Published in final edited form as:

J Insect Physiol. 2020 ; 121: 104015. doi:10.1016/j.jinsphys.2020.104015.

Involvement of Glutamate and Serotonin Transmitter Systems in Male Sex Determination in *Daphnia pulex*.

Allison A. Camp^a, Jeonga Yun^a, Samantha A. Chambers^a, Maher H. Haeba^a, Gerald A. LeBlanc^{a,b}

^aNorth Carolina State University, Department of Biological Sciences, Raleigh, NC 27695 USA

Abstract

Environmental sex determination occurs in many organisms, however the means by which environmental stimuli are translated into endocrine messages remains poorly understood. The N-methyl-D-aspartate receptor (NMDAR) was evaluated as a candidate neural sensor of environmental signals linking environmental cues to endocrine responses using the crustacean *Daphnia pulex*. NMDAR agonists, modulators, and antagonists were evaluated for their ability to impact *D. pulex* male sex determination during early stages of reproductive maturity under conditions that simulated seasonal change. The antagonists MK-801 and desipramine significantly increased male sex determination. Both chemicals are also modulators of serotonergic and noradrenergic systems, thus, we evaluated several modulators of monoamine neurotransmission in an effort to discern which signaling pathways might contribute to male sex determination. Compounds that altered serotonergic signaling also stimulated male sex determination. The involvement of the glutamate and monoamine signaling in male sex determination was supported by the increase in mRNA levels of related receptors and transporters under conditions that stimulate male sex determination. Further, mRNA levels of components of the terminal endocrine pathway responsible for male sex determination were also elevated under stimulatory conditions. Overall, we provide evidence that glutamatergic and serotonergic systems function upstream of the endocrine regulation of male sex determination in early life stage daphnids.

Graphical Abstract:

^bCorresponding author: Campus Box 7633, 850 Main Campus Drive, Raleigh, NC 27605-7633, Gerald_leblanc@ncsu.edu.

Authors' contributions:

AAC, SAC, MH, and conducted male sex determination experiments. JY conducted BRET assays. AAC evaluated changes in mRNA levels along the male sex determination signaling pathway, performed statistical analyses of the data, and drafted the manuscript. GAL conceived of the study, contributed to data interpretation, and prepared revisions of the manuscript. All authors approved of the final draft of the manuscript for publication.

Competing interests:

The authors declare no competing interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Environmental sex determination; endocrine cascade; juvenile hormones; zooplankton; abiotic stimuli; N-methyl-D-aspartate receptor

1. 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. While external cues drive their sex determination, genetic factors are involved in the process as well. For instance, daphnids have retained several genes that are known to be involved in insect sex determination, including the *Sex Lethal* gene (*Sxl*), *Transformer* (*Tra*), and the *Doublesex* gene (*Dsx*) (Colbourne et al., 2011; Kopp, 2012; LeBlanc and Medlock, 2015). The *Doublesex* gene has sexually dimorphic transcript abundance such that male daphnids have higher levels of *Dsx* transcripts in sex-specific structures, suggesting that this gene is critical in phenotypic differentiation between the sexes (Kato et al., 2011). More recently, an additional dominant gene has been discovered downstream of the methyl farnesoate pathway. When present, methyl farnesoate has no effect and all female offspring are produced (Ye et al., 2019). These genetic factors help explain the diversity in daphnid environmental sex determination, including sensitivity to cues, cyclicity, and in some cases resistance to environmental cues.

Many daphnid species are cyclic parthenogens and reproduce both asexually and sexually. During asexual reproduction, daphnids clonally produce female offspring (Hebert, 1978).

Environmental cues activate the methyl farnesoate signaling pathway resulting in the production of male offspring (male sex determination) to enable sexual reproduction (Hobek and Larsson, 1990; Kleiven et al., 1992; Korpelainen, 1986). Sexual reproduction during times of seasonal change or adverse conditions marks an opportunity to genetically diversify the population via fertilized winter eggs (LeBlanc and Medlock, 2015). The neuroendocrine linkage between the environmental stimuli and methyl farnesoate signaling remains unknown; however, evidence from RNAseq experiments with *D. pulex* has implicated ionotropic glutamate receptors in male sex determining processes (Toyota et al., 2015a).

One ionotropic glutamate receptor, the N-methyl-D-aspartate receptor (NMDAR), 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 a 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). For instance, ethanol is a NMDAR modulator that has been shown to inhibit NMDAR function (Hoffman et al., 1989; Lovinger et al., 1989; Wirkner et al., 1999; Wirkner et al., 2000; Woodward, 1999). Another class of glutamate receptors, 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 Pfeiffer-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). The NMDAR is widely accepted as a powerful driver of plasticity and adaptive responses within the nervous system and serves as a powerful modulator of physiology. Taken together, these factors make this receptor a strong candidate neural sensor in daphnids to facilitate neuroendocrine processes in the male sex determination pathway that occur at times that necessitate physiological change.

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 (Camp et al., 2019; Hobek and Larsson, 1990; Toyota et al., 2017). We have previously demonstrated the degree to which temperature and photoperiodic cues influence male sex determination in newly reproductive *D. pulex* under conditions mimicking seasonal transition, and this work builds on that existing knowledge (Camp et al., 2019). 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) produce methyl farnesoate, the male sex-determining hormone in daphnids (Olmstead and LeBlanc, 2002; Toyota et al., 2015a; Xie et al., 2016). Methyl farnesoate activates the methyl farnesoate receptor (MfR)

by binding to the transcription factor methoprene-tolerant (Met) and stimulating its recruitment of steroid receptor co-activator (SRC) (Kakaley et al., 2017; LeBlanc et al., 2013; Miyakawa et al., 2013; Toyota et al., 2015b). The activated MfR functions as a transcription factor to regulate expression of male sex determining genes as well as other genes (Rider et al., 2005).

