ABSTRACT

CAMP, ALLISON ANN. A Neural Sensor of Environmental Stimuli and its Disruption by Neuroactive Chemicals in the Freshwater Microcrustacean *Daphnia pulex*. (Under the direction of Dr. Gerald A. LeBlanc).

Environmental sex determination is a phenomenon in which environmental cues govern offspring sex. The means by which critical stimuli are sensed, transduced, and translated into endocrine outcomes to influence offspring sex remains poorly understood. We used the model genus *Daphnia* spp. to examine a candidate neural target that may serve to connect sensory systems to endocrine cascades.

We hypothesized that an upstream neural sensor of environmental stimuli initiates changes in the terminal hormone pathway leading to male sex determination in daphnids. We additionally hypothesized that this processor is subject to disruption by neuroactive exogenous chemicals. To approach these questions, we established the environmental stimuli required by our model organism to reliably induce male sex determination. Next, we utilized male sex determination assays to evaluate neuroactive compounds to establish a candidate neural processor. This neural processor and male sex determining genes were further examined through the measurement of mRNA levels under varying environmental conditions and neuroactive chemical exposure.

We first hypothesized that photoperiod and temperature co-regulate male sex determination in *Daphnia magna* and *D. pulex*. We assessed a short (10:14 hr Light:Dark) and long (16:8 hr L:D) day photoperiod and temperatures 16, 18, 20, and 22°C. Short day photoperiod rendered both species susceptible to male sex determination and temperature further modulated the magnitude of male production in a species-specific manner. We additionally hypothesized that permissive conditions would activate elements of the male sex determination signaling pathway. mRNA levels were examined for *JHAMT*, *FAMT*, *Met*, and *SRC.* A

permissive photoperiod significantly increased *Met* mRNA levels. Additionally, mRNA levels of these gene products fluctuated temporally.

Next, we investigated a candidate neural sensor of environmental cues, the N-methyl-D-aspartate receptor (NMDAR). NMDAR-targeting chemicals were screened to examine its involvement in male sex determination for *D. pulex*. The antidepressant drugs MK-801 and desipramine significantly increased male production under permissive conditions. Subsequently, we probed the extent to which non-permissive and permissive conditions impacted mRNA levels for NMDAR subunits, *JHAMT*, *FAMT*, *Met*, and *SRC*. *NMDAR-b*, *Met*, and *SRC* mRNA levels were increased under permissive conditions.

Additionally, we tested other antidepressant pharmaceuticals to further define the neurological control of male sex determination. We found that drugs targeting serotonin and norepinephrine (octopamine in invertebrates) signaling stimulated male offspring production. Further, these compounds elevated elements relating to serotonin and octopamine transmission indicative of increased signaling by these neurotransmitters. Antidepressant efficacy has been linked to impacts on NMDAR function, therefore within that context, these results are congruent with our findings with NMDAR antagonists.

Finally, we investigated whether exposure to MK-801 influenced NMDAR subunits and male sex determining genes by assessing mRNA levels under permissive and non-permissive photoperiods. MK-801 lowered mRNA levels for *NMDAR-b*, *Met*, and *SRC* under a permissive photoperiod, while the opposite was true under non-permissive photoperiod. These results indicate that expression of *NMDAR-b*, *Met*, and *SRC* may be co-regulated. Results also corroborate previous observations that MK-801 likely stimulates male sex determination through a process independent of photoperiodic regulation.

Overall, we demonstrate the importance of photoperiod and temperature in the co-regulation of male sex determination for *D. pulex* and *D. magna*, and advance our understanding of how environmental cues impact neuroendocrine pathways in daphnids. We also provide evidence that glutamate signaling through the NMDAR is involved in male sex determining processes, while demonstrating the need for further research to clarify how chemicals such as MK-801 exert their effects on sex ratios. Overall, we provide compelling evidence for the role of the NMDAR in the conversion of environmental cues to the activation of the methyl farnesoate signaling pathway that results in male sex determination in daphnids.

A Neural Sensor of Environmental Stimuli and its Disruption by Neuroactive Chemicals in the Freshwater Microcrustacean *Daphnia pulex*.

by
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APPROVED BY:

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DEDICATION

This dissertation is dedicated to all the small animals of the world, that have no voice in matters of pollution or destruction of the environment.

BIOGRAPHY

Allison Camp, a west coast native, spent the first 18 years of her life in the small, quaint town of Walla Walla, Washington. Afforded a very large backyard with a pond, creek, and hundreds of trees, Allison spent most of her childhood outside exploring the yard while collecting various critters and specimens. She was known to bring her variety of nature treasures into the house, and at any given time her parents would find shed snake skin, bones, egg shells, rocks, feathers, owl pellets, leaves, and acorns in her bedroom. These formative years created an indelible love of nature that would follow her throughout her life.

In school, Allison was always an enthusiastic student, especially while in science classes. She was also lucky enough to have wonderful science teachers along her path, including a very encouraging 7th grade science teacher named Mrs. Parrish, a bubbly AP Biology teacher named Mrs. Swant, and a quirky physics teacher named Mr. Ahrens. Upon entering college, Allison pursued science further, entering the Neurobiology and Psychology programs at University of Washington. In college Allison also discovered her love of running, which became an important social and energetic outlet for her through college and beyond.

After graduating from University of Washington, Allison began working in the ecotoxicology program and NOAA's Northwest Fisheries Science Center. There she experienced applied neurobiology in the context of toxicology, while working with fish and insects. Simultaneously, she began an internship at Washington Toxics Coalition, a non-profit aimed to reduce human exposure to harmful chemicals by conducting research and lobbying lawmakers. These experiences opened up Allison's eyes to the world of toxicology and she decided to pursue graduate school.

Allison made a cross-country move in order to begin her doctoral program at NC State in August of 2012. She had little idea of what would be in store for her over the next six years, and there were many hard years, personally and professionally. There were also gleaming bright moments, including meeting her now husband and fellow toxicologist AtLee Watson, running her personal best in the marathon (3:03:43), starting a small business to honor her love of nature, and building a new life and a new group of friends in North Carolina.

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I would like to specially thank Dr. LeBlanc, who welcomed me into his lab at a time when we were both uncertain I would make it through the program. I am eternally grateful for his support, kindness, and willingness to take a chance on me.

I would also like to thank current members of the lab including Stephanie Eytcheson, Stephanie Street, Jeonga Yun, as well as past members of the LeBlanc lab including Elizabeth Medlock, Gwuijun Kwon, Charisse Holmes, past and present visiting scholars, and a whole host of undergraduates.

Special thanks to my family, who supported me throughout my doctoral program and asked far too often when I would be finished. Special thanks to friends, who encouraged me relentlessly even through my darkest times. Special thanks to my running friends, who couldn't care less about toxicology, but listened to my graduate school stories nonetheless.

Special thank you to my husband, AtLee Watson, who has been by my side as my biggest cheerleader in all aspects of life. Also, to Brownie and Fudgie who bring us endless laughter.

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CHAPTER 1

INTRODUCTION

Environmental Sex Determination

Over evolutionary time, two broad means of reproduction have formed. Asexual reproduction, where offspring are clonally produced by one individual, and sexual reproduction which requires two different sexes in order to produce offspring. While asexual reproduction has the capacity to yield more offspring than sexual reproduction, opportunities for genetic diversification are limited (Uyenoyama, 1984). Sexual reproduction on the other hand, benefits from genetic recombination, which can contribute to purging deleterious mutations from the population while also increasing prevalence of useful mutations (Butlin, 2002). The benefits of genetic reproduction are robust enough that most animal groups have both male and female sexes and utilize sexual reproduction.

In order to have both male and female sexes within a population, there must be a means by which sex is determined. The two major strategies for sex determination are genetic sex determination (GSD) and environmental sex determination (ESD). GSD dictates offspring sex through the transfer of genes that govern offspring sex. For example, in humans the XX and XY genotypes result in female and male offspring, respectively. In this sex determining method, the Y male chromosome carries genes that are critical for the development of the male morphology. In some groups, the females possess the heterogametic pair of sex chromosomes (Bull, 1985).

Alternatively, in environmental sex determination (ESD), the sex of offspring is influenced by external, abiotic cues. This phenomenon can occur in a wide range of taxa including annelids (Korpelainen, 1990), arthropods (Korpelainen, 1990), reptiles (Ciofi and Swingland, 1997), and fish (Devlin and Nagahama, 2002; F. W. H. Beamish, 1993). There are clear benefits of ESD for

certain groups of animals. Charnov and Bull (1977) proposed that the ESD is most beneficial for organisms in which males and females best thrive at different environmental conditions. Sex of offspring might be determined by temperature such that females are produced at temperatures suited to female neonatal survival; while, males are produced at temperatures suited to male neonatal survival.

Depending on the taxonomic group, different abiotic cues influence programming into male or female offpsring. In turtles and crocodilian groups, temperature is the most informative abiotic cue in ESD (temperature sex determination, TSD); however, groups respond differently to increasing temperature as it relates to ultimate sex ratios (i.e. positive, negative, bell-shaped) (Ciofi and Swingland, 1997; Lang and Andrews, 1994). Similarly, fish species that are subject to ESD are also commonly sensitive to temperature (Luckenbach et al., 2009). Additional abiotic cues such as density, nutritional status, pH, photoperiod, are associated with ESD (Korpelainen, 1990).

The means by which ESD transpires at the molecular level differs between groups. In flatfish such as flounder, temperature and potentially stress have been shown to influence the expression of the cytochrome P450 enzyme *cyp19a1* (aromatase), which metabolizes testosterone to 17β-estradiol, an estrogen responsible for promoting ovarian development and female phenotypic traits (Devlin and Nagahama, 2002; Luckenbach et al., 2009). While the molecular mechanisms linking temperature to *cyp19a1a* expression are unknown, the *cyp19a1a* gene possesses several upstream response elements suggesting that environmental conditions may increase or decrease transcription factor levels that control the expression of *cyp19a1a*. Further, there is evidence that epigenetic programming may influence *cyp19a1a* expression in fish (Luckenbach et al., 2009). In crocodilians subject to TSD, there is a critical window of gonadal differentiation during which temperature conditions influence sex hormone levels, thus

influencing sex (Lang and Andrews, 1994). In all of these examples of organizational events early in development, the precise mechanism by which temperature regulates critical enzyme levels remains unknown.

Susceptibility of ESD to Chemical Contaminants

ESD involves the programming of offspring sex during a critical window of development via precise regulation of sex steroid hormone levels. Exposure to exogenous chemicals in the environment during this critical window can disrupt normal ESD processes. There are many means by which exogenous chemicals can disrupt critical hormone levels, including mimicking the endogenous hormone, increasing or decreasing activity of enzymes in the metabolic cascade that produces sex-determining hormones, or acting as an antagonist at the hormone receptor site. Disruptions may result in a manipulation of offspring sex and subsequently alter sex ratios within the population. Endocrine disruption may also result in organizational effects in an organism that may result in intersex individuals or other abnormal phenotypes at the cellular or gross levels (Guillette Jr. et al., 1995).

There are many examples of exogenous chemicals impacting animals with ESD. One notable example is the population decline of American alligators in Lake Apopka, FL. A spill of wastewater containing dicofol and DDT metabolites, which both exhibit estrogenic activity, occurred in the lake and resulted in Superfund site designation (Guillette et al., 1994). Male alligators in the lake after the spill displayed gross phenotypic abnormalities (de-masculinization) as well as altered hormone levels that lowered reproductive success and ultimately led to a sharp population decline following exposure (Guillette et al., 1994; Guillette et al., 1999). Further studies with American alligators found that topical application of estrogenic chemicals to eggs incubated at male-inducing temperatures resulted in female offspring, thus

demonstrating the ability of estrogenic chemicals to impact ESD in the animals (Guillette Jr. et al., 1995). A similar example has been noted in the fish *Castostomus commersoni* (white sucker) in Colorado downstream of a waste water treatment plant releasing estrogenic effluent. The white sucker population showed skewed sex ratios, incidence of intersex, reduced sperm counts in males, and altered ovarian development in exposed individuals (Vajda, Alan et al., 2008).

Another example of chemical contaminants interfering with ESD can be found within crustaceans. Some members of the insecticide class termed insect growth regulators (IGRs) mimic juvenile hormone, an insect hormone responsible for maturation and reproductive processes within insects (Nijhout and Williams, 1974). This hormone mediates the transition from larval to adult forms in insects, making it an appealing pesticide target for insects with destructive adult forms. Further, juvenile hormone plays a role in reproduction once insects have reached maturity (Jindra et al., 2013). IGRs can perturb endocrine function in non-target invertebrate species such as the crustacean *Daphnia* spp. In daphnids, IGRs mimic the juvenile hormone analog methyl farnesoate, the hormone responsible for male sex determination of offspring (Olmstead and LeBlanc, 2003). IGR insecticides such as pyriproxyfen and methoprene can induce male offspring under environmental conditions where asexual reproduction is expected (Olmstead and LeBlanc, 2003). *In vitro* molecular studies have demonstrated that IGRs directly interact with the hormone receptor in daphnids, confirming their action as a hormone mimic (Medlock Kakaley et al., 2017).

Hypothesis

A defining feature of ESD is that environmental signals must be sensed and transduced into a molecular signaling event to affect sex determination. It follows that a neuroendocrine connection (i.e. a neural processor) exists to link sensory systems to hormonal signaling cascades. Overall, we hypothesized that an upstream neural processor of environmental signals initiates the

cascade leading to environmental sex determination. Further, this processor is subject to disruption by neuroactive exogenous chemicals.

Daphnia as a Model to Study ESD

To test this hypothesis, we used the model aquatic invertebrate *Daphnia pulex*, which is a small freshwater crustacean used in many research fields including ecology and functional genomics (Colbourne et al., 2011). In freshwater lentic environments, zooplankton such as *Daphnia* spp. are keystone invertebrates. Daphnids control algae and bacteria levels, while also serving as prey for both other invertebrates (e.g. insects) and vertebrates (e.g. fish) (Lampert, 2006). Daphnids are also widely used in toxicity testing as a model invertebrate due to their sensitivity to chemical perturbation and amenability to laboratory experiments (rapid generation time, translucent bodies, low cost husbandry) (U.S. EPA, 2002). Moreover, daphnids are subject to ESD and environmental cues trigger the production of male offspring. Despite their widespread use as a test organism, the factors contributing to their ESD, marked by a transition from asexual to sexual reproduction, have not been well established. Further, their fully sequenced genome and elucidated hormonal cascades makes *Daphnia pulex* an ideal model to elucidate the neuroendocrine mechanisms responsible for ESD, and how exposure to exogenous chemicals may dysregulate different components of the pathways therein.

Daphnids reproduce via asexual parthenogenetic reproduction under conditions that are favorable for rapid population growth (e.g. summer) to create a population of clonal females (Hebert, 1978). As environmental signals change to suggest the onset of adverse conditions, daphnids introduce males into the environment, thus increasing male:female sex ratios. Once males are present, sexual reproduction can occur to produce diapausing fertilized eggs that are resistant to harsh conditions (e.g. desiccation or freezing) (Hebert, 1978). Several environmental

cues have been implicated in daphnid male sex determination including photoperiod (Korpelainen, 1986; Stross, 1969a; Toyota et al., 2015), temperature (Brown and Banta, 1932; Korpelainen, 1986), crowding (Hobek and Larsson, 1990; Kleiven et al., 1992; Olmstead and LeBlanc, 2001; Stross, 1969b), and food availability (Kleiven et al., 1992; Zhang and Baer, 2000); however, it is unclear which cues are most influential in initiating male offspring or whether cues function in an interactive manner. Varying methods, combinations of cues, and different test species have made unequivocal conclusions difficult to draw from these studies.

The endocrine pathway responsible for male sex determination in crustaceans has been largely elucidated. The enzymes juvenile hormone acid o-methyltransferase (JHAMT) and farnesoic acid o-methyltransferase (FAMT) contribute to the conversion of farnesoic acid to methyl farnesoate (Xie et al., 2016). Methyl farnesoate is a sesquiterpenoid hormone equivalent to juvenile hormone in insects (Laufer and Biggers, 2001). In crustaceans, methyl farnesoate controls sexual maturation and morphological changes within immature offspring (Laufer and Biggers, 2001). Once produced, methyl farnesoate associates with the protein methoprene-tolerant (Met) which then heterodimerizes with steroid receptor co-activator (SRC) (Kakaley et al., 2017). Together Met and SRC comprise the methyl farnesoate receptor (MfR) complex. The MfR acts as a transcription factor and initiates downstream expression of hemoglobin (*hb2*) and male sex determining genes such as the doublesex gene (Kato et al., 2011; Rider et al., 2005).

Methyl farnesoate is required for male offspring production by programming developing oocytes into males (male sex determination) (Lampert et al., 2012; Olmstead and LeBlanc, 2002). The critical window for methyl farnesoate to program oocytes occurs between 60-72 hours of the molt cycle within the ovary of *D. magna*. Further, the developing oocytes are very sensitive to methyl farnesoate, requiring as little as 30 nM exposure concentration to elicit male production,

and 400 nM to elicit 100% male production (Olmstead and LeBlanc, 2002). Chemicals that mimic methyl farnesoate, such as the IGR pyriproxyfen have been shown to significantly induce male sex determination in daphnids (80% male offspring) at 0.3 nM (Olmstead and LeBlanc, 2003). Thus, exposure to IGRs could result in inappropriate seasonal timing of male offspring and have the potential to disrupt population dynamics. While the key components of the terminal hormone cascade have been elucidated, the neuroendocrine connection has not been established. One promising linkage involves an ionotropic glutamate receptor, the N-methyl-D-aspartate receptor (NMDAR), which has been found to be differentially expressed in daphnids producing male versus female offspring (Toyota et al., 2015).

Evidence for the NMDAR as a Neuroendocrine Link

The NMDAR is ionotropic glutamate receptor composed of four peptide subunits each with a transmembrane domain, ligand binding domain, and an amino terminal domain. The NMDAR is a coincident detector within the nervous system, requiring the neurotransmitter glutamate, a depolarized membrane to remove a Mg^{2+} block, and a cofactor (e.g. glycine) for the receptor pore to open (Rousseaux, 2008). In vertebrates, there are three classes of peptide subunits, NR1, NR2, and NR3 (Rousseaux, 2008). The NR2 family contains the glutamate binding site within the ligand binding domain, and NR1 subunits contain the cofactor binding site (Traynelis et al., 2010). The amino terminal domain contains both positive and negative allosteric modulator binding sites (Hackos and Hanson, 2016; Monaghan et al., 2012; Zhu et al., 2016). Calcium ions are responsible for most NMDAR effects on the post-synaptic cell as calcium activates several downstream cellular processes, such as neuronal nitric oxide synthase (nNOS) activation to produce nitric oxide (NO), a potent signaling molecule (Cossenza et al., 2014).

The NMDAR is an important link between sensory systems and neuroendocrine pathways in mammals. For instance, in the neural circuity linking photoperiod cues to circadian centers in the vertebrate nervous system, phototransduction by the retina activates the retinohypothalamic tract and relays photoperiod information to the suprachiasmatic nuclei in the brain (Tosini et al., 2014). Glutamate signaling and NMDARs play a critical role at this junction and influence alterations in light-related biological rhythms (Ding et al., 1994; Tosini et al., 2014). Additionally, glutamate signaling through the NMDAR plays a critical role in reproduction, regulating melatonin levels in the pineal gland to influence gonadal function of seasonally reproducing mammals, such as hamsters (Colwell et al., 1991). Further, estrogen receptors and NMDARs within the vertebrate brain localize on the same neurons within regions relating to hormone regulation (e.g. hypothalamus) (Kia et al., 2002). It has also been established that glutamate-NO signaling via the NMDAR is involved with a variety of neuroendocrine processes such as the release of gonadotropin releasing hormone, luteinizing hormone releasing hormone, as well as the timing and onset of puberty (Bhat et al., 1998; Dhandapani and Brann, 2000).

NMDARs were first characterized in invertebrates with studies conducted in crayfish (Pfeiffer-Linn and Glantz, 1991) and their role in environmental stimuli integration has been demonstrated in a range of invertebrates. For instance, NMDARs are associated with learning and memory processes in *Drosophila* (Xia et al., 2005), *Apis mellifera* (Si et al., 2004), *C. elegans* (Kano et al., 2008; Mellem et al., 2002), and *Alpysia* (Ezzeddine and Glanzman, 2003). In *D. pulex*, there are two identified subtypes of NMDAR subunit, a and b, which are similar in coding sequence to *Drosophila* NMDAR subunit sequences, and equivalent to vertebrate subtypes NR1 and NR2, respectively (McCoole et al., 2012). There is evidence that the NMDAR is involved in the sensing of environmental stimuli in *D. pulex*. Specifically, environmental conditions that

stimulate male offspring production have been associated with increased expression of genes relating to ionotropic glutamate receptors (Toyota et al., 2015). Further, NMDAR function is related to reproductive processes in other invertebrate groups. In both crickets and butterflies, blocking NMDAR signaling decreased reproductive output (Geister et al., 2008). In the flesh fly, reducing NMDAR signaling reduced vitellogenin production, a protein associated with oocyte development and maturation (Begum et al., 2004). In cockroaches, NMDARs have been associated with mediating juvenile hormone synthesis in mature females (Chiang et al., 2002). Although NMDAR function has not been well studied in invertebrates for its potential role in connecting sensory systems and endocrine function, its role in synaptic plasticity and dynamic processes such as seasonal reproduction makes it a strong candidate for involvement in ESD.

NMDAR in Relation to Mood Disorders

Beyond sensory transduction, the role of glutamate and NMDARs has been pursued as a possible driver of mood disorders, such as major depression. Classic models of depression implicate the dysregulation of small monoamine signaling molecules (e.g. dopamine, serotonin, norepinephrine) (Schildkraut, 1965) and as a result traditional depression treatments target monoamine pathways. However, one disadvantage associated with these therapeutics is the multiple-week delay in symptom alleviation (Heninger et al., 1996). A landmark study using the NMDAR antagonist ketamine found that administration of this drug rapidly ameliorated symptoms of depression, even in patients resistant to treatment with monoamine-targeting therapies, suggesting that glutamate dysregulation may be a key element of depressive mood disorders (Berman et al., 2000; Zarate et al., 2006). Additionally, a growing body of literature connects stress to increased glutamate signaling (Musazzi et al., 2011). Together, this established a link connecting stress and subsequent dysregulation of glutamate signaling as likely initiating events in the

pathophysiology of depression (Musazzi et al., 2011). This newer model of depression is termed the neuroplasticity hypothesis.

Evidence in support of the neuroplasticity hypothesis has revealed many associations between glutamate signaling and depression (Tokita et al., 2012). For instance, administering NMDAR antagonists has been found to reduce depressive behavior in rodent studies (Koike et al., 2011; Kordjazy et al., 2016; Neis et al., 2015; Rosa et al., 2003; Yang et al., 2016), and patients with depression have been found to have elevated levels of plasma glutamate (Mitani et al., 2006). There are no therapeutic drugs on the market that specifically target glutamatergic systems for the treatment of depression, and ketamine is not a viable agent due to its dissociative psychoactive effects in humans (Sanacora et al., 2012). Thus, monoamine-targeting antidepressants continue to be the treatment of choice and are widely prescribed. There is evidence that these drugs may dampen glutamatergic signaling by downregulating glutamate receptor subunit expression (Boyer et al., 1998; Sanacora et al., 2012). The connection between depression, glutamatergic signaling, and antidepressant chemicals raises questions about whether these pharmaceuticals may impact neuroendocrine connections in animals that utilize the NMDAR to link neural and endocrine signaling pathways.

Research Outline

We hypothesized that the environmental cues of temperature and photoperiod function together, in the invertebrate *Daphnia pulex,* to dictate the sex of offspring, and that differences in gene expression within the terminal endocrine cascade would be present in animals exposed to male sex determining conditions. Next, we hypothesized that an upstream neural processor of environmental signals initiates the hormonal cascade leading to male sex determination in *D. pulex*. Further, we hypothesized that this processor is subject to disruption by neuroactive

exogenous chemicals (Fig. 1). These overall hypotheses will be addressed in the following chapters.

Chapter 2 addresses the hypothesis that a combination of both temperature and photoperiod are required to initiate male sex determination in *D. pulex*. To test the relative importance of these abiotic cues, we tested combinations of long day (16:8 L:D) or short day (10:14 L:D) photoperiods at either 16, 18, 20 or 22°C on the incidence of male sex determination. Assays were also conducted with the sympatric species *D. magna* for comparison. We also examined mRNA levels in animals under environmental conditions that stimulated either the production of males or females to assess whether male-stimulating conditions altered expression of key elements of the putative male sex determination endocrine signaling cascade.

In Chapter 3, with the environmental conditions required to reliably initiate male production in *D. pulex* established in Chapter 2, we assessed whether the NMDAR was a viable candidate neural processor of environmental signals. We tested a variety of NMDAR agonists, antagonists, and modulators to determine their effect on male production in male sex determination assays. Pharmaceuticals that stimulated male sex determination (antidepressants) were further evaluated using Bioluminescence Resonance Energy Transfer (BRET) to determine whether they were stimulating male sex determination by interacting directly with the terminal hormone receptor (MfR). We also evaluated whether active pharmaceuticals functioned as surrogates of either environmental stimuli, photoperiod and temperature. Finally, we evaluated mRNA levels of the NMDAR subunits and genes involved in the hormonal induction of male sex determination under, environmental conditions that stimulated female or male sex determination, to further delineate the role of the NMDAR in this pathway.

We hypothesized in Chapter 4 that pharmaceuticals, with different molecular targets, might serve useful to further define the neurological pathway that transduces environmental cues to endocrine control of male sex determination in *D. pulex*. Based on these findings, we assessed whether relative gene expression of specific receptors and transporters targeted by the pharmaceuticals were influenced by environmental cues for male sex determination.

