, the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. In turn, their protein complex CRY-PER inhibits the transcriptional activity of CLOCK-BMAL1 heterodimers, thus inhibiting their own gene regulation (Zhang and Kay, 2010). Within this feedback loop, there is a daily oscillation in the levels of clock gene RNA and proteins. In mammals, the master circadian pacemaker for controlling behavioral rhythms is located in the suprachiasmatic nuclei of the anterior hypothalamus (SCN; Herbert, 1994; Reppert and Weaver, 2002). This master circadian pacemaker produces a rhythmic oscillation of approximately 24 hours and can be entrained by external cues, synchronizing circadian time to the environment. Master circadian pacemakers synchronize the timing of peripheral oscillators scattered throughout the body that regulate local rhythms in physiology and behavior via neural and hormonal signals (Bartness et al., 2001; Balsalobre, 2002). Timekeeping in fish is much less understood, as the existence of a master circadian pacemaker has not been proven, although key components of the internal circadian system have been identified in the brain and pineal gland of several species, including salmon, zebrafish, and the golden rabbitfish (Park et al., 2007; Davie et al., 2009; Sanchez and Sanchez-Vazquez, 2009).

As sea lamprey, *Petromyzon marinus,* progress through the several stages of their life cycle, their locomotor activity changes between a nocturnal rhythm and arrhythmicity multiple times (Kleerekoper et al., 1961; Sterba, 1962; Thomas, 1962; Hardisty and Potter 1971a, 1971b; Manion and Smith, 1978; Moore and Mallatt, 1980). In this species the light-sensitive pineal complex has been shown to regulate activity rhythms, as its removal abolishes locomotor rhythmicity (Tomotsu and Morita, 1986; Morita et al., 1992). Because these rhythms in

locomotor activity persist even under constant conditions (Morita et al., 1992), it appears to be regulated by an internal circadian oscillator. Exogenous factors, including temperature, have been shown to have an effect on these rhythms (Binder and McDonald, 2007), and in previous studies, we have demonstrated that olfactory cues in the form of sex pheromone compounds released from spermiated males affect the locomotor activity and patterns of pre-ovulated and ovulated adult females (Dissertation Chapter 1; Walaszczyk et al, 2013). Pheromones play a crucial role in communication among individuals of a species. They are released by an individual (sender) and received by conspecifics (receivers). For sea lamprey, pheromones are a vital part of successful reproduction (Buchinger et al., 2015). From our previous studies, we know that pheromones can alter the locomotor activity pattern. To date, however, what role sex pheromone compounds have as exogenous cues for the endogenous timekeeping system that influences locomotor activity rhythms remains unknown in vertebrates.

Information regarding the expression of the genes and protein products involved in circadian timing is lacking in the sea lamprey and other extant jawless fish species. Clock mechanisms in lamprey are of interest from a comparative standpoint and given their place on an evolutionary timescale. Additionally, understanding of the circadian system offers an opportunity to elucidate the cellular and molecular events that connect genes to behavior. Based on our previous studies showing that sex pheromone compounds can affect the locomotor activity amounts and timing pre-ovulated and ovulated females (Dissertation Chapters 1 and 2; Walaszczyk et al., 2013), and as a first step towards understanding the roles of the endogenous timekeeping system in the timing of locomotor activity in this species, we test the hypothesis that sex pheromones released from mature males influences the circadian clock genes and proteins controlling circadian rhythmicity in the brain and pineal gland of adult females.

The aims of this study were to provide the first examination of clock genes and proteins in the brain and pineal gland of adult female sea lamprey, and to elucidate the effects of olfactory exposure to sex pheromone compounds on these endogenous components. We investigated the effects of two specific sex pheromone compounds, 3-keto petromyzonol sulfate (3kPZS) and petromyzonol sulfate (PZS), as well as the entire suite of sex pheromones released from spermiated males via washings (spermiated male washings; SMW), on the transcriptional levels of four genes involved in the transcriptional clock loop (*Clock*, *Bmal1*, *Per1*, and *Cry*) as well as the proteins BMAL1 and CRY within the adult pre-ovulated female brain and pineal gland using real-time quantitative PCR (RT-QPCR) and immunocytochemistry (ICC) techniques. These genes were selected because they are known to be the major components of the molecular circadian clockwork (Reppert and Weaver, 2001, 2002). Here, we present the first study that documents the expression of these genes in the sea lamprey as well as provides a unique example of how a sex pheromone can have an effect on the circadian clock transcriptional-translational feedback loops within a vertebrate species.

METHODS

Animals

Sea lamprey were captured in mechanical traps by the U.S. Fish and Wildlife service and the Department of Fisheries and Oceans, Canada in the St. Mary's River, an outflow of Lake Superior. The Institutional Committee on Animal Use and Care of Michigan State University approved the standard operating procedures for transporting, maintaining, handling, and euthanizing sea lamprey (AUF#02/13-041-00). The protocol conducted by Vladykov (1949) was used to separate females from males. Spermiated males were used to collect washings (SMW)

and were identified as such based on the animal having a dorsal ridge and expressing sperm when manual pressure was applied to the abdomen.

Experimental Design

Treatment experiments were conducted at the University Research Containment Facility at Michigan State University as previously described (Dissertation Chapter 2). Adult pre-ovulated females were acclimated together for 6 days prior to experiments in a large, circular flow through tank (120 cm diameter, 50 cm high) equipped with an air stone (25.4 cm, Penn-Plax, Hauppauge, NY, U.S.A.). At the end of the acclimation period, females were separated into four equal groups and placed into four identical experimental tanks. Both experimental tanks and the acclimation tanks had identical dimensions and conditions. Tank water was adjusted to a constant temperature of 15°C and held under a 14:10 h light-dark cycle (lights on 0600 h, lights off 2000 h) using light timers. Each tank was exposed to 500 lux on average across the surface of the water of 6500K light during the daytime hours and <1 lux dim red light (740 nm) during nighttime hours. PVC pipes (10 cm diameter, 35.5 cm long) were placed at the bottom of each tank to serve as a light refuge. Modified trolling motors (Minn Kota Model Endura C2 Transom-Mount; Johnson's Outdoor Inc., Racine, WI, U.S.A.) were used to imitate slow moving stream conditions (Mean ± SE = 0.04 ± 0.01 m/sec). Flow rates were determined using a portable flow meter (Model 2000; Hach Marsh-McBirney, Loveland, CO, U.S.A.). Trolling motors were turned on for 24 h for the third and sixth day of acclimation and remained on for the duration of the experiment.

On the morning of each experiment, pre-ovulated females were placed in groups into tanks (N = 7 per tank, N = 28 total animals for each experiment) at 0800 h and allowed to acclimate for four hours. At 1200 h, one of four treatments was introduced to each tank (either a

final tank concentration of 10^{-10} M 3kPZS (Bridge Organics, Vicksburg, MI, U.S.A.), 10^{-10} M PZS (Bridge Organics), SMW with an equivalent amount of 10^{-10} M 3kPZS and average amount of methanol as other treatments, or an equivalent amount of methanol used as a vehicle control (MeOH, Sigma-Aldrich, St. Louis, MO, U.S.A.). Treatments selected for each tank were chosen randomly. SMW used for experiments were collected by placing 20 spermiated males into a tank of 200 L of aerated water with no flow for 36 hours. 1 L water samples were collected and frozen at -20°C until use. Natural 3kPZS concentrations in SMW were quantified using ultra-high-performance liquid chromatography-tandem mass spectrometry according to the protocol described by Li et al. (2011). Initial doses of treatment were added to bring the concentrations up to the desired level. Solutions made based on each tank's volume and flow through velocity were prepared and pumped into each tank during the duration of experiments via reef dosing pumps (Model The Sentry; Innovative Aquatics, Cumming, GA, U.S.A.) to account for the outflow and keep the treatment concentrations at a stable level. Pre-ovulated females were treated for 24 hours and then euthanized with 0.05% MS222 (Tricaine methanesulfonate; Sigma-Aldrich).

Identification of Clock Gene Sequences

We used genomic resources, including the nucleotide and protein sequences of the sea lamprey, together with a sea lamprey transcriptome assembly of 89 RNA-Seq samples across different developmental stages to identify the clock genes of interest (See Supplemental Table S4.1 for full cDNA sequences). Available clock proteins of fruitfly (*Drosophila melanogaster*), zebrafish (*Danio rerio*), and mouse (*Mus musculus*) were retrieved from GenBank (Kersey et al., 2013; Pruitt et al., 2014) and used as queries in standalone BLASTP or TBLASTN searches against the lamprey genomic resources. The coding regions in the retrieved RNA-seq transcripts were predicted using the GETORF program in the EMBOSS online tool (Rice et al., 2000;

EMBOSS GUI v1.14; http://imed.med.ucm.es/cgi-bin/emboss.pl?_ action=input&_ app=getorf), and the retrieved genomic sequences were subjected to *ab initio* gene prediction using the Augustus program (Stanke et al., 2005; http://bioinf.uni-greifswald.de/augustus/) and protein-based similarity gene prediction using FGENESH+ web server (Solovyev 2007; http://www.softberry.com/). The final protein-coding sequences were validated by using them as queries in BLASTP searches against the NCBI non-redundant protein sequence database (nr).