Here we hypothesize that the NMDAR is a candidate neural processor of environmental signals and may function in linking environmental cues to endocrine responses during seasonal transitions. We tested this hypothesis using a variety of NMDAR agonists, modulators, and antagonists to determine their ability to modulate male sex determination in newly mature *D. pulex*. Further, we assessed whether neuroactive chemicals targeting monoamine transmitter systems had the potential to alter male production. Finally, we evaluated whether environmental cues that lead to male sex determination impacted mRNA levels of neural targets and putative constituents of the male sex determination signaling cascade in recently matured daphnids.

2. Materials and Methods

2.1 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 (Camp et al., 2019; Hannas et al., 2010). *D. pulex* WTN6 strain were originally obtained from the Center for Genomics and Bioinformatics (Indiana University, IN, USA) and were maintained by researchers at the National Institute for Basic Biology in Aichi, Japan, before they were gifted to us. They have since been cultured in our laboratory for 4 years.

Daphnids were collected from culture as < 24-hour old neonates and transferred to either short (10:14 hr L:D) winter-like or long (16:8 hr L:D) summer-like photoperiods at 18°C prior to chemical exposures. These conditions are known to stimulate and not stimulate male sex determination, respectively, in developing daphnids (Camp et al., 2019). Neonates were individually reared in 50 mL beakers containing 40 mL of daphnid media. Daphnid media consisted of reconstituted deionized water (192 mg L⁻¹ CaSO₄·H₂O, 192 mg L⁻¹ NaHCO₃, 120 mg L⁻¹ MgSO₄, 8.0 mg L⁻¹ KCl, 1.0 µg L⁻¹ vitamin B₁₂ and 1.0 µg L⁻¹ selenium). Daphnids were fed daily 1.4 × 10⁸ 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 chemicals once they reached reproductive maturity, marked by the first deposition of embryos into their brood chamber. Each treatment group consisted of ten individually-housed daphnids. Stock solutions of N-methyl-D-aspartate (NMDA), glycine, AMPA, AP-5, (+)-MK-801 hydrogen maleate, desipramine hydrochloride, fluoxetine hydrochloride (Prozac®), citalopram hydrobromide (Celexa®), bupropion (Wellbutrin®), and nisoxetine hydrochloride were prepared in deionized water. Pure ethanol (200 proof) was added directly to the test media. Nomifensine was prepared in DMSO. DMSO concentration (0.1% v/v) was equal across exposure and control groups for nomifensine assays. Concentrations were selected based on preliminary toxicity assays (data

not shown). Media was refreshed 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 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.

2.2 Bioluminescence resonance energy transfer (BRET) assays

Drosophila Schneider 2 (S2) cells (Life Technologies, Carlsbad, CA, USA) were cultured with Schneider's *Drosophila* Medium (Gibco, Carlsbad, CA, USA) in T25 flasks containing 10% heat-inactivated fetal bovine serum and supplemented with streptomycin sulfate (50 µg) and penicillin G (50 units). Cells were maintained at $26 \pm 0.5^\circ\text{C}$ with >50% relative humidity. S2 cells were seeded at a cell density of 7.5×10^5 cells·cm⁻² into 60 cm² culture dishes containing 10 mL of medium for the assay. Cells were transiently transfected using calcium phosphate, with 45 µg of total plasmid DNA containing the *D. pulex* MfR subunit fusion 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 CuSO₄ (800 µM) 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), and MK-801 or desipramine, delivered in 100 µL phosphate-buffered saline. Solutions were incubated for 5 minutes at $26 \pm 0.5^\circ\text{C}$. Emissions were then 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 *Renilla* luciferase substrate DeepBlueC™ (5.0 µM). The BRET ratio was calculated as emissions at 535/410 nm 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*.

2.3 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 (embryos in brood chamber), all animals were molt-synchronized and collected at 0, 24, 36, or 48 hours post-molt. Molt-synchronizing entailed monitoring daphnids every two hours for molting to occur. Once an animal molted, that established time zero for that individual. Four replicates each containing 3–5 daphnids were collected at each time point. Daphnids from individual replicates were transferred to RNeasy lysis buffer (100 µL) 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, USA). RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to manufacturer recommendations. cDNA was synthesized using ImProm-II™ Reverse Transcription System with oligo (dT) primers (Promega). mRNA levels of *D. pulex nmdar-a*, *nmdar-b*, *sert-a*, *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 *nmdar-a*, *nmdar-b*, and *sert-a*, were developed using nucleotide sequences provided by the Department of Energy Joint Genome Institute, accessed via NCBI and designed using Primer3 (v. 0.4.0) software. Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Primer specificity was confirmed based on amplicon length and nucleotide sequence (Eton Bioscience Inc, San Diego, CA, USA). Primer sequences for *nmdar-a* were Forward: GTCGTCGGTGATGTGAGATG, Reverse: AACAAAGAGCGGACAGAAA and *nmdar-b* were Forward: AGCCATGGAGTACCTTGTCG and Reverse: ACTTTGGGTGCTCCACTCTG. Primer sequences for *sert-a* were Forward: AGTCTATGCTCGGCTTCCAA, Reverse: ACCGACTTTGATGGACCAAG. PCR was performed with the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using 2x SYBR™ Green Premix. 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, USA) was used to analyze relative levels of gene expression by normalizing to two housekeeping genes, *actin* and *GAPDH*.