Finally, in Chapter 5 we utilized MK-801, an antidepressant drug, to assess whether exposure to this chemical altered mRNA levels associated with NMDAR function and the putative male-sex determining cascade.

Taken together, we elucidate the role of temperature and photoperiod in male sex determination for two species and contributing important information regarding the expression and timing of key signaling elements within the terminal hormone cascade for *D. pulex*. We additionally reveal that the NMDAR is integral for neuroendocrine processes and have found evidence for its involvement in daphnid male sex determination. Further, we highlight the potential of other neuroactive chemicals such as antidepressants to impact neuroendocrine processes. Overall, our results have established foundational knowledge of the neuroendocrine connections that link sensory systems to endocrine cascades leading to male sex determination within daphnids and demonstrate its susceptibility to neuroactive exogenous chemicals.

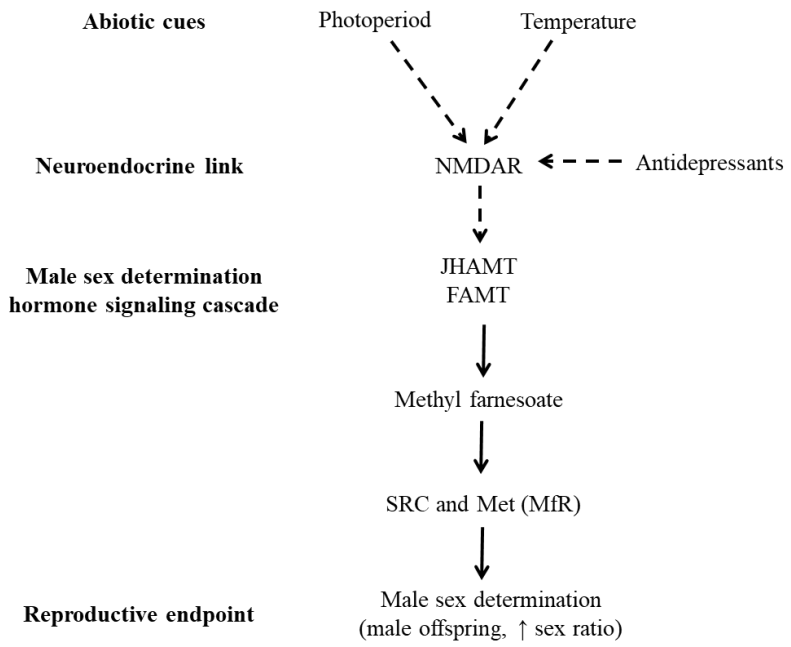

Figure 1: Hypothesized neuro-endocrine male sex determination pathway of *D. pulex* evaluated in the research presented here. Solid lines represent known aspects of the signaling cascade. Dotted lines represent hypothesized connections.

REFERENCES

Begum, M., Breuer, M., Kodrik, D., Rahman, M. and De Loof, A. (2004). The NMDA receptor antagonist MK-801 inhibits vitellogenesis in the flesh fly *Neobellieria bullata* and in the desert locust *Schistocerca gregaria*. *J Insect Physiol* **50**, 927–934.

Berman, R., Cappiello, A. and Anand, A. (2000). Antidepressant effects of ketamine in depressed patients. *Biol. Psychiatry* **3223**,.

Bhat, G. K., Mahesh, V. B., Ping, L., Chorich, L., Wiedmeier, V. T. and Brann, D. W. (1998). Opioid-glutamate-nitric oxide connection in the regulation of luteinizing hormone secretion in the rat. *Endocrinology* **139**, 955–960.

Boyer, P.-A., Skolnick, P. and Fossom, L. (1998). Chronic administration of imipramine and citalopram alters the expression of NMDA receptor subunit mRNAs in mouse brain. *J. Mol. Neurosci.* **10**, 219–233.

Brown, L. A. and Banta, A. M. (1932). Sex control in Cladocera. VII. Male production in relation to temperature. *Physiol. Zool.* **5**, 218–229.

Bull, J. J. (1985). Sex determining mechanisms: An evolutionary perspective. *Experientia* **41**, 1285–1296.

Butlin, R. (2002). The costs and benefits of sex: new insights from old asexual lineages. *Nat. Rev. Genet.* **3**, 311–317.

Charnov, E. L. and Bull, J. J. (1977). When is sex environmentally determined? *Nature* **267**, 192–193.

Chiang, A., Pszczolkowski, M., Liu, H. and Lin, S. (2002). Ionotropic glutamate receptors mediate juvenile hormone synthesis in the cockroach, *Diploptera punctata*. *Insect Biochem. Mol. Biol.* **32**, 669–78.

Ciofi, C. and Swingland, I. R. (1997). Environmental sex determination in reptiles. *Appl. Anim. Behav. Sci.* **51**, 251–265.

Colbourne, J. K., Pfrender, M. E., Gilbert, D., Thomas, W. K., Tucker, A., Oakley, T. H., Tokishita, S., Aerts, A., Arnold, G. J., Basu, M. K., et al. (2011). The ecoresponsive genome of *Daphnia pulex*. *Science (80-.).* **331**, 555–561.

Colwell, C. S., Max, M., Hudson, D. and Menaker, M. (1991). Excitatory amino acid receptors may mediate the effects of light on the reproductive system of the golden hamster. *Biol.Reprod.* **44**, 604–608.

Cossenza, M., Socodato, R., Portugal, C. C., Domith, I. C. L., Gladulich, L. F. H., Encarnação, T. G., Calaza, K. C., Mendonça, H. R., Campello-Costa, P. and Paes-de-Carvalho, R. (2014). *Nitric Oxide in the Nervous System: Biochemical, Developmental, and Neurobiological Aspects*.

Devlin, R. H. and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* **208**, 191–364.

Dhandapani, K. M. and Brann, D. W. (2000). The role of glutamate and nitric oxide in the reproductive neuroendocrine system. *Biochem. cell Biol.* **78**, 165–79.

Ding, J. M., Chen, D., Weber, E. T., Faiman, L. E., Rea, M. A. and Gillette, M. U. (1994). Resetting the biological clock: Mediation of nocturnal circadian shifts by glutamate and NO. *Science (80-.).* **266**, 1713–1717.

Ezzeddine, Y. and Glanzman, D. L. (2003). Prolonged habituation of the gill-withdrawal reflex in *Aplysia* depends on protein synthesis, protein phosphatase activity, and postsynaptic glutamate receptors. *J. Neurosci.* **23**, 9585–9594.

F. W. H. Beamish (1993). Environmental Sex Determination in Southern Brook Lamprey, *Ichthyomyzon gagei*. *Can. J. Fish. Aquat. Sci.* **50**, 1299–1307.

Geister, T. L., Lorenz, M. W., Hoffmann, K. H. and Fischer, K. (2008). Effects of the NMDA receptor antagonist MK-801 on female reproduction and juvenile hormone biosynthesis in the cricket *Gryllus bimaculatus* and the butterfly *Bicyclus anynana*. *J. Exp. Biol.* **211**, 1587–93.

Guillette, L. J., Gross, T. S., Masson, G. R., Matter, J. M., Percival, H. F. and Woodward, A. R. (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ. Health Perspect.* **102**, 680–688.

Guillette, L. J., Brock, J. W., Rooney, A. A. and Woodward, A. R. (1999). Serum concentrations of various environmental contaminants and their relationship to sex steroid concentrations and phallus size in juvenile American alligators. *Arch. Environ. Contam. Toxicol.* **36**, 447–455.

Guillette Jr., L. J., Crain, D. A., Rooney, A. A. and Pickford, D. B. (1995). Organization verses activation: The role of endocrine disrupting contaminants (EDCs) during embryonic development in wildlife. *Environ. Health Perspect.* **103**, 1164–1457.

Hackos, D. H. and Hanson, J. E. (2016). Diverse modes of NMDA receptor positive allosteric modulation: Mechanisms and consequences. *Neuropharmacology* **112**, 34–45.

Hebert, P. D. N. (1978). The population biology of *Daphnia* (Crustacea, Daphnidae). *Biol. Rev.* **53**, 387–426.

Heninger, G. R., Delgado, F. I. and Charney, D. (1996). The revised monoamine theory of depression: a modulatory role for monoamines based on new findings from monoamine

depletion experiments. *Pharmacopsychiatry* **29**, 2–11.

Hobek, A. and Larsson, P. (1990). Sex determination in *Daphnia magna*. *Ecology* **71**, 2255–2268.

Jindra, M., Palli, S. R. and Riddiford, L. M. (2013). The Juvenile Hormone Signaling Pathway in Insect Development. *Annu. Rev. Entomol.* **58**, 181–204.

Kakaley, E. K. M., Wang, H. Y. and LeBlanc, G. A. (2017). Agonist-mediated assembly of the crustacean methyl farnesoate receptor. *Sci. Rep.* **7**, 45071.

Kano, T., Brockie, P. J., Sassa, T., Fujimoto, H., Kawahara, Y., Iino, Y., Mellem, J. E., Madsen, D. M., Hosono, R. and Maricq, A. V. (2008). Memory in Caenorhabditis elegans is mediated by NMDA-type ionotropic glutamate receptors. *Curr. Biol.* **18**, 1010–1015.

Kato, Y., Kobayashi, K., Watanabe, H. and Iguchi, T. (2011). Environmental sex determination in the branchiopod crustacean *Daphnia magna*: Deep conservation of a Doublesex gene in the sex-determining pathway. *PLoS Genet.* **7**,.

Kia, H. K., Yen, G., Krebs, C. J. and Pfaff, D. W. (2002). Colocalization of estrogen receptor alpha and NMDA-2D mRNAs in amygdaloid and hypothalamic nuclei of the mouse brain. *Mol. Brain Res.* **104**, 47–54.

Kleiven, O. T., Larsson, P. and Hobek, A. (1992). Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* **65**, 197–206.

Koike, H., Iijima, M. and Chaki, S. (2011). Involvement of AMPA receptor in both the rapid and sustained antidepressant-like effects of ketamine in animal models of depression. *Behav. Brain Res.* **224**, 107–111.

Kordjazy, N., Haj-Mirzaian, A., Amiri, S., Ostadhadi, S., Amini-Khoei, H. and Dehpour, A. R. (2016). Involvement of N-methyl-D-aspartate receptors in the antidepressant-like effect

of 5-hydroxytryptamine 3 antagonists in mouse forced swimming test and tail suspension test. *Pharmacol. Biochem. Behav.* **141**, 1–9.

Korpelainen, H. (1986). The effects of temperature and photoperiod on life history parameters of *Daphnia magna* (Crustacea: Cladocera). *Freshw. Biol.* **16**, 615–620.

Korpelainen, H. (1990). Sex ratios and conditions required for environmental sex determination in animals. *Biol. Rev. Camb. Philos. Soc.* **65**, 147–184.

Lampert, W. (2006). *Daphnia*: Model herbivore, predator and prey. *Polish J. Ecol.* **54**, 607–620.

Lampert, W., Lampert, K. P. and Larsson, P. (2012). Induction of male production in clones of *Daphnia pulex* by the juvenoid hormone methyl farnesoate under short photoperiod. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* **156**, 130–133.

Lang, J. W. and Andrews, H. V (1994). Temperature-dependent sex determination in crocodilians. *J. Exp. Zool.* **270**, 28–44.

Laufer, H. and Biggers, W. J. (2001). Unifying concepts learned from methyl farnesoate for invertebrate reproduction and post-embryonic development. *Am. Zool.* **41**, 442–457.

Luckenbach, J., Borski, R., Daniels, H. and Godwin, J. (2009). Sex determination in flatfishes: Mechanisms and environmental influences. *Semin. Cell Dev. Biol.* **20**, 256–263.

McCoole, M. D., D'Andrea, B. T., Baer, K. N. and Christie, A. E. (2012). Genomic analyses of gas (nitric oxide and carbon monoxide) and small molecule transmitter (acetylcholine, glutamate and GABA) signaling systems in *Daphnia pulex*. *Comp. Biochem. Physiol. - Part D Genomics Proteomics* **7**, 124–160.

Medlock Kakaley, E. K., Eytcheson, S. A. and LeBlanc, G. A. (2017). Ligand-mediated receptor assembly as an end point for high-throughput chemical toxicity screening. *Environ.*

Sci. Technol. **51**, 9327–9333.

Mellem, J., Brockie, P., Zheng, Y., Madsen, D. and Maricq, A. (2002). Decoding of polymodal sensory stimuli by postsynaptic glutamate receptors in *C. elegans*. *Neuron* **36**, 933–944.

Mitani, H., Shirayama, Y., Yamada, T., Maeda, K., Ashby, C. R. and Kawahara, R. (2006). Correlation between plasma levels of glutamate, alanine and serine with severity of depression. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* **30**, 1155–1158.

Monaghan, D. T., Irvine, M. W., Costa, B. M., Fang, G. and Jane, D. E. (2012). Pharmacological modulation of NMDA receptor activity and the advent of negative and positive allosteric modulators. *Neurochem. Int.* **61**, 581–592.

Musazzi, L., Racagni, G. and Popoli, M. (2011). Stress, glucocorticoids and glutamate release: Effects of antidepressant drugs. *Neurochem. Int.* **59**, 138–149.

Neis, V. B., Moretti, M., Manosso, L. M., Lopes, M. W., Leal, R. B. and Rodrigues, A. L. S. (2015). Agmatine enhances antidepressant potency of MK-801 and conventional antidepressants in mice. *Pharmacol. Biochem. Behav.* **130**, 9–14.

Nijhout, H. F. and Williams, C. M. (1974). Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last instar larva and the decision to pupate. *J. Exp. Biol.* **61**, 481–491.

Olmstead, A. and LeBlanc, G. (2001). Temporal and quantitative changes in sexual reproductive cycling of the cladoceran *Daphnia magna* by a juvenile hormone analog. *J. Exp. Zool.* **290**, 148–155.

Olmstead, A. W. and LeBlanc, G. A. (2002). Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. *J. Exp. Zool.* **293**, 736–739.

Olmstead, A. W. and LeBlanc, G. A. (2003). Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. *Environ. Health Perspect.* **111**, 919–924.

Pfeiffer-Linn, C. and Glantz, R. M. (1991). An arthropod NMDA receptor. *Synapse* **9**, 35–42.

Rider, C. V, Gorr, T. A., Olmstead, A. W., Wasilak, B. A. and LeBlanc, G. A. (2005). Stress signaling: coregulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean. *J. Exp. Biol.* **208**, 15–23.

Rosa, A. O., Lin, J., Calixto, J. B., Santos, A. R. S. and Rodrigues, A. L. S. (2003). Involvement of NMDA receptors and L-arginine-nitric oxide pathway in the antidepressant-like effects of zinc in mice. *Behav. Brain Res.* **144**, 87–93.

Rousseaux, C. G. (2008). A Review of Glutamate Receptors I: Current Understanding of Their Biology. *J. Toxicol. Pathol.* **21**, 25–51.

Sanacora, G., Treccani, G. and Popoli, M. (2012). Towards a glutamate hypothesis of depression: An emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology* **62**, 63–77.

Schildkraut, J. (1965). The catecholamine hypothesis of affective disorders. *J. Neuropsychiatry Clin. Neurosci.* **7**, 524–533.

Si, A., Helliwell, P. and Maleszka, R. (2004). Effects of NMDA receptor antagonists on olfactory learning and memory in the honeybee (*Apis mellifera*). *Pharmacol. Biochem. Behav.* **77**, 191–197.

Stross, R. G. (1969a). Photoperiod Control of Diapause in *Daphnia*. II. Induction of Winter Diapause in the Arctic. *Biol. Bull.* **136**, 264–273.

Stross, R. G. (1969b). Photoperiod control of diapause in *Daphnia*. III. Two-stimulus control of

long-day, short-day induction. *Biol. Bull.* **137**, 359–374.

Tokita, K., Yamaji, T. and Hashimoto, K. (2012). Roles of glutamate signaling in preclinical and/or mechanistic models of depression. *Pharmacol. Biochem. Behav.* **100**, 688–704.

Tosini, G., Iuvone, P. M., McMahon, D. G. and Collin, S. P. eds. (2014). The Retina and Circadian Rhythms. 27–48.

Toyota, K., Miyakawa, H., Yamaguchi, K., Shigenobu, S., Ogino, Y., Tatarazako, N., Miyagawa, S. and Iguchi, T. (2015). NMDA receptor activation upstream of methyl farnesoate signaling for short day-induced male offspring production in the water flea, *Daphnia pulex*. *BMC Genomics* **16**, 186.

Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Odgen, K. K., Hansen, K. B., Yuan, H., Myers, S. J. and Dingledine, R. (2010). Glutamate receptor ion channels: structure, regulation and function. *Pharmacol. Rev.* **62**, 405–496.

U.S. EPA (2002). *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*.

Uyenoyama, M. K. (1984). On the Evolution of Parthenogenesis: A genetic representation of the "cost of meiosis." *Evolution (N. Y).* **38**, 87–102.

Vajda, Alan, M., Barber, L. B., Gray, J. L., Lopez, E. M., Woodling, J. D. and Norris, D. O. (2008). Reproductive disruption in fish downstream from an estrogenic wastewater effluent. *Environ. Sci. Technol.* **42**, 3407–3414.

Xia, S., Miyashita, T., Fu, T.-F., Lin, W.-Y., Wu, C.-L., Pyzocha, L., Lin, I.-R., Saitoe, M., Tully, T. and Chiang, A.-S. (2005). NMDA receptors mediate olfactory learning and memory in *Drosophila*. *Curr. Biol.* **15**, 603–15.

Xie, X., Tao, T., Liu, M., Zhou, Y., Liu, Z. and Zhu, D. (2016). The potential role of juvenile

hormone acid methyltransferase in methyl farnesoate (MF) biosynthesis in the swimming crab, *Portunus trituberculatus*. *Anim. Reprod. Sci.* **168**, 40–49.

Yang, B., Ren, Q., Ma, M., Chen, Q. X. and Hashimoto, K. (2016). Antidepressant effects of (+)-MK-801 and (-)-MK-801 in the social defeat stress model. *Int. J. Neuropsychopharmacol.* **19**, 1–5.

Zarate, C. A., Singh, J. B., Carlson, P. J., Brutsche, N. E., Ameli, R., Luckenbaugh, D. A., Charney, D. S. and Manji, H. K. (2006). A Randomized Trial of an N-methyl-D-aspartate Antagonist in Treatment-Resistant Major Depression. *Arch. Gen. Psychiatry* **63**, 856–864.

Zhang, L. and Baer, K. . (2000). The influence of feeding, photoperiod and selected solvents on the reproductive strategies of the water flea, *Daphnia magna*. *Environ. Pollut.* **110**, 425–430.

Zhu, S., Stein, R. A., Yoshioka, C., Lee, C. H., Goehring, A., McHaourab, H. S. and Gouaux, E. (2016). Mechanism of NMDA Receptor Inhibition and Activation. *Cell* **165**, 704–714.

CHAPTER 2

Complementary Roles of Photoperiod and Temperature in Environmental Sex Determination in *Daphnia* spp (Crustacea: Cladocera)

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ABSTRACT

Zooplankton communities are fundamental to the success of freshwater lentic habitats and groups such as *Daphnia* spp. are considered a keystone genus. Daphnids exhibit a unique reproductive strategy (environmental sex determination) wherein environmental conditions dictate sex of the offspring and whether they reproduce asexually or sexually. The introduction of males into a population denotes the first step in the switch from asexual parthenogenetic reproduction to sexual reproduction. We tested the hypothesis that photoperiod and temperature function together to regulate male sex determination and that these environmental stimuli would activate elements of the male sex determination signaling cascade. Results showed that photoperiod was a critical cue in creating permissive conditions for male production. Further, under photoperiod-induced permissive conditions, male sex determination was temperature dependent. The two daphnid species tested, *Daphnia. pulex* and *D. magna,* exhibited different temperature dependencies with *D. pulex* producing a lower percentage of male offspring with increasing temperatures between 16-22°C. In contrast, *D. magna* produced a higher percentage of males with increasing temperatures. We found consistent patterns of gene expression within the molt cycle of *D. pulex* for genes within the male sex determination signaling cascade independent of environmental stimuli. For juvenile hormone acid o-methyltransferase (JHAMT), an enzyme responsible for the synthesis of the sex-determining hormone methyl farnesoate, mRNA levels were elevated early in the reproductive cycle. Subsequently, mRNA levels of the methyl farnesoate receptor subunits, *Met* and *SRC*, were elevated. Environmental conditions conducive to male offspring production increased *Met* mRNA levels. Results indicate that male sex determination in daphnids is under the permissive control of photoperiod and the regulatory control of temperature. Further, these

environmental cues may stimulate male sex determination by increasing levels of the *Met* subunit of the methyl farnesoate receptor.

INTRODUCTION

Zooplankton such as *Daphnia* spp. are keystone invertebrates in freshwater lentic environments. Daphnids graze on algae and bacteria, while serving as prey for other invertebrates and vertebrates (Lampert, 2006). Daphnids are also widely used in a variety of scientific fields including toxicology, ecology, and functional genomics due to their global distribution in both permanent and transient water bodies, sensitivity to environmental perturbation, responsiveness to environmental cues, and amenability to laboratory experiments (Colbourne et al., 2011; Gillooly and Dodson, 2000; U.S. EPA, 2002).

Daphnids are subject to environmental sex determination in that environmental cues influence the sex of their offspring. Most daphnid species reproduce via cyclic parthenogenesis, and under environmental conditions favorable for rapid population growth, daphnids reproduce asexually creating a clonal population of females (Hebert, 1978; Hobek and Larsson, 1990). As environmental cues signal the onset of unfavorable conditions, male offspring are introduced into the population, denoting the beginning of the sexual reproduction cycle. Sexual reproduction ultimately results in the production of fertilized diapausing eggs (resting eggs) that are resistant to harsh conditions (e.g. desiccation or freezing) (Hebert, 1978). Resting eggs can remain dormant for decades, and resume development once environmental conditions are favorable (Brendonck and De Meester, 2003; Schwartz and Hebert, 1987; Stross, 1966).

In daphnids, photoperiod is critical for egg hatching in the spring (Dupuis and Hann, 2009; Stross, 1966; Vandekerkhove et al., 2005), and has also been shown to influence male production

(Korpelainen, 1986; Stross, 1969a; Toyota et al., 2015a; Zhang and Baer, 2000). Other cues such as temperature (Brown and Banta, 1932; Korpelainen, 1986) and crowding (Banta and Brown, 1929; Hobek and Larsson, 1990; Kleiven et al., 1992; Olmstead and LeBlanc, 2001) have also been shown to influence both male offspring and resting egg production. Many studies have used resting egg production as an indicator of male production, however the mechanism by which resting egg development occurs in unknown and separate from that of male production (i.e. daphnids can produce broods of male offspring without producing diapausing eggs). Despite numerous studies, the critical environmental cues to initiate male sex determination have remained equivocal.

Methyl farnesoate is the crustacean hormone responsible for programming oocytes into male offspring (LeBlanc and Medlock, 2015; Olmstead and LeBlanc, 2002; Toyota et al., 2015b). Methyl farnesoate is a sesquiterpenoid hormone produced by the enzymes farnesoic acid o-methyltransferase (FAMT) and juvenile hormone acid o-methyltransferase (JHAMT) (Xie et al., 2016). Methyl farnesoate activates the methyl farnesoate receptor (MfR) which is a heterodimer of the proteins methoprene-tolerant (Met) and steroid receptor co-activator (SRC) (Kakaley et al., 2017; Toyota et al., 2015b). The activated MfR functions as a transcription factor to regulate expression of male sex determining genes and other genes including hemoglobin genes (Fig. 1A) (Rider et al., 2005).

We hypothesized that both photoperiod and temperature are required to initiate male sex determination in *D. pulex* and *D. magna*. Further, we hypothesized that exposure to a combination of environmental cues that elicited male production would increase expression in the key genes of the male sex determination signaling endocrine cascade in *D. pulex*.

MATERIALS AND METHODS

Male sex determination

D. pulex (clone WTN6) and *D. magna* (clone NCSU1) cultures were maintained in the laboratory at 20°C, 16:8 Light:Dark (L:D) using methods described previously (Hannas et al., 2010). Under these conditions, both species reproduce parthenogenetically. Daphnids were collected from culture as < 24 hour old neonates and transferred to their respective experimental conditions: short photoperiod (10:14 L:D) or long photoperiod (16:8 L:D) and either 16, 18, 20, 22°C. Neonates were individually reared in 50 mL beakers containing 40 mL culture media, which consists of reconstituted deionized water (192 mg/L $CaSO_4 \cdot H_2O$, 192 mg/L $NaHCO_3$, 120 mg/L $MgSO_4$, 8.0 mg/L KCl, 1.0 µg/L selenium and 1.0 µg/L vitamin B_{12}). Daphnids were fed daily 100 µL *Pseudokirchneriella subcapitata* suspension (1.4 x 10^8 cells) and 50 µl Tetrafin® fish food suspension (Pet International, Blacksburg, VA, USA) prepared as described previously (Hannas et al., 2010). All treatment combinations were replicated with ten individual daphnids. Media was changed every other day. Mature daphnids were monitored daily for brood release and offspring were removed on the day observed, counted, and sex determined by the length of the first antennae (Fig. 1B) (Olmstead and LeBlanc, 2000). Six broods of offspring were collected per organism.