Real-Time Quantitative PCR (RTQ-PCR)

Experiments for collection of tissues for RTQ-PCR using the SYBR Green System (Applied Biosystems, Foster City, CA, U.S.A.) were conducted from 21-May-14 to 22-May-14. Sea lamprey were sacrificed at 1200 h after 24 hours of treatment ($N = 7$ animals per treatment). Forebrain, hindbrain, and pineal complex tissues were removed from the animal, separated, and immediately snap frozen using liquid nitrogen. Tissues were then transferred into -80°C until the time of processing. Frozen brains or pineal complexes were analyzed for transcripts of *Clock*, *Bmal1*, *Per1*, *Cry*, and 60S ribosomal mRNA concentrations with RTQ-PCR during July 2014. RTQ-PCR followed the procedure described by Chung-Davidson et al. (2008). Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.), treated with a TURBO DNA-free kit (Applied Biosystems), and then reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) and random hexamers (Promega, Madison, WI, U.S.A.). Synthetic oligos were used as standards and run on the sample plate. 60S ribosomal RNA was used as an internal standard and was confirmed to have no change in the expression level between treatments. Reactions were analyzed on an ABI 7900 real-time PCR thermal cycler (MSU Genomics Technology Support Facility, MI, U.S.A.). RTQ-PCR data was analyzed by one-way ANOVA followed by Bonferroni post-hoc tests if the ANOVA showed significant

treatment differences ($p < 0.05$). The sequences for primers and standards (Sigma-Aldrich) for each mRNA are listed in Table 1.

Immunocytochemistry (ICC)

Experiments for collection of tissues for ICC were conducted from 26-May-15 to 27-May-15 and 30-May-15 to 31-May-15. Sea lamprey were sacrificed at 1200 h or 0000 h after 24 hours of treatment ($N = 7$ animals per treatment). The brain and pineal complex were dissected out and placed into 4% paraformaldehyde (in 0.1M phosphate buffer, pH 7.4) for two nights at 4°C and then transferred to 20% sucrose for cryoprotection. Tissue was then sectioned into 20 µm slices using a Leica CM1850 cryostat. Sections were mounted onto Superfrost Plus slides (Fisher Scientific, MA, USA) and immunostained for either BMAL1 (1:200, BMAL1: ab93806, Abcam, Cambridge, MA, U.S.A.) or CRY (1:200, CRY1: sc-3317, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.). Negative controls for immunocytochemistry (lacking primary antibody of interest) were performed simultaneously (See Supplemental File 2).

The general procedure for immunocytochemistry followed the protocol by Chung-Davidson et al. (2004). Briefly, sections were washed in Tris buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.2) 3 times (5 min each) in between each step. Sections were first reacted with 0.03% H_2O_2 (DAB substrate kit, Vector, Burlingame, CA, U.S.A.) for 30 minutes to eliminate the endogenous peroxidase activity, incubated in the primary antibody (1:200 for both rabbit anti-BMAL1 and rabbit anti-CRY in TBS with 0.05% Triton X-100) and normal goat serum (Vectastain ABC Elite Kit for rabbit IgG, Vector) at 4°C for 48 hours, reacted with biotinylated secondary antibody (goat-anti-rabbit IgG, Vectastain ABC kit, Vector) in TBS with 0.05% Triton- for 2 hours, and followed by ABC solution (Vectastain ABC kit, Vector) in 0.05% Triton X-100 for 2 hours. Sections were then reacted with 3,3′-diaminobenzidine and $NiCl_2$

(DAB substrate kit, Vector) for 13 minutes, dehydrated through an ethanol series (70%, 95%, and 100%, 2 min each), clarified twice by xylene (5 min and 10 min), and finally covered with glass using DPX mounting media (Sigma-Aldrich, MO, U.S.A.). A Zeiss Axioskop II (Carl Zeiss, Thornwood, NY, U.S.A.) was used to visualize the stained sections and images were capture using a digital camera (Carl Zeiss) and AxioVision Rel. 4.2 software (Carl Zeiss). Stained areas were identified according to Nieuwenhuys and Nicholson (1998).

In our initial analysis to determine changes in the pineal gland, the primary antibody dilutions used for brain sections were too concentrated to allow us to compare between treatment groups. We proceeded with this experiment, therefore, using a more diluted primary antibody (1:500 for both CRY and BMAL1) and a less sensitive imaging technique (immunofluorescence). Slides went through the same aforementioned protocol until after the primary antibody incubation. Following this, slides were washed with TBS 3 times (5 min each), incubated with the secondary antibody, Alexafluor 488 anti-rabbit IgG containing 5 mM sodium azide, and normal goat serum (Life Technologies, Gaithersburg, MD, U.S.A.), washed again with TBS (3x, 5 min each) , and coverslipped with ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Slides were reviewed between the methanol vehicle control at 1200 h and 0000 h to investigate if BMAL1 and CRY proteins appeared to be cycling in a rhythm.

RESULTS

Sex Pheromone Effects on Clock Gene Expression

In the forebrain, treatment group affected the expression of all genes examined: *Clock* (ANOVA, $F_{3,24} = 3.9$, $p = 0.02$), *Bmal1* (ANOVA, $F_{3,24} = 5.2$, $p = 0.002$), *Cry* (ANOVA, $F_{3,24} = 3.9$, $p = 0.003$), and *Per1* (ANOVA, $F_{3,24} = 4.4$, $p = 0.01$). Spermiated male washings (SMW), 3kPZS, and PZS treatments had similar effects, reducing transcription levels compared to the

methanol vehicle control for all four genes examined (Figure 4.1, Bonferroni post hoc test, $p < 0.05$) in the forebrain.

In the pineal complex, only the gene *Bmal1* was affected by treatment (ANOVA, $F_{3,24} = 4.1$, $p = 0.02$). Further analysis revealed that only SMW affected the expression of this gene compared to the vehicle control (Figure 4.2, Bonferroni post hoc test, SMW vs. MeOH: $p = 0.03$). In contrast with the other tissues examined, treatment did not have a significant effect on gene expression in the hindbrain of *Clock* (ANOVA, $F_{3,24} = 3.9$, $p = 0.21$), *Bmal1* (ANOVA, F $F_{3,24} = 1.6$, $p = 0.2$), *Cry* (ANOVA, $F_{3,24} = 2.5$, $p = 0.08$), or *Per1* (ANOVA, $F_{3,24} = 0.432$, $p = 0.07$). Our internal control, the ribosomal subunit 60S, did not show differences in expression levels between treatment groups in the forebrain (ANOVA, $F_{3,24} = 1.6$, $p = 0.2$) or the hindbrain (ANOVA, $F_{3,24} = 2.4$, $p = 0.1$).

Sex Pheromone Effects on Protein Expression

Both CRY and BMAL1 were found in similar areas within the forebrain (Table 2) and the hindbrain. Females treated with SMW had the greatest number of areas with reduced immunoreactivity compared to the vehicle control for both CRY and BMAL1. Although there was minor staining, no changes in immunoreactivity between treatment groups were seen in the hindbrain for either protein. Females treated with SMW or 3kPZS had reduced staining for the CRY protein in the hypothalamus, habenula, medial pallium, striatum, and torus semicircularis areas of the brain (See Figure 4.3 for representative examples). Fish treated with SMW had additional decreases in immunoreactivity in the lateral pallium, dorsal pallium areas surrounding the lateral ventricle, and the mammillary area. PZS affected fewer areas than the other two treatments. For this treatment, immunoreactivity for the CRY protein was only reduced in the hypothalamus and the striatum compared to the vehicle control.

Similar patterns were seen in immunoreactivity for the BMAL1 protein (Table 2; See Figure 4.3 for representative example), except that there was no staining in the hypothalamus or the mammillary area for the vehicle control. All areas that stained positive for fish treated with the vehicle control were reduced in fish exposed to SMW. The 3kPZS treatment also reduced immunoreactivity for BMAL1 in all areas except for the torus semicicularis and the stratum griseum periventricular. In contrast, fish treated with PZS showed positive staining for BMAL1 in all areas examined, except for the hypothalamus. Experiments for the pineal gland showed increased BMAL1 immunoreactivity in those females treated with SMW compared to the other treatment groups (Figure 4.4), but no difference in CRY immunoreactivity between treatment groups.

To examine if there was evidence for rhythmic expression of clock proteins, we compared BMAL1 and CRY proteins at 1200 h and 0000 h between the vehicle control samples (see Figure 4.5 for representative example). BMAL1 had greater immunofluorescence at 0000 h than 1200 h. Oppositely, CRY had greater expression at 1200 h than 0000 h.

DISCUSSION

The results presented here demonstrate that sex pheromone compounds affect the endogenous circadian system within adult female sea lamprey as evidenced by alterations in clock gene mRNA and protein products. Levels of *Bmal1* were increased in the pineal gland for those females treated with the entire suite of sex pheromone compounds released by spermiated males into the water. In addition, all four clock genes examined within the forebrain of those fish treated with SMW, 3kPZS, or PZS treatments showed a reduction in transcription levels. Furthermore, we demonstrated that there were protein level effects for BMAL1, a positive element of the molecular feedback loop, and CRY, a negative element. Within several forebrain

regions, the immunostaining for these proteins is reduced after 24 h exposure to sex pheromones. Finally, we demonstrate that there are increases in BMAL1 protein expression in the pineal gland, a structure implicated in circadian locomotor rhythmicity. Although in most organisms the major environmental synchronizer is the daily light-dark cycle (Aschoff, 1981, Brown and Schibler, 1999), other non-photic cues are capable of modulating this photic synchronization (Menaker and Eskin, 1966; Hau and Gwinner 1996; Hastings et al., 1998). It is most likely a complex integration of environmental and physiological cues that acts to synchronize the circadian timing system. While there are many examples that fish exhibit circadian rhythms and use pheromones for communication and reproduction, there have been no studies examining the role of pheromones in the regulation of circadian rhythms in fish (Baghel and Pati, 2015). Here, we present the first example that sex pheromones can affect the endogenous circadian molecular framework in a vertebrate species.