2.4 Data Analyses

Treatment-related differences in the percentage male offspring produced were evaluated using One-way ANOVA with Dunnett's multiple comparisons test when variances were no different among treatments. If variances were significantly different (Brown-Forsythe and/or Bartlett's test), comparisons of male offspring production were assessed with the Kruskal-Wallis test with Dunn's post-hoc multiple comparisons test. Animals that perished prior to completing six broods of offspring were excluded from analyses. Final number of animals per experiment were as follows: NMDA (n=8–10), glycine (n=10), NMDA and glycine (n=9–10), ethanol (n=9–10), AMPA (n=9–10), AP-5 (n=9–10), MK-801 (n=8–10), desipramine (n=9–10), fluoxetine (n=9–10; n=4 at 1 µM), citalopram (n=8–10), bupropion (n=9–10; n=5 at 1 µM), nomifensine (n=9–10), and nisooxetine (n=7–10). BRET assay results (n=6) were analyzed by One-way ANOVA with Tukey's multiple comparisons test. For gene expression experiments, raw Ct values and fold change values were assessed for outliers with the Grubbs test. Outliers were excluded from subsequent analyses. Differences in relative expression at each time point were analyzed using two-tailed Student's t-tests if variances were equal. If variances were significantly different (F test), the Mann-Whitney test was used. mRNA levels were normalized to the respective mRNA measured in the long photoperiod control group at time zero. Error bars denote standard error of the mean and the alpha level was 0.05 for all analyses. Statistical analyses were performed with Prism (v7.02, GraphPad Software, Inc).

3. Results

3.1 Male sex determination with NMDAR-targeting chemicals

Two NMDAR agonists N-methyl-D-aspartate (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 newly mature 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 modulators assessed, ethanol nor AMPA, significantly altered male production at the concentrations tested (Figs. 2A, B).

We next evaluated the ability of the NMDAR antagonists, MK-801, desipramine, and AP-5, to modulate male sex determination. MK-801 and desipramine are NMDAR channel blockers (Huettnner and Bean, 1988; 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 (Fig. 3A). MK-801 significantly increased male production at 1, 3, and 10 μM ($p < 0.05$) (Fig. 3B). Likewise, desipramine significantly stimulated the production of male offspring in a concentration-dependent manner with 0.1, 0.3, and 1.0 μM resulting in higher male production ($p < 0.05$, Fig. 3C). These compounds had no effect on male sex determination (i.e. did not initiate male production) when evaluated under the non-permissive (16:8 hr L:D) photoperiod (Figs. 3B, C). Thus, MK-801 and desipramine stimulated male offspring production under conditions permissive of male sex determination, but these compounds could not supplant the photoperiodic cue in newly mature daphnids.

3.2 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 some other exogenous compounds (Olmstead and LeBlanc, 2003). Bioluminescence resonance energy transfer (BRET) assays were performed to determine whether MK-801 and desipramine stimulated dimerization of MfR subunits, an early event in the activation of the MfR. The positive control, methyl farnesoate, significantly stimulated the dimerization of Met and SRC ($p < 0.001$), however, concentrations of MK-801 and desipramine as high as 100 μM had no effect on receptor assembly (Fig. 4). These results indicated that MK-801 and desipramine act at a location within the male sex determination pathway other than the MfR.

3.3 Male sex determination with neurotransmitter modulators

The two chemicals that stimulated male sex determination are also known inhibit monoamine transporters. MK-801 is known to inhibit serotonin, noradrenaline, and dopamine reuptake transporters (Nishimura et al., 1998), while desipramine inhibits noradrenergic reuptake transporters (Brunello et al., 2002). To assess whether the effects of these chemicals may have been due to their ability to inhibit monoamine reuptake transporters, we tested a suite of pharmaceuticals that targeted monoamine systems to evaluate their impact on male sex determination. Two selective serotonin reuptake inhibitors

(SSRIs) were evaluated, fluoxetine hydrochloride (Prozac®) and citalopram hydrobromide (Celexa®) (Brunello et al., 2002; Pörzgen et al., 2001). Under the short-day photoperiod, both fluoxetine (Fig. 5A) and citalopram (Fig. 5B) exposure increased male production at the highest concentration tested (1 μ M). Neither compound stimulated male production under the long photoperiod (Figs. 5A, B).

Next, we evaluated three pharmaceuticals that target dopaminergic and noradrenaline signaling. Bupropion (Wellbutrin®) and nomifensine are nonselective inhibitors of dopaminergic and noradrenaline reuptake (Dwoskin et al., 2006; Tatsumi et al., 1997). Nisoxetine is a selective noradrenaline reuptake inhibitor (Tejani-Butt et al., 1990). None of the monoamine uptake inhibitors significantly altered male production under the short- or long-day photoperiod (Figs. 6A-C).

3.4 Impact of photoperiod on selected mRNA levels

We evaluated selected mRNA levels in molt synchronized animals reared under both long-day (summer-like, non-permissive) and short-day (winter-like, permissive) photoperiods. These daphnids were newly reproductively mature, thus capturing early adult mRNA levels within the animals. mRNA levels of neural receptors and transporters associated with impacts on male production in male sex determination assays were evaluated, including *nmdar-a* and *-b* (McCoole et al., 2012a), and the serotonin reuptake transporter *sert-a* (McCoole et al., 2012b).

mRNA levels for both NMDAR subunits increased over time, under both photoperiods, during the initial 48 hrs post-molt (Fig. 7A, B). mRNA levels for *nmdar-a* were not influenced by photoperiod (Fig. 7A); however, *nmdar-b* mRNA levels were significantly elevated (5.5 to 103 times higher) under the short-day, permissive photoperiod as compared to the long-day, non-permissive photoperiod (Fig. 7B, $p = 0.029$). Daphnids reared under the short-day photoperiod produced significantly higher mRNA levels of *sert-a* at 24 ($p = 0.0001$), 36 ($p = 0.029$), and 48 ($p = 0.015$) hours of the molt cycle as compared to long-day photoperiod (Fig. 7C). No differences were observed for *sert-a* between photoperiods at 0 hours post molt (Fig. 7C).