MfR signaling pathway mRNA levels

D. pulex (≤ 24 hours old) were reared in either long or short photoperiod, at 18°C using methods described above. Once daphnids reached sexual maturity (eggs in brood chamber), all animals were molt-synchronized such that animals could be collected at 0, 24, 36, and 48 hours post-molt. Four replicates of 3-5 daphnids were collected per time point. Daphnids from individual replicates were transferred to 100 µL RNAlater® and held at 4°C for 24 hours, then stored at -

80°C until used for RNA extraction. Whole animals were homogenized using Next Advance Bullet Blender® and zirconium oxide beads (1.0mm diameter, Next Advance, Troy, NY). RNA isolation was conducted using the SV Total RNA Isolation System (Promega, Madison, WI) according to manufacturer recommendations. Synthesis of cDNA was conducted using ImProm-II™ Reverse Transcription System with oligo (dT) primers (Promega, Madison, WI).

mRNA levels of *JHAMT*, *FAMT*, *Met*, and *SRC* were measured by RT-qPCR. Primer sequences for *JHAMT* (Miyakawa et al., 2010), *FAMT* (Toyota et al., 2015b), *Met* (Miyakawa et al., 2010), and *SRC* (Toyota et al., 2015b) were used to amplify mRNA sequences. RT-qPCR was performed with the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) using 2x SYBR™ Green Premix (Fisher Scientific, Hampton, NH). A single melting peak was detected for each sample with an amplification efficiency of >96%, indicating amplification occurred only for the target sequence. Genex software (BioRad, Hercules, CA) was used to analyze relative levels of gene expression by normalizing to two housekeeping genes, *actin* and *GAPDH*.

Data Analyses

Male sex determination was calculated as mean percentage male offspring for ten individuals for six consecutive broods. Differences in male offspring production were assessed with One-Way ANOVA with Tukey's adjustment for multiple comparisons for *D. pulex* under short photoperiod. Remaining comparisons of male offspring production were assessed with the Kruskal-Wallis test with Dunn's post-hoc multiple comparisons test due to significantly different variances. Differences in fecundity between daphnids reared under long and short photoperiods were evaluated using Student's t-tests. For gene expression experiments, differences in relative expression at each time point were analyzed using Student's t-tests. Gene expression was

normalized to long photoperiod control group at time 0. Error bars denote SEM. The alpha level was set at 0.05 for all analyses. Statistical analyses were performed with Prism (v7.02, GraphPad Software, Inc).

RESULTS

Male Sex determination

We hypothesized that requisite photoperiodic and temperature cues regulate male sex determination in *D. pulex* and *D. magna*. *D. pulex* did not produce male offspring under a long day photoperiod at any temperature evaluated (Fig. 2A). In contrast, under a short day photoperiod, *D. pulex* produced male offspring at all temperatures with the percentage of males significantly increasing with decreasing temperature (Fig. 2B). The increase in the percentage of male offspring under the short day photoperiod was not an artifact associated with a decrease in total offspring produced. Rather, total offspring produced was significantly higher under short photoperiod conditions for most temperatures assessed (Fig. 3).

Similar experiments were performed with the often sympatric species *D. magna* (Östman, 2011; U.S. EPA, 2002) to determine whether this complementary effect of photoperiod and temperature on male sex determination was species-specific. Again, male offspring were not produced at any temperature evaluated under a long day photoperiod (Fig. 4A), but males were produced at 18 to 22°C under the short day photoperiod (Fig. 4B). In contrast to *D. pulex*, the proportion of male offspring increased with increasing temperature (Fig. 4B). Further, the total number of neonates produced decreased with increasing temperature under a short day photoperiod. This trend was not evident under the long day photoperiod. The number of neonates

produced under the long day photoperiod was comparable to or significantly higher than under the short photoperiod (Fig. 5).

To summarize, a short day photoperiod rendered both *D. pulex* and *D. magna* susceptible to temperature-dependent male sex determination. However, optimum temperature for male sex determination varied between species. At temperatures between 16 and 22°C, the proportion of male offspring produced decreased with increasing temperature for *D. pulex*, while the opposite occurred with *D. magna*.

Environmental activation of the methyl farnesoate signaling pathway

We have previously shown that the hormone methyl farnesoate stimulates male sex determination in daphnids (Olmstead and LeBlanc, 2001; Rider et al., 2005), though it is unknown whether environmental cues utilize this signaling pathway in orchestrating male sex determination. *D. pulex* were reared at 18°C under a long day, non-permissive photoperiod and a short day, permissive photoperiod. mRNA levels were measured for *JHAMT* and *FAMT* – the enzymes responsible for producing methyl farnesoate, along with *Met* and *SRC*, the subunits of the methyl farnesoate receptor.

mRNA levels within the methyl farnesoate signaling cascade varied over the course of the molt/reproductive cycle. *JHAMT* mRNA levels were elevated at 24 hours post-molt among daphnids reared under both long and short day photoperiods (Fig. 6A). *JHAMT* mRNA levels were significantly higher in the long day photoperiod group at this time (Fig. 6A). *FAMT* mRNA levels remained unchanged under both photoperiods throughout the time course (Fig. 6B). *Met* mRNA levels also were elevated at 24 hours and progressively declined thereafter. *Met* mRNA levels were significantly higher under the short day photoperiod at 0 and 24 hours post-molt (Fig. 6C). *SRC*

attained maximum mRNA levels at 24 hours post-molt under both photoperiods with levels progressively declining thereafter. No significant differences in *SRC* mRNA levels were observed between photoperiods throughout the time course. (Fig. 6D).

To summarize, *JHAMT*, *Met* and *SRC* mRNA levels were elevated early in the reproductive cycle; while, *FAMT* mRNA levels were relatively constant throughout the cycle. Photoperiod had no effect on *FAMT* or *SRC* mRNA level. The short day photoperiod significantly increased *Met* mRNA levels. Photoperiod had mixed effects on *JHAMT* mRNA levels.

DISCUSSION

We hypothesized that both photoperiod and temperature are required to initiate male sex determination in *D. pulex* and *D. magna*. Our findings revealed that both photoperiod and temperature requirements must be met for these species to produce male offspring. While, both species require a short-day photoperiod to produce male offspring, temperature requirement differed between species. Among the temperatures evaluated, the maximum percentage of male offspring produced by *D. pulex* occurred at 16°C, while, maximum percentage produced by *D. magna* occurred at 22°C.

Day length is a powerful cue for animals that are seasonally reproductive or are subject to environmental sex determination (Korpelainen, 1990; Reiter, 1993). Photoperiod influences many processes related to reproduction in daphnids, such as the production of resting eggs (Carvalho and Hughes, 1983; Stross, 1966; Stross, 1969a; Stross, 1969b; Stross and Hill, 1968), and their hatching (Schwartz and Hebert, 1987; Vandekerkhove et al., 2005). Previous studies have implicated photoperiod in male sex determination (Kleiven et al., 1992; Korpelainen, 1986; Toyota et al., 2015a; Toyota et al., 2017) and in other closely related processes in crustaceans such as

molting (Chang and Mykles, 2011). The permissive photoperiod used here, 10:14 hr L:D, occurs in temperate and artic regions and both *D. pulex* and *D. magna* are both distributed in these climates (Crease et al., 2012; Ferrari and Hebert, 1982; Mitchell and Lampert, 2000).

Unlike photoperiod, temperature modulated male sex determination in a species-specific manner. Our results suggest that the geographic origins of the organisms used in the study may have been sufficiently different that the confluence of photoperiod and temperature for temporally optimum male sex determination significantly varies between the species. These results imply a species difference in responsiveness to temperature. However, we cannot exclude the possibility that clones of the same species derived from geographically distinct regions may also exhibit significant differences in temperature responsiveness.

Our temporal assessment of mRNA levels within the methyl farnesoate signaling pathway revealed that *JHAMT* mRNA levels were maximally expressed at 24 hrs post-molt under both photoperiods. These results suggest that methyl farnesoate levels also increase early in the reproductive cycle. The early expression of *JHAMT* is consistent with observations of Toyota et al. (2015). However, these investigators observed that the increase in *JHAMT* mRNA occurred only under the short day photoperiod. We observed that *JHAMT* mRNA levels were actually lower under the short-day photoperiod at 24 hrs post molt. This difference in studies may reflect differences in time points analyzed. Toyota et al. (2015) noted significantly elevated *JHAMT* mRNA levels under a short day photoperiod at 30 hrs post-ovulation. We noted elevated but significantly lower levels under the short day photoperiod, as compared to the long day photoperiod, at 24 hrs post-molt. At 36 hrs, levels were no longer elevated under either photoperiod in our study, though levels were now significantly elevated under the short-day

photoperiod as compared to the long day photoperiod. We may have missed the peak in *JHAMT* mRNA levels under short day photoperiod that was noted by Toyota et al (2015).

Met mRNA levels were elevated under the permissive short day photoperiod, as compared to the nonpermissive long day photoperiod, early in the time course. The Met protein is in the beta-helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) family of transcriptional regulators and is conserved among insects and crustaceans (Li et al., 2011; Miyakawa et al., 2014). In daphnids, methyl farnesoate binds to Met which then recruits the protein SRC forming an active transcription factor (Kakaley et al. 2017). *Met* mRNA levels have been shown previously in daphnids to increase early in the reproductive cycle prior to the sensitive window of oocyte sex programming (Kakaley et al., 2017). The pattern of expression we see here in *D. pulex* agrees with these previously findings, wherein *Met* maximum mRNA levels are attained early in the reproductive cycle. Further, we observed a significant increase in *Met* mRNA levels at both 0 and 24 hours post-molt under a permissive photoperiod which may reflect an increased sensitivity to methyl farnesoate under these conditions in anticipation of oocyte programming.

The general timing and trends in gene expression observed within the reproductive cycle of *D. pulex* reared under both long and short photoperiod are consistent with the putative male sex determination signaling pathway (Fig. 1A). Expression of *JHAMT*, one of the genes responsible for producing methyl farnesoate, showed highest expression levels early in the molt/reproductive cycle (0-24 hours), likely reflecting preparation for oocyte programming for the subsequent brood of offspring. The lack of oscillation in *FAMT* expression is consistent with findings from other researchers (Toyota et al., 2015b) and suggests that JHAMT is primarily responsible for the induction of methyl farnesoate synthesis. The MfR subunits Met and SRC displayed increases in mRNA levels over a broad time range. These general trends in mRNA levels were observed under

both permissive and non-permissive photoperiods, suggesting that regardless of environmental cues, key signaling elements are expressed in an oscillatory manner throughout the reproductive cycle and are likely involved in aspects of reproductive maturation other than sex determination.

Taken together, our results established the importance of photoperiod in male sex determination in *D. pulex* and *D. magna*. Further, we identified species-specific differences in the magnitude of male production in relation to temperature, shedding insights on phenological differences between species or between clones and their response to thermal cues. We also identified photoperiod-independent trends in gene expression along the male sex determination signaling cascade. Finally, we discovered that the photoperiodic cue that renders daphnids responsive to the stimulatory action of temperature on male sex determination causes an elevation in *Met* mRNA levels. A subsequent elevation in Met protein levels may be a determining factor in male sex determination.

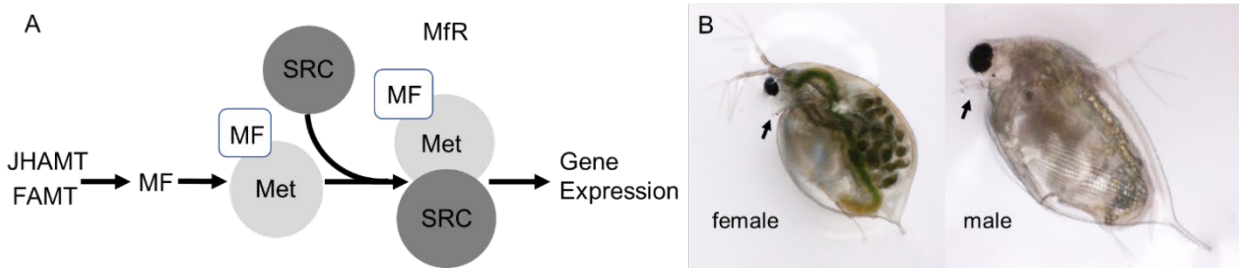

Figure 1: Endocrine cascade controlling male sex determination in daphnids. Panel A: The putative signaling cascade leading to male sex determination. The enzymes JHAMT and FAMT contribute to MF synthesis. MF associates with the transcription factor Met and stimulates its recruitment of SRC. Together these proteins comprise the methyl farnesoate receptor (MfR) complex. The activated MfR initiates downstream expression of male sex determining genes. Panel B: Morphological differences in the 1st antennae of female and male *D. pulex*. JHAMT: juvenile hormone acid o-methyltransferase, FAMT: farnesoic acid o-methyltransferase, MF: methyl farnesoate, Met: methoprene-tolerant, SRC: steroid receptor co-activator.

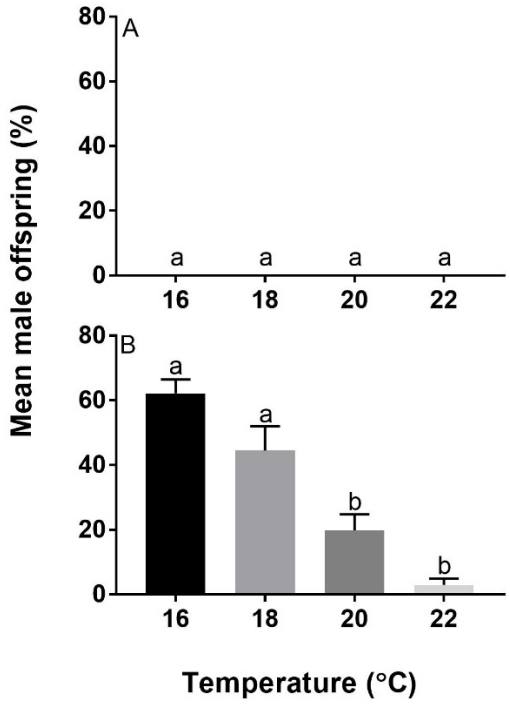

Figure 2: Mean percentage male offspring produced by *D. pulex* under long day (16:8 hrs L:D) and short day (10:14 hrs L:D) photoperiods across temperatures 16-22°C. Panel A: Male offspring production under a long day photoperiod. Panel B: Male offspring production under a short day photoperiod. Data are presented as means and standard error, n=8-10. Different letters denote significant differences among treatments ($p \leq 0.05$).

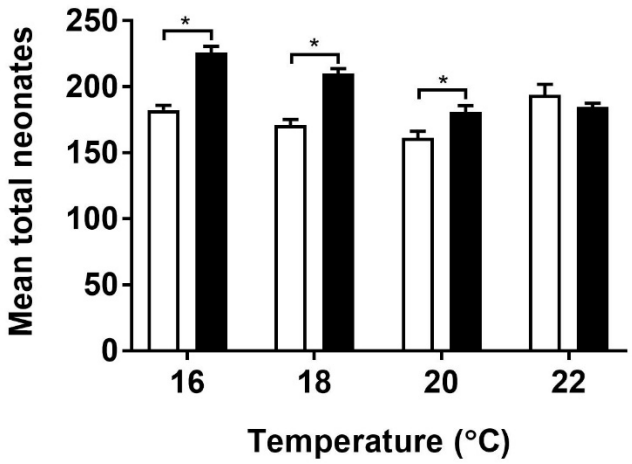

Figure 3: Fecundity of *D. pulex* under conditions permissive and non-permissive of male production across temperatures. White bars denote the non-permissive long day photoperiod (16:8 hrs L:D) and black bars denote short day photoperiod (10:14 hrs L:D). Fecundity was calculated as the mean and SEM total neonate production over 6 broods of offspring (n=8-10). An asterisk denotes a significant difference between treatments ($p \leq 0.05$).

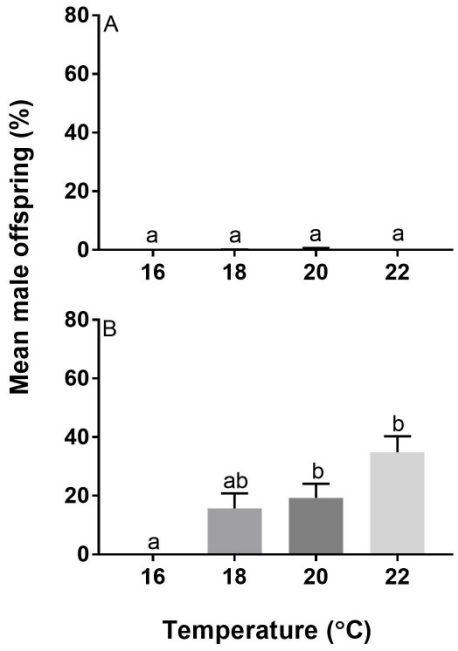

Figure 4: Mean percentage male offspring produced by *D. magna* under long day (16:8 hrs L:D) and short day (10:14 hrs L:D) photoperiods across temperatures 16-22°C. Panel A: Male offspring production under a long day photoperiod. Panel B: Male offspring production under a short day photoperiod. Data are presented as means and standard error, n=7-10. Different letters denote significant differences among treatments.

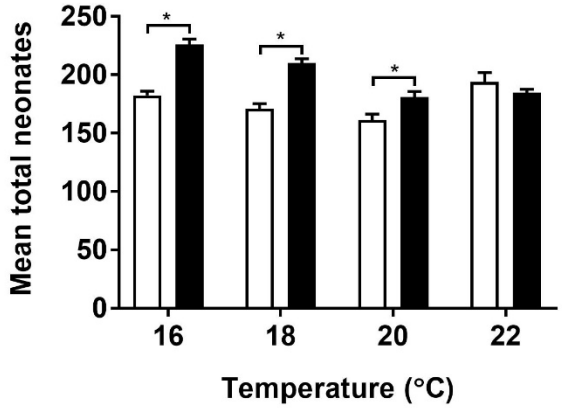

Figure 5: Fecundity of *D. magna* under conditions permissive and non-permissive of male production across temperatures. White bars denote the non-permissive long day photoperiod (16:8 hrs L:D) and black bars denote short day photoperiod (10:14 hrs L:D). Fecundity was calculated as the mean and SEM total neonate production over 6 broods of offspring (n=7-10). An asterisk denotes a significant difference between treatments.

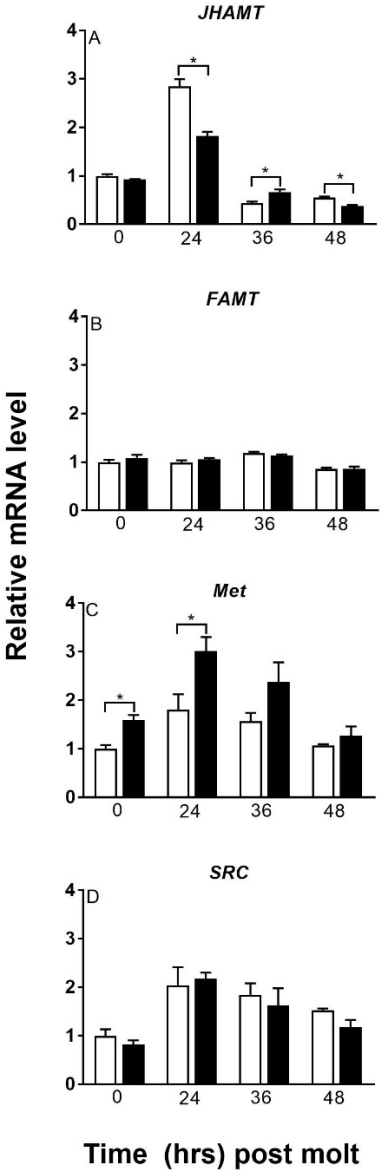

Figure 6: mRNA expression levels of *JHAMT*, *FAMT*, *Met*, and *SRC* under long and short day photoperiods at 18°C. White bars denote the long day photoperiod (16:8 hrs L:D) and black bars denote the short day photoperiod (10:14 L:D). Panel A: JHAMT. Panel B: FAMT. Panel C: Met. Panel D: SRC. mRNA levels, respectively. Panels C and D show mRNA levels for *Met* and *SRC*, respectively. Data are presented as mean and SEM mRNA levels normalized to respective levels under the long day photoperiod at time 0 (n=4). An asterisk denotes a significant difference between treatments.

REFERENCES

Banta, A. M. and Brown, L. A. (1929). Control of sex in Cladocera. I. Crowding the mothers as a means of controlling male production. *Physiol. Zool.* **2**, 80–92.

Brendonck, L. and De Meester, L. (2003). Egg banks in freshwater zooplankton: Evolutionary and ecological archives in the sediment. *Hydrobiologia* **491**, 65–84.

Brown, L. A. and Banta, A. M. (1932). Sex control in Cladocera. VII. Male production in relation to temperature. *Physiol. Zool.* **5**, 218–229.

Carvalho, G. R. and Hughes, R. N. (1983). The effect of food availability, female culture-density and photoperiod on ephippia production in *Daphnia magna Straus* (Crustacea: Cladocera). *Freshw. Biol.* **13**, 37–46.

Chang, E. S. and Mykles, D. L. (2011). Regulation of crustacean molting: A review and our perspectives. *Gen. Comp. Endocrinol.* **172**, 323–330.

Colbourne, J. K., Pfrender, M. E., Gilbert, D., Thomas, W. K., Tucker, A., Oakley, T. H., Tokishita, S., Aerts, A., Arnold, G. J., Basu, M. K., et al. (2011). The ecoresponsive genome of *Daphnia pulex*. *Science.* **331**, 555–561.

Crease, T. J., Omilian, A. R., Costanzo, K. S. and Taylor, D. J. (2012). Transcontinental Phylogeography of the *Daphnia pulex* Species Complex. *PLoS One* **7**,.

Dupuis, A. P. and Hann, B. J. (2009). Climate change, diapause termination and zooplankton population dynamics: An experimental and modelling approach. *Freshw. Biol.* **54**, 221–235.

Ferrari, D. C. and Hebert, P. D. N. (1982). The induction of sexual reproduction in *Daphnia magna*: Genetic differences between arctic and temperate populations. *Can. J. Zool.* **60**, 2143–2148.

Gillooly, J. and Dodson, S. (2000). Latitudinal patterns in the size distribution and seasonal

dynamics of new world, freshwater cladocerans. *Limnol. Oceanogr.* **45**, 22–30.

Hannas, B. R., Das, P. C., Li, H. and LeBlanc, G. a (2010). Intracellular conversion of environmental nitrate and nitrite to nitric oxide with resulting developmental toxicity to the crustacean Daphnia magna. *PLoS One* **5**, e12453.

Hebert, P. D. N. (1978). The population biology of *Daphnia* (Crustacea, Daphnidae). *Biol. Rev.* **53**, 387–426.

Hobek, A. and Larsson, P. (1990). Sex determination in *Daphnia magna*. *Ecology* **71**, 2255–2268.

Kakaley, E. K. M., Wang, H. Y. and LeBlanc, G. A. (2017). Agonist-mediated assembly of the crustacean methyl farnesoate receptor. *Sci. Rep.* **7**, 45071.

Kleiven, O. T., Larsson, P. and Hobek, A. (1992). Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* **65**, 197–206.

Korpelainen, H. (1986). The effects of temperature and photoperiod on life history parameters of *Daphnia magna* (Crustacea: Cladocera). *Freshw. Biol.* **16**, 615–620.

Korpelainen, H. (1990). Sex ratios and conditions required for environmental sex determination in animals. *Biol. Rev. Camb. Philos. Soc.* **65**, 147–184.

Lampert, W. (2006). *Daphnia*: Model herbivore, predator and prey. *Polish J. Ecol.* **54**, 607–620.

LeBlanc, G. A. and Medlock, E. K. (2015). Males on demand: the environmental-neuro-endocrine control of male sex determination in daphnids. *FEBS J.* 1–14.

Li, M., Mead, E. A. and Zhu, J. (2011). Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. *Proc. Natl. Acad. Sci.* **108**, 638–643.

Mitchell, S. E. and Lampert, W. (2000). Temperature adaptation in a geographically

widespread zooplankter, *Daphnia magna*. *J. Evol. Biol.* **13**, 371–382.

Miyakawa, H., Imai, M., Sugimoto, N., Ishikawa, Y., Ishikawa, A., Ishigaki, H., Okada, Y., Miyazaki, S., Koshikawa, S., Cornette, R., et al. (2010). Gene up-regulation in response to predator kairomones in the water flea, *Daphnia pulex*. *BMC Dev. Biol.* **10**, 45.

Miyakawa, H., Toyota, K., Sumiya, E. and Iguchi, T. (2014). Comparison of JH signaling in insects and crustaceans. *Curr. Opin. Insect Sci.* **1**, 81–87.

Olmstead, A. W. and LeBlanc, G. A. (2000). Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. *Environ. Toxicol. Chem.* **19**, 2107–2113.

Olmstead, A. and LeBlanc, G. (2001). Temporal and quantitative changes in sexual reproductive cycling of the cladoceran *Daphnia magna* by a juvenile hormone analog. *J. Exp. Zool.* **290**, 148–155.

Olmstead, A. W. and LeBlanc, G. A. (2002). Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. *J. Exp. Zool.* **293**, 736–739.

Östman, Ö. (2011). Interspecific competition affects genetic structure but not genetic diversity of *Daphnia magna*. *Ecosphere* **2**, 1–9.

Reiter, R. J. (1993). The melatonin rhythm: both a clock and a calendar. *Experientia* **49**, 654–664.

Rider, C. V, Gorr, T. A., Olmstead, A. W., Wasilak, B. A. and LeBlanc, G. A. (2005). Stress signaling: coregulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean. *J. Exp. Biol.* **208**, 15–23.

Schwartz, S. S. and Hebert, P. D. N. (1987). Methods for the activation of the resting eggs of *Daphnia*. *Freshw. Biol.* **17**, 373–379.

Stross, R. G. (1966). Light and temperature requirements for diapause development and release in *Daphnia*. *Ecology* **47**, 368–374.

Stross, R. G. (1969a). Photoperiod control of diapause in *Daphnia*. III. Two-stimulus control of long-day, short-day induction. *Biol. Bull.* **137**, 359–374.

Stross, R. G. (1969b). Photoperiod Control of Diapause in *Daphnia*. II. Induction of Winter Diapause in the Arctic. *Biol. Bull.* **136**, 264–273.

Stross, R. G. and Hill, J. C. (1968). Photoperiod Control of Winter Diapause in the Fresh-Water Crustacean, *Daphnia*. *Biol. Bull.* **134**, 176–198.