We have demonstrated that sex pheromones affect the clock gene system in the pineal complex. In the sea lamprey, pineal gland cells are classical photoreceptor cells with structural and functional similarities to retinal photoreceptors (Cole and Youson, 1982). The pineal gland transduces photic information and relay messages to other areas of the brain via neural and neuroendocrine systems. The pineal gland in the lamprey can be considered a master circadian clock because its removal results in a disruption of the rhythmic behavior of locomotor activity (Morita et al., 1992; Binder and McDonald, 2007). This differs from mammals, in which the master clock is within the suprachiasmatic nuclei (SCN) of the hypothalamus that receives light inputs via the retina (Reppert and Weaver, 2002). In both cases, the master clock can synchronize the timing of peripheral oscillators found in other brain regions that contribute to the production of rhythms in physiology and behavior (Bartness et al., 2001; Balsalobre, 2002).

Our results show that of the four clock genes we examined, only *Bmal1* was affected within the pineal gland, and that these effects were only seen in females treated with SMW. This is consistent with results in mammals that BMAL1 is an essential component of the master circadian pacemaker in mammals. Loss of this protein in mice results in complete and immediate loss of circadian rhythmicity in constant darkness (Bunger et al., 2000). In addition, in double knock-out *Bmal1* mice, the locomotor activity in light-dark cycles is impaired and activity levels are reduced (Bunger et al., 2000). Furthermore, *Bmal1* is a gene of particular interest because its transcription is directly regulated by an additional feedback loop that has been shown to direct *Bmal1* transcription, providing robustness and stability to the core loop of autoregulatory transcriptional-translational clock genes (Emery and Reppert, 2004), whose expression and translation in the brain have direct consequences on the locomotor activity output (Masubchi et al., 2000).

Our results demonstrate that all four of the clock genes examined had reduced transcription levels in the forebrain of adult female sea lamprey exposed to a suite of sex pheromones (SMW), or an individual sex pheromone compound (3kPZS or PZS). These results were further confirmed on the protein level through examination of the corresponding protein products of BMAL1 and CRY. In adult females exposed to any of the three pheromone treatments, there was a reduction in CRY staining in the hypothalamic area (see Figure 4.3 for representative). Given the dominant role of the suprachiasmatic nuclei (SCN) in the circadian system of other vertebrates, it is possible peripheral oscillators within this hypothalamic region play a role in the circadian timekeeping system of the lamprey and other teleost species. This area could be directly under the control of the pineal gland, which projects to the dorsal hypothalamus and the ventral thalamus areas of the brain (Puzdrowski and Northcutt, 1989;

Yanez et al., 1993). In future studies, it would be interesting to clarify the exact roles of the pineal gland and hypothalamus as well as their interactions in circadian timekeeping.

In addition to their effects in the hypothalamus, SMW and 3kPZS reduced the BMAL1 immunoreactivity in the habenula of the adult female brain compared to the control (see Figure 4.3 for representative example). In lamprey, this region acts as an integrative center between the striatum and the limbic and motor systems (Yañez and Anadón, 1994). In lower vertebrates, the habenular complex is strongly associated with the pineal gland (Guglielmotti and Cristino, 2006), and in lamprey there are extensive connections to the habenula from the pineal complex (Yáñez and Anadón, 1999). In addition, the pineal tract connects the habenula to the dorsal mesencephalic tegmentum motor region (Yáñez et al., 1993). The habenula has been implicated in several roles, including behavioral state, motor activity, emotion, and motivation (For review, see Hikosaka, 2010). In the mammal species the Syrian hamster, it has been shown that transecting the major output pathway of the habenula alters the daily amount of locomotor activity. In these animals, which normally have a bout of elevated activity followed by shorter bursts, their activity is spread out more in a homogenous manner throughout the night (Paul et al., 2011). Given these previous studies, we speculate that the habenula is receiving signals from the pineal gland and is involved in one of the output pathways from this main circadian pacemaker that contributes to the overall timing of locomotor activity.

Alterations in *Cry or Per* transcriptions have been shown to have direct consequences in the locomotor activity output of other species. For example, *Cry1/Cry2* double knock-out mice have complete arrhythmicity in constant conditions (van der Horst et al., 1999; Vitaterna et al., 1999; Bae et al., 2001; Zheng et al., 2001). In addition, mice with targeted disruption of the *mPer2* gene have strongly abnormal behavioral rhythms in which their free-running period is

very short and the mice become arrhythmic after a variable number of days in constant conditions. Mice with inactivated *mPer1* genes have a period that is shorter by 1 h than their wild-type littermates as well as changes in the clock genes of peripheral tissues (Okamura et al., 1999; Zheng et al., 1999; Cermakian et al., 2001). These results suggest that different clock genes can have different and/or complementary roles in the regulation of behavioral rhythms depending on the species and the environmental conditions.

The effects of sex pheromone compounds on clock gene transcription and translation in the forebrain are contrasting to the effects we saw within the pineal gland in that the level of transcription in all four genes is reduced. Although the same basic oscillator components exist in central (in sea lamprey the pineal gland) and peripheral (other regions of the brain) oscillators, and both have been shown to be capable of cell-autonomous oscillations, there can be differences between them in clock gene expression and intercellular coupling. In addition, the non-mammalian vertebrate timekeeping system can include peripheral clocks that have independent pacemakers and their own responses to exogenous cues. For example, in zebrafish, the peripheral clocks are directly entrainable by light (Whitmore et al., 1998). This also can occur in mammalian species. In rodents, feeding time can affect the peripheral oscillator phases without affecting the master clock (Damiola et al., 2000; Stokkan et al., 2001). Because the circadian system is evolutionarily conserved in animals, including in the clock genes involved in rhythm generation, comparative studies are important and could reveal endogenous mechanisms that enable some animals to be plastic in their behavioral rhythms.

The global reduction in clock gene expression within the forebrain could be due to several factors, including a reduction in gene transcription, a reduction in the amplitude of gene transcript oscillations, or due to a phase-shifting of these rhythms. This phenomenon has been

seen in several species and was dependent on environmental conditions. In honeybees, activity rhythms can be plastic. The nurse bees care for the brood across all hours of the day and night, while forager bees are behaviorally rhythmic. In nurse bees, brain oscillations in gene expression of the four putative clock genes, *Period, Cryptochrome-m, Cycle,* and *Timeout* are attenuated or completely suppressed compared to the foragers (Shemesh et al., 2007). In European hamsters, there is a halt in clock gene expression rhythmicity during hibernation periods, as the clock genes *Per1, Per2,* and *Bmal1* are constantly expressed in the SCN (Revel et al, 2007). In this study, we confirm there is an effect on clock gene transcription levels in the forebrain, however, we only reviewed one time point and future studies are needed to elucidate the full effects on the gene transcription rhythms of these genes.

Here, we demonstrate the effects of sex pheromone compounds in a jawless fish species, the sea lamprey, on the endogenous clock in the form of changes in clock gene transcription levels and their translational counterparts in the brain and pineal gland. This is the first investigation of genes and proteins involved in the circadian transcriptional-translational autoregulatory feedback loops within a lamprey species as well as the possible role of sex pheromones as an environmental cue involved in modulation of these feedback loops. In this study, we provide evidence that, like in other non-vertebrate and vertebrate species, the clock proteins have different rhythmic expression in the pineal gland and it is likely that this is the case of other brain regions given the nature of the feedback loops of which they are a part. Future studies should focus on elucidating the rhythms in expression of these genes and proteins within this species, and investigating the role of pheromones in the regulation of circadian rhythms and their behavioral outputs.

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APPENDIX

Table 4.1: Sequences of designed primers and standards used for real-time quantitative PCR (RT-QPCR). Primer sequences listed in the standard 5' to 3' direction.

Gene	5' Primer	3' Primer	Standard
Clock	CTGGCGCCGAGTTCACA	TGATCCAAGAAGAGGAACTTCCA	CTGGCGCCGAGTTCACATCCAGACACAGCCTGGAGTGGAAGTTCCTCTTCTTGGATCA
Bmal1	ATGGCGGTGCAGCACAT	TGTAGTTGGCCTCCGTGTACAC	ATGGCGGTGCAGCACATGAAGACGCTGCGCGGCGCCACTAACGTGTACACGGAGGCCAACTACA
Cry	CGCCTGTTCAAGGAGTGGAA	CGAATGGCTCAGAGTCATACTCA	CGCCTGTTCAAGGAGTGGAACATCAGCCGGCTCACGTTTGAGTATGACTCTGAGCCATTCG
Per1	CGCCGTGCACAAGAAGATCT3	CGAGCAGAAGCGGAATGG	CGCCGTGCACAAGAAGATCTTGAAGTTTGTGGGGCAGCCATTCGACCACTCCCCATTCCGCTTCTGCTCG
60S	CGCATCCGCGCAATG	GTCGGGTATGTCCACGATCTG	CGCATCCGCGCAATGAAGACCATCCAGAGCAATCAGATCGTGGACATACCCGAC

Table 4.2: Summary of immunoreactive staining in the adult pre-ovulated female brain for CRY and BMAL1 proteins after treatment exposure to spermiated male washings (SMW with equivalent amount of 10^{-10} M 3kPZS) , 10^{-10} M 3kPZS, 10^{-10} M PZS, or the methanol control (MeOH) for 24 h. + represents positively stained cells and - represents lack of stained cells in each corresponding brain area. MeOH= methanol control; SMW= spermiated male washings.