Photoperiodic modulation of gene expression for components of the methyl farnesoate signaling pathway, the terminal component of the male sex determining pathway were also assessed. Specifically, mRNA levels for the enzymes that contribute to methyl farnesoate synthesis, *JHAMT* and *FAMT*, and the MfR subunits *Met* and *SRC* were assessed under permissive and non-permissive photoperiodic conditions. *JHAMT* mRNA levels attained maximum levels at 24 hrs post-molt under both photoperiods (Fig. 8A), while *FAMT* mRNA levels exhibited a slight rhythmic pattern under both photoperiods (Fig. 8B). *Met* and *SRC* mRNA attained maximum levels at 24 hrs post-molt under both photoperiods, then decreased to various degrees (Fig. 8C, D).

Photoperiod elicited a varied and sometimes profound effect on mRNA levels for the constituents of the male sex determining signaling pathway. *JHAMT* mRNA levels were elevated under the short photoperiod during the initial 36 hrs post-molt (Fig. 8A, 24 hrs $p = 0.0005$, 36 hrs $p = 0.0015$); while, *FAMT* mRNA levels were slightly increased during the

initial 24 hrs post molt (Fig. 8B, 0 hr $p = 0.0011$, 24 hrs $p = 0.013$). *Met* mRNA levels also were significantly elevated in the short photoperiod as compared to the long photoperiod (24 hrs $p = 0.013$, 36 hrs $p = 0.0001$, 48 hrs $p = 0.029$, Fig. 8C). *SRC* mRNA levels were significantly elevated at 24 ($p = 0.029$), 36 ($p = 0.029$), and 48 ($p < 0.0001$) hours in the short photoperiod. The magnitude of difference in mRNA levels between photoperiods was much greater for *SRC* than for *Met* (Fig. 8C, D).

To summarize, results are consistent with the short-day, permissive photoperiod stimulating the expression of the *nmdar-b* subunit and *sert-a* which may subsequently influence the increased expression of the male sex determination pathway genes. The robust increase in *nmdar-b* mRNA levels suggests that this receptor may be the primary factor in initiating downstream effects and play an important role in producing the physiological changes associated with seasonal transitions.

4. Discussion

We hypothesized that the NMDAR is a viable candidate neural sensor of environmental signals and may function in linking environmental cues to endocrine responses in daphnids during seasonal transitions. We demonstrated that the NMDAR agonists, modulators, and the antagonist AP-5 had no effect on this phenomenon during early life stages. MK-801, desipramine, fluoxetine and citalopram, however, all stimulated male sex determination and have multiple impacts on glutamate and monoamine transmitter systems, suggesting that both systems may be involved in regulating male sex determination.

Our findings with NMDAR-targeting chemicals are distinct from previous studies that examined the influence of the same chemicals on *D. pulex*. Toyota and colleagues (2015b) investigated the impact of NMDA, AMPA, and MK-801 on male sex determination with older animals well-acclimated to environmental conditions. Further, they assessed one brood of offspring from briefly exposed mothers. They found that NMDA and AMPA stimulated male production and MK-801 suppressed male production under a 14:10 hr L:D photoperiod (Toyota et al., 2015a). Conversely, our study design initiated environmental conditions when daphnids were neonates, and the maturing daphnids were continuously exposed to treatments while broods were assessed. Thus, we assessed a different life stage, type of stressor, and duration of stressor. Additionally, we examined five consecutive broods of offspring rather than one brood. The daphnids used in the present work would physiologically resemble daphnids developing during the seasonal transition into fall, whereas the daphnids used by Toyota and colleagues (2015b) would more closely resemble mid-season daphnids. The dissimilar results observed with NMDA, AMPA, and MK-801 exposure were likely due to these key differences in experimental design. These disparate results shed insight into the shifting physiology of *D. pulex* over time, and it is possible that *D. pulex* during early reproductive weeks express different levels of NMDAR subunits or distinctive receptor composition than older daphnids. Taken together these studies suggest that young daphnids undergoing physiological changes during seasonal transitions may respond differently than older, more mature daphnids to similar chemical exposures.

The NMDAR antagonists, MK-801 and desipramine, that significantly altered male production in the work performed here, also impact serotonergic and noradrenergic transmitter systems through reuptake inhibition, respectively (Brunello et al., 2002; Nishimura et al., 1998). Thus, it is possible that changes in male sex determination observed with these compounds were in part due to their effects on serotonin or noradrenaline transmission rather than glutamate. This would provide an explanation as to why the NMDAR agonists, modulators, and the other antagonist were unable to alter male production, while serotonin-targeting chemicals were able to increase male production. Interestingly, the efficacy of monoamine-targeting antidepressants is considered to be due to their effects on glutamatergic pathways (Du et al., 2006; Liu et al., 2017; Zarate et al., 2010) since dysregulated glutamate signaling is implicated in the pathophysiology of mood disorders such as depression (Musazzi et al., 2011; Racagni and Popoli, 2008; Sanacora et al., 2012; Tokita et al., 2012). Thus, the close links between these transmitter systems makes it probable that both serotonergic and glutamatergic pathways may both be involved in neural signaling upstream of male sex determining endocrine pathways.