Toyota, K., Miyakawa, H., Yamaguchi, K., Shigenobu, S., Ogino, Y., Tatarazako, N., Miyagawa, S. and Iguchi, T. (2015a). NMDA receptor activation upstream of methyl farnesoate signaling for short day-induced male offspring production in the water flea, *Daphnia pulex*. *BMC Genomics* **16**, 186.

Toyota, K., Miyakawa, H., Hiruta, C., Furuta, K., Ogino, Y., Shinoda, T., Tatarazako, N., Miyagawa, S., Shaw, J. R. and Iguchi, T. (2015b). Methyl farnesoate synthesis is necessary for the environmental sex determination in the water flea *Daphnia pulex*. *J. Insect Physiol.* **80**, 22–30.

Toyota, K., Sato, T., Tatarazako, N. and Iguchi, T. (2017). Photoperiodism of male offspring production in the water flea *Daphnia pulex*. *Zoolog. Sci.* **34**, 312–317.

U.S. EPA (2002). *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*.

Vandekerkhove, J., Declerck, S., Brendonck, L., Conde-Porcuna, J. M., Jeppesen, E. and De Meester, L. (2005). Hatching of cladoceran resting eggs: Temperature and photoperiod. *Freshw. Biol.* **50**, 96–104.

Xie, X., Tao, T., Liu, M., Zhou, Y., Liu, Z. and Zhu, D. (2016). The potential role of juvenile hormone acid methyltransferase in methyl farnesoate (MF) biosynthesis in the swimming crab, *Portunus trituberculatus*. *Anim. Reprod. Sci.* **168**, 40–49.

Zhang, L. and Baer, K. . (2000). The influence of feeding, photoperiod and selected solvents on the reproductive strategies of the water flea, *Daphnia magna*. *Environ. Pollut.* **110**, 425–430.

CHAPTER 3

Evidence for a Role of the N-methyl-D-aspartate Receptor in Male Sex Determination in *Daphnia pulex*.

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ABSTRACT

The N-methyl-D-aspartate receptor (NMDAR) is a complex ionotropic glutamate receptor involved in a variety of processes including learning, memory, and neuroendocrine function. We tested the hypothesis that the NMDAR is a viable candidate neural processor of environmental signals and may function in linking environmental cues to endocrine responses involved in male sex determination in the freshwater microcrustacean *Daphnia pulex*. The NMDAR agonists, N-methyl-D-aspartate and glycine, were evaluated for their ability to alter the production of male offspring individually and in co-exposure. Additionally, NMDAR activity modulators ethanol and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), and the antagonists AP-5, MK-801, and desipramine were tested individually. Results showed that the antagonists MK-801 and desipramine significantly altered (increased) male production in a concentration-dependent manner. MK-801 and desipramine were further evaluated to determine whether their ability to stimulate male sex determination was due to downstream activation of the methyl farnesoate receptor (MfR). Using bioluminescence resonance energy transfer to measure the agonist-mediated dimerization of the MfR subunits Met and SRC, we demonstrated that the sex-determining hormone methyl farnesoate stimulated dimerization but the NMDAR antagonists had no effect. We concluded that MK-801 and desipramine stimulated male sex determination upstream of the action of the methyl farnesoate and may function by inhibiting the activity of the NMDAR. Further experiments were conducted with MK-801 to determine whether MK-801 mimicked the action of photoperiod or temperature, two environmental regulators of male sex determination. MK-801 stimulated male sex determination only at a photoperiod that was permissive of male sex determination and appeared to function additively with temperature to stimulate male sex determination. Finally, we assessed mRNA levels of NMDAR subunits and

the signaling elements in the male sex determination signaling cascade under male stimulating and non-male stimulating environmental conditions. mRNA levels of *NMDAR-b* and *SRC* were dramatically increased under short day photoperiod as compared to long day. *JHAMT*, *FAMT*, and *Met* mRNA levels also were increased with short day photoperiod though to a lesser degree. Overall, we provide evidence that modulation of the NMDAR-b subunit by photoperiod may mediate the action of the NMDAR in regulating male sex determination.

INTRODUCTION

Environmental sex determination, the phenomenon where environmental cues influence the sex ratio of offspring, has evolved in several distantly related groups throughout evolutionary time. Environmental sex determination occurs in invertebrate and vertebrate groups including annelids, rotifers (Korpelainen, 1990), fish (Devlin and Nagahama, 2002; F. W. H. Beamish, 1993), crustaceans (Hebert, 1978), and reptiles (Ciofi and Swingland, 1997; Lang and Andrews, 1994). A variety of environmental cues regulate environmental sex determination depending on the taxonomic group including temperature, nutritional status, pH, crowding cues, and photoperiod (Ciofi and Swingland, 1997; Korpelainen, 1990). Studies to date indicate that environmental sex determination involves the sensory transduction of environmental stimuli and subsequent modification of hormonal pathways in order to influence offspring sex (Devlin and Nagahama, 2002; Korpelainen, 1990).

The microcrustacean *Daphnia* spp. is a keystone genus in freshwater environments and is subject to environmental sex determination. Many daphnid species are cyclic parthenogens and reproduce both asexually and sexually. During asexual reproduction, daphnids clonally produce female offspring (Hebert, 1978). Environmental cues stimulate daphnids to produce male offspring

(male sex determination) to enable sexual reproduction (Hobek and Larsson, 1990; Kleiven et al., 1992; Korpelainen, 1986). As with all environmental sex determination, a neuroendocrine connection must exist such that abiotic cues can be sensed, transduced, and translated into endocrine messages.

The neuroendocrine linkage that mediates the transition from asexual to sexual reproduction in daphnids remains unknown, however, evidence from RNAseq experiments with *D. pulex* implicates ionotropic glutamate receptors in male sex determining processes (Toyota et al., 2015a). The N-methyl-D-aspartate receptor (NMDAR) is an ionotropic glutamate receptor that is a coincident detector within the nervous system, requiring several simultaneous factors for the receptor to open (glutamate, glycine, and a depolarized membrane to remove a Mg^{2+} block) (Rousseaux, 2008; Traynelis et al., 2010). The NMDAR requires both the NR1 subunit, which contains the co-factor binding site, as well as an NR2 subunit, which contains the glutamate binding site, for a functional receptor (Rousseaux, 2008).

The NMDAR also possesses several allosteric binding sites through which NMDAR function may be modulated (Dingledine et al., 1999; Monaghan et al., 2012; Reynolds, 1990; Rousseaux, 2008). Ethanol, a NMDAR modulator, has been widely studied regarding its effects on glutamatergic signaling. It has been shown to inhibit NMDAR function, thus acting in an antagonistic manner (Hoffman et al., 1989; Lovinger et al., 1989; Wirkner et al., 1999; Wirkner et al., 2000; Woodward, 1999). Another glutamate receptor, α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors, are often co-localized with NMDARs and are a faster opening glutamate receptor that contributes to the depolarization of the post-synaptic membrane to facilitate NMDAR opening (Riedel et al., 2003; Rousseaux, 2008).

The NMDAR is a dynamic receptor involved in complex behaviors such as learning and memory in invertebrates (Glantz and Pfeifferr-linn, 1992; Kano et al., 2008; Si et al., 2004; Xia et al., 2005). It has also been shown to be involved in invertebrate reproductive function (Begum et al., 2004; Chiang et al., 2002; Geister et al., 2008; Huang et al., 2015; Toyota et al., 2015a) and has been implicated in environmental stimuli integration (Kano et al., 2008; Mellem et al., 2002), making it a strong candidate in daphnids as a potential neuroendocrine link contributing to daphnid male sex determination pathways.

We and others have previously established that photoperiod is required to reliably initiate male sex determination processes in *D. pulex* and that temperature further modulates the magnitude of the response (Hobek and Larsson, 1990; Toyota et al., 2017). We and others have also determined the putative elements of the male sex determination hormone signaling cascade. The enzymes farnesoic acid o-methyltransferase (FAMT) and juvenile hormone o-methyltransferase (JHAMT) (Xie et al., 2016) produce methyl farnesoate, the male sex-determining hormone in daphnids (Olmstead and LeBlanc, 2002; Toyota et al., 2015a). Methyl farnesoate activates the methyl farnesoate receptor (MfR) by binding the Met subunit of the MfR and stimulating its recruitment of steroid receptor co-activator (SRC) (Kakaley et al., 2017; Toyota et al., 2015b). The activated MfR functions as a transcription factor to regulate expression of male sex determining genes.

Here we hypothesize that the NMDAR is a viable candidate neural processor of environmental signals and may function in linking environmental cues to endocrine responses. We tested the hypothesis using a variety of NMDAR agonists, modulators, and antagonists to determine their ability to modulate male sex determination in *D. pulex*. Further, we addressed whether chemicals with the ability to impact male production behaved more like the photoperiod

or temperature cue, both of which influence male production. Finally, we evaluated the influence of photoperiod on mRNA levels of NMDAR subunits and putative constituents of the male sex determination signaling cascade.

MATERIALS AND METHODS

Male Sex Determination

D. pulex (clone WTN6) cultures were maintained in the laboratory at 20°C, 16:8 hr Light:Dark (L:D) using methods described previously (Hannas et al., 2010). Daphnids were collected from culture as < 24-hour old neonates and transferred to either long (16:8 hr L:D) or short (10:14 hr L:D) day photoperiods at 18°C prior to chemical exposures, known to be non-male stimulating and male stimulating conditions, respectively (Chapter 2). Neonates were individually reared in 50 mL beakers containing 40 mL of daphnid media. Daphnid media consisted of reconstituted deionized water (192 mg/L $CaSO_4 \cdot H_2O$, 192 mg/L $NaHCO_3$, 120 mg/L $MgSO_4$, 8.0 mg/L KCl, 1.0 µg/L selenium and 1.0 µg/L vitamin B_{12}). Daphnids were fed daily 1.4 x 10^8 cells *Pseudokirchneriella subcapitata* and Tetrafin® fish food suspension (4 mg dry weight) (Pet International, Blacksburg, VA, USA) as described previously (Hannas et al., 2010).

Daphnids were exposed to the candidate modulators of NMDAR activity upon deposition of the first brood of embryos into their brood chamber. Each treatment group consisted of ten individual daphnids. Stock solutions of N-methyl-D-aspartate (NMDA) (Sigma Aldrich, St. Louis, MI), glycine (Sigma Aldrich), AMPA (Fisher Scientific, Hampton, NH), AP-5 (Sigma Aldrich), (+)-MK-801 hydrogen maleate (Fisher Scientific), and desipramine hydrochloride (Fisher Scientific) were prepared in deionized water. Pure ethanol (200 proof) (Sigma Aldrich) was added directly to the test media. Concentrations were selected based on preliminary toxicity assays (data

not shown). Media was changed every other day. Offspring produced by the isolated daphnids were counted, and sex was determined by the length of the first antennae on the day they were released (Olmstead and LeBlanc, 2000). Six broods of offspring were collected for each animal, and broods 2-6 were combined and used in analyses since the first brood was not fully exposed to the test materials.

Bioluminescence resonance energy transfer (BRET) assays

Drosophila Schneider 2 (S2) cells (Life Technologies, Carlsbad, CA) were cultured with Schneider's *Drosophila* Medium (Gibco, Carlsbad, CA) in T25 flasks containing 10% heat-inactivated fetal bovine serum (Gibco) and supplemented with 50 µg streptomycin sulfate (Fisher Scientific) and 50 units penicillin G (Fisher Scientific). Cells were maintained at 25.5-26.5°C with >50% relative humidity. S2 cells were seeded at a cell density of 7.5 x 10^5 cells/cm^2 into 60 cm^2 culture dishes for the assay. Cells were transiently transfected, with calcium phosphate, with 45 µg of total plasmid DNA containing two fusion proteins of the *D. pulex* MfR subunit proteins: *pMT:Rluc2-SRC* and *pMT:mAmetrine-Met.* Fusion protein construction is described elsewhere (Kakaley et al., 2017). The ratio of *SRC:Met* fusion proteins was 1:6 (Kakaley et al., 2017). Transcription of the transfected genes was induced with 800 µM $CuSO_4$ 24 hours after transfection, and harvested at 48 hours post-transfection. Transfected cells were exposed to carrier control (0.01% DMSO used to deliver methyl farnesoate), positive control (10 µM methyl farnesoate, Echelon Biosciences, Salt Lake City, UT), MK-801, or desipramine, delivered in 100 µL phosphate-buffered saline. Solutions were incubated for 5 min incubation at 25.5-26.5°C. Emissions were measured at 410 nm (*Renilla* luciferase 2 (Rluc2)) and 535 nm (mAmetrine) with a FLUOstar® Omega microplate reader (BMG Labtech, Germany) in the presence of 5.0 µM

Renilla luciferase substrate DeepBlueC™ (Gold Biotechnology, Inc). The BRET ratio was calculated as emissions at 535/410 nm and corrected for background emissions (Kakaley et al., 2017). Individual BRET assays were replicated 6 times. The BRET ratio represented the level of agonist-mediated binding of *Met* and *SRC* as indicated by the fluorescence emitted by *mAmetrine-Met* following excitation by the light emitted by *Rluc2-SRC*.

mRNA Analysis

D. pulex (≤ 24 hours old) were reared under conditions that either stimulated (10:14 hr L/D photoperiod, 18°C) or did not stimulate (16:8 hr L:D photoperiod, 18°C) male sex determination using methods described above. Once daphnids reached sexual maturity (eggs in brood chamber), all animals were molt-synchronized (Mu and LeBlanc, 2002) and collected at 0, 24, 36, or 48 hours post-molt. Four replicates each containing 3-5 daphnids were collected at each time point. Daphnids from individual replicates were transferred to 100 µL RNAlater® and held at 4°C for 24 hours, then stored at -80°C until used for RNA extraction. Whole animals were homogenized using a Next Advance Bullet Blender® and zirconium oxide beads (1.0 mm diameter, Next Advance, Troy, NY). RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI) according to manufacturer recommendations. cDNA was synthesized using ImProm-II™ Reverse Transcription System with oligo (dT) primers (Promega, Madison, WI).

mRNA levels of *NMDAR-a*, *NMDAT-b*, *JHAMT*, *FAMT*, *Met*, and *SRC* were measured by Real Time-qPCR. Primer sequences for *JHAMT* (Miyakawa et al., 2010), *FAMT* (Toyota et al., 2015b), *Met* (Miyakawa et al., 2010), and *SRC* (Toyota et al., 2015b) were used to amplify mRNA sequences. Primer sets for *NMDAR-a* and *NMDAR-b* were developed using nucleotide sequences provided by the Department of Energy Joint Genome Institute, accessed via NCBI (Bethesda, MD)

and designed using Primer3 (v. 0.4.0). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Primer specificity was confirmed based on amplicon length and nucleotide sequence (Eton Bioscience Inc, San Diego, CA). *NMDAR-a* primers were Forward: GTCGTCGGTGATGTGAGATG and Reverse: AACAAGAAGGCGGACAGAAA and *NMDAR-b* primers were Forward: AGCCATGGAGTACCTTGTCG and Reverse: ACTTTGGGTCGTCCACTCTG. PCR was performed with the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) using 2x SYBR™ Green Premix (ThermoFisher Scientific). A single melting peak was detected for each sample with an amplification efficiency of >93%, indicating amplification occurred only for the target sequence. Genex software (Bio-Rad Laboratories, Hercules, CA) was used to analyze relative levels of gene expression by normalizing to two housekeeping genes, *actin* and *GAPDH*.

Data Analyses

Treatment-related differences in the percentage male offspring produced were evaluated using One-way ANOVA with Tukey's multiple comparisons test when variances were not different among treatments. The Kruskal-Wallis test with Dunn's post hoc comparison was used when variances were significantly different among treatments. Differences in male production in the temperature-dependent male sex determination assays with MK-801 were evaluated using Student's t-test. BRET assay results were analyzed by One-way ANOVA with Tukey's multiple comparisons test. Treatment differences in mRNA levels were evaluated using Student's t-test when variances were not different among treatments. The Mann-Whitney test was used when variances were significantly different among treatments. Time-related differences in mRNA levels for each gene and photoperiod were evaluated using One-way ANOVA with Tukey's multiple

comparisons test when variances were not different among treatments. The Kruskal-Wallis test with Dunn's post hoc comparison was used when variances were significantly different among treatments. Significance was set at an alpha level of 0.05. Error bars represent SEM. Statistical analyses were performed with Prism (v7.02, GraphPad Software, Inc).

RESULTS

Male sex determination

Two NMDAR agonists NMDA and glycine were first evaluated individually for their ability to stimulate or suppress male sex determination in *D. pulex* under environmental conditions at which the maternal organisms would be expected to produce 20-50% males (10:14 hr L:D photoperiod, 18°C). Neither NMDA (Fig. 1A) nor glycine (Fig. 1B) significantly impacted the percentage male offspring produced. Since both NMDA and glycine are required for NMDAR activation, these compounds were also evaluated together. Co-exposure to both NMDAR agonists did not significantly alter the percentage of male offspring produced (Fig. 1C). Likewise, neither NMDAR modulator (ethanol and AMPA) significantly altered male production at the concentrations tested (Fig. 2A, B).

We next evaluated the ability of the NMDAR antagonists, MK-801, desipramine, and AP-5, to modulate male sex determination. MK-801 is an uncompetitive antagonist and NMDAR channel blocker (Huettner and Bean, 1988). Desipramine is an open channel blocker (Sernagor et al., 1989; Szasz et al., 2007). AP-5 is a competitive antagonist at the glutamate binding site on the NMDAR (Rousseaux, 2008). AP-5 had no influence on the proportion of male offspring produced at the concentrations tested (Fig. 3A), while both MK-801 (Fig. 3B) and desipramine (Fig. 3C) significantly stimulated the production of male offspring in a concentration-dependent manner

under a 10:14 hr L:D photoperiod that is permissive of male sex determination. These compounds had no effect on male sex determination under the non-permissive photoperiod (Fig. 3B, C). These results suggested that MK-801 and desipramine required the appropriate photoperiodic cue to stimulate male sex determination and could not supplant this cue. Additional experiments were performed with MK-801 to determine if this compound could stimulate male sex determination under a temperature gradient that provided a range in the expected magnitude of male sex determination (Chapter 2). MK-801 increased the proportion of male offspring produced at all temperature by ~40 to ~150% with significant increases detected at temperatures ranging from 16 to 20°C (Fig. 4). These results suggest that under a permissive photoperiod, MK-801 can augment the stimulatory action of temperature on male sex determination.

MfR activation

We considered that the action of MK-801 and desipramine could be a consequence of these compounds activating the methyl farnesoate receptor (MfR), recognizing that male sex determination is regulated by the activation of this receptor by its hormonal ligand (Olmstead and LeBlanc, 2002). Additionally, the MfR has been shown to be susceptible to activation by other exogenous compounds (Olmstead and LeBlanc, 2003). BRET assays were performed to determine whether MK-801 and desipramine stimulated dimerization of the MfR subunits, an early event in the activation of the MfR. The positive control, methyl farnesoate, significantly stimulated the dimerization of Met and SRC, however, concentrations of MK-801 and desipramine as high as 100 μM had no effect on receptor assembly (Fig. 5). These results indicated that MK-801 and desipramine acted at a location within the male sex determination pathway other than the MfR.

Impact of photoperiod on selected mRNA levels

The impact of photoperiod on mRNA levels of the NMDAR subunits a and b, enzymes that contribute to methyl farnesoate synthesis juvenile hormone acid o-methyltransferase (JHAMT) and farnesoic acid o-methyltransferase (FAMT), and the MfR subunits Met and SRC were evaluated at several timepoints during the molt cycle. Our goal was to identify components of the putative male sex determination pathway that are elevated or suppressed in response to photoperiod.

mRNA levels for both NMDAR subunits increased over time during the initial 48 hrs post-molt (Figs. 6A, B, 7A, B). *JHAMT* mRNA levels attained maximum levels at 24 hrs post-molt (Figs. 6C, 7C). *FAMT* mRNA levels exhibited a slight rhythmic pattern with levels decreasing slightly between 0 and 24 hrs, increasing between 24 and 36 hrs, then decreasing between 36 and 48 hrs post-molt (Figs. 6D, 7D). *Met* and *SRC* mRNA attained maximum levels at 24 hrs post-molt, then decreased to various degrees (Figs. 6E, F, 7E, F).

Photoperiod elicited a varied and sometimes profound effect on mRNA levels for the putative constituents of the male sex determining signaling pathway. *NMDAR-a* levels were not influenced by photoperiod (Fig. 8A); however, *NMDAR-b* mRNA levels were 5.5 to 103 times higher in the short day, permissive, 10:14 hr photoperiod as compared to the long day, non-permissive, 16:8 hr photoperiod (Fig. 8B). *JHAMT* mRNA levels were elevated in the 10:14 hr photoperiod during the initial 36 hrs post-molt (Fig. 8C); while, *FAMT* mRNA levels were slightly increased during the initial 24 hrs post molt (Fig. 8D). *Met* and *SRC* mRNA levels also were elevated in the 10:14 hr photoperiod as compared to the 16:8 hr photoperiod, however, the magnitude of difference in mRNA levels between photoperiods was much greater for *SRC* (Fig. 8E, F).

To summarize, results are consistent with the short day, permissive photoperiod stimulating the expression of the NMDAR-b subunit which provides for a functional NMDAR that stimulates elements of the male sex determination pathway.

DISCUSSION

We hypothesized that the NMDAR is a viable candidate as a neural processor of environmental signals and may function in linking environmental cues to endocrine responses in daphnids. We demonstrated that the NMDAR agonists NMDA and glycine, while empirically reducing the incidence of male sex determination, had no statistically significant effect on this phenomenon. Similarly, the NMDAR modulators ethanol and AMPA did not influence male sex determination. However, the NMDAR antagonists MK-801 and desipramine, stimulated male sex determination. Further experiments revealed that the stimulatory activity of MK-801 presented only under a photoperiod that renders organism responsive to the stimulatory effect of temperature, and acted in combination with temperature to stimulate male sex determination. We conclude that these compounds interact with the NMDAR in a manner that stimulates the male sex determining pathway, possibly utilizing the same components of the pathway as does temperature.

Our findings contradict those of other studies, which found that NMDA and AMPA stimulated male production under a photoperiod intermediate to those used in the present study (14:10 hr L:D) (Toyota et al., 2015a). Additionally, Toyota and colleagues found that MK-801 suppressed male production, which also is contrary to our findings (Toyota et al., 2015a). This may be attributed to differences in experimental designs. We assessed percentage male production under continuous exposure over five consecutive broods, while Toyota and colleagues (2015a) assessed male production for only one brood of offspring. We opted to use the cumulative results

from five consecutive broods because we found brood to brood variability relating to male sex determination to be unacceptably high.

The methyl farnesoate receptor (MfR) is a well-documented target of hormones and other small molecules that stimulate male sex determination in daphnids (Olmstead and LeBlanc, 2002; Olmstead and LeBlanc, 2003). The hormone methyl farnesoate binds to the protein Met stimulating it to dimerize with SRC and forming the active MfR transcription factor (Kakaley et al., 2017). We considered the possibility that MK-801 and desipramine stimulate male sex determine by acting as a Met ligand and stimulating MfR assembly. This possibility was evaluated using bioluminescence resonance energy transfer (BRET) technology. While the hormone methyl farnesoate readily stimulated dimerization of Met and SRC in our system, neither MK-801 nor desipramine were active. We conclude that these compounds are acting elsewhere along the male sex determination pathway, possibly the NMDAR.

Exposure to MK-801 and desipramine, under a permissive, short day photoperiod significantly increased male production; while, exposures conducted under the long, non-permissive photoperiod were unable to initiate male production. Further, under the permissive photoperiod, MK-801 functioned in concert with temperature to increase male sex determination. These findings suggest that MK-801 and desipramine stimulate the male sex determining pathway in a manner similar to temperature and distinct from the manner in which photoperiod stimulates the pathway.

Results revealed mRNA levels of *NMDAR-b* are highly malleable and subject to modulation by photoperiodic cues. *NMDAR-b* was highly expressed under the permissive short day photoperiod as compared to the non-permissive long day photoperiod. This suggests that the

induction of NMDAR-b by photoperiod may result in the assembly of competent NMDAR which is required to stimulate male offspring production.

The resilience of *NMDAR-a* to modulation by photoperiod and the susceptibility of *NMDAR-b* to photoperiod suggest that these receptor subunits function similarly to the NMDAR-1 (NR1) and NMDAR 2 (NR2) in vertebrates wherein NR1 expression is tightly regulated (Rousseaux, 2008). In contrast, NR2 expression is more plastic and plays a modulatory role in regulating receptor NMDAR activity. Changes in NR2 expression and subsequently NMDAR structure (i.e. receptor heterodimeric composition) have been shown to alter receptor kinetics and pharmacology (Gielen et al., 2009; Yuan et al., 2009). Thus, the differential expression of *NMDAR-b* under different photoperiods likely reflects a fundamental shift in NMDAR function within *D. pulex*. We conclude that *NMDAR-b* expression is responsive to photoperiod causing changes in glutamate signaling in response to this environmental cue.

JHAMT mRNA levels were appreciably higher early in the molt/reproductive cycle under the short day photoperiod as compared to the long day photoperiod. These results are consistent with those reported by Toyota et al. (2015), who also demonstrated that *D. pulex* JHAMT efficiently catalyzes the conversion of farnesoic acid to methyl farnesoate. We conclude that short day photoperiod elevates levels of methyl farnesoate. Subsequent to the induction of *JHAMT*, *Met* and *SRC* mRNA levels increased and were continually elevated through at least 48 hrs post molt. This elevation of MfR subunit mRNAs likely reflects increases in the respective subunit protein levels. MfR subunits are also elevated by short day photoperiod either due to induction by methyl farnesoate or co-regulation with methyl farnesoate. Regardless, under environmental conditions permissive of male sex determination the male-determining hormone and its receptor are present at levels greater than under non-permissive environmental conditions.