	CRY				**BMAL1**			
Brain Region	MeOH	SMW	3kPZS	PZS	MeOH	SMW	3kPZS	PZS
Surounding Lateral Ventricle	+	-	+	+	+	-	-	+
Lateral Pallium	+	-	+	+	+	-	-	+
Dorsal Pallium	+	-	+	+	+	-	-	+
Medial Pallium	+	-	-	+	+	-	-	+
Striatum	+	-	-	-	+	-	-	+
Habenula	+	-	-	+	+	-	-	+
Hypothalamus	+	-	-	-	-	-	-	-
Thalamus	+	-	-	+	+	-	-	+
Stratum Griseum Periventricular	+	+	+	+	+	-	+	+
Torus Semicircularis	+	-	-	+	+	-	+	+
Mammillary Area	+	-	+	+	-	-	-	+

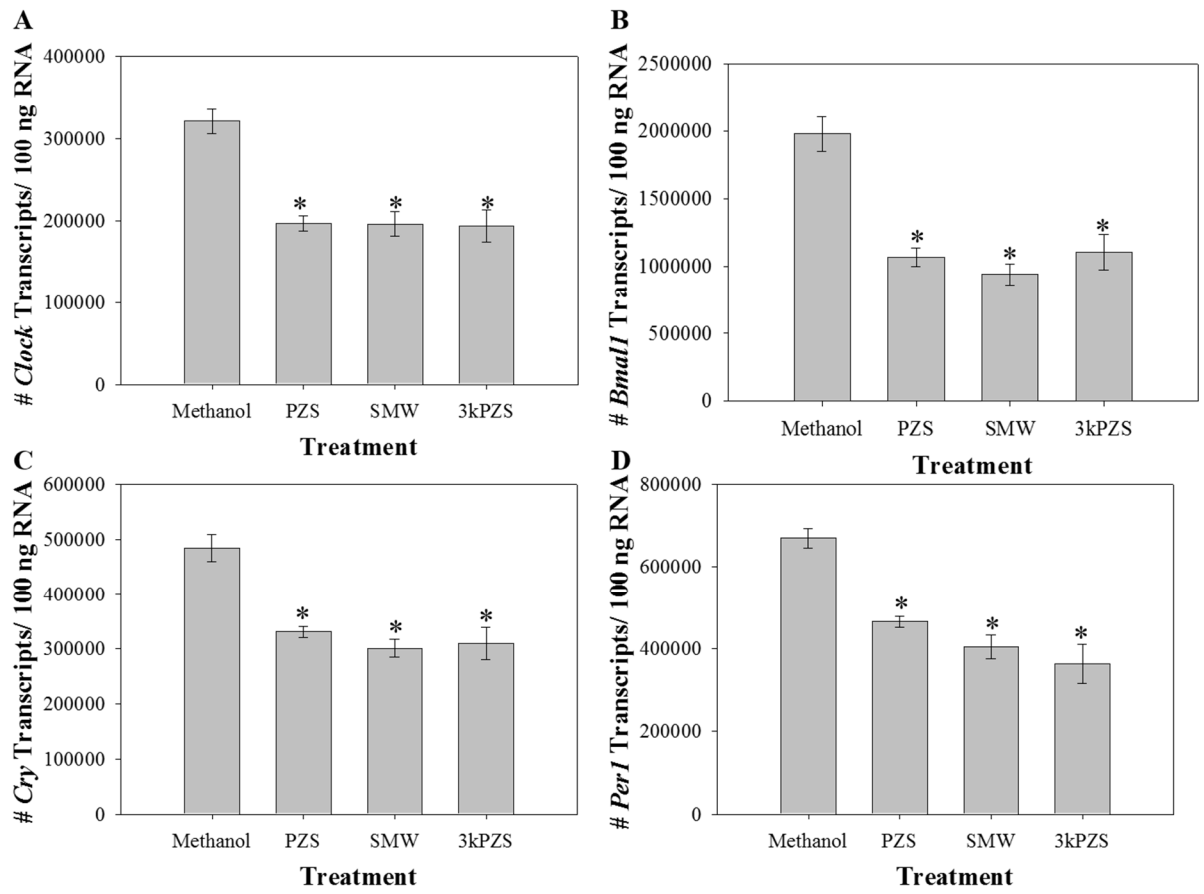

Figure 4.1: Sex pheromone compounds reduce the transcription levels of (A) *Clock,* (B) *Bmal1*, (C) *Cry*, and (D) *Per1* in the forebrain of adult female ($N = 7$ per treatment per gene examined) sea lamprey treated with spermiated male washings (SMW with equivalent amount of 10^{-10} M 3kPZS), 10^{-10} M 3kPZS, 10^{-10} M PZS, or the methanol control (MeOH) for 24 h. The asterisk denotes significant difference between control and the treatment ($p < 0.05$). Error bars indicate SEM.

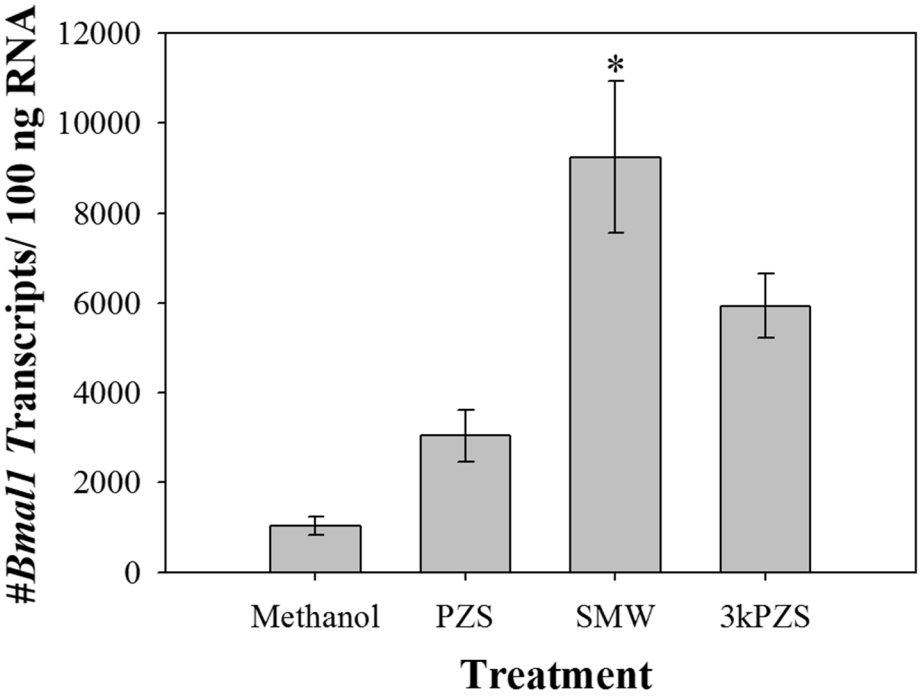

Figure 4.2: Spermiated male washings (SMW) reduce the transcription level of *Bmal1* in the pineal gland of adult female sea lamprey ($N = 7$ per treatment). Sea lamprey treated with SMW (with equivalent amount of 10^{-10} M 3kPZS), 10^{-10} M 3kPZS, 10^{-10} M PZS, or the methanol control (MeOH) for 24 h. The asterisk denotes significant difference between control and the treatment ($p < 0.05$). Error bars indicate SEM.