Assessments of mRNA levels also supported a role for both glutamate and monoamine signaling in male sex determination. Altered *sert-a* mRNA levels were observed under environmental conditions permissive of male sex determination. Serotonin is involved in a variety of crustacean growth and reproduction processes. In crabs, serotonin impacts levels of molting-related hormones, and is involved in ovarian development (Girish et al., 2017; Richardson et al., 1991; Robert et al., 2016; Sainath and Reddy, 2011). Girish and colleagues (2017) observed that serotonin elevated methyl farnesoate levels in crabs, and since methyl farnesoate is the male sex determining hormone in daphnids (Olmstead and LeBlanc, 2002), this could provide an explanation as to how increased availability of serotonin by the serotonin reuptake inhibitors tested here could elevate male offspring production.

mRNA levels of NMDAR subunits revealed that the *nmdar-a* subunit was not subject to modulation by photoperiod, while the *nmdar-b* subunit was highly responsive to photoperiodic cues. This suggests that these receptor subunits function similarly to the NMDAR-1 (NR1) and NMDAR-2 (NR2) in vertebrates, respectively, wherein NR1 expression is more tightly regulated (Rousseaux, 2008) while NR2 expression is malleable and modulated by a variety of stimuli including chemical exposure, environmental stimuli, and stress (Kopp et al., 2007; Paoletti, 2011). Changes in NR2 expression and subsequently NMDAR structure (i.e. receptor heteromeric composition) have been shown to alter receptor kinetics and pharmacology (Gielen et al., 2009; Paoletti and Neyton, 2007). Specifically, greater NR2 presence within a receptor population increases the probability of the receptor to be open (P_{open}) such that increased ion flow occurs prior to inactivation (Gielen et al., 2009; Yuan et al., 2009). Calcium, one of the ions that moves through the NMDAR, elicits a variety of powerful downstream effects on the postsynaptic cell to modulate plasticity of the nervous system. Thus, the robust increase in expression of *nmdar-b* under a short photoperiod likely reflects a fundamental shift in NMDAR functionality and receptor dynamics within *D. pulex*. We conclude that *nmdar-b* expression is responsive to seasonal change resulting in alterations in glutamate signaling and plasticity of the daphnid nervous system within young daphnids. It is possible that this increase in subunit expression is no

longer present in older, more mature daphnids, or in daphnids that have not experienced recent fluctuations in stimuli.

Genes within the male sex determination endocrine pathway also exhibited altered mRNA levels in the presence of permissive environmental conditions in young daphnids. *JHAMT* mRNA levels were appreciably higher early in the reproductive cycle under the short-day photoperiod. These results are consistent with those reported by Toyota et al (2015a), who demonstrated that *D. pulex* JHAMT is the primary contributor to the conversion of farnesoic acid to methyl farnesoate in daphnids. We conclude that short-day photoperiod elevated levels of methyl farnesoate through the induction of JHAMT. Subsequent to the induction of JHAMT, *Met* and *SRC* mRNA levels were 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, we surmise, 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 sex determining hormone and its receptor are present at levels greater than under non-permissive environmental conditions in these daphnids. The trends observed in mRNA levels for these genes across the reproductive cycle are consistent with previous research, although the magnitude of the response is more pronounced here than in previous work (Camp et al., 2019).

There are several directions for future work within this research area. One logical next step is to evaluate mRNA levels of neuroendocrine-related genes after exposure to neuroactive chemicals that increase male production. Additionally, research on daphnid neurobiology, including receptor characterization and behavior would be useful to clarify the degree to which daphnid NMDARs and other receptors behave similarly to analogous insect receptors. Further, an analysis of the impact of neuroactive chemicals on other genes related to daphnid sex determination (e.g. *Dsx*) would also be a useful line of inquiry to elucidate their role in environmental sex determination.

5. Conclusions

Overall, results support a role for glutamate and serotonin neurotransmitter systems in male sex determination processes as elucidated through whole animal and molecular responses. Additionally, we show that key enzymes and transcription factors involved in male sex determination are influenced by environmental cues in young daphnids and are elevated under conditions representative of seasonal change. Finally, we highlight that neuroactive chemicals have the ability to influence endocrine processes in daphnids and alter sex ratios in newly reproductively mature *D. pulex*.

Acknowledgments:

The authors would like to thank the LeBlanc laboratory members for their assistance.

Funding:

The work was funded by NSF grant IOS-1350998, NIEHS grant R25-ES028974, and a fellowship from the Scholar Rescue Fund, Institute of International Education to MHH.

AAC, SAC, MH, JY: Investigation. AAC: Formal Analysis, Writing – Original Draft, Visualization. GAL: Conceptualization, Resources, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition.

Abbreviations:

(NMDAR)	N-methyl-D-aspartate receptor
(ser _t -a)	serotonin reuptake transporter
(JHAMT)	juvenile hormone acid O-methyltransferase
(FAMT)	farnesoic acid O-methyltransferase
(Met)	methoprene-tolerant
(SRC)	steroid receptor co-activator
(AMPA)	α -amino-3hydroxy-5-methylisoxazole-4-propionate
(MfR)	methyl farnesoate receptor
(MF)	methyl farnesoate
(SSRIs)	selective serotonin reuptake inhibitors