Notably, the NMDAR antagonists that significantly altered male production in *D. pulex*, MK-801 and desipramine, have antidepressant activity in vertebrate systems. There is a growing body of evidence that dysregulated glutamate signaling is involved in mood disorders such as depression (Sanacora et al., 2012; Tokita et al., 2012), and studies have shown that NMDAR antagonists can alleviate depressive symptoms within animal models of depression (Ghasemi et al., 2010; Murrough, 2015; Neis et al., 2015; Padovan and Guimarães, 2004). While the effects of some antidepressants have been evaluated in crustaceans, the majority of research has focused on monoamine targeting drugs, making our finding an important contribution to this field (Fong and Ford, 2014).

While many NMDAR-targeting chemicals used here did not alter male production in *D. pulex*, our results with MK-801 and desipramine support a role for the NMDAR in male sex determination processes. Additionally, we demonstrated that environmental conditions that yield male offspring increase expression of the NMDAR-b subunit, further implicating this receptor in male sex determining processes. We also show that the key enzymes and transcription factors involved in the production of the male sex determining hormone methyl farnesoate are influenced by photoperiod cues. Finally, we provide that evidence that MK-801 acts in a complementary fashion to the stimulatory effect of temperatures, and we propose that temperature may also be integrated through NMDAR signaling. Overall, we provide foundational evidence for neuroendocrine links in *D. pulex* as they relate to male sex determination processes.

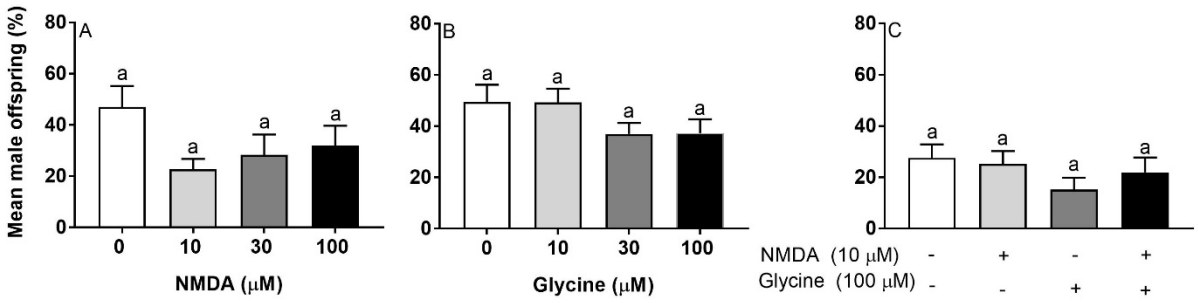

Figure 1: Male offspring production by *D. pulex* exposed to NMDAR agonists NMDA, glycine, and co-exposures. Panel A: Male production with exposure to the agonist NMDA. Panel B: Male production with exposure to the co-factor glycine. Panel C: Male production with individual and co-exposure to 10 μM NMDA and 100 μM glycine. Data are presented as means and standard error, n=8-10. Different letters denote significant differences.

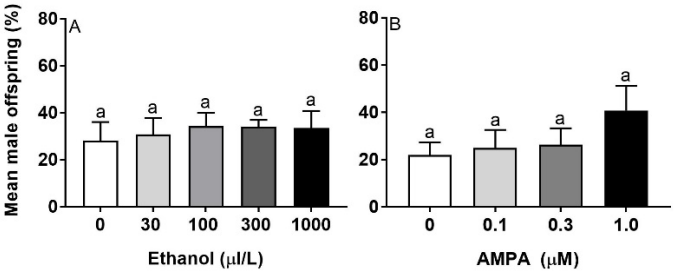

Figure 2. Male offspring production by *D. pulex* exposed to NMDAR modulators ethanol and AMPA. Panel A: Male production with exposure to ethanol. Panel B: Male production with exposure to AMPA. Data are presented as means and standard error, n=9-10. Different letters denote significant differences ($p \leq 0.05$).

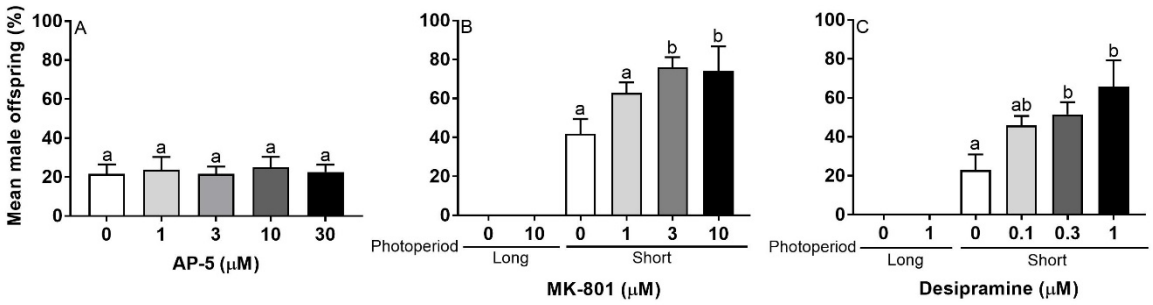

Figure 3: Male offspring production by *D. pulex* exposed to NMDAR antagonists AP-5, MK-801, and desipramine. Panel A: Male production with exposure to AP-5 under a short day photoperiod. Panel B: Male production with exposure to MK-801 under long and short day photoperiods. Panel C: Male production with exposure to desipramine under long and short day photoperiods. Short photoperiod is 10:14 hr L:D, and long photoperiod is 16:8 hr L:D. Data are presented as means and standard error, n=9-10. Different letters denote significant differences ($p \leq 0.05$).

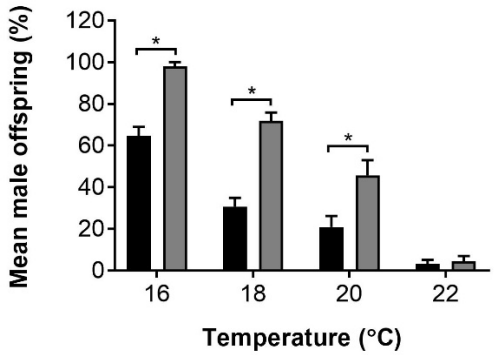

Figure 4. Male offspring production by *D. pulex* exposed to the NMDAR antagonist MK-801 at various temperatures. Black bars represent short photoperiod control groups, and grey bars represent short photoperiod MK-801-exposed groups. Data are presented as means and standard error, n=8-10. Asterisks denote significant differences.

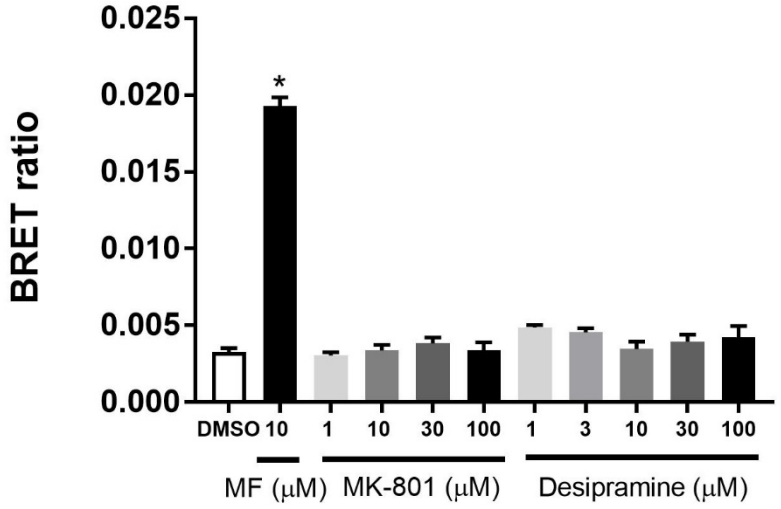

Figure 5: Ligand-mediated Met and SRC assembly as measured using BRET. Data are present as means and standard error (n=6). An asterisk denotes a significant difference from the DMSO control. MF: methyl farnesoate (positive control).

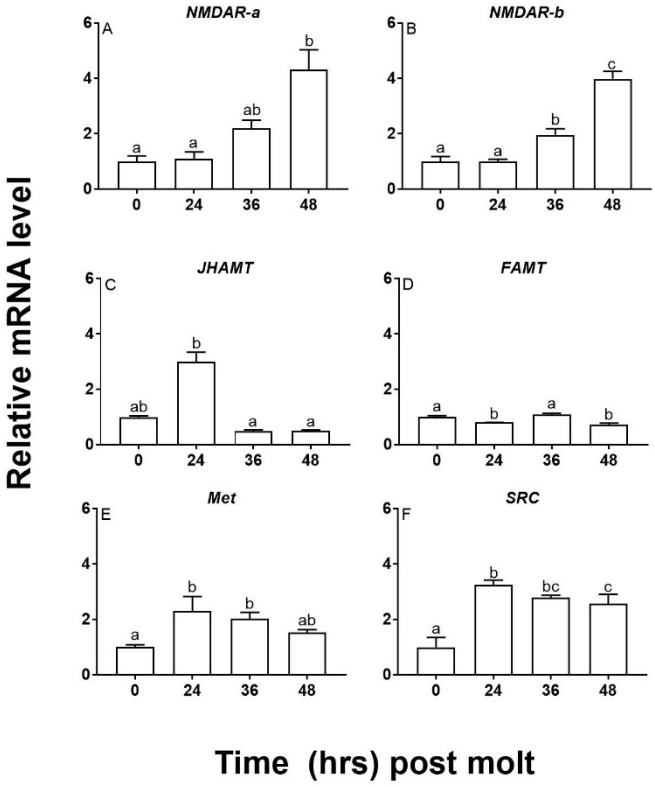

Figure 6. mRNA levels for *D. pulex* reared under long day (16:8 hr) photoperiod during the intermolt period. Panel A: *NMDAR-a*, Panel B: *NMDAR-b*, Panel C: *JHAMT*, Panel D: *FAMT*, Panel E: *Met*, Panel F: *SRC*. Groups are normalized to the long photoperiod control time 0 group. Different letters denote significant differences.

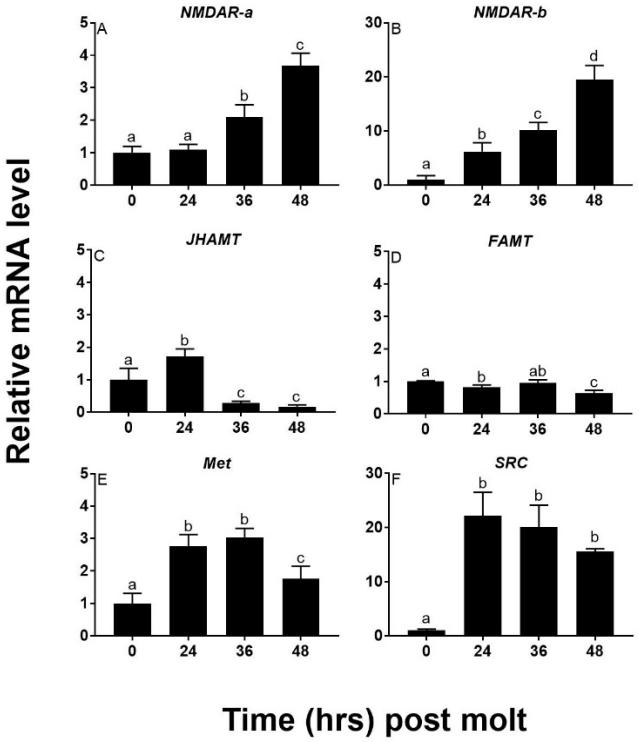

Figure 7: mRNA levels for *D. pulex* reared under short day (10:14 hr) photoperiod during the intermolt period. Panel A: *NMDAR-a*, Panel B: *NMDAR-b*, Panel C: *JHAMT*, Panel D: *FAMT*, Panel E: *Met*, Panel F: *SRC*. Groups are normalized to the short photoperiod control time 0 group. Different letters denote significant differences.

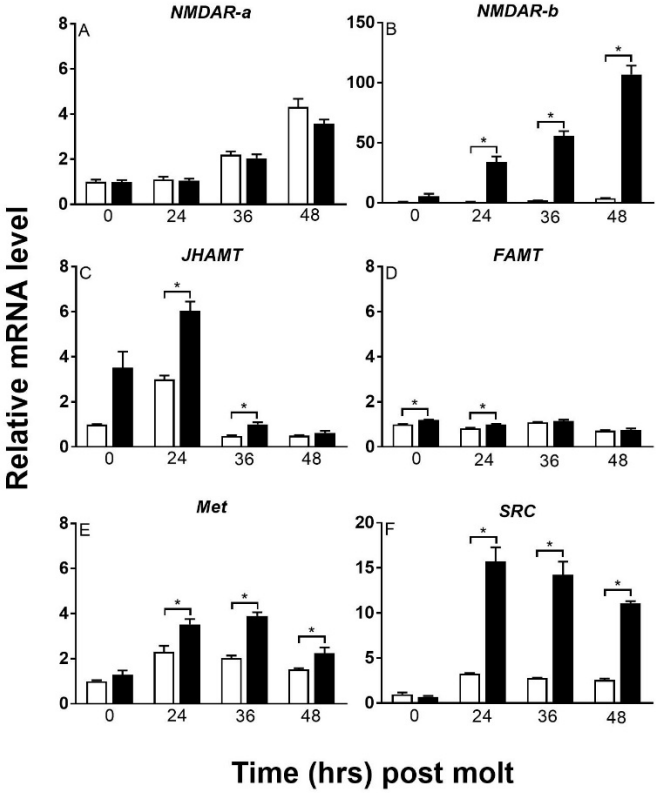

Figure 8. Relative mRNA levels for *D. pulex* reared under long day (16:8 hr, white bar) and short day (10:14 hr, black bar) photoperiods during the intermolt period. Panel A: *NMDAR-a*, Panel B: *NMDAR-b*, Panel C: *JHAMT*, Panel D: *FAMT*, Panel E: *Met*, Panel F: *SRC*. Groups are normalized to the long photoperiod control 0 hour group. An asterisk denotes significant differences.

REFERENCES

Begum, M., Breuer, M., Kodrik, D., Rahman, M. and De Loof, A. (2004). The NMDA receptor antagonist MK-801 inhibits vitellogenesis in the flesh fly *Neobellieria bullata* and in the desert locust *Schistocerca gregaria*. *J Insect Physiol* **50**, 927–934.

Chiang, A.-S., Lin, W.-Y., Liu, H.-P., Pszczolkowski, M., Fu, T.-F., Chiu, S.-L. and Holbrook, G. (2002). Insect NMDA receptors mediate juvenile hormone biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 37–42.

Ciofi, C. and Swingland, I. R. (1997). Environmental sex determination in reptiles. *Appl. Anim. Behav. Sci.* **51**, 251–265.

Devlin, R. H. and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* **208**, 191–364.

Dingledine, R., Traynelis, S. F. F., Borges, K., Bowie, D. and Traynelis, S. F. F. (1999). The glutamate receptor ion channels. *Pharmacol. Rev.* **51**, 7.

F. W. H. Beamish (1993). Environmental Sex Determination in Southern Brook Lamprey, *Ichthyomyzon gagei*. *Can. J. Fish. Aquat. Sci.* **50**, 1299–1307.

Fong, P. P. and Ford, A. T. (2014). The biological effects of antidepressants on the molluscs and crustaceans: A review. *Aquat. Toxicol.* **151**, 4–13.

Geister, T. L., Lorenz, M. W., Hoffmann, K. H. and Fischer, K. (2008). Effects of the NMDA receptor antagonist MK-801 on female reproduction and juvenile hormone biosynthesis in the cricket *Gryllus bimaculatus* and the butterfly *Bicyclus anynana*. *J. Exp. Biol.* **211**, 1587–93.

Ghasemi, M., Raza, M. and Dehpour, A. R. (2010). NMDA receptor antagonists augment

antidepressant-like effects of lithium in the mouse forced swimming test. *J. Psychopharmacol.* **24**, 585–594.

Gielen, M., Siegler Retchless, B., Money, L., Johnson, J. W. and Paoletti, P. (2009). Mechanism of differential control of NMDA receptor activity by NR2 subunits. 703–708.

Glantz, R. M. and Pfeifferr-linn, C. (1992). NMDA Receptors in invertebrates. *Comp. Biochem. Physiol.* **103**, 243–248.

Hannas, B. R., Das, P. C., Li, H. and LeBlanc, G. a (2010). Intracellular conversion of environmental nitrate and nitrite to nitric oxide with resulting developmental toxicity to the crustacean *Daphnia magna*. *PLoS One* **5**, e12453.

Hebert, P. D. N. (1978). The population biology of *Daphnia* (Crustacea, Daphnidae). *Biol. Rev.* **53**, 387–426.

Hobek, A. and Larsson, P. (1990). Sex determination in *Daphnia magna*. *Ecology* **71**, 2255–2268.

Hoffman, P. L., Rabe, C. S., Moses, F. and Tabakoff, B. (1989). N-methyl-D-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. *J. Neurochem.* **52**, 1937–1940.

Huang, J., Hult, E. F., Marchal, E. and Tobe, S. S. (2015). Identification and characterization of the NMDA receptor and its role in regulating reproduction in the cockroach *Diploptera punctata*. *J. Exp. Biol.* **218**, 983–90.

Huettner, J. E. and Bean, B. P. (1988). Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: Selective binding to open channels. *Proc. Natl. Acad. Sci.* **85**, 1307–1311.

Kakaley, E. K. M., Wang, H. Y. and LeBlanc, G. A. (2017). Agonist-mediated assembly of

the crustacean methyl farnesoate receptor. *Sci. Rep.* **7**, 45071.

Kano, T., Brockie, P. J., Sassa, T., Fujimoto, H., Kawahara, Y., Iino, Y., Mellem, J. E., Madsen, D. M., Hosono, R. and Maricq, A. V. (2008). Memory in *Caenorhabditis elegans* is mediated by NMDA-type ionotropic glutamate receptors. *Curr. Biol.* **18**, 1010–1015.

Kleiven, O. T., Larsson, P. and Hobek, A. (1992). Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* **65**, 197–206.

Korpelainen, H. (1986). The effects of temperature and photoperiod on life history parameters of *Daphnia magna* (Crustacea: Cladocera). *Freshw. Biol.* **16**, 615–620.

Korpelainen, H. (1990). Sex ratios and conditions required for environmental sex determination in animals. *Biol. Rev. Camb. Philos. Soc.* **65**, 147–184.

Lang, J. W. and Andrews, H. V (1994). Temperature-dependent sex determination in crocodilians. *J. Exp. Zool.* **270**, 28–44.

Lovinger, D. M., White, G. and Weight, F. F. (1989). Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science* **243**, 1721–1724.

Mellem, J., Brockie, P., Zheng, Y., Madsen, D. and Maricq, A. (2002). Decoding of polymodal sensory stimuli by postsynaptic glutamate receptors in *C. elegans*. *Neuron* **36**, 933–944.

Miyakawa, H., Imai, M., Sugimoto, N., Ishikawa, Y., Ishikawa, A., Ishigaki, H., Okada, Y., Miyazaki, S., Koshikawa, S., Cornette, R., et al. (2010). Gene up-regulation in response to predator kairomones in the water flea, *Daphnia pulex*. *BMC Dev. Biol.* **10**, 45.

Monaghan, D. T., Irvine, M. W., Costa, B. M., Fang, G. and Jane, D. E. (2012). Pharmacological modulation of NMDA receptor activity and the advent of negative and

positive allosteric modulators. *Neurochem. Int.* **61**, 581–592.

Mu, X. and LeBlanc, G. A. (2002). Developmental toxicity of testosterone in the crustacean *Daphnia magna* involves anti-ecdysteroidal activity. *Gen. Comp. Endocrinol.* **129**, 127–133.

Murrough, J. W. (2015). Glutamate NMDA receptor modulators for the treatment of depression: trials and tribulations. *Psychopharmacology (Berl).*

Neis, V. B., Moretti, M., Manosso, L. M., Lopes, M. W., Leal, R. B. and Rodrigues, A. L. S. (2015). Agmatine enhances antidepressant potency of MK-801 and conventional antidepressants in mice. *Pharmacol. Biochem. Behav.* **130**, 9–14.

Olmstead, A. W. and LeBlanc, G. A. (2000). Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. *Environ. Toxicol. Chem.* **19**, 2107–2113.

Olmstead, A. W. and LeBlanc, G. A. (2002). Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. *J. Exp. Zool.* **293**, 736–739.

Olmstead, A. W. and LeBlanc, G. A. (2003). Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. *Environ. Health Perspect.* **111**, 919–924.

Padovan, C. M. and Guimarães, F. S. (2004). Antidepressant-like effects of NMDA-receptor antagonist injected into the dorsal hippocampus of rats. *Pharmacol. Biochem. Behav.* **77**, 15–19.

Reynolds, J. (1990). Arcaine uncovers dual interactions of polyamines with the N-methyl-D-aspartate receptor. *J. Pharmacol. Exp. Ther.* **255**, 1001–1007.

Riedel, G., Platt, B. and Micheau, J. (2003). Glutamate receptor function in learning and

memory. *Behav. Brain Res.* **140**, 1–47.

Rousseaux, C. G. (2008). A Review of Glutamate Receptors I: Current Understanding of Their Biology. *J. Toxicol. Pathol.* **21**, 25–51.

Sanacora, G., Treccani, G. and Popoli, M. (2012). Towards a glutamate hypothesis of depression: An emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology* **62**, 63–77.

Sernagor, E., Kuhn, D., Vyklicky, L. and Mayer, M. L. (1989). Open channel block of NMDA receptor responses evoked by tricyclic antidepressants. *Neuron* **2**, 1221–7.

Si, A., Helliwell, P. and Maleszka, R. (2004). Effects of NMDA receptor antagonists on olfactory learning and memory in the honeybee (*Apis mellifera*). *Pharmacol. Biochem. Behav.* **77**, 191–197.

Szasz, B. K., Mike, A., Karoly, R., Gerevich, Z., Illes, P., Vizi, E. S. and Kiss, J. P. (2007). Direct Inhibitory Effect of Fluoxetine on N-Methyl-D-Aspartate Receptors in the Central Nervous System. *Biol. Psychiatry* **62**, 1303–1309.

Tokita, K., Yamaji, T. and Hashimoto, K. (2012). Roles of glutamate signaling in preclinical and/or mechanistic models of depression. *Pharmacol. Biochem. Behav.* **100**, 688–704.

Toyota, K., Miyakawa, H., Yamaguchi, K., Shigenobu, S., Ogino, Y., Tatarazako, N., Miyagawa, S. and Iguchi, T. (2015a). NMDA receptor activation upstream of methyl farnesoate signaling for short day-induced male offspring production in the water flea, *Daphnia pulex*. *BMC Genomics* **16**, 186.

Toyota, K., Miyakawa, H., Hiruta, C., Furuta, K., Ogino, Y., Shinoda, T., Tatarazako, N., Miyagawa, S., Shaw, J. R. and Iguchi, T. (2015b). Methyl farnesoate synthesis is necessary for the environmental sex determination in the water flea *Daphnia pulex*. *J. Insect*

Physiol. **80**, 22–30.

Toyota, K., Sato, T., Tatarazako, N. and Iguchi, T. (2017). Photoperiodism of male offspring production in the water flea *Daphnia pulex*. *Zoolog. Sci.* **34**, 312–317.

Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Odgen, K. K., Hansen, K. B., Yuan, H., Myers, S. J. and Dingledine, R. (2010). Glutamate receptor ion channels: structure, regulation and function. *Pharmacol. Rev.* **62**, 405–496.

Wirkner, K., Poelchen, W., Köles, L., Mühlberg, K., Scheibler, P., Allgaier, C. and Illes, P. (1999). Ethanol-induced inhibition of NMDA receptor channels. *Neurochem. Int.* **35**, 153–162.

Wirkner, K., Eberts, C., Poelchen, W., Allgaier, C. and Illes, P. (2000). Mechanism of inhibition by ethanol of NMDA and AMPA receptor channel functions in cultured rat cortical neurons. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **362**, 568–576.

Woodward, J. J. (1999). Ionotropic glutamate receptors as sites of action for ethanol in the brain. *Neurochem. Int.* **35**, 107–113.

Xia, S., Miyashita, T., Fu, T.-F., Lin, W.-Y., Wu, C.-L., Pyzocha, L., Lin, I.-R., Saitoe, M., Tully, T. and Chiang, A.-S. (2005). NMDA receptors mediate olfactory learning and memory in *Drosophila*. *Curr. Biol.* **15**, 603–15.

Xie, X., Tao, T., Liu, M., Zhou, Y., Liu, Z. and Zhu, D. (2016). The potential role of juvenile hormone acid methyltransferase in methyl farnesoate (MF) biosynthesis in the swimming crab, *Portunus trituberculatus*. *Anim. Reprod. Sci.* **168**, 40–49.

Yuan, H., Hansen, K. B., Vance, K. M., Ogden, K. K. and Traynelis, S. F. (2009). Control of NMDA Receptor Function by the NR2 Subunit Amino-Terminal Domain. *J. Neurosci.* **29**, 12045–12058.