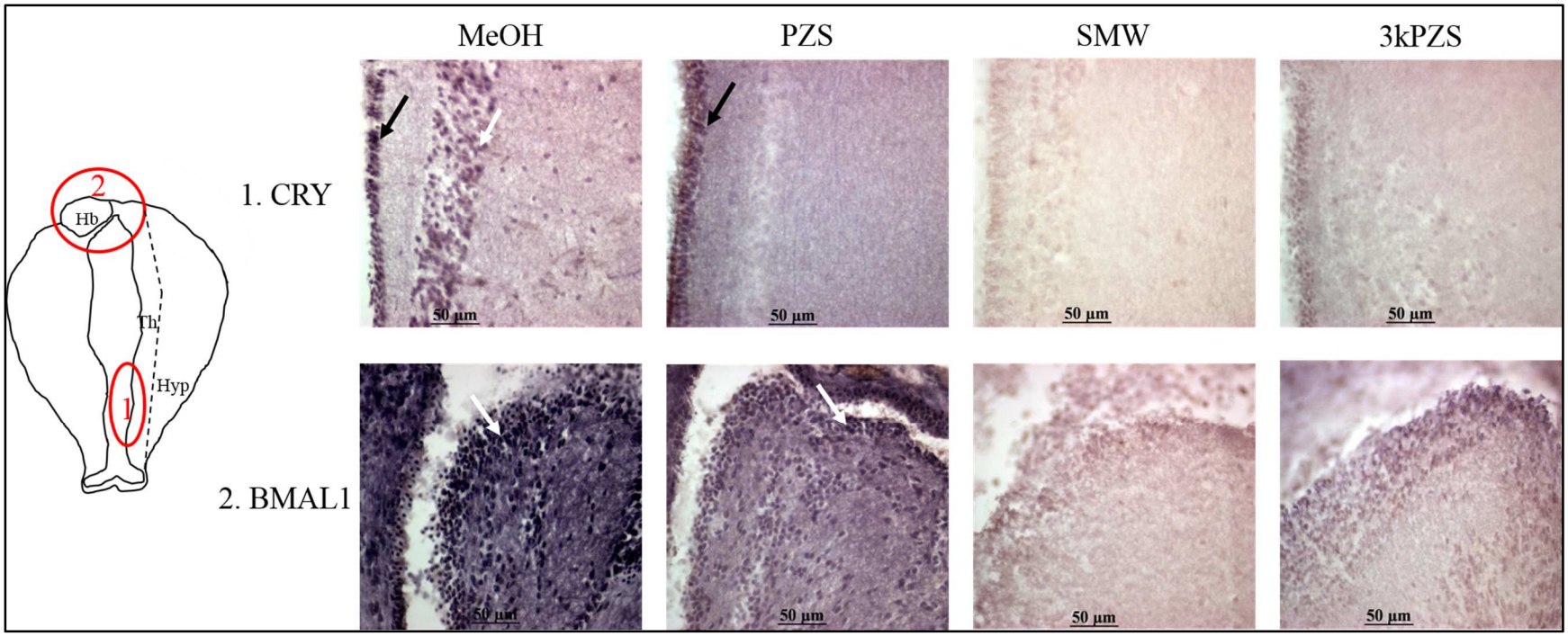

Figure 4.3: Representative example of decreased immunoreactivity of (1) CRY protein in the hypothalamic area (white arrows) after 24 h treatments (SMW with equivalent amount of 10^{-10} M 3kPZS), 3kPZS (10^{-10} M), or PZS (10^{-10} M). Additional loss of staining in ependymal cells lining the 3rd ventricle (black arrows) were also lost in those females treated with SMW or 3kPZS and (2) Decreased immunoreactivity of BMAL1 in the habenula (white arrows = examples of stained cells) following 24 h treatment of SMW or 3kPZS. Schematic to left represents area seen in pictures. Hyp= Hypothalamus, Th= Thalamus, Hb= Habenula. SMW= spermiated male washings, MeOH= methanol control.

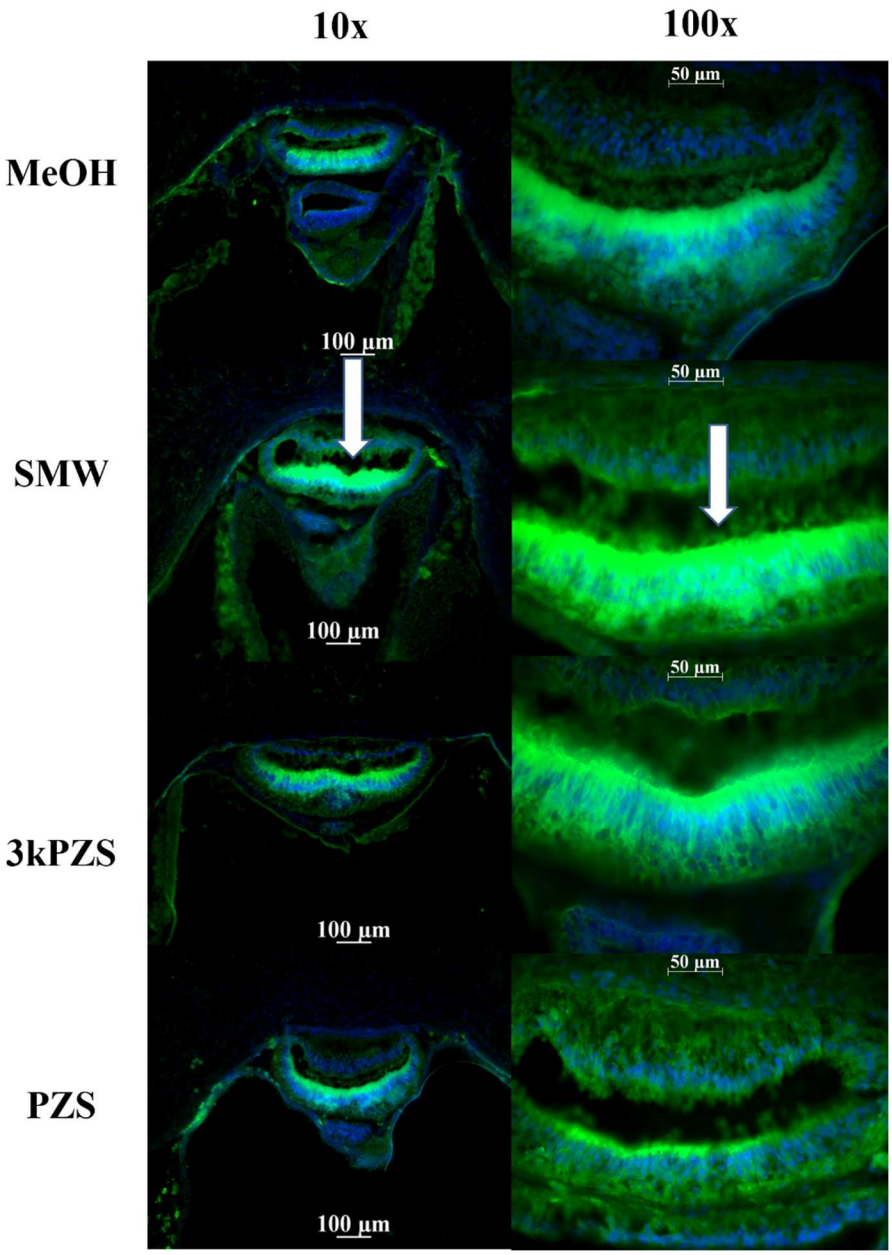

Figure 4.4: Representative images of immunofluorescence staining of pineal gland cells for BMAL1 protein (green) and DAPI (blue) at 10x and 40x magnifications. Increased immunofluorescent staining (white arrows) seen in adult female sea lamprey treated for 24 h with spermiated male washings (SMW containing equivalent amount of 10^{-10} M 3kPZS) compared to individual pheromone compounds (10^{-10} M 3kPZS or 10^{-10} M PZS) or the vehicle control (MeOH).

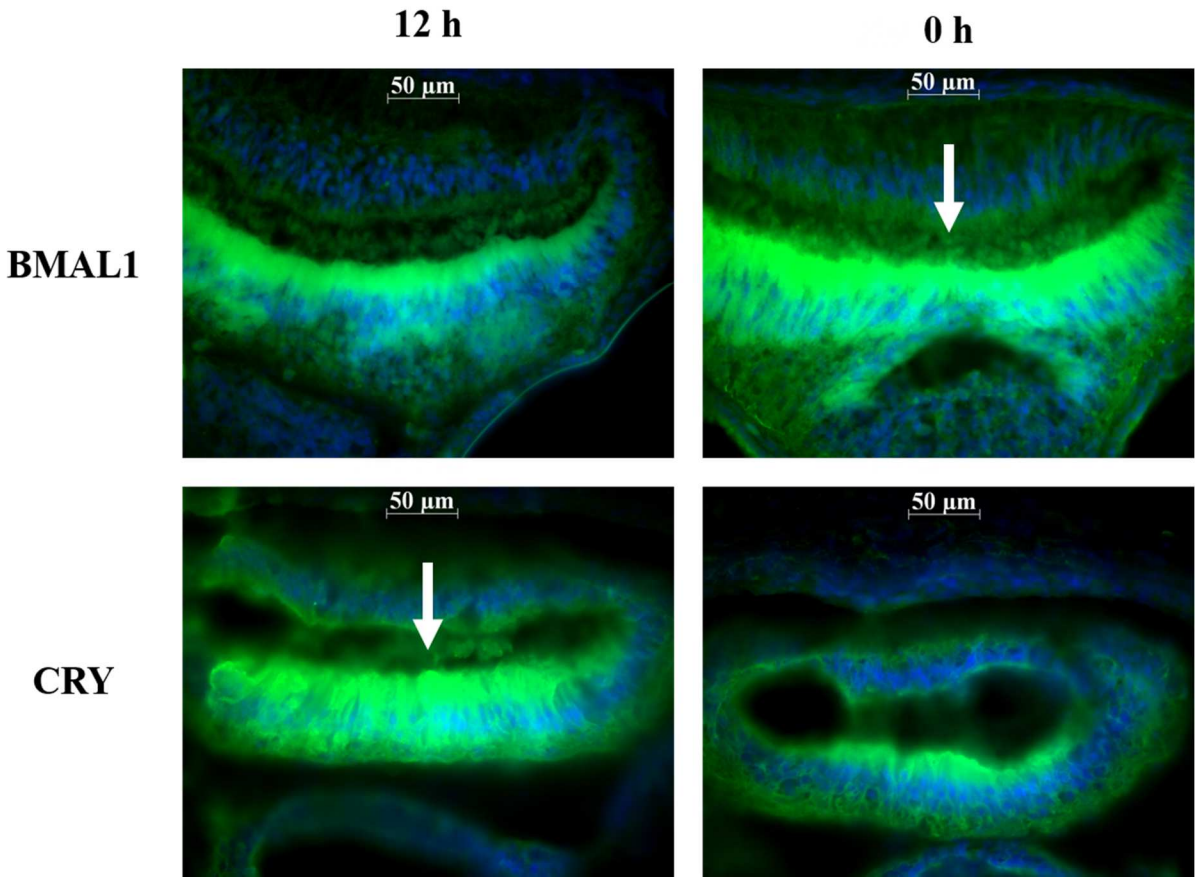

Figure 4.5: Representative images of immunofluorescence staining of pineal gland cells for BMAL1 or CRY protein (green) and DAPI (blue) at 40x magnification. Evidence for rhythmic changes in protein expression in adult female sea lamprey treated for 24 h with vehicle control (MeOH). Increased immunofluorescent staining in BMAL1 at 0 h compared to 12 h and increased staining in CRY at 12 h compared to 0 hr.