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*. *Journal of Insect Physiology* 50, 927–934. [PubMed: 15518660]
- Brunello N, Mendlewicz J, Kasper S, Leonard B, Montgomery S, Nelson JC, Paykel E, Versiani M. and Racagni G. (2002). The role of noradrenaline and selective noradrenaline reuptake inhibition in depression. *European Neuropsychopharmacology* 12, 461–475. [PubMed: 12208564]
- Camp AA, Haeba MH and LeBlanc GA (2019). Complementary roles of photoperiod and temperature in environmental sex determination in *Daphnia* sp (Crustacea: Cladocera). *Journal of Experimental Biology* 222, jeb195289.
- 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. *Proceedings of the National Academy of Sciences* 99, 37–42.
- Ciofi C. and Swingland IR (1997). Environmental sex determination in reptiles. *Applied Animal Behavior Science* 51, 251–265.
- Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A, Oakley TH, Tokishita S, Aerts A, Arnold GJ, Basu MK, et al. (2011). The ecoresponsive genome of *Daphnia pulex*. *Science* 331, 555–561. [PubMed: 21292972]
- Devlin RH 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 SFF, Borges K, Bowie D. and Traynelis SFF (1999). The glutamate receptor ion channels. *Pharmacology Reviews* 51, 7.
- Du J, Machado-Vieira R, Maeng S, Martinowich K, Manji HK and Zarate CA (2006). Enhancing AMPA to NMDA throughput as a convergent mechanism for antidepressant action. *Drug Discovery Today* 3, 519–526. [PubMed: 25411578]
- Dwoskin LP, Rauhut AS, King-Pospisil KA and Bardo MT (2006). Review of the pharmacology and clinical profile of bupropion, an antidepressant and tobacco use cessation agent. *CNS Drug Reviews* 12, 178–207. [PubMed: 17227286]

- Beamish FWH (1993). Environmental Sex Determination in Southern Brook Lamprey, *Ichthyomyzon gagei*. *Canadian Journal of Fisheries and Aquatic Sciences* 50, 1299–1307.
- Geister TL, Lorenz MW, Hoffmann KH 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*. *Journal of Experimental Biology* 211, 1587–93. [PubMed: 18456886]
- Gielen M, Siegler Retchless B, Money L, Johnson JW and Paoletti P. (2009). Mechanism of differential control of NMDA receptor activity by NR2 subunits. *Nature* 459, 703–708. [PubMed: 19404260]
- Girish BP, Swetha CH and Reddy PS (2017). Serotonin induces ecdysteroidogenesis and methyl farnesoate synthesis in the mud crab, *Scylla serrata*. *Biochemistry and Biophysics Research Communications* 490, 1340–1345.
- Glantz RM and Pfeiffer-linn C. (1992). NMDA Receptors in invertebrates. *Comparative Biochemistry and Physiology* 103, 243–248.
- Hannas BR, Das PC, Li H. and LeBlanc GA (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 PDN (1978). The population biology of *Daphnia* (Crustacea, Daphnidae). *Biological Reviews* 53, 387–426.
- Hobek A. and Larsson P. (1990). Sex determination in *Daphnia magna*. *Ecology* 71, 2255–2268.
- Hoffman PL, Rabe CS, Moses F. and Tabakoff B. (1989). N-methyl-D-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. *Journal of Neurochemistry* 52, 1937–1940. [PubMed: 2542453]
- Huang J, Hult EF, Marchal E. and Tobe SS (2015). Identification and characterization of the NMDA receptor and its role in regulating reproduction in the cockroach *Diploptera punctata*. *Journal of Experimental Biology* 218, 983–90. [PubMed: 25657209]
- Huettnner JE and Bean BP (1988). Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: Selective binding to open channels. *Proceedings of the National Academy of Sciences* 85, 1307–1311.
- Kakaley EKM, Wang HY and LeBlanc GA (2017). Agonist-mediated assembly of the crustacean methyl farnesoate receptor. *Scientific Reports* 7, 45071.
- Kano T, Brockie PJ, Sassa T, Fujimoto H, Kawahara Y, Iino Y, Mellem JE, Madsen DM, Hosono R. and Maricq AV (2008). Memory in *Caenorhabditis elegans* is mediated by NMDA-type ionotropic glutamate receptors. *Current Biology* 18, 1010–1015. [PubMed: 18583134]
- 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 Genetics* 7, e1001345.
- Kleiven OT, Larsson P. and Hobek A. (1992). Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* 65, 197–206.
- Kopp A. (2012). Dmrt genes in the development and evolution of sexual dimorphism. *Trends in Genetics*. 28, 175–184. [PubMed: 22425532]
- Kopp C, Longordo F. and Lüthi A. (2007). Experience-dependent changes in NMDA receptor composition at mature central synapses. *Neuropharmacology* 53, 1–9. [PubMed: 17499817]
- Korpelainen H. (1986). The effects of temperature and photoperiod on life history parameters of *Daphnia magna* (Crustacea: Cladocera). *Freshwater Biology* 16, 615–620.
- Korpelainen H. (1990). Sex ratios and conditions required for environmental sex determination in animals. *Biological Reviews of the Cambridge Philosophical Society* 65, 147–184.
- Lang JW and Andrews HV (1994). Temperature-dependent sex determination in crocodilians. *Journal of Experimental Zoology* 270, 28–44.
- LeBlanc GA and Medlock EK (2015). Males on demand: the environmental-neuroendocrine control of male sex determination in daphnids. *FEBS Journal* 1–14. [PubMed: 26140664]
- LeBlanc GA, Wang YH, Holmes CN, Kwon G. and Medlock EK (2013). A Transgenerational Endocrine Signaling Pathway in Crustacea. *PLoS One* 8,.