CHAPTER 4

Pharmacologic Modulators Implicate Octopamine and Serotonin in Regulating Male Sex Determination in *Daphnia pulex*

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ABSTRACT

Many species of the freshwater crustacean *Daphnia* are cyclic parthenogens and produce males when environmental conditions necessitate the production of fertilized sexual eggs. Previously, we determined that under environmental conditions permissive to male production, two chemicals that are known to have antidepressant activity significantly increased sex ratios in *Daphnia pulex*. We hypothesized that other pharmaceuticals with antidepressant activity may also impact the male sex determination in *D. pulex*. We evaluated a suite of antidepressants (fluoxetine, citalopram, bupropion, nomifensine, and nisoxetine) for their ability to modulate male sex determination in *D. pulex* under environmental conditions permissive and non-permissive of male production. We hypothesized that neuroactive modulators would provide insight into neurological pathways that regulate this process. Citalopram and nisoxetine significantly increased male production at 1.0 μM, and fluoxetine elicited a concentration-response in male production over the range of concentrations evaluated. The antidepressants that altered male production target the serotonin and norepinephrine systems within the vertebrate nervous system, equivalent to serotonin and octopamine in invertebrates. We further assessed changes in mRNA levels in these neurotransmitter systems under conditions permissive and non-permissive to male production. Results show that environmental conditions permissive to male production significantly increased mRNA levels of the octopamine-alpha-a receptor (*OctαR-a*) and the serotonin re-uptake transporter (*SERT-a*). Overall, we show that some antidepressants have the potential to stimulate male sex determination and provide evidence that serotonergic and octopaminergic signaling likely plays a role in male sex determining processes.

INTRODUCTION

Zooplankton such as *Daphnia* spp are keystone invertebrates in freshwater lentic environments (Lampert, 2006). Daphnids are subject to environmental sex determination in that environmental cues influence the sex of their offspring. Most daphnid species reproduce via cyclic parthenogenesis, and under environmental conditions advantageous for rapid population growth, daphnids reproduce asexually generating a clonal population of females. When environmental cues signal the onset of disadvantageous conditions, male offspring are produced such that fertilized diapausing eggs can be generated (Hebert, 1978; Hobek and Larsson, 1990). Methyl farnesoate is the crustacean hormone responsible for programming oocytes into male offspring (LeBlanc and Medlock, 2015; Olmstead and LeBlanc, 2002; Toyota et al., 2015b).

Elements of the signaling cascade resulting in methyl farnesoate production and subsequent activation of the methyl farnesoate receptor (MfR) have been determined (Kakaley et al., 2017; Miyakawa et al., 2014; Olmstead and LeBlanc, 2002; Toyota et al., 2015b; Toyota et al., 2015a; Xie et al., 2016), however, questions remain about the pathway that connects the neuro-sensory elements of the signaling cascade to the endocrine system. We have previously shown that *D. pulex* exposed to the N-methyl-D-aspartate receptor (NMDAR) antagonists MK-801 and desipramine produce significantly more males when reared under conditions permissive to male sex determination (Chapter 3). Further, we demonstrated that both chemicals were acting upstream of methyl farnesoate and its receptor. Both of these compounds have antidepressant activity and specifically act on neural targets (Brunello et al. 2002; Ghasemi et al. 2010; Murrough 2015; Padovan & Guimarães 2004). MK-801 is an NMDAR uncompetitive antagonist and acts as an open channel blocker at the receptor (Huettner and Bean, 1988). Desipramine, a tricyclic antidepressant, inhibits norepinephrine re-uptake transporters and increases norepinephrine levels

in the synapse, and also acts as an NMDAR antagonist (Brunello et al., 2002; Sernagor et al., 1989).

Given that male sex determination in daphnids necessitates a neuroendocrine pathway to connect sensory systems to hormonal signaling cascades, we postulated that other antidepressant pharmaceuticals may have the capacity to disrupt the neuroendocrine processes involved in male sex determination. Here we hypothesized that that active modulators would provide insight into neurological pathways that regulate this process. We tested the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and citalopram, the norepinephrine and dopamine transporter inhibitors bupropion and nomifensine, as well as the selective norepinephrine reuptake inhibitor nisoxetine. Further, we hypothesized that male stimulating environmental conditions would alter levels of mRNA relating to the neurotransmitter systems targeted by active antidepressant pharmaceuticals in animals reared under permissive as compared to non-permissive environmental conditions for male sex determination.

MATERIALS AND METHODS

Male Sex Determination

D. pulex (clone WTN6) cultures were maintained in the laboratory at 20°C, 16:8 Light:Dark (L:D) photoperiod using methods described previously (Hannas et al., 2010). Daphnids were collected from culture as < 24-hour old neonates and transferred to either long day (16:8 L:D) or short day (10:14 L:D) photoperiods at 18°C prior to chemical exposures. Neonates were individually reared in 50 ml beakers containing 40 ml of daphnid media. Daphnid media consisted of reconstituted deionized water (192 mg/L $CaSO_4 \cdot H_2O$, 192 mg/L $NaHCO_3$, 120 mg/L $MgSO_4$, 8.0 mg/L KCl, 1.0 µg/L selenium and 1.0 µg/L vitamin B_{12}). Daphnids were

fed daily the unicellular green algae *Pseudokirchneriella subcapitata* (1.4 x 10^8 cells) and 50 µL Tetrafin® fish food suspension (Pet International, Blacksburg, VA, USA) as described previously (Hannas et al., 2010).

Daphnids were exposed to pharmaceuticals upon deposition of the first brood of embryos into the brood chamber. Each treatment group consisted of ten individual daphnids. Stock solutions of fluoxetine hydrochloride (Prozac, Fisher Scientific, Hampton, NH), citalopram hydrobromide (Celexa, Sigma Aldrich, St. Louis, MO), bupropion (Wellbutrin, Fisher Scientific), and nisoxetine hydrochloride (Fisher Scientific) were prepared in deionized water. Nomifensine (Fisher Scientific) was prepared in DMSO. DMSO concentration (0.1% v/v) was equal across exposure and control groups for nomifensine assays. Media was changed every other day. Offspring produced by the isolated daphnids were counted, and sex was determined by the length of the first antennae (Olmstead and LeBlanc, 2000). Six broods of offspring were assessed for each animal, and broods 2-6 were combined and used in analyses since the first brood was not fully exposed to the test materials.

mRNA Level Measurements

D. pulex were reared as described above in long day and short day photoperiods. After six days of development, daphnids were molt synchronized such that animals could be collected at 0, 24, 36, and 48 hours post-molt under each photoperiod. Four replicates of 3-5 daphnids were collected per time point. Daphnids from individual replicates were transferred to 100 µL RNAlater® and held at 4°C for 24 hours, then stored at -80°C until RNA extraction. Animals were homogenized using Next Advance Bullet Blender® and zirconium oxide beads (1.0 mm diameter, Next Advance, Troy, NY). RNA isolation was performed using the SV Total RNA Isolation

System (Promega, Madison, WI) according to manufacturer recommendations. Synthesis of cDNA was conducted using ImProm-II™ Reverse Transcription System with oligo (dT) primers (Promega, Madison, WI).

mRNA levels of the octopamine receptor *OctαR-A* and the serotonin transporter *SERT-a* were measured by RT-qPCR. Primer sets for *SERT-a* and *OctαR-A* were developed using nucleotide sequences provided by the Department of Energy Joint Genome Institute, accessed via NCBI (Bethesda, MD), and made using Primer3 (v. 0.4.0). Primer specificity was confirmed based on amplicon length and nucleotide sequence (Eton Bioscience Inc, San Diego, CA). Primer sequences for *OctαR-A* were: Forward: GAGACCAAAGCTGCCAAGAC, Reverse: CGATCTTCTGGAAGGCGTAG and for *SERT-a* were Forward: AGTCTATGCTCGGCTTCCAA, Reverse: ACCGACTTTGATGGACCAAG. Primer specificity was assessed by confirming amplicon length and amplicon sequence (Eton Bioscience Inc, San Diego, CA). RT-qPCR was performed with the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) using 2x SYBR™ Green Premix (Fisher Scientific). A single melting peak was detected for each sample with an amplification efficiency of >95.0%, indicating amplification occurred only for the target sequence. Genex software (Bio-Rad Laboratories, Hercules, CA) was used to calculate relative levels of mRNAs by normalizing mRNA levels to two housekeeping genes, *actin* and *GAPDH*.

Data Analyses

Treatment-related differences in the proportion of male offspring were assessed by determining the percentage male offspring produced in broods 2 through 6 for each maternal organism. Significant differences among treatments were evaluated using One-way ANOVA with

Tukey's multiple comparisons test ($p \leq 0.05$) when variances were no different among treatments. The Kruskal-Wallis test with Dunn's post hoc comparison was used when variances were significantly different among treatments. Fecundity was assessed by comparing the mean total neonate production for broods 2 through 6 for each maternal organism. Significant differences for long day photoperiod exposures were assessed in the same manner as male sex determination assays. Differences in neonate production for long day photoperiod-reared animals were assessed using Student's t-tests. Differences in mRNA levels at a given time point were analyzed using Student's t-tests, and all mRNA values were normalized to the long day photoperiod control group at 0 hours post molt. Error bars denote SEM. The alpha level was 0.05 for all analyses. Statistical analyses were performed with Prism (v7.02, GraphPad Software, Inc).

RESULTS

Male sex determination

All antidepressants were evaluated for their ability to stimulate or suppress male sex determination in *D. pulex* under environmental conditions at which the maternal organisms would be expected to produce 20-50% males (18°C, 10:14 L:D photoperiod). Two antidepressants that target serotonergic systems were evaluated, fluoxetine hydrochloride and citalopram hydrobromide. Both are selective serotonin reuptake inhibitors, with citalopram having a higher specificity for the serotonin transporter (Pörzgen et al., 2001). Neither serotonin-targeting antidepressant initiated male production under long day photoperiod conditions (Fig. 1A, B). Under the short day photoperiod, fluoxetine exposure tended to increase male production, however, not significantly ($p = 0.08$) (Fig. 1A). Fluoxetine significantly reduced fecundity at 1.0 μM for both short day photoperiod and long day photoperiod assays (Fig. 1C).

Under the short day photoperiod, citalopram significantly increased male production at the highest concentration assessed while also significantly increasing neonate production at the lowest concentration (0.01 μM) (Fig. 1B, D). Citalopram significantly decreased fecundity at the highest concentration in the long day photoperiod assay (Fig. 1D).

Next, we evaluated two pharmaceuticals that are considered atypical antidepressants in that they target multiple transmitter systems. Bupropion (Wellbutrin) is a nonselective inhibitor of dopaminergic and norepinephrine reuptake (Dwoskin et al., 2006), while nomifensine also targets dopamine and norepinephrine reuptake but has higher affinity at both targets (Tatsumi et al., 1997). Neither antidepressant initiated male production in *D. pulex* under long day photoperiod conditions (Fig. 2A, B). Under the short day photoperiod, bupropion did not significantly alter male production or fecundity (Fig. 2A, C). However, under the long day photoperiod, buproprion significantly decreased fecundity at 1.0 μM (Fig. 2C).

Finally, we assessed nisoxetine, a selective norepinephrine re-uptake inhibitor (Tejani-Butt et al., 1990). Under a long day photoperiod, nisoxetine was unable to initiate male production (Fig. 3A). Under the short day photoperiod, exposure tended to increase male production, and, significant differences were found between the lowest and highest concentration groups (Fig. 3A). Fecundity was not impacted by nisoxetine exposure under long day photoperiod conditions however significantly decreased fecundity at the highest concentration under the short day photoperiod (Fig. 3B).

mRNA Levels

We evaluated selected mRNA levels in molt synchronized animals reared under both long day and short day photoperiods. The evaluations of anti-depressant effects on male sex

determination implicated serotonergic signaling and norepinephrine signaling may be involved in male sex determination processes. Octopamine is the equivalent of norepinephrine in invertebrates (Balfanz et al., 2005). Thus, we assessed two genes involved with transmission of these neurotransmitters, *SERT-a*, a serotonin re-uptake transporter, and α-adrenergic-like octopamine receptor (*OctαR-A*) (McCoole et al., 2012). Short day photoperiod produced significantly higher mRNA levels for both *OctαR-A* and *SERT-a* at 24, 36, and 48 hours of the reproductive cycle as compared to long day photoperiod, while no differences were observed between photoperiods at 0 hours post molt (Fig. 4A, B).

DISCUSSION

Results indicated that two classes of antidepressant pharmaceuticals have the potential to stimulate male sex determination, SSRIs and chemicals targeting norepinephrine (octopamine in invertebrates) reuptake.

Other researchers have examined the impacts of antidepressants on crustacean life history outcomes, with the majority of studies focusing on fluoxetine. Multiple studies have observed that exposure to fluoxetine increases neonate production in *D. magna* (Campos et al., 2016; Flaherty and Dodson, 2005). In the present study, fluoxetine had no effect on *D. pulex* neonate production at lower concentrations, and significantly decreased neonate production at the highest concentration assayed. However, our experiments with citalopram did yield significantly higher neonate production at the lowest concentration assessed. Fluoxetine has also been shown to impact *D. magna* feeding rates and molting at lowest observed effect concentrations of 1.7 and 2.9 μM fluoxetine, respectively, which are higher than the concentrations assessed here (Jordão et al., 2016). The incongruous results may reflect differences in experimental design, since other

researchers conducted longer assays with a different species and under a different temperature (warmer). We have previously shown that fecundity and temperature sensitivity differ between *D. pulex* and *D. magna* (Chapter 2).

Our SSRI results agree with work performed by others that have deemed fluoxetine to have higher toxicity than citalopram. In both 48 hour LC_{50} assays and immobility EC_{50} assays, fluoxetine has been found to have higher toxicity (Christensen et al., 2007; Henry et al., 2004). In our results, the highest concentration of citalopram resulted in no adverse effects in the short photoperiod group, while fluoxetine exposure reduced fecundity. Additionally, higher rates of mortality were observed in fluoxetine exposed groups at the highest concentration. SSRIs inhibit the removal of serotonin from the synapse to promote serotonin binding to the post-synaptic cell. We show that the serotonin re-uptake transporter (*SERT-a*) is significantly higher expressed under environmental conditions that are permissive of male production, suggesting that serotonin signaling is involved in male sex determination.

Our findings that altered serotonin signaling can impact reproductive processes are consistent with other studies that have found serotonin to be involved in crustacean reproduction. In crabs, serotonin impacts levels of hormones involved with molting (Girish et al., 2017; Robert et al., 2016; Sainath and Reddy, 2011) as well as levels of methyl farnesoate (Girish et al., 2017). Serotonin has also been implicated in ovarian development in crabs (Richardson et al., 1991). Girish et al. (2017) observed that serotonin elevated methyl farnesoate levels in crabs. Methyl farnesoate is the male sex determining hormone in daphnids (Olmstead and LeBlanc, 2002), thus its elevation by serotonin would be expected to increase the production of male offspring.

Very little is known about the impacts of atypical antidepressants such as bupropion and nomifensine on crustacean reproduction, thus our findings here represent an important

contribution to basic toxicity data for these chemicals and are informative when considering other chemicals with similar modes of action. Likewise, nisoxetine previously had no information regarding daphnid toxicity or life history.

The octopamine-α receptor A (*OctαR-A*) mRNA levels were elevated under environmental conditions that are permissive to male production as compared to non-permissive conditions, further implicating octopaminergic systems in male sex determination processes. Octopamine and its role in neuromodulation has been studied in a range of invertebrates including insects and crustaceans. Octopamine is critical in a variety of sensory based behaviors such as learning, memory, feeding, courtship, motor control, and visual processes (Christie, 2011; Farooqui, 2007; Roeder, 1999; Verlinden et al., 2010). Octopamine has also been associated with neuroendocrine processes, and administration of octopamine has been found to increase levels of juvenile hormone (equivalent to methyl farnesoate in crustaceans) in the corpora allata of honey bees and locusts (Lafon-Cazal and Baehr, 1988; Rachinsky, 1994; Roeder, 1999). Octopamine levels have also been found to be influenced by photoperiod, and in crickets a peak of brain octopamine was observed after the onset of scotophase (darkness) (Woodring et al., 1988). The effects of nisoxetine seen here, which targets the vertebrate equivalent of octopamine re-uptake transporters, may result in increased synaptic octopamine signaling.

Recent advances in mood disorder research implicates dysregulated glutamate signaling in the pathophysiology of depression (Musazzi et al., 2011; Racagni and Popoli, 2008; Sanacora et al., 2012), and there is evidence that commonly prescribed antidepressants (e.g SSRIs and serotonin-norepinephrine reuptake inhibitors, SNRIs) may elicit antidepressant symptom relief through modulation of glutamatergic systems (Du et al., 2006; Liu et al., 2017; Sanacora et al.,

2012; Zarate et al., 2010). For instance, chronic administration of citalopram to mice resulted in altered NMDAR subunit mRNA levels within the brain (Boyer et al., 1998). Additionally SRRIs and SNRIs administration has been shown to alter NMDAR function as well as expression levels in rat hippocampal neurons (Pittaluga et al., 2007). Thus, the SSRIs and norepinephrine-targeting pharmaceuticals assessed here may be exerting their impact on male sex determination via modulation of NMDAR function and glutamate transmission. This would be consistent with our prior findings that NMDAR antagonists increase male production in *D. pulex* (Chapter 3).

Overall, we provide evidence that antidepressants targeting serotonin and octopamine transmitter systems have the potential to stimulate male sex determination in *D. pulex* under permissive environmental conditions. We also provide novel information for the atypical antidepressants, bupropion and nomifensine, regarding their impacts on daphnid life history endpoints. These results, in combination with previous studies (Chapter 3), implicate serotonin, octopamine, and NMDAR signaling in linking environmental cues to endocrine processes that determine sex of offspring in daphnids.

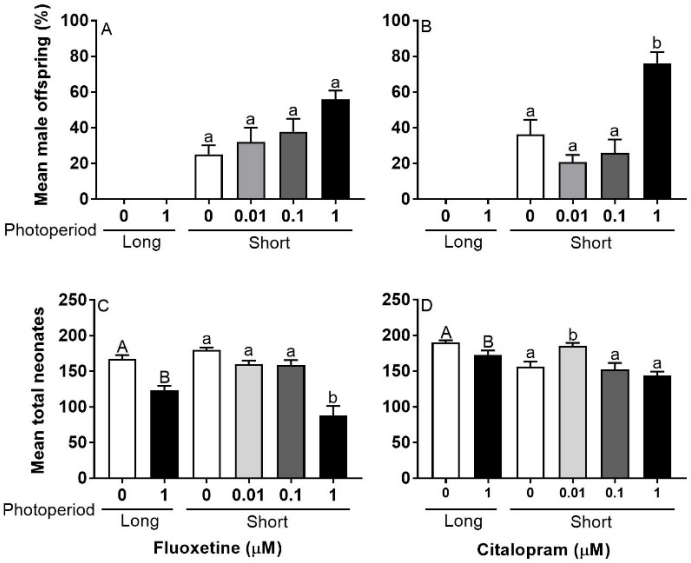

Figure 1. *D. pulex* male sex determination assays with antidepressants targeting serotonergic systems. Panel A and B show male offspring production for fluoxetine and citalopram, respectively, under both long day (16:8 L:D) and short day (10:14 L:D) photoperiods. Panel C and D show neonate production for fluoxetine and citalopram, respectively, during the male sex determination experiments. Different capital letters denote significant differences in long day photoperiod assays, different lower-case letters denote significant differences in short day photoperiod assays.

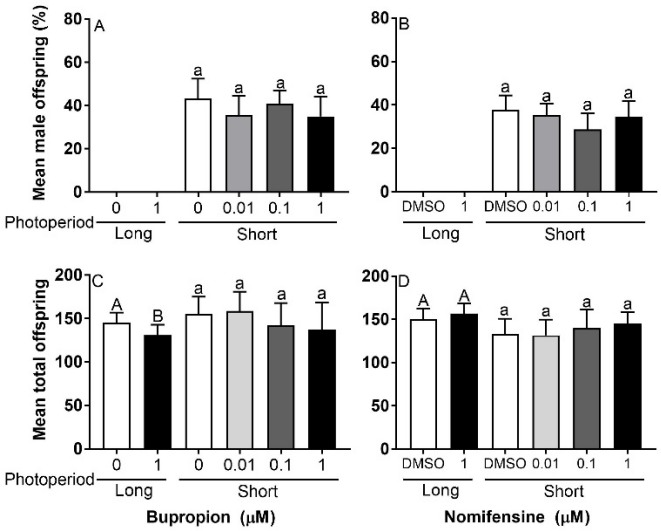

Figure 2. *D. pulex* male sex determination assays with antidepressants targeting dopaminergic and octopaminergic systems. Panel A and B show male offspring production for bupropion and nomifensine, respectively, under both long day (16:8 L:D) and short day (10:14 L:D) photoperiods. Panel C and D show neonate production for bupropion and nomifensine, respectively, during the male sex determination experiments. Different capital letters denote significant differences in long day photoperiod assays, different lower-case letters denote significant differences in short day photoperiod assays.

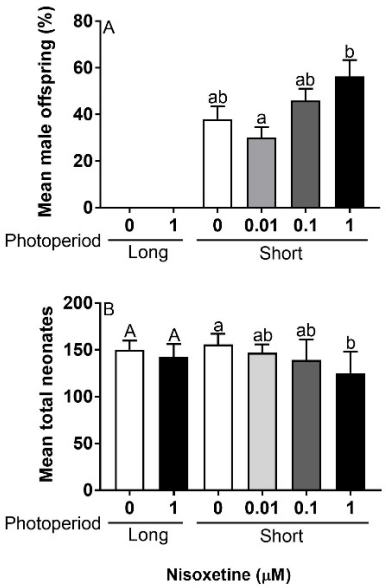

Figure 3. *D. pulex* male sex determination assays with nisoxetine, an octopaminergic system-targeting pharmaceutical. Panel A shows male offspring production for nisoxetine, under both long day (16:8 L:D) and short day (10:14 L:D) photoperiods. Panel B shows neonate production for nisoxetine during the male sex determination experiments. Different capital letters denote significant differences in long day photoperiod assays, different lower-case letters denote significant differences in short day photoperiod assays.

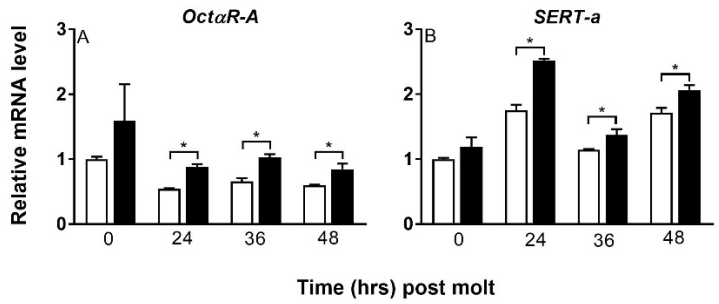

Figure 4. *OctαR-A* receptor and *SERT-a* transporter mRNA levels in *D. pulex* reared under long day and short day photoperiods across the reproductive cycle. White bars represent long day photoperiod and black bars represent short day photoperiod. Panels A: mRNA levels of the octopamine receptor *OctαR-A*. Panel B: mRNA levels of the serotonin transporter *SERT-a*. All groups were normalized to the long day photoperiod control group at time 0. Asterisks denote significant differences between groups.

REFERENCES

Balfanz, S., Strünker, T., Frings, S. and Baumann, A. (2005). A family of octapamine receptors that specifically induce cyclic AMP production or Ca2+ release in *Drosophila melanogaster*. *J. Neurochem.* **93**, 440–451.

Boyer, P.-A., Skolnick, P. and Fossom, L. (1998). Chronic administration of imipramine and citalopram alters the expression of NMDA receptor subunit mRNAs in mouse brain. *J. Mol. Neurosci.* **10**, 219–233.

Brunello, N., Mendlewicz, J., Kasper, S., Leonard, B., Montgomery, S., Nelson, J. C., Paykel, E., Versiani, M. and Racagni, G. (2002). The role of noradrenaline and selective noradrenaline reuptake inhibition in depression. *Eur. Neuropsychopharmacol.* **12**, 461–475.

Campos, B., Rivetti, C., Kress, T., Barata, C. and Dircksen, H. (2016). Depressing antidepressant: Fluoxetine affects serotonin neurons causing adverse reproductive responses in *Daphnia magna*. *Environ. Sci. Technol.*

Christensen, A. M., Faaborg-Andersen, S., Ingerslev, F. and Baun, A. (2007). Mixture and single-substance acute toxicity of selective serotonin reuptake inhibitors in *Ceriodaphnia dubia*. *Environ. Toxicol. Chem.* **26**, 85–91.

Christie, A. E. (2011). Crustacean neuroendocrine systems and their signaling agents. *Cell Tissue Res.* **345**, 41–67.

Du, J., Machado-Vieira, R., Maeng, S., Martinowich, K., Manji, H. K. and Zarate, C. A. (2006). Enhancing AMPA to NMDA throughput as a convergent mechanism for antidepressant action. *Drug Discov. Today* **3**, 519–526.

Dwoskin, L. P., Rauhut, A. S., King-Pospisil, K. A. and Bardo, M. T. (2006). Review of the pharmacology and clinical profile of bupropion, an antidepressant and tobacco use cessation

agent. *CNS Drug Rev.* **12**, 178–207.

Farooqui, T. (2007). Octopamine-mediated neuromodulation of insect senses. *Neurochem. Res.* **32**, 1511–1529.

Flaherty, C. M. and Dodson, S. I. (2005). Effects of pharmaceuticals on *Daphnia* survival, growth, and reproduction. *Chemosphere* **61**, 200–207.

Ghasemi, M., Raza, M. and Dehpour, A. R. (2010). NMDA receptor antagonists augment antidepressant-like effects of lithium in the mouse forced swimming test. *J. Psychopharmacol.* **24**, 585–594.

Girish, B. P., Swetha, C. H. and Reddy, P. S. (2017). Serotonin induces ecdysteroidogenesis and methyl farnesoate synthesis in the mud crab, *Scylla serrata*. *Biochem. Biophys. Res. Commun.* **490**, 1340–1345.

Hannas, B. R., Das, P. C., Li, H. and LeBlanc, G. a (2010). Intracellular conversion of environmental nitrate and nitrite to nitric oxide with resulting developmental toxicity to the crustacean *Daphnia magna*. *PLoS One* **5**, e12453.

Hebert, P. D. N. (1978). The population biology of *Daphnia* (Crustacea, Daphnidae). *Biol. Rev.* **53**, 387–426.

