© 2019. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2019) 222, jeb195289. doi:10.1242/jeb.195289

RESEARCH ARTICLE

Complementary roles of photoperiod and temperature in environmental sex determination in *Daphnia* spp.

Allison A. Camp, Maher H. Haeba and Gerald A. LeBlanc*

ABSTRACT

Daphnia spp., a keystone genus in freshwater lentic habitats, are subject to environmental sex determination wherein environmental conditions dictate offspring sex 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 co-regulate male sex determination and that these environmental stimuli would activate elements of the male sex determination signaling cascade. The results revealed 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 evaluated, *Daphnia pulex* and *Daphnia magna*, exhibited different temperature dependencies. *Daphnia pulex* produced fewer males with increasing temperatures between 16 and 22°C, and *D. magna* exhibited the opposite trend. We found consistent expression patterns of key genes along the male sex-determining signaling pathway in *D. pulex* independent of environmental stimuli. mRNA levels for the enzyme responsible for synthesis of the male sex-determining hormone, methyl farnesoate, were elevated early in the reproductive cycle, followed by increased mRNA levels of the methyl farnesoate receptor subunits *Met* and *SRC*. Environmental conditions that stimulated male offspring production significantly increased *Met* mRNA levels. The 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.

KEY WORDS: Zooplankton, Endocrine cascade, Methyl farnesoate receptor, Juvenoids, Methyl farnesoate

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 because of 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; US 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., 2015b; 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 is 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.

The hormone methyl farnesoate influences a variety of developmental processes in crustaceans including growth and reproductive maturation (Laufer and Biggers, 2001). In daphnids, methyl farnesoate is responsible for programming developing oocytes into male offspring (LeBlanc and Medlock, 2015; Olmstead and LeBlanc, 2002; Toyota et al., 2015a). 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; LeBlanc et al., 2013; Miyakawa et al., 2013; Toyota et al., 2015a). The activated MfR functions as a transcription factor to regulate the expression of male sex-determining genes and other genes including hemoglobin in daphnids (Fig. 1A) (Rider et al., 2005). Exposing daphnids to increasing concentrations of methyl farnesoate results in corresponding increases in male offspring production (Olmstead

Department of Biological Sciences, North Carolina State University, Raleigh, NC 27695, USA.

*Author for correspondence (Gerald_leblanc@ncsu.edu)

A.A.C., 0000-0002-0318-4402; M.H.H., 0000-0002-9713-2346; G.A.L., 0000-0002-9711-9109

Received 26 October 2018; Accepted 7 January 2019

Journal of Experimental Biology (2019) 222, jeb195289. doi:10.1242/jeb.195289

List of symbols and abbreviations

FAMT	farnesoic acid *O*-methyltransferase
JHAMT	juvenile hormone acid *O*-methyltransferase
Met	methoprene-tolerant
MF	rmethyl farnesoate
MfR	methyl farnesoate receptor
SRC	steroid receptor co-activator

and LeBlanc, 2002); thus, endogenous mRNA levels of genes related to methyl farnesoate production and its subsequent action may be elevated under conditions that promote male offspring production.

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 elicit male production would increase expression of the key genes of the male sex determination signaling endocrine cascade in *D. pulex*.

MATERIALS AND METHODS

Male sex determination

Daphnia pulex and *D. magna* were evaluated for responsiveness to photoperiodic and temperature cues because these species have been used extensively to evaluate male sex determination in response to environmental stimuli, often with equivocal results (summarized in LeBlanc and Medlock, 2015). Our intent was to provide clarity to these observations. *Daphnia pulex* Leydig 1860 (clone WTN6) and *D. magna* Straus 1820 (clone NCSU1) cultures were used in experiments. *Daphnia magna* were originally acquired from the US Environmental Protection Agency, Mid-Continent Ecology Division, Duluth, MN, USA, and have been cultured in our laboratory for over 20 years. *Daphnia 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. *Daphnia pulex* have since been cultured in our laboratory for 4 years. Both species are capable of male offspring production and were maintained in the laboratory at 20°C, on a 16 h:8 h light:dark photoperiod (L:D) using methods described previously (Hannas et al., 2010). Under these conditions, both species reproduce parthenogenetically. Female daphnids were collected from culture as <24 h old neonates and transferred to their respective experimental conditions (n=10): short (10 h:14 h L:D) or long photoperiod (16 h:8 h L:D) and 16, 18, 20 or 22°C. Neonates were individually reared in 50 ml beakers containing 40 ml culture media, which consists of reconstituted deionized water [192 mg l^{-1} $CaSO_4{\cdot}H_2O$, 192 mg l^{-1} $NaHCO_3$, 120 mg l^{-1} $MgSO_4$, 8.0 mg l^{-1} KCl, 1.0 µg l^{-1} vitamin B_{12} (Sigma Aldrich, St Louis, MO, USA) and 1.0 µg l^{-1} selenium (Alfa Aesar, Haverhill, MA, USA)]. Daphnids were fed daily 100 µl *Pseudokirchneriella subcapitata* suspension (1.4×10^8 cells) and 50 µl TetraFin® fish food suspension (Tetra®, Melle, Germany), prepared as described previously (Hannas et al., 2010). All treatment combinations were replicated with 10 individual daphnids. Media were 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.