- Liu B, Liu J, Wang M, Zhang Y. and Li L. (2017). From Serotonin to Neuroplasticity: Evolvement of Theories for Major Depressive Disorder. *Frontiers in Cellular Neuroscience* 11, 1–9. [PubMed: 28154525]
- Lovinger DM, White G. and Weight FF (1989). Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science* 243, 1721–1724. [PubMed: 2467382]
- McCoole MD, D'Andrea BT, Baer KN and Christie AE (2012a). Genomic analyses of gas (nitric oxide and carbon monoxide) and small molecule transmitter (acetylcholine, glutamate and GABA) signaling systems in *Daphnia pulex*. *Comparative Biochemistry and Physiology - Part D Genomics Proteomics* 7, 124–160.
- McCoole MD, Atkinson NJ, Graham DI, Grasser EB, Joselow AL, McCall NM, Welker AM, Wilsterman EJ, Baer KN, Tilden AR, et al. (2012b). Genomic analyses of aminergic signaling systems (dopamine, octopamine and serotonin) in *Daphnia pulex*. *Comparative Biochemistry and Physiology - Part D Genomics Proteomics* 7, 35–58.
- 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. [PubMed: 12467596]
- 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 Developmental Biology* 10, 45. [PubMed: 20433737]
- Miyakawa H, Toyota K, Hirakawa I, Ogino Y, Miyagawa S, Oda S, Tatarazako N, Miura T, Colbourne JK and Iguchi T. (2013). A mutation in the receptor Methoprene-tolerant alters juvenile hormone response in insects and crustaceans. *Nature Communications* 4, 1856–1857.
- Monaghan DT, Irvine MW, Costa BM, Fang G. and Jane DE (2012). Pharmacological modulation of NMDA receptor activity and the advent of negative and positive allosteric modulators. *Neurochemistry International* 61, 581–592. [PubMed: 22269804]
- Musazzi L, Racagni G. and Popoli M. (2011). Stress, glucocorticoids and glutamate release: Effects of antidepressant drugs. *Neurochemistry International* 59, 138–149. [PubMed: 21689704]
- Nishimura M, Sato K, Okada T, Schloss P, Shimada S. and Tohyama M. (1998). MK-801 blocks monoamine transporters expressed in HEK cells. *FEBS Letters* 423, 376–380. [PubMed: 9515743]
- Olmstead AW and LeBlanc GA (2000). Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. *Environmental Toxicology and Chemistry* 19, 2107–2113.
- Olmstead AW and LeBlanc GA (2002). Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. *Journal of Experimental Zoology* 293, 736–739. [PubMed: 12410602]
- Olmstead AW and LeBlanc GA (2003). Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. *Environmental Health Perspectives* 111, 919–924. [PubMed: 12782492]
- Paoletti P. (2011). Molecular basis of NMDA receptor functional diversity. *European Journal of Neuroscience* 33, 1351–1365. [PubMed: 21395862]
- Paoletti P. and Neyton J. (2007). NMDA receptor subunits: function and pharmacology. *Current Opinions in Pharmacology* 7, 39–47.
- Pörzgen P, Park SK, Hirsh J, Sonders MS and Amara SG (2001). The antidepressant-sensitive dopamine transporter in *Drosophila melanogaster*: a primordial carrier for catecholamines. *Molecular Pharmacology* 59, 83–95.
- Racagni G. and Popoli M. (2008). Cellular and molecular mechanisms in the long-term action of antidepressants. *Dialogues in Clinical Neuroscience* 10, 385–400. [PubMed: 19170396]
- Reynolds J. (1990). Arcaine uncovers dual interactions of polyamines with the N-methyl-D-aspartate receptor. *Journal of Pharmacology and Experimental Therapeutics* 255, 1001–1007. [PubMed: 2148185]
- Richardson GH, Deecaraman M. and Fingerman M. (1991). The effect of biogenic amines on ovarian development in the fiddler crab, *Uca pugilator*. *Comparative Biochemistry and Physiology* 99C, 53–56.

- Rider CV, Gorr TA, Olmstead AW, Wasilak BA and LeBlanc GA (2005). Stress signaling: coregulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean. *Journal of Experimental Biology* 208, 15–23. [PubMed: 15601873]
- Riedel G, Platt B. and Micheau J. (2003). Glutamate receptor function in learning and memory. *Behavioral Brain Research* 140, 1–47.
- Robert A, Monsinjon T, Delbecq JP, 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. *Aquatic Toxicology* 175, 192–204. [PubMed: 27060239]
- Rousseaux CG (2008). A review of glutamate receptors I: Current understanding of their biology. *Journal of Toxicologic Pathology* 21, 25–51.
- Sainath SB and Reddy PS (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. [PubMed: 21827775]
- Sernagor E, Kuhn D, Vyklicky L. and Mayer ML (1989). Open channel block of NMDA receptor responses evoked by tricyclic antidepressants. *Neuron* 2, 1221–7. [PubMed: 2483111]
- Si A, Helliwell P. and Maleszka R. (2004). Effects of NMDA receptor antagonists on olfactory learning and memory in the honeybee (*Apis mellifera*). *Pharmacology Biochemistry and Behavior* 77, 191–197.
- Szasz BK, Mike A, Karoly R, Gerevich Z, Illes P, Vizi ES and Kiss JP (2007). Direct Inhibitory Effect of Fluoxetine on N-Methyl-D-Aspartate Receptors in the Central Nervous System. *Biological Psychiatry* 62, 1303–1309. [PubMed: 17659262]
- Tatsumi M, Groshan K, Blakely RD and Richelson E. (1997). Pharmacological profile of antidepressants and related compounds at human monoamine transporters. *European Journal of Pharmacology* 340, 249–258. [PubMed: 9537821]
- Tejani-Butt SM, Brunswick DJ and Frazer A. (1990). [³H]Nisoxetine: a new radioligand for norepinephrine uptake sites in brain. *European Journal of Pharmacology* 191, 239–243. [PubMed: 2086242]
- Tokita K, Yamaji T. and Hashimoto K. (2012). Roles of glutamate signaling in preclinical and/or mechanistic models of depression. *Pharmacology Biochemistry and Behavior* 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. [PubMed: 25867484]
- Toyota K, Miyakawa H, Hiruta C, Furuta K, Ogino Y, Shinoda T, Tatarazako N, Miyagawa S, Shaw JR and Iguchi T. (2015b). Methyl farnesoate synthesis is necessary for the environmental sex determination in the water flea *Daphnia pulex*. *Journal of Insect Physiology* 80, 22–30. [PubMed: 25721056]
- Toyota K, Sato T, Tatarazako N. and Iguchi T. (2017). Photoperiodism of male offspring production in the water flea *Daphnia pulex*. *Zoological Sciences* 34, 312–317.
- Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Odgen KK, Hansen KB, Yuan H, Myers SJ and Dingledine R. (2010). Glutamate receptor ion channels: structure, regulation and function. *Pharmacology Reviews* 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. *Neurochemistry International* 35, 153–162. [PubMed: 10405999]
- 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-Schmiedeberg's Archives of Pharmacology* 362, 568–576. [PubMed: 11138850]
- Woodward JJ (1999). Ionotropic glutamate receptors as sites of action for ethanol in the brain. *Neurochemistry International* 35, 107–113. [PubMed: 10405994]