Henry, T. B., Kwon, J.-W., Armbrust, K. L. and Black, M. C. (2004). Acute and chronic toxicity of five selective serotonin reuptake inhibitors in *Ceriodaphnia dubia*. *Environ. Toxicol. Chem.* **23**, 2229–2233.

Hobek, A. and Larsson, P. (1990). Sex determination in *Daphnia magna*. *Ecology* **71**, 2255–2268.

Huettner, J. E. and Bean, B. P. (1988). Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: Selective binding to open channels. *Proc. Natl. Acad. Sci.* **85**,

1307–1311.

Jordão, R., Garreta, E., Campos, B., Lemos, M. F. L., Soares, A. M. V. M., Tauler, R. and Barata, C. (2016). Compounds altering fat storage in *Daphnia magna*. *Sci. Total Environ.* **545**–**546**, 127–136.

Kakaley, E. K. M., Wang, H. Y. and LeBlanc, G. A. (2017). Agonist-mediated assembly of the crustacean methyl farnesoate receptor. *Sci. Rep.* **7**, 45071.

Lafon-Cazal, M. and Baehr, J. C. (1988). Octopaminergic control of corpora allata activity in an insect. *Experientia* **44**, 895–896.

Lampert, W. (2006). *Daphnia*: Model herbivore, predator and prey. *Polish J. Ecol.* **54**, 607–620.

LeBlanc, G. A. and Medlock, E. K. (2015). Males on demand: the environmental-neuro-endocrine control of male sex determination in daphnids. *FEBS J.* 1–14.

Liu, B., Liu, J., Wang, M., Zhang, Y. and Li, L. (2017). From Serotonin to Neuroplasticity: Evolvement of Theories for Major Depressive Disorder. *Front. Cell. Neurosci.* **11**, 1–9.

McCoole, M. D., Atkinson, N. J., Graham, D. I., Grasser, E. B., Joselow, A. L., McCall, N. M., Welker, A. M., Wilsterman, E. J., Baer, K. N., Tilden, A. R., et al. (2012). Genomic analyses of aminergic signaling systems (dopamine, octopamine and serotonin) in *Daphnia pulex*. *Comp. Biochem. Physiol. - Part D Genomics Proteomics* **7**, 35–58.

Miyakawa, H., Toyota, K., Sumiya, E. and Iguchi, T. (2014). Comparison of JH signaling in insects and crustaceans. *Curr. Opin. Insect Sci.* **1**, 81–87.

Murrough, J. W. (2015). Glutamate NMDA receptor modulators for the treatment of depression: trials and tribulations. *Psychopharmacology (Berl).*

Musazzi, L., Racagni, G. and Popoli, M. (2011). Stress, glucocorticoids and glutamate release:

Effects of antidepressant drugs. *Neurochem. Int.* **59**, 138–149.

Olmstead, A. W. and LeBlanc, G. A. (2000). Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. *Environ. Toxicol. Chem.* **19**, 2107–2113.

Olmstead, A. W. and LeBlanc, G. A. (2002). Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. *J. Exp. Zool.* **293**, 736–739.

Padovan, C. M. and Guimarães, F. S. (2004). Antidepressant-like effects of NMDA-receptor antagonist injected into the dorsal hippocampus of rats. *Pharmacol. Biochem. Behav.* **77**, 15–19.

Pittaluga, A., Raiteri, L., Longordo, F., Luccini, E., Barbiero, V. S., Racagni, G., Popoli, M. and Raiteri, M. (2007). Antidepressant treatments and function of glutamate ionotropic receptors mediating amine release in hippocampus. *Neuropharmacology* **53**, 27–36.

Pörzgen, P., Park, S. K., Hirsh, J., Sonders, M. S. and Amara, S. G. (2001). The antidepressant-sensitive dopamine transporter in *Drosophila melanogaster*: a primordial carrier for catecholamines. *Mol. Pharmacol.* **59**, 83–95.

Racagni, G. and Popoli, M. (2008). Cellular and molecular mechanisms in the long-term action of antidepressants. *Dialogues Clin. Neurosci.* **10**, 385–400.

Rachinsky, A. (1994). Octopamine and serotonin influence on corpora allata activity in honey bee (*Apis mellifera*) larvae. *J. Insect Physiol.* **40**, 549–554.

Richardson, G. H., Deecaraman, M. and Fingerman, M. (1991). The effect of biogenic amines on ovarian development in the fiddler crab, *Uca pugilator*. *Comp. Biochem. Physiol.* **99C**, 53–56.

Robert, A., Monsinjon, T., Delbecque, J. P., Olivier, S., Poret, A., Foll, F. Le, Durand, F.

and Knigge, T. (2016). Neuroendocrine disruption in the shore crab *Carcinus maenas*: Effects of serotonin and fluoxetine on chh- and mih-gene expression, glycaemia and ecdysteroid levels. *Aquat. Toxicol.* **175**, 192–204.

Roeder, T. (1999). Octopamine in invertebrates. *Prog. Neurobiol.* **59**, 533–561.

Sainath, S. B. and Reddy, P. S. (2011). Effect of selected biogenic amines on reproduction in the fresh water edible crab, *Oziotelphusa senex senex*. *Aquaculture* **313**, 144–148.

Sanacora, G., Treccani, G. and Popoli, M. (2012). Towards a glutamate hypothesis of depression: An emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology* **62**, 63–77.

Sernagor, E., Kuhn, D., Vyklicky, L. and Mayer, M. L. (1989). Open channel block of NMDA receptor responses evoked by tricyclic antidepressants. *Neuron* **2**, 1221–7.

Tatsumi, M., Groshan, K., Blakely, R. D. and Richelson, E. (1997). Pharmacological profile of antidepressants and related compounds at human monoamine transporters. *Eur. J. Pharmacol.* **340**, 249–258.

Tejani-Butt, S. M., Brunswick, D. J. and Frazer, A. (1990). [3H]Nisoxetine: a new radioligand for norepinephrine uptake sites in brain. *Eur. J. Pharmacol.* **191**, 239–243.

Toyota, K., Miyakawa, H., Hiruta, C., Furuta, K., Ogino, Y., Shinoda, T., Tatarazako, N., Miyagawa, S., Shaw, J. R. and Iguchi, T. (2015b). Methyl farnesoate synthesis is necessary for the environmental sex determination in the water flea *Daphnia pulex*. *J. Insect Physiol.* **80**, 22–30.

Toyota, K., Miyakawa, H., Yamaguchi, K., Shigenobu, S., Ogino, Y., Tatarazako, N., Miyagawa, S. and Iguchi, T. (2015a). NMDA receptor activation upstream of methyl farnesoate signaling for short day-induced male offspring production in the water flea,

Daphnia pulex. *BMC Genomics* **16**, 186.

Verlinden, H., Vleugels, R., Marchal, E., Badisco, L., Pflüger, H. J., Blenau, W. and Broeck, J. Vanden (2010). The role of octopamine in locusts and other arthropods. *J. Insect Physiol.* **56**, 854–867.

Woodring, J. P., Meier, O. W. and Rose, R. (1988). Effect of development, photoperiod, and stress on octopamine levels in the house cricket, *Acheta domesticus*. *J. Insect Physiol.* **34**, 759–765.

Xie, X., Tao, T., Liu, M., Zhou, Y., Liu, Z. and Zhu, D. (2016). The potential role of juvenile hormone acid methyltransferase in methyl farnesoate (MF) biosynthesis in the swimming crab, *Portunus trituberculatus*. *Anim. Reprod. Sci.* **168**, 40–49.

Zarate, C., MacHado-Vieira, R., Henter, I., Ibrahim, L., Diazgranados, N. and Salvadore, G. (2010). Glutamatergic modulators: The future of treating mood disorders? *Harv. Rev. Psychiatry* **18**, 293–303.

CHAPTER 5

Influence of the N-methyl-D-aspartate receptor antagonist MK-801 on gene expression relating to male sex determination in the freshwater crustacean *Daphnia pulex* (Crustacea: Cladocera)

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ABSTRACT

The N-methyl-D-aspartate receptor (NMDAR) antagonist MK-801 has been shown to stimulate male sex determination in daphnids under environmental conditions permissive of male sex determination. We tested the hypothesis that MK-801 exerted this effect on male sex determination by altering mRNA levels of NMDAR subunits and putative downstream signaling elements of the male sex determining signaling pathway in the freshwater crustacean *Daphnia pulex*. We assessed mRNA levels of NMDAR-a, NMDAR-b, the methyl farnesoate synthesizing enzymes JHAMT and FAMT, and the methyl farnesoate receptor subunits Met and SRC under photoperiods permissive (short day photoperiod, 10:14 hr L:D) and non-permissive (long day photoperiod, 16:8 hr L:D) of male sex determination. Under the short day photoperiod, MK-801 exposure significantly decreased mRNA levels of *NMDAR-b, Met*, and *SRC.* Conversely, under the long day photoperiod, MK-801 exposure significantly increased mRNA levels of *NMDAR-b, Met*, and *SRC.* MK-801 had no effect on mRNA levels of *NMDAR-a, JHAMT,* and *FAMT.* These results indicate that MK-801 modulates the expression of the NMDAR-b subunit, as well as the subunits of the methyl farnesoate receptor. However, the effect on these mRNAs differ under photoperiods that are either permissive or non-permissive of male sex determination. Further, the effects of MK-801 are incongruous with previously demonstrated effects of photoperiod on these mRNAs, suggesting additional complexity to the regulation of male-sex determination.

INTRODUCTION

Environmental sex determination, the phenomenon where environmental cues influence the sex ratio of offspring, occurs in a wide range of taxonomic groups including nematodes, insects (Korpelainen, 1990), fish (Devlin and Nagahama, 2002; F. W. H. Beamish, 1993), crustaceans

(Hebert, 1978), and reptiles (Ciofi and Swingland, 1997; Lang and Andrews, 1994). Studies to date indicate that environmental sex determination involves the sensory transduction of environmental cues and subsequent alteration of hormonal processes in order to influence offspring sex (Devlin and Nagahama, 2002; Korpelainen, 1990). A variety of environmental cues regulate environmental sex determination depending on the taxonomic group including temperature, nutritional status, pH, crowding cues, and photoperiod (Ciofi and Swingland, 1997; Korpelainen, 1990).

The crustacean *Daphnia sp* are keystone freshwater invertebrates that are subject to environmental sex determination in that many species are cyclic parthenogens and reproduce both asexually and sexually (Hobek and Larsson, 1990; Innes and Singleton, 2000; Kleiven et al., 1992; Lampert et al., 2012). Daphnids clonally produce female offspring when environmental conditions are favorable for population growth (Hebert, 1978). Male offspring are produced when environmental cues signal adverse conditions to enable sexual reproduction (Hobek and Larsson, 1990; Kleiven et al., 1992; Korpelainen, 1986). We have recently shown that the environmental cues of photoperiod and temperature co-regulate male sex determination in *Daphnia pulex*, such that a short day (10:14 hr Light:Dark) photoperiod creates permissive conditions and temperature further modulates the response (Chapter 2).

We and others have also determined the putative elements of the male sex determination hormone signaling cascade. The enzymes juvenile hormone acid o-methyltransferase (JHAMT) and farnesoic acid o-methyltransferase (FAMT) (Xie et al., 2016) produce methyl farnesoate, the male sex-determining hormone in daphnids (Olmstead and LeBlanc, 2002; Toyota et al., 2015a). Methyl farnesoate activates the methyl farnesoate receptor (MfR) by binding the Met subunit of the MfR and stimulating recruitment of steroid receptor co-activator (SRC) to create a heterodimer

of Met-SRC (Kakaley et al., 2017; Toyota et al., 2015b). The activated MfR functions as a transcription factor to regulate expression of male sex determining genes and other genes (Kato et al., 2011; Rider et al., 2005).

The neuroendocrine linkage that mediates the transition from asexual to sexual reproduction in daphnids remains unknown, however, we have provided evidence for the involvement of the N-methyl-D-aspartate receptor (NMDAR) and its sensitivity to photoperiodic cues (Chapter 3). The NMDAR is an ionotropic glutamate receptor that is a coincident detector within the nervous system, requiring several factors to be present for the receptor to open (Rousseaux, 2008; Traynelis et al., 2010). The NMDAR requires a NR1 (also called NMDAR-a) subunit, which contains the co-factor binding site, as well as an NR2 (also called NMDAR-b) subunit, which contains the glutamate binding site, for a functional receptor (Rousseaux, 2008).

We have previously shown that the NMDAR antagonist MK-801 increases male sex determination in *D. pulex* under conditions permissive of male production (Chapter 3), suggesting that MK-801 has the potential to alter signaling in the daphnid male sex determining signaling cascade. Here we hypothesized that MK-801 alters mRNA levels of NMDAR subunits and signaling elements in the male sex determining signaling cascade.

MATERIALS AND METHODS

Animal Rearing and Exposures

D. pulex (clone WTN6) cultures were maintained in the laboratory at 20°C, 16:8 hr Light:Dark (L:D) using methods described previously (Hannas et al., 2010). Daphnids were collected from culture as < 24-hour old neonates and transferred to either long (16:8 L:D) or short (10:14 L:D) day photoperiods at 18°C prior to chemical exposures. Neonates were individually

reared in 50 mL beakers containing 40 mL of daphnid media. Daphnid media consisted of reconstituted deionized water (192 mg/L $CaSO_4 \cdot H_2O$, 192 mg/L $NaHCO_3$, 120 mg/L $MgSO_4$, 8.0 mg/L KCl, 1.0 µg/L selenium and 1.0 µg/L vitamin B_{12}). Daphnids were fed daily 1.4 x 10^8 cells *Pseudokirchneriella subcapitata* and Tetrafin® fish food suspension (4 mg dry weight) (Pet International, Blacksburg, VA, USA) as described previously (Hannas et al., 2010). Daphnids were exposed to 10 µM MK-801 upon deposition of the first brood of embryos into their brood chamber. The stock solutions of (+)-MK-801 hydrogen maleate (Fisher Scientific, Hampton, NH) were prepared in deionized water. Exposure solutions were changed every other day.

mRNA Level Measurements

All animals were molt synchronized (Mu and LeBlanc, 2002) at the release of their first brood of offspring and collected at 0, 24, 36, or 48 hours post-molt. Four replicates each containing 3-5 daphnids were collected at each time point. Daphnids from individual replicates were transferred to 100 µL RNAlater® and held at 4°C for 24 hours, then stored at -80°C until used for RNA extraction. Whole animals were homogenized using a Next Advance Bullet Blender® and zirconium oxide beads (1.0mm diameter, Next Advance, Troy, NY). RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI) according to manufacturer recommendations. cDNA was synthesized using ImProm-II™ Reverse Transcription System with oligo (dT) primers (Promega, Madison, WI).

mRNA levels of *NMDAR-a*, *NMDAR-b, JHAMT*, *FAMT*, *Met*, and *SRC* were measured by RT-qPCR. Primer sequences for *NMDAR-a* (Chapter 3), *NMDAR-b* (Chapter 3), *JHAMT* (Miyakawa et al., 2010), *FAMT* (Toyota et al., 2015b), *Met* (Miyakawa et al., 2010), and *SRC* (Toyota et al., 2015b) were used to amplify mRNA sequences. RT-qPCR was performed with the

7300 Real Time PCR System (Applied Biosystems, Foster City, CA) using 2x SYBR™ Green Premix (ThermoFisher Scientific). A single melting peak was detected for each sample with an amplification efficiency of >93%, indicating amplification occurred only for the target sequence. Genex software (Bio-Rad Laboratories, Hercules, CA) was used to analyze relative levels of gene expression by normalizing to two housekeeping genes, *actin* and *GAPDH*.

Data Analyses

Treatment differences in mRNA levels were evaluated using Student's t-test when variances were not different among treatments. The Mann-Whitney test was used when variances were significantly different between groups. Significance was set at an alpha level of 0.05. Statistical analyses were performed with Prism (v7.02, GraphPad Software, Inc).

RESULTS

Impact of MK-801 on selected mRNA levels

Experiments were performed to evaluate whether MK-801 stimulated male offspring production by manipulating mRNA levels of components of the male sex determining signaling pathway. Under the photoperiod non-permissive of male sex determination, MK-801 had no impact on *NMDAR-a* mRNA levels (Fig. 1A), while *NMDAR-b* mRNA levels were significantly higher with MK-801 exposure (Fig. 1B). MK-801 had little effect on *JHAMT* and *FAMT* mRNA levels under the non-permissive photoperiod (Figs. 1C, D). Consistent with impacts on *NMDAR-b* mRNA levels, *Met* and *SRC* mRNA levels were significantly elevated as compared to levels in untreated organisms under the non-permissive photoperiod (Figs. 1E, F).

Under the permissive 10:14 L:D photoperiod, MK-801 had no impact on *NMDAR-a* mRNA levels (Fig. 2A). *NMDAR-b* mRNA levels were highly responsive to MK-801 exposure,

however, under the permissive photoperiod, *NMDAR-b* levels were significantly lower with MK-801 treatment as compared to untreated organisms (Fig. 2B). MK-801 had little effect on *JHAMT* and *FAMT* mRNA levels under the permissive photoperiod (Figs. 2C, D). Consistent with the suppressive action of MK-801 on *NMDAR-b* under the permissive photoperiod, levels of *Met* and *SRC* mRNA also were significantly lower among MK-801 treated organisms as compared to untreated daphnids (Figs. 2E, F).

In summary, results demonstrate sensitivity of *NMDAR-b*, *Met*, and *SRC* mRNA levels to MK-801 treatment. Results provide evidence to suggest that *Met* and *SRC* expression may be co-regulated with *NMDAR-b* or regulated by a functional NMDAR resulting from synthesis of the NMDAR-*b* subunit.

DISCUSSION

We hypothesized that the vertebrate NMDAR antagonist MK-801, which is able to increase sex ratios in *D. pulex*, would alter mRNA levels of the NMDAR subunits as well as key genes in the male sex determination signaling cascade. We demonstrated that MK-801 increased mRNA levels of *NMDAR-b*, *Met*, and *SRC* in animals reared under a long day, non-permissive photoperiod, and decreased mRNA levels of the same genes in animals reared under a short day, permissive photoperiod. MK-801 did not impact *NMDAR-a*, *JHAMT* or *FAMT* mRNA levels.

We found that MK-801 was unable to alter *NMDAR-a* mRNA levels. This result was consistent with our previous observation *NMDAR-a* mRNA levels were consistent despite varying environmental conditions (Chapter 3). Together these results support the conclusion that NMDAR-a expression is not readily perturbed by environmental stimuli nor chemical exposure to NMDAR-targeting compounds.

Our results show that expression of the enzymes responsible for producing the male sex determining hormone methyl farnesoate were not increased by MK-801 exposure under conditions permissive to male production. However, male:female sex ratios are increased in animals exposed to MK-801 (Chapter 3). This suggests that glutamate signaling through the NMDAR is not the primary driver of male sex determination. Moreover, our results suggest that *Met* and *SRC* mRNA levels are also not driving this response since MK-801 decreased levels of those genes as well in conditions permissive to male production. While these results together support a relationship between *NMDAR-b*, *Met*, and *SRC* expression, it is unclear why short day photoperiod in addition to MK-801 exposure decreases mRNA levels for these genes.

One other researcher has found instances of chemical exposure altering expression of *Met*. Similar to daphnids, insect Met is critical to growth and development, as it binds to juvenile hormone, the insect equivalent to methyl farnesoate (Jindra et al., 2013; Miura et al., 2005; Miyakawa et al., 2013). *Chironomus riparius* exposed to chemicals frequently found in personal care products exhibited increased expression of *Met* (Ozáez et al., 2016).

The effects of MK-801 on mRNA levels along the male sex determining pathway exhibit a lack of concordance with previous observations on the effect of photoperiod on these mRNA levels. Results suggests that MK-801 would render organisms reared under a non-permissive photoperiod, susceptible to temperature-induced male sex determination and likewise, would render organisms reared under a permissive photoperiod, non-susceptible to temperature-induced male sex determination. Yet, previous studies revealed that permissiveness, as determined by photoperiod, is not subject to alteration by MK-801 (Chapter 3).

Temperature also is a known regulator of male sex determination (Chapter 2). Further, we previously provided evidence that MK-801 and temperature could function together to

stimulate male sex determination. Thus, effects of MK-801 on pathway components that are subject of modulation by photoperiod may be secondary and insignificant when compared to its possible effects on pathway components that are subject to modulation by temperature. This aspect of the male sex determination pathway was not evaluated in the present study.

In conclusion, results from this study, along with previous results (Chapter 3), demonstrate that components of the putative male sex determining pathway, specifically NMDAR-b, Met, and SRC, are subject to modulation by external influences that may regulate or dysregulate population sex ratios. Similarities in induction trends and magnitudes suggest that *NMDAR-b* and *SRC* are co-regulated. Alternatively, induction of *NMDAR-b* may result in the assembly of functional NMDAR whose activity increases expression of *SRC*.

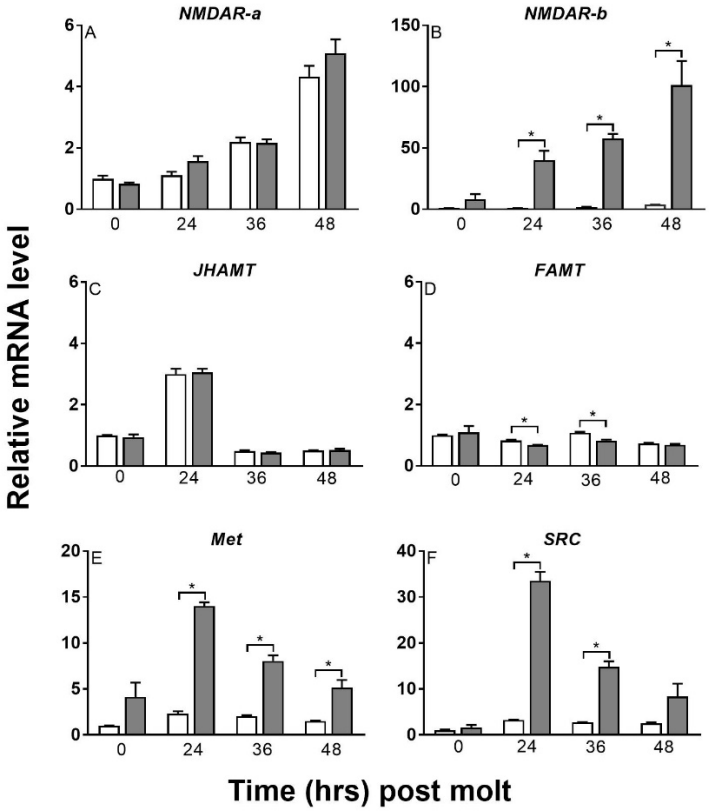

Figure 1. Relative mRNA levels for *D. pulex* reared under long day photoperiod with 10 µM MK-801 exposure. White bars represent long day photoperiod and grey bars represent long day photoperiod MK-801-exposed. Panels A and B: Levels of the NMDAR subunits, a and b, respectively. Panels C and D: Levels of the enzymes responsible for methyl farnesoate production, JHAMT and FAMT, respectively. Panels E and F: Levels of the MfR subunits, Met and SRC, respectively. All groups are normalized to the respective control long day photoperiod at 0 hours. Data are presented as mean and standard error (n=4). As asterisk denotes a significant difference ($p \leq 0.05$).

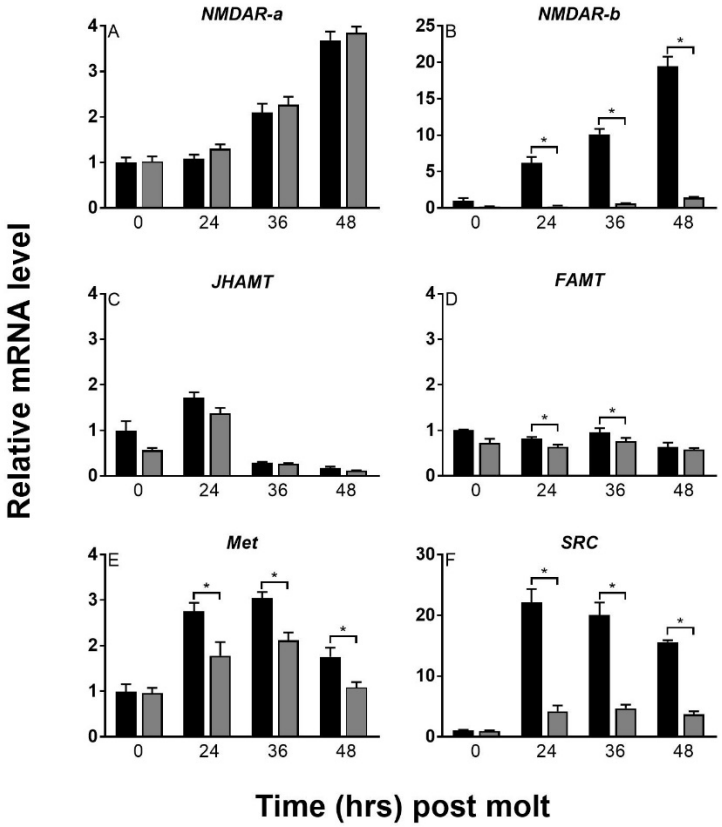

Figure 2. Relative mRNA levels for *D. pulex* reared under short day photoperiod with 10 µM MK-801 exposure. Black bars represent short day photoperiod and grey bars represent short day photoperiod MK-801-exposed. Panels A and B: Levels of the NMDAR subunits, a and b, respectively. Panels C and D: Levels of the enzymes responsible for methyl farnesoate production, JHAMT and FAMT, respectively. Panels E and F: Levels of the MfR subunits, Met and SRC, respectively. All groups are normalized to the respective control long day photoperiod at 0 hours. An asterisk denotes significant differences. Data are presented as mean and standard error (n=4). As asterisk denotes a significant difference ($p \leq 0.05$).