SUPPLEMENTAL INFORMATION

Table S4.1: cDNA sequences for annotated clock gene sequences used to develop primers and standards for real-time quantitative PCR experiments.

1. *Brain and muscle aryl hydrocarbon receptor nuclear translocator- like (BMAL1)*

CCGACGGAGAGGAGAACGCGGTGGCTTTGGCTGCGAAGGTGGTTGCGGTAACGCGC
GCGCGCGCACGCACGCACGCGATGTCCTTCGCGACGCAGCCGGCGTTCATCCCCGA
CTCGTTTCACGAAGATGAAGCGATGGCGGACCAGCGCATGGACATCAGCTCGACGA
TGAGCGAGTTCCTGGCGCCTGGCTCCTCGGGGCTGATGCCCCGCACGCTCAGCAGCG
CCGCCCTCGACTTCAACCGCAAGCGGCGCGGCAGCTCCCCCGACTACGACCTGGAT
GGCTTTTCATTCGAGGACTGCATGGACACGGACAGAGACGACCAACTCAACAGAGT
GGAAGGCACGGATCAGCAGTGCAGAAACAAATACTCCAAGGAGGCTCACAGTCAG
ATCGAGAAGCGGCGGCGGGACAAGATGAACAGCTACATCGACGAGCTGGCGTCCAT
GGTGCCCACGTGCAGCGCCATGTCGCGCAAGCTGGACAAGCTCACGGTGCTGCGCA
TGGCGGTGCAGCACATGAAGACGCTGCGCGGCGCCACTAACGTGTACACGGAGGCC
AACTACAAACCGGCGTTTCTGTCCGACGACGAGCTCAAAAATCTGATCCTGCGGGCC
TCCGAGGGGTTCCTCTTTGTGGTCGGATGTGATCGTGGGAAAATCCTCTTCGTCTCA
GAGTCGGTCTCCAAGATCCTAAACTTCGGCCAGAATGACCTGATTGGGCAGAGTCTC
TTTGACTACCTTCATCCCAAGGACATTGCCAAGGTGAAGGAACAGCTGTCCTCGTCA
GACACGGCTCCTAGGGAGAGGCTTATAGACGCTAAGACGGGCTTGCCGGTGAAGAC
GGACGTGCTGGCGAGACCCAGCCGCCTGTGCTCGGGCGCCCGGCGAGC

2. *Circadian locomotor output cycles kaput (CLOCK)*

ATGTCAGAAGGCTTAGATGAAACGGGACGCAGAGATGAGAGAACCAGAGCGAAAC
GCGCTCAGCGGATCCGTTCGGAGAAGAAGCGGCGGAAGCAGTTCAACGAACTCATC
AGCGAGTTGGGGGCGCTGCTGCCCGATGGCCAGTCCCGAATGGACAAGCCCACGGT
CCTGCAGCGGACGCTGCACTTCTTTAGCAGCCACCACGAGCTGAACGTGGAACAGG
AGACCAACGAGCCTCCTCCGCCGTGGAAGCCAGCTTTTCTCAGCAACGACAACTTCA
CGCAGCTCATTCTCGAGGCCATGGACTGCTTCGTAATTGTCGTGATGAGCAGTGGCG
AGATCTCGTTCGTGTCCGAGAGCGTCACGTCGCTCCTCGGTTACCTGCCAAGTGAGC
TCCTCAGACAGAACCTCTTCGATTTCGTGCCGGAGCACGAACAAGCGGAGCTGCGCT
GCCTTTTCCAGGCTGACCTGAGCCAGGTGCAGGAGGGACCCCCCGAAGCCCCGCAA
GCGGGCGCGCCGAATGAGCGGCACTTCGAGTTGTGCTGCCACCTGCAGCGCGGAGA
GGCACTCGCGCACTTCGCTGCGGGACAGACGCCGCCGCCGCCGGTGGTGGTGGCGG
CGGCGTCGCGCCCCGCCGAGGAGGCGCCGACTTACGAGCGAGTGAAGCTCAGCGGC
TTCCTGCGCCTGCACCTGCACGACGAGCAAGCGGAGGTGGCGGCGGGGCCTTGCCT
GTCGGAGGAGCCTTCGGGGACAGAGGAGCCAGCGACCACCCCGGGGGTGCCAGGG
GGTCCGCCGCGTCCGCCTCCCTGCCTCGTCGCCTCGGTCCACCTCCTCACGTCGCAGT
TCATCAAGGAGCTGTCCTCACTGGAGGAAGCTGGCGCCGAGTTCACATCCAGACAC
AGCCTGGAGTGGAAGTTCCTCTTCTTGGATCACAGGGCACCGCCCATCATCGGCTAC
ATGCCCTTCGAGGTCCTGGGAACGTCCGGCTACGACTACTACCACGTGGACGACCTG
GAGAGCATCACGCAGTGCCACAGGGACTTGAAGCAGTCTGGGAAGGGAAAGTCCTG

Table S4.1 (cont'd)

CCACTACCGCTTTCTGACCAAAGGGCAGCAGTGGATCTGGCTGCAGACCAAGTACTT
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ACTCAGCCAACCGGCGAGCCAGGCGGTCGTTCAGAACGTCGGCCAGCCGATTGGCC
AGACCGTGCATCAGCAGCACATCGGCCCACCCTGGCAACAGCAGCTCGTCGTGAGC
CCCAACATCGGCCAGCCGGGCAGGCAGGCTCTTGGCCAGAGCTCGAGCCAGACCGT
CGACCAGAGGCTTGGCCAGCAGAGCGCCATGCAGAGACTGAAGGAGCAGCTGGAG
CAGAGGACGCACGTGCTGCAGACGGTGATCCTGCAGCAGCAGGAGGAGCTCCGCCA
CATCCAGCAGCAGCTTGCCATGCCCCATCAGGTGGTGACCACGTTAAGTGGTACTCC
CATCCTGGTTTCAGCTTCCATTATGATGCCGTCCTTCACAACCTTTAGTCAACAACCG
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TCAGCAGCACCAGCATCAGCAGCACCAGCAGCACCAGCATCAGCAGCATCAGCAGC
AACAGCATCAGCAGCAACAGCATCAACAGCACCAAATAATTGACCAACAGTCACAA
GCGCAGCAGAGCTGTGCTCAAAACAAGGGACAGCACTTGAGGGTGCAGGCCCAACG
GAGAGCAGAAGGGCACCTGCAGCCGACACTGCTGATGCCGACCATGCTGCAGATAC
AACAGCAGCAGCAACAGCAGCATCAACAGCAGCAACAGCAGCATCAACAACAGCA
TCAACAACACCAGCAGCACCAGCAGCATCAACAGCAGCACCAACAGCACCAACAGC
AGCAGCAACAGCAGCAACAACGGCAGCAGTGCAGGCGATCATCCTCAGAGAAGCA
CCAGTCCCCATCGCCACGGCATCAGCAGCAGCACCAGCAAGAGCACACATTGCGCT
CTCCAAATGTGCAGTATCACCGTTCTATGAGCGCGGAGCCGCAACAGAGAAGTCAT
CAGCAGCATCAGCAACCACAGCAGCAGCAGCAGCAATTGCTATCTCAAGAGAGGCT
GTTGTGGATGGCGAGTGGACAGCAGGATTCCCAGGGCTATCTGCACGCCACGTACC
ACGCGCCCCATGCGGTGGGCGGTGTGGCTGGGAGGCAGCGCCTGTTGCACGCGAGG
TCGAGCGGGCGGCAGCAGCCACAGCCGCCTGTCGACCGCGTGCAGCGACAGCATCC
CGCGGTGCAAACACAGCAACAGCAGCAACAGCAGCAGCAGCAACAGAGCCCCAGG
TGA