- 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*. *Current Biology* 15, 603–15. [PubMed: 15823532]
- 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*. *Animal Reproductive Science* 168, 40–49.
- Ye Z, Molinier C, Zhao C, Haag CR and Lynch M. (2019). Genetic control of male production in *Daphnia pulex*. *Proceedings of the National Academy of Sciences* 116, 15602–15609.
- Yuan H, Hansen KB, Vance KM, Ogden KK and Traynelis SF (2009). Control of NMDA Receptor Function by the NR2 Subunit Amino-Terminal Domain. *Journal of Neuroscience* 29, 12045–12058.
- Zarate C, MacHado-Vieira R, Henter I, Ibrahim L, Diazgranados N. and Salvatore G. (2010). Glutamatergic modulators: The future of treating mood disorders? *Harvard Reviews in Psychiatry* 18, 293–303.

Camp et al, Highlights

- Short photoperiod (10:14hr L:D) stimulates male sex determination in *Daphnia pulex*.
- Glutamate- and monoamine-targeting chemicals increased male production.
- mRNA levels of neural targets were elevated under male sex determination.
- mRNA levels of endocrine targets were elevated under male-producing conditions.

Figure 1: Male offspring production by *D. pulex* exposed to NMDAR agonists NMDA, glycine, and co-exposures.

A: Male production with exposure to the agonist NMDA. B: Male production with exposure to the co-factor glycine. C: Male production with individual and co-exposure to 10 μM NMDA and 100 μM glycine. Data represent mean ± SE. Asterisks denote significant differences from controls.

Figure 2. Male offspring production by *D. pulex* exposed to NMDAR modulators ethanol and AMPA.

A: Male production with exposure to ethanol. B: Male production with exposure to AMPA.

Data represent mean $\pm$ SE. Asterisks denote significant differences from controls.

Figure 3: Male offspring production by *D. pulex* exposed to NMDAR antagonists AP-5, MK-801, and desipramine.

A: Male production with exposure to AP-5 under a short-day photoperiod. B: Male production with exposure to MK-801 under short- and long-day photoperiods. C: Male production with exposure to desipramine under short- and long-day photoperiods. Short-day photoperiod was 10:14 hr L:D, and long-day photoperiod was 16:8 hr L:D. Data represent mean $\pm$ SE. Asterisks denote a significant difference from the control.

Figure 4: Ligand-mediated Met and SRC assembly as measured using BRET.

Data represent mean $\pm$ SE. An asterisk denotes a significant difference from the control. MF: methyl farnesoate (positive control).

Figure 5. *D. pulex* male sex determination assays with antidepressants targeting serotonergic systems.

A: Male offspring production with exposure to fluoxetine. B: Male offspring production with exposure to citalopram. Long-day photoperiod was 16:8 L:D and short-day photoperiod was 10:14 L:D. An asterisk denotes a significant difference from the control. Data represent mean $\pm$ SE.

Figure 6. *D. pulex* male sex determination assays with antidepressants targeting dopaminergic and octopaminergic systems.

A: Male offspring production with exposure to bupropion. B: Male offspring production with exposure to nomifensine. C: Male offspring production with exposure to nisoxetine. Long-day photoperiod was 16:8 L:D and short-day photoperiod was 10:14 L:D. An asterisk denotes a significant difference from the control. Data represent mean $\pm$ SE.

Figure 7. Relative mRNA levels at defined times during the molt cycle for *nmdar-a*, *nmdar-b*, and *sirt-a* among *D. pulex* reared under long- and short- day photoperiods.

White bars denote long photoperiod 16:8 hr L:D and black bars denote short photoperiod 10:14 hr L:D. A: *nmdar-a* mRNA levels. B: *nmdar-b* mRNA levels. C: *sirt-a* mRNA levels. Groups are normalized to the long photoperiod control at time zero. Data represent mean $\pm$ SE and asterisks denote significant differences ($p < 0.05$).

Figure 8. Relative mRNA levels at defined times during the molt cycle for *JHAMT*, *FAMT*, *Met*, and *SRC* among *D. pulex* reared under long- and short- day photoperiods.

White bars denote long photoperiod 16:8 hr L:D and black bars denote short photoperiod 10:14 hr L:D. A: *JHAMT* mRNA levels. B: *FAMT* mRNA levels. C: *Met* mRNA levels. D: *SRC* mRNA levels. Groups are normalized to the long photoperiod control at time zero. Data represent mean $\pm$ SE and asterisks denote significant differences ($p < 0.05$).