REFERENCES

Ciofi, C. and Swingland, I. R. (1997). Environmental sex determination in reptiles. *Appl. Anim. Behav. Sci.* **51**, 251–265.

Devlin, R. H. and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* **208**, 191–364.

F. W. H. Beamish (1993). Environmental Sex Determination in Southern Brook Lamprey, *Ichthyomyzon gagei*. *Can. J. Fish. Aquat. Sci.* **50**, 1299–1307.

Hannas, B. R., Das, P. C., Li, H. and LeBlanc, G. a (2010). Intracellular conversion of environmental nitrate and nitrite to nitric oxide with resulting developmental toxicity to the crustacean *Daphnia magna*. *PLoS One* **5**, e12453.

Hebert, P. D. N. (1978). The population biology of *Daphnia* (Crustacea, Daphnidae). *Biol. Rev.* **53**, 387–426.

Hobek, A. and Larsson, P. (1990). Sex determination in *Daphnia magna*. *Ecology* **71**, 2255–2268.

Innes, D. J. and Singleton, D. R. (2000). Variation in allocation to sexual and asexual reproduction among clones of cyclically parthenogenetic *Daphnia pulex* (Crustacea: Cladocera). *Biol. J. Linn. Soc.* **71**, 771–787.

Jindra, M., Palli, S. R. and Riddiford, L. M. (2013). The Juvenile Hormone Signaling Pathway in Insect Development. *Annu. Rev. Entomol.* **58**, 181–204.

Kakaley, E. K. M., Wang, H. Y. and LeBlanc, G. A. (2017). Agonist-mediated assembly of the crustacean methyl farnesoate receptor. *Sci. Rep.* **7**, 45071.

Kato, Y., Kobayashi, K., Watanabe, H. and Iguchi, T. (2011). Environmental sex

determination in the branchiopod crustacean *Daphnia magna*: Deep conservation of a Doublesex gene in the sex-determining pathway. *PLoS Genet.* **7**,.

Kleiven, O. T., Larsson, P. and Hobek, A. (1992). Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* **65**, 197–206.

Korpelainen, H. (1986). The effects of temperature and photoperiod on life history parameters of *Daphnia magna* (Crustacea: Cladocera). *Freshw. Biol.* **16**, 615–620.

Korpelainen, H. (1990). Sex ratios and conditions required for environmental sex determination in animals. *Biol. Rev. Camb. Philos. Soc.* **65**, 147–184.

Lampert, W., Lampert, K. P. and Larsson, P. (2012). Induction of male production in clones of *Daphnia pulex* by the juvenoid hormone methyl farnesoate under short photoperiod. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* **156**, 130–133.

Lang, J. W. and Andrews, H. V (1994). Temperature-dependent sex determination in crocodilians. *J. Exp. Zool.* **270**, 28–44.

Miura, K., Oda, M., Makita, S. and Chinzei, Y. (2005). Characterization of the Drosophila Methoprene-tolerant gene product: Juvenile hormone binding and ligand-dependent gene regulation. *FEBS J.* **272**, 1169–1178.

Miyakawa, H., Imai, M., Sugimoto, N., Ishikawa, Y., Ishikawa, A., Ishigaki, H., Okada, Y., Miyazaki, S., Koshikawa, S., Cornette, R., et al. (2010). Gene up-regulation in response to predator kairomones in the water flea, *Daphnia pulex*. *BMC Dev. Biol.* **10**, 45.

Miyakawa, H., Toyota, K., Hirakawa, I., Ogino, Y., Miyagawa, S., Oda, S., Tatarazako, N., Miura, T., Colbourne, J. K. and Iguchi, T. (2013). A mutation in the receptor Methoprene-tolerant alters juvenile hormone response in insects and crustaceans. *Nat. Commun.* **4**, 1856–1857.

Mu, X. and LeBlanc, G. A. (2002). Developmental toxicity of testosterone in the crustacean *Daphnia magna* involves anti-ecdysteroidal activity. *Gen. Comp. Endocrinol.* **129**, 127–133.

Olmstead, A. W. and LeBlanc, G. A. (2002). Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. *J. Exp. Zool.* **293**, 736–739.

Ozáez, I., Aquilino, M., Morcillo, G. and Martínez-Guitarte, J. (2016). UV filters induce transcriptional changes of different hormonal receptors in *Chironomus riparius* embryos and larvae. *Environ. Pollut.* **214**, 239–247.

Rider, C. V, Gorr, T. A., Olmstead, A. W., Wasilak, B. A. and LeBlanc, G. A. (2005). Stress signaling: coregulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean. *J. Exp. Biol.* **208**, 15–23.

Rousseaux, C. G. (2008). A Review of Glutamate Receptors I: Current Understanding of Their Biology. *J. Toxicol. Pathol.* **21**, 25–51.

Toyota, K., Miyakawa, H., Yamaguchi, K., Shigenobu, S., Ogino, Y., Tatarazako, N., Miyagawa, S. and Iguchi, T. (2015a). NMDA receptor activation upstream of methyl farnesoate signaling for short day-induced male offspring production in the water flea, *Daphnia pulex*. *BMC Genomics* **16**, 186.

Toyota, K., Miyakawa, H., Hiruta, C., Furuta, K., Ogino, Y., Shinoda, T., Tatarazako, N., Miyagawa, S., Shaw, J. R. and Iguchi, T. (2015b). Methyl farnesoate synthesis is necessary for the environmental sex determination in the water flea *Daphnia pulex*. *J. Insect Physiol.* **80**, 22–30.

Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Odgen, K. K., Hansen, K. B., Yuan, H., Myers, S. J. and Dingledine, R. (2010). Glutamate receptor

ion channels: structure, regulation and function. *Pharmacol. Rev.* **62**, 405–496.

Xie, X., Tao, T., Liu, M., Zhou, Y., Liu, Z. and Zhu, D. (2016). The potential role of juvenile hormone acid methyltransferase in methyl farnesoate (MF) biosynthesis in the swimming crab, *Portunus trituberculatus*. *Anim. Reprod. Sci.* **168**, 40–49.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Environmental sex determination (ESD) is a strategy utilized by many genera of organisms, though relatively little is known of the processes that regulate the phenomenon. Research has focused largely upon: a) environmental factors that influence sex determination (Ciofi and Swingland, 1997; Kleiven et al., 1992; Korpelainen, 1986; Korpelainen, 1990), b) endocrine processes that dictate sex of offspring (Crews and Bergeron, 1994; Luckenbach et al., 2009; Olmstead and LeBlanc, 2002), and c) ability of environmental chemicals to disrupt sex determination (Guillette Jr. et al., 1995; Olmstead and LeBlanc, 2000; Vajda, Alan et al., 2008). Processes responsible for the reception of environmental cues and their conversion to endocrine signals remains largely unknown. In this research program, we have provided compelling evidence for neurological processes that fill this knowledge gap.

Daphnids are an excellent organism with which to study processes relating to ESD given their sequenced genome, rapid development time, ease of husbandry, and cyclic parthenogenesis reproductive strategy of many species (Christie and McCoole, 2012; Colbourne et al., 2005; Colbourne et al., 2011). Daphnids are broadly used in a variety of scientific fields and are relied on as a representative genus for freshwater invertebrates (U.S. EPA, 2002). Their extensive use in toxicity testing has established daphnids as a model organism, however, despite their status as a surrogate aquatic invertebrate in research, their ability to switch from clonal reproduction to sexual reproduction is an aspect of their biology that remains poorly understood.

Some aspects of daphnid male sex determination have been established, such as the terminal hormone signaling cascade. We and others have determined that methyl farnesoate is the hormone required to program developing oocytes into male offspring within daphnids (Olmstead

and LeBlanc, 2002; Toyota et al., 2015a). Methyl farnesoate, which is produced by two enzymes JHAMT and FAMT, binds to the transcription factor Met in order to initiate heterodimerization with another transcription factor SRC (Kakaley et al., 2017; Xie et al., 2016). Together these comprise the methyl farnesoate receptor and orchestrate downstream gene expression (Kato et al., 2011; Rider et al., 2005). However, both an equivocal understanding of the critical environmental cues that initiate the switch to male production, and the neural processing that mediates this response was lacking and required further inquiry. We hypothesized that an upstream neural processor of environmental signals initiates the cascade leading to environmental sex determination. We further proposed that this processor is subject to disruption by neuroactive exogenous chemicals.

The research in Chapter Two sought to characterize the environmental stimuli that lead to male sex determination in two species of daphnids (*Daphnia magna* and *D. pulex*), and hypothesized that photoperiod and temperature would co-regulate the response. We found that photoperiod was critical in creating permissive conditions for male production, and temperature modulated the response in a species-specific manner. Upon establishing stimulatory cues, we further hypothesized that environmental stimuli alone would impact mRNA levels of genes known to be involved in the male sex determining process. We discovered that environmental stimuli significantly increased mRNA levels of the transcription factor Met in *D. pulex*, which binds to methyl farnesoate when it is present to initiate methyl farnesoate receptor assembly. The increased mRNA levels for Met likely reflects increased sensitivity to methyl farnesoate in the animals under male-stimulating conditions.

Additionally, we contributed valuable knowledge regarding the oscillation of mRNA levels of genes within the male sex determining cascade. Peaks in mRNA levels agreed with the

sequence of the putative signaling cascade, and we discovered that general trends were consistent across the reproductive cycle regardless of the photoperiod. This suggests that these genes are important for other processes involved with reproduction and molting such that they are expressed in a consistent manner regardless of whether male sex determination occurs. The practice of molt-synchronizing animals prior to collection minimized variability due to the infradian rhythm, and this technique should be taken into consideration when developing standard methods for evaluating chemical toxicity to daphnids.

Establishing the environmental stimuli that led to male sex determination in two species of daphnids provides valuable information for the purposes of future toxicity testing and ecological studies that seek to capture seasonal transitions or relevant life history endpoints. Both temperature and photoperiod were important cues, confirming other research that has found one or both of these cues to be involved in male sex determination (Brown and Banta, 1932; Kleiven et al., 1992; Korpelainen, 1986; Lampert et al., 2012; Toyota et al., 2017). A six-degree temperature span was assessed in our assays, however this does not capture the full range of temperatures these species may experience based on their geographic distributions (U.S. EPA, 2002). Further research for both species at a broader range of temperatures will elucidate the full extent of temperature modulation on male sex determination processes.

The degree to which environmental stimuli impact hormonal signaling cascades (e.g. enzyme activity, circulating hormone levels) has been somewhat explored for other animals with ESD (Crain et al., 1997; Crews and Bergeron, 1994; Desvages and Pieau, 1992), however, few studies directly measure mRNA levels of key signaling elements. Here we were able to examine mRNA levels for several key sex determining genes in part due to the fully sequenced genome of *D. pulex* (Colbourne et al., 2005). Our results corroborated similar data for the *D. pulex* terminal

hormone signaling cascade (Kakaley et al., 2017; Toyota et al., 2015a). Research of this nature is imperative to fully understanding the process of male sex determination and the factors that control its occurrence.

In Chapter Three, we hypothesized that there was a neural processor of environmental signals that coordinated subsequent endocrine changes. We proposed the N-methyl-D-aspartate receptor (NMDAR), a glutamate receptor, was a viable candidate based on prior RNAseq experiments conducted by other researchers (Toyota et al., 2015b). We assayed a suite of chemicals that target NMDAR function to assess their impact on male sex determination. Two chemicals, MK-801 and desipramine, were found to significantly impact *D. pulex* sex ratios and are both antagonists of the NMDAR. From this discovery, we further probed the involvement of the NMDAR in male sex determination processes by investigating mRNA levels of NMDAR subunits under permissive and non-permissive photoperiods. We revealed that NMDAR-b levels were highly malleable in response to photoperiodic cues. Based on the structural requirements for NMDARs, this finding suggests that NMDAR functionality may be increased in the presence of environmental cues permissive to male sex determination. Further research is necessary to determine whether the increase in NMDAR-b subunit occurs broadly across the daphnid nervous system or is concentrated in specific regions. This information would help discern whether specific neural structures within daphnids contain concentrated expression of NMDAR-b subunits and in turn may be responsible for orchestrating neuroendocrine cascades.

In Chapter Four, we evaluated several additional anti-depressant pharmaceuticals having different modes of action in an effort to further discern neurological signaling pathways that regulate male sex determination. Antidepressants that targeted serotonin signaling and norepinephrine signaling (equivalent to octopamine in invertebrates) both increased male:female

sex ratios. Examination of mRNA levels of a serotonin re-uptake transporter and an octopamine receptor revealed that environmental conditions permissive to male sex determination significantly increased these levels.

Dysregulated glutamate signaling has been implicated in the pathophysiology of depression (Musazzi et al., 2011; Racagni and Popoli, 2008; Sanacora et al., 2012). Evidence in vertebrate systems suggests that SSRIs and serotonin-norepinephrine reuptake inhibitors (SNRIs) function as antidepressants by altering glutamate transmission through the NMDAR (Du et al., 2006; Liu et al., 2017; Sanacora et al., 2012; Zarate et al., 2010). As such, the SSRIs and norepinephrine-targeting chemicals assessed here may be impacting male sex determination by modulating NMDAR function and glutamate transmission, consistent with our prior findings that NMDAR antagonists increase male production in *D. pulex* (Chapter 3). Further experiments assessing NMDAR subunit mRNA levels in daphnids exposed to SSRIs or norepinephrine-targeting pharmaceuticals would further elucidate the means by which these chemicals influence male sex determination.

Concern regarding the growing number of human pharmaceuticals released from waste water treatment plants has grown in recent decades (Glassmeyer et al., 2017; Metcalfe et al., 2010; Schultz et al., 2010; Writer et al., 2013). Fish and other aquatic species exposed to neuroactive pharmaceuticals can display a range of disrupted behaviors, including disrupted mating, aggression, escape, and locomotive behaviors (Sehonova et al., 2018). The majority of neuroactive pharmaceuticals have not been adequately evaluated for their impact on behavior or reproductive processes on non-target organisms. As such we establish groundwork information relating to the potential of certain classes of antidepressant drugs to perturb sex determining processes in daphnids.

In Chapter Five, we hypothesized that exposure to MK-801, one of the chemicals that increased sex ratios in *D. pulex*, exerted this effect by altering mRNA expression in NMDAR subunits and genes within the male sex determining signaling cascade. We evaluated MK-801's effects under both non-male stimulating and male stimulating conditions. Results showed that NMDAR-b was highly responsive to MK-801 exposure, however, the directionality of the response depended on environmental conditions. Notably, under conditions non-permissive to male production, NMDAR-b, Met, and SRC mRNA levels were elevated, while the opposite was true under permissive conditions.

When considering these findings in the context of our results from Chapter Three, MK-801's effect on NMDAR-b, Met, and SRC under non-permissive conditions is similar to the response we observed in animals exposed to permissive environmental conditions alone. However, animals exposed to MK-801 under a long photoperiod do not produce male offspring despite the increased mRNA levels of these genes. Conversely, MK-801 increases male production in short photoperiod conditions while decreasing expression of *NMDAR-b*, *Met*, and *SRC*. These findings suggest that MK-801 does not exert its effects on *D. pulex* sex ratios exclusively through glutamate signaling. Further, MK-801 was unable to alter expression of *JHAMT* or *FAMT*, suggesting that MK-801 impacts another factor that contributes to male sex determination that was not evaluated here.

MK-801 likely impacts the male sex determination pathway through temperature-related factors, since Chapter Three demonstrated that MK-801 acted in an additive fashion with temperature to increase male offspring production. However, we did not assess temperature's impact on mRNA levels within the proposed and putative male sex determination signaling cascade, nor did we assess the impacts of MK-801 on mRNA levels at multiple temperatures. In

other taxa subject to ESD, specifically those subject to temperature sex determination, the mechanistic underpinnings of temperature's influence on sex determining pathways is also not well understood and is the subject of ongoing research (Devlin and Nagahama, 2002; Lang and Andrews, 1994; Santos et al., 2017). Likewise, the mechanism by which temperature modulates male production in daphnids remains unknown and is an avenue for further investigation.

One major finding of this research is that *D. pulex* has differential sensitivity to chemicals based on the environmental conditions alone, which is an essential consideration when estimating the impact of an exposure. For all the chemicals that elicited changes in daphnid sex ratios, a short day photoperiod was required to observe altered sex ratios. Experiments conducted under conditions similar to standard laboratory conditions did not yield differences, meaning effects would be missed when using standard testing protocols. The use of standard conditions has been debated in the context of ecological risk assessment, wherein recommended laboratory conditions capture a narrow range of physiological states (Bednarska et al., 2013). Conversely, expanding test conditions poses limits the rate at which research can be completed, since the list of chemicals of concern in the environment is already extensive and difficult to complete with current time and resource allocation.

Overall our results contribute important information regarding the life history of two species of daphnids and their sensitivity to environmental stimuli as it relates to male sex determination. We established mRNA level oscillations in relationship to the infradian reproductive cycle and the impact of environmental stimuli on those levels. We also provide evidence in support of the NMDAR as a neural sensor of environmental stimuli, however, further research is needed to clarify and fully understand its role. Results will inform experimental

designs for future research projects in regard to the molt/reproductive cycle of a critical freshwater invertebrate.

REFERENCES

Bednarska, A. J., Jevtić, D. M. and Laskowski, R. (2013). More ecological ERA: Incorporating natural environmental factors and animal behavior. *Integr. Environ. Assess. Manag.* **9**, e39–e46.

Brown, L. A. and Banta, A. M. (1932). Sex control in Cladocera. VII. Male production in relation to temperature. *Physiol. Zool.* **5**, 218–229.

Christie, a. E. and McCoole, M. D. (2012). From genes to behavior: investigations of neurochemical signaling come of age for the model crustacean *Daphnia pulex*. *J. Exp. Biol.* **215**, 2535–2544.

Ciofi, C. and Swingland, I. R. (1997). Environmental sex determination in reptiles. *Appl. Anim. Behav. Sci.* **51**, 251–265.

Colbourne, J. K., Singan, V. R. and Gilbert, D. G. (2005). wFleaBase: The *Daphnia* genome database. *BMC Bioinformatics* **6**, 1–5.

Colbourne, J. K., Pfrender, M. E., Gilbert, D., Thomas, W. K., Tucker, A., Oakley, T. H., Tokishita, S., Aerts, A., Arnold, G. J., Basu, M. K., et al. (2011). The ecoresponsive genome of *Daphnia pulex*. *Science (80-.).* **331**, 555–561.

Crain, D. A., Guillette, L. J., Rooney, A. A. and Pickford, D. B. (1997). Alterations in Steroidogenesis in Alligators (*Alligator mississippiensis*) Exposed Naturally and Experimentally to Environmental Contaminants. *Environ. Health Perspect.* **105**, 528–533.

Crews, D. and Bergeron, J. M. (1994). Role of reductase and aromatase in sex determination in the red-eared slider (*Trachemys scripta*), a turtle with temperature-dependent sex determination. *J. Endocrinol.* **443**, 279–289.

Desvages, G. L. E. and Pieau, C. (1992). Aromatase activity in the gonads of turtle embryos as

a function of the incubation temperature of eggs. *J. Steriod Biochem. Mol. Biol.* **41**, 851–853.

Devlin, R. H. and Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* **208**, 191–364.

Du, J., Machado-Vieira, R., Maeng, S., Martinowich, K., Manji, H. K. and Zarate, C. A. (2006). Enhancing AMPA to NMDA throughput as a convergent mechanism for antidepressant action. *Drug Discov. Today* **3**, 519–526.

Glassmeyer, S. T., Furlong, E. T., Kolpin, D. W., Batt, A. L., Benson, R., Boone, J. S., Conerly, O., Donohue, M. J., King, D. N., Kostich, M. S., et al. (2017). Nationwide reconnaissance of contaminants of emerging concern in source and treated drinking waters of the United States. *Sci. Total Environ.* **581**–**582**, 909–922.

Guillette Jr., L. J., Crain, D. A., Rooney, A. A. and Pickford, D. B. (1995). Organization verses activation: The role of endocrine disrupting contaminants (EDCs) during embryonic development in wildlife. *Environ. Health Perspect.* **103**, 1164–1457.

Kakaley, E. K. M., Wang, H. Y. and LeBlanc, G. A. (2017). Agonist-mediated assembly of the crustacean methyl farnesoate receptor. *Sci. Rep.* **7**, 45071.

Kato, Y., Kobayashi, K., Watanabe, H. and Iguchi, T. (2011). Environmental sex determination in the branchiopod crustacean *Daphnia magna*: Deep conservation of a Doublesex gene in the sex-determining pathway. *PLoS Genet.* **7**,.

Kleiven, O. T., Larsson, P. and Hobek, A. (1992). Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* **65**, 197–206.

Korpelainen, H. (1986). The effects of temperature and photoperiod on life history parameters

of *Daphnia magna* (Crustacea: Cladocera). *Freshw. Biol.* **16**, 615–620.

Korpelainen, H. (1990). Sex ratios and conditions required for environmental sex determination in animals. *Biol. Rev. Camb. Philos. Soc.* **65**, 147–184.

Lampert, W., Lampert, K. P. and Larsson, P. (2012). Induction of male production in clones of *Daphnia pulex* by the juvenoid hormone methyl farnesoate under short photoperiod. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* **156**, 130–133.

Lang, J. W. and Andrews, H. V (1994). Temperature-dependent sex determination in crocodilians. *J. Exp. Zool.* **270**, 28–44.

Liu, B., Liu, J., Wang, M., Zhang, Y. and Li, L. (2017). From Serotonin to Neuroplasticity: Evolvement of Theories for Major Depressive Disorder. *Front. Cell. Neurosci.* **11**, 1–9.

Luckenbach, J., Borski, R., Daniels, H. and Godwin, J. (2009). Sex determination in flatfishes: Mechanisms and environmental influences. *Semin. Cell Dev. Biol.* **20**, 256–263.

Metcalfe, C. D., Chu, S., Judt, C., Li, H., Oakes, K. D., Servos, M. R. and Andrews, D. M. (2010). Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. *Environ. Toxicol. Chem.* **29**, 79–89.

Musazzi, L., Racagni, G. and Popoli, M. (2011). Stress, glucocorticoids and glutamate release: Effects of antidepressant drugs. *Neurochem. Int.* **59**, 138–149.

Olmstead, A. W. and LeBlanc, G. A. (2000). Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. *Environ. Toxicol. Chem.* **19**, 2107–2113.

Olmstead, A. W. and LeBlanc, G. A. (2002). Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. *J. Exp. Zool.* **293**, 736–739.

Racagni, G. and Popoli, M. (2008). Cellular and molecular mechanisms in the long-term action

of antidepressants. *Dialogues Clin. Neurosci.* **10**, 385–400.

Rider, C. V, Gorr, T. A., Olmstead, A. W., Wasilak, B. A. and LeBlanc, G. A. (2005). Stress signaling: coregulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean. *J. Exp. Biol.* **208**, 15–23.

Sanacora, G., Treccani, G. and Popoli, M. (2012). Towards a glutamate hypothesis of depression: An emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology* **62**, 63–77.

Santos, D., Luzio, A. and Coimbra, A. M. (2017). Zebrafish sex differentiation and gonad development: A review on the impact of environmental factors. *Aquat. Toxicol.* **191**, 141–163.

Schultz, M. M., Werner, S. L., Schoenfuss, H. L., Barber, L. B., Blazer, V. S., Norris, D. O. and Vajda, A. M. (2010). Antidepressant Pharmaceuticals in Two U. S. Effluent-Impacted Streams: Occurrence and Fate in Water and Sediment, and Selective Uptake in Fish Neural Tissue. *Environ. Sci. Technol.* **44**, 1918–1925.

Sehonova, P., Svobodova, Z., Dolezelova, P., Vosmerova, P. and Faggio, C. (2018). Effects of waterborne antidepressants on non-target animals living in the aquatic environment: A review. *Sci. Total Environ.* **631**–**632**, 789–794.

Toyota, K., Miyakawa, H., Hiruta, C., Furuta, K., Ogino, Y., Shinoda, T., Tatarazako, N., Miyagawa, S., Shaw, J. R. and Iguchi, T. (2015a). Methyl farnesoate synthesis is necessary for the environmental sex determination in the water flea *Daphnia pulex*. *J. Insect Physiol.* **80**, 22–30.

Toyota, K., Miyakawa, H., Yamaguchi, K., Shigenobu, S., Ogino, Y., Tatarazako, N., Miyagawa, S. and Iguchi, T. (2015b). NMDA receptor activation upstream of methyl

farnesoate signaling for short day-induced male offspring production in the water flea, *Daphnia pulex*. *BMC Genomics* **16**, 186.

Toyota, K., Sato, T., Tatarazako, N. and Iguchi, T. (2017). Photoperiodism of male offspring production in the water flea *Daphnia pulex*. *Zoolog. Sci.* **34**, 312–317.

U.S. EPA (2002). *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*.

Vajda, Alan, M., Barber, L. B., Gray, J. L., Lopez, E. M., Woodling, J. D. and Norris, D. O. (2008). Reproductive disruption in fish downstream from an estrogenic wastewater effluent. *Environ. Sci. Technol.* **42**, 3407–3414.

Writer, J. H., Ferrer, I., Barber, L. B. and Thurman, E. M. (2013). Widespread occurrence of neuro-active pharmaceuticals and metabolites in 24 Minnesota rivers and wastewaters. *Sci. Total Environ.* **461**–**462**, 519–527.

Xie, X., Tao, T., Liu, M., Zhou, Y., Liu, Z. and Zhu, D. (2016). The potential role of juvenile hormone acid methyltransferase in methyl farnesoate (MF) biosynthesis in the swimming crab, *Portunus trituberculatus*. *Anim. Reprod. Sci.* **168**, 40–49.

Zarate, C., MacHado-Vieira, R., Henter, I., Ibrahim, L., Diazgranados, N. and Salvadore, G. (2010). Glutamatergic modulators: The future of treating mood disorders? *Harv. Rev. Psychiatry* **18**, 293–303.