3. *Cryptochrome (CRY)*

CTCGGCGCGCAGTCACTGAGACGCTGCGCTGAGGGAGCGACGAGTCGCATCGCATA
AAGTACGCGGCGCCAATAGGAAAATATTTATTCACACGCACGCACGGCAGAGTATA
GGCGTAATCTGTGAGAGGAATTGCAGGTTTGTTTCCCCCCCCCCGCCTCATGAATATA
CACGCGCGCATAAGAAATCGATCTCTCTTACCGCGCGTCATAAAACGATTATCGGGG
AATCAAATTGCAAATCTCTGGGTTTGATCCGCGGCGTATACATTACGTGGTCGCTGT
TGATAGCGGGTGGCAGCGATATATAACGTAAAAAATAGCGCAAAATGGAGCGTTAC
AACTCTTCGTTGTGCACACCGAGAGAGCGAATGACGCCTGACTTATAGATTTCGGTA
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AAAAGCACCTGCCGTTACCGACAAACTACCGCGCCTGTCGCTATATACATATATAAA
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TCGACGTCAAGTAACGAAGAGAGAGAGAGAGGCAGGCGAGAGAGACGAGGCTTGT
GGCAGGTGGTGGTGGTGGTGTGGCAATGGTGCTCAACTCGATCCACTGGTTCCGCAA
GGGACTGCGCCTTCACGACAACCCGGCGCTGCGCGAGGCGCTGCAGGGCGCCGACA
CCGTCCGCTGCGTCTACATCCTCGACCCCTGGTTCGCGGGCGCCTCCAACGTGGGCA
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Table S4.1 (cont'd)

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GCTGGTGCGCATCTCCCACACACTCTACAACCTTGACAGAATAATCGAGTTGAACGA
AGGGCAGGCACCACTCACATACAAGCGCTTCCAGGCGCTGGTGAGCCGCATGGAGC
CGCCCGAGAGGCCCGTGGGAACCATCACGAGCGAGGTGATGGGCCCCTGCCGCACA
CCGCTCTGGGAGGACCACGACGAACGCTATGGGGTACCTTCCCTCGAGGAGCTCGG
TTTTGACACTGATGGCCTAACGACTGCCGTCTGGCAGGGAGGCGAGTCCGAGGCCCT
GACCCGCTTGGACAGACACCTGGAGAGGAAGGCATGGGTGGCAAACTTTGAGCGCC
CAAGGATGAACGCCAATTCGCTGTTGGCGAGCCCCACGGGACTCAGCCCATACCTG
CGGTTCGGCTGCCTTTCCTGCCGTCTTTTTCACTACAAGCTCACAGAGCTCTACAAGA
AGGTAAAGAAGAACAGCTCTCCACCGCTTTCGCTGTACGGCCAGCTCCTGTGGAGG
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CCCCATCTGTGTCCAGATCCCCTGGGACCGCAACCCCGAGGCCCTTGCCAAGTGGGC
GGAGGGCCGTACAGGCTACCCCTGGATAGATGCCATCATGACACAGTTACGGCAGG
AGGGCTGGATCCATCACCTGGCACGACACGCAGTCGCCTGCTTCCTGACACGCGGA
GACCTCTGGGTATCCTGGGAGGAGGGCATGAAGGTATTTGAGGAACTGCTGCTGGA
TGCTGACTGGAGCGTGAATGCAGGCAGTTGGATGTGGCTGTCCTGCTCCTCCTTCTTT
CAGCAGTTCTTCCACTGCTACTGCCCAGTGGGATTTGGCCGACGCACCGATCCAAAC
GGGGACTACATACGCCGCTACATTCCGCTGCTACGAGCCTTCCCGGCCAAGTATATC
TACGACCCATGGAACGCTCCGGAGAGCGTACAGAAGACGGCACGCTGCATAATAGG
CGTGGACTACCCCAAGCCCATGGCCAACCACGCCGAGGCCAGTCGCCTCAACATCG
AGCGCATGAAGCAGATCTACCAGCACCTGTCACGCTACCGCGGCCTCTGTCTGTTGG
CGTCTGTGCCCTCCAACGGCAATGGTGGGATGTCCGGCTATGGCCAGGGTGATGGCC
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AACGGTGGAGATGATGGAGCCACTTGCTCCTACCAAAAGGACCAACAGCCGTGCGC
AAGTGGCTCATTGCGGCAGCAACCGAGAGAAATGGCGGCGCCAGCTGGTGTAGGCA
GCCCGGGTGCGCTGGACCCCCTGGGGGTGCTGAAACGGGACCGTCTGTGCCGGATG
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CATGGCGGGTAACTGAACATCAACTGTTTGAAGCTTATAGAAATGTTACGGAAAGCT
TATCGTGGGATGCAAAAGAATTCCACAGAAGAAAAACCCTCGAATCATCAGTTAAC
ATCGAAGCTCAAGTGAACATGAGTTGAAGTGTGTAAAAGCGCATCTGGATTTCTTAC
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CTAAGGTGGTGTTGAAATTTTGTGACCCGTAAACACTTATGTTAAATGATCAGCACA
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AAGAGAAAAAAATAAAAACAATCTGGAGATACAGCATCCATGAGGTACAGTTCAGA
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CGTGAGTTTTTTTTGCACAGCCCCCAGGTATCTGCGCTGTTAAGTGGTTGCTTTTGCA
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ACTGTGGCCCCTGTATGCTCTAAGTCAGGCAGGGCCTCCTGATGATATAACCCCATA
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Table S4.1 (cont'd)

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AATATTTTATATTTTCTTTAACATGTTGATGTGTTGCCTTTAAAATGCGTAGTGAAAT
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TTGTACAGGACTCGTATTTGTGTGCGCTTATAAAAGAATACAGTGCAATACTCGTCG
TTACCACATTGATAAGAGCAATGTTGTATAGTCAGGGTATGAGCGATATTATTGTTT
CCGTGTTATTTATTATATTTATTCTTATAATAACGACATTGATATATCAGAATGGGAA
ATGTATTGCTCCTTAAGTGTTTATACGCGAGTCATAATGCATATTTGGGAAAACAAA
AATGGCTTCAC

4. *Period1 (PER1)*

GGAGCACGATGGCCATCCATTTACAGACACCCGCGACAAATAATTAAAACTCATTTC
ACTTGATAACCATTGATAAAGTCAATAAAATCCAAGCCAGAGGCGATTCGTGATTTT
GACTAAAAAAAACAACAATTGTCTTACCGGCGCGGGTGCCCAGAGGCTAATCCTGC
TCCGAAAGCGGTGCCGACCATTCCCGATGGACACGGAGCAGTGCGAGGCGAGCGAC
GCGATGGCCATGCCGGCGGGCACCGCGGGCAACGACCCCTGCCGTCGCGACGACGA
ACCGTCACATCCGAGGGACCACGAGGAGGAGCCCGGACCACCGGCCGATGACGTGG
ACATGAAGAGCAACGGCTCGCAGGGGAACGACTCGAGCGGGAACGATGAGCTCAA
TGGCAGCGAGTCGAACGGTACGGGGTCGCATGATGGCGCGTCGCGCGGCGCCGGCT
CCAGCGGCCGCTCGAGGGGCAGCGGCAAGGACTCTGCGCTGATGCTGCTCACGTCC
GAGAGCAACAAGAGCTCAAATTCCCAGAGCCCGTCTCCTCCAAGCAGCTCCAATGC
CTTCAGTCTCCTGAGCAGCTCCGAGCAAGACCACCCATCCACATCCGGTTGCAGCAG
TGAGCAGTCGATGAAGGCCAAGACACAGAAGGCCCTGCTCAAGACACTCAAGGACT
TGAAGGCCCGGCTGCCGCCTGAGCGACGCGTCAAGGGCAAGTCGTCAACGCTGGCG
ACGCTGCAGTACGCGGTCTCGTGCATCAAGCAGATTCGAGCCAACGAGGAGTACTA
CCAGCTGTGGACGGTGGATGATGGGAAAACGGCAACGCTCGACATCGGCACCTATA
CCATGGAGGAGCTGGAGACGATCACCTCTGAGTACACACCCAAAAGCACCGACACT
TTTGCGATGGTGGTGTCGCTCATCTCGGGCCGCATGATGTTCTCGTCGGAGCACACA
GCCTGGACGCTCGGCTGTCGAAAGGAGCTGCTGTCCGATGTGAAGTTCGTGGAGAT
GCTGGCGCCACAGGACGTGAGCGTCTTCTACAGCGCCACGGCGCCGGGGAAGCTCC
ACCATGGGGCAGCATGCACGAGGCCGCTGCTGCTGCATTTGAATATCCCCAGGAGA
AGCCGTTCTTTTGCCGAATCCGGGGGGGCGGCAGTAAGGACGAGGCGGTGTACCTG
CCGTTCCGGCTCACCCCGTACCTCATGAAGGTTCGCGAGGGCGAGGACGGCGCCGA

Table S4.1 (cont'd)