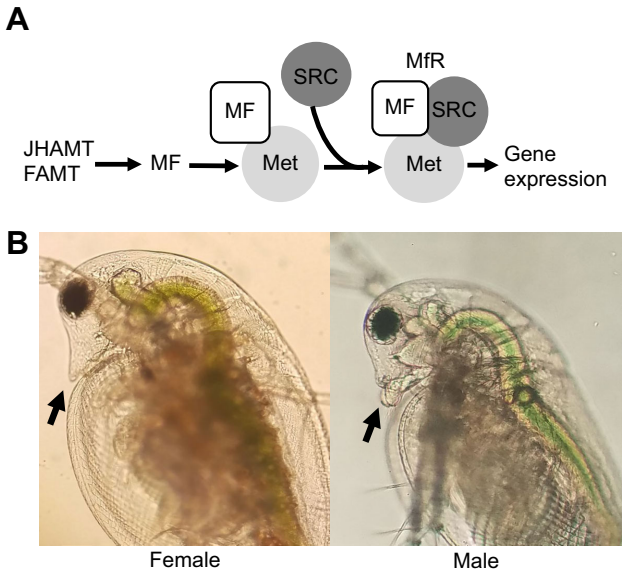

Fig. 1. Endocrine cascade controlling male sex determination in daphnids. (A) The putative signaling cascade leading to male sex determination. The enzymes juvenile hormone acid *O*-methyltransferase (JHAMT) and farnesoic acid *O*-methyltransferase (FAMT) contribute to methyl farnesoate (MF) synthesis.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

MfR signaling pathway mRNA levels

Daphnia pulex female neonates (<24 h old) were reared on either a long or a short photoperiod, at 18°C using the methods described above. Once daphnids reached sexual maturity (eggs in brood chamber), all animals were molt synchronized with the release of their first brood of offspring, such that animals could be collected at 0, 24, 36 and 48 h post-molt. Molt synchronization entailed monitoring daphnids every 2 h for molting to occur. Once an animal molted, that established time zero for that individual. Four replicates of 3–5 daphnids were collected per time point; thus, each time point represents 12–20 daphnids. Daphnids from individual replicates were transferred to 100 µl RNAlater® (Fisher Scientific, Hampton, NH, USA) and held at 4°C for 24 h, then stored at −80°C until used for RNA extraction. Whole animals were homogenized using Next Advance Bullet Blender® and zirconium oxide beads (1.0 mm diameter) (Next Advance, Troy, NY, USA). RNA isolation was conducted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Synthesis of cDNA was conducted using ImProm-II™ Reverse Transcription System with oligo (dT) primers (Promega).

mRNA levels of *JHAMT*, *FAMT*, *Met* and *SRC* were measured by reverse transcription-quantitative PCR (RT-qPCR). Primer sequences for *JHAMT* (Miyakawa et al., 2010), *FAMT* (Toyota et al., 2015a), *Met* (Miyakawa et al., 2010) and *SRC* (Toyota et al., 2015a) were used to amplify mRNA sequences. RT-qPCR was performed with the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using 2× SYBR™ Green Premix (Fisher Scientific, Hampton, NH, USA). A single melting peak was detected for each sample with an amplification efficiency

Journal of Experimental Biology (2019) 222, jeb195289. doi:10.1242/jeb.195289

of >96%, indicating amplification occurred only for the target sequence. Genex software (BioRad, Hercules, CA, USA) was used to analyze relative levels of gene expression by normalizing to two housekeeping genes, *actin* and *GAPDH*.

Data analysis

Male sex determination was calculated as the mean percentage male offspring for