CGCGGGTGGAGAGCCATGCTGCTTGGTGCTTGCTGAGCGCGTGCACTCGGGCTACGA
AGCGCCACGCATCCCCCCGGAGAAGCGAATCTTCACGACGACGCACACGCCGGGCT
GCGTCTTCCTGGACGTGGACGAGAGGGCGATCCCCCTGCTGGGATACCTGCCCCAG
GACCTAGTGGGGACCCCCATGCTGACGTACATCCACCCCAACGACCGGCACCTGAT
GCTCGCCGTGCACAAGAAGATCTTGAAGTTTGTGGGGCAGCCATTCGACCACTCCCC
ATTCCGCTTCTGCTCGTACAACGGGGACTATGTGACCATCGATACGAGCTGGTCGAG
CTTCGTAAACCCGTGGAGCCGCAAGGTGGCCTTCATCATCGGCCGCCACAAAGTCCG
CACAGGCCCCCTCAACGAGGACGTGTTTGCGAGCCCCGCGGCCGCGGTGGTTGGCG
AAGTGGAGCCGCGGGCTCCGGACTCGGACGTCGCTGACATCCAGGAGAGGATCCAC
AAACTCCTGCTGCAGCCTGTGCACAATAATGGCTCGAGCGGCTACGGGAGCCTCAT
GGGCAGCAACGGGCATGACTCGCACGGGCACCGGCTGAGCCTCGCTTCCTCCAGCG
AGGGCACCGGCGCTGCACCTGATGACGGCCACGCCGACAAACCCATGTCATTCGCC
CAGATCTGCGTGGACGTGCAGCAGCTGACGAACCCCGGGCGGCAGCAGCAGCAGCG
CTTCCCGGAGCCCCTGGGCCGGCAGCAGCAGCAGCAGCTGGGAGCGCATGTGGGAG
ATCAAGGCCGGATGGTGCTGCCCAAGGCCGTGCTGGAGATGGGCCAAGAATGCCCG
CGGGCCGGACGTAAGCGCTCAACCAGAGCCGTCACGGATGATACCGGCAAGAAGG
AGCAGACGGCCATGCCGTCCTACCAGCAGATCAACTGCCTCTACTCCATCATACGAT
ACCTGGAGAGCTGCAACGTGCCCAAGGCGGAGAAGCGCAAGTGCCCGGCCTTGTGC
GAGGCCTCCTCCTCAACCTCGGACGACGAGAAGCCGGACGTGGCCGCACAGGATCC
GAGCGGAGCCGAGATGGACCCGGAAGCAGTGATGGAGGCGGAGCCCGAGATGGCG
GTGGAGCCCGTGGAGCCGGTGGCGCCGCCCGCCATGGCGACGCCGCCGCCGCCTCC
GCCGCCGCTGGCCCCGCTGCCACTGCCCCTGAGCAAGGCGGAGAGCGCCATGTCGG
TGACGAGTCAGTGCAGCTACAGCAGCACCATCGTTCACGTGGGCGACCGCGACAAA
AAGAACCAGCCGCCCGACTCCGAAATCGCAGCTCTGGAGGACACGCCCAACATGGG
GGGCGGAGACCCTCCCGAGATGCCCGGCAAGGGGGGCACGGCCCCCCCACCGGAG
AAGGAGCGCGAGAGGGAGGAGTACAAGAAGGTGGGCCTCACCAAGGAGGTGCTGT
CGGCCCACACGCTCAAGGAGGAGCAGACGTACATCCAGCGCTTCCTGGCGCGCGAC
GGCACCACCAACTTCTGGGCGTTCGGCTCCCCGCACCGCTTTTGCCTACCGAGCCAA
CACCGCGGCCGCTCACGCGACAGAGTGGTGGCGCAGTCGCCGCGTGTTAAGAAGGA
TGCCAGCGAAGAGGGGAACAACAACGACACAATGTCCAATTCCAGCGACCTCCTGG
ACCTGCTGCTGCAGGAGGACTCCCACTCGGGCACGGACTCGGCCGCCTCGGGCTCG
GGGTCCTCGGGCTCGGGGTCGTCGGGTTCGCGCTCGCTCGGCTCCGCGGGCTCGGGC
GGCTCCAACGCCTGCGACACGTCGCGCAGCGGCACAGGCAGCACCAGCAACACCAG
CCAGTACTTTGCAAGCAATGACTCATCGGAGGTTGACCAGAAAGAGCGCAAGGAGA
GGGAGGAGGACAACAAGTTCAAGCAGCTGTTGCTGCAGGACCCCATCTGGCTGCTC
ATGGCCAACACCAACGAGTCAGTCATGATGACCTATCAGCTGCCCTCCCGGGACGT
GGAGACCGTTCTCAAGGAGGACCGCGAGAAGCTCAAGGAGATGCAGAAGCTGCAG
CCTCGCTTCACGGAAGCACAGCGCCGGGAGCTGTGTGAGGTGCACCCATGGCTCAA
GAAAGGAGGGCTCCCCGTGGCTATCAACATCCAGGTGTGTGAGTGCGGCAGCACCA
CGAGCACCAAGGCCTCGGCCCCGTTTGACGTGGAGACGCACGAGATGGACGATGGT
GGGATGCTCGAACTAGGCGAGGAGGGCTCGGGCGTGGCCCAGCAGGCCGGCACGGT

Table S4.1 (cont'd)

GGCACCCCAGCCCATGCTGCTGCCCCCGCCGCCGCCAGCTCCGTTTGGCGGCACCGT
TTCTGCGGCGATGCGTGCGGCCCACAAGACCGCGCAGGCCTCTGAGGGCGTTGCCA
TGGAGACGGGGGTGTTGAGGAGGGACTGGCAGCAGCGAGGACAAATTGCGTCACCG
GCGAAACGAGCGCAGCAGCAGCAGTCGGAGGCATCCTCTGAATTTGCGCGGCCCTC
AGCGTGCAAGTAG

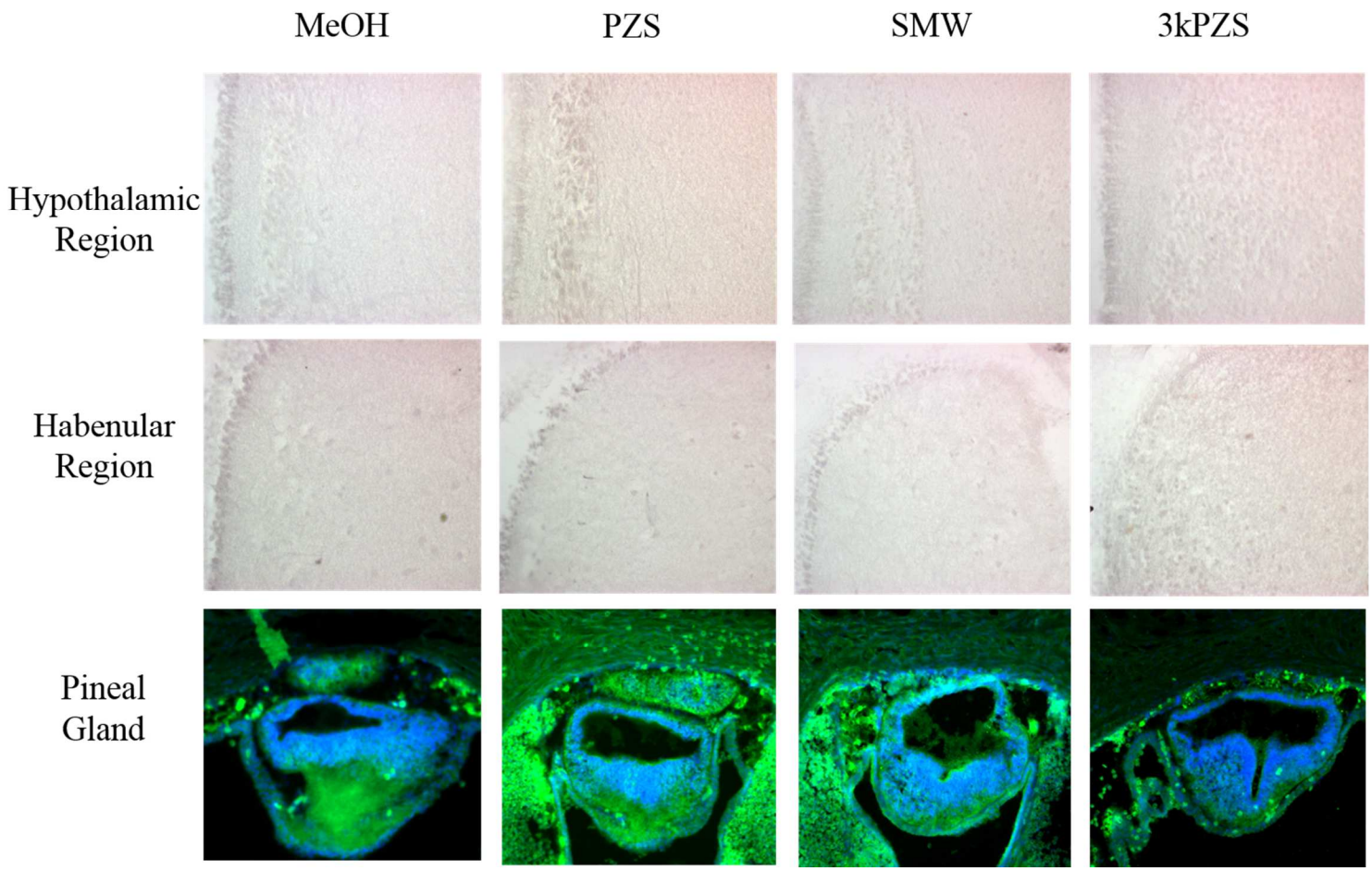

Figure S4.1: Representative normal goat serum controls for habenular, hypothalamic, and pineal gland regions of pre-ovulated females treated for 24 h with spermiated male washings (SMW containing equivalent amount of 10^{-10} M 3kPZS), 10^{-10} M 3kPZS, 10^{-10} M PZS, or the vehicle methanol control (MeOH) at 10x magnification. Hypothalamic and habenular region samples treated with DAB and additional nickel. Pineal gland samples stained with Alexafluor 488 anti-rabbit IgG (green) and DAPI (blue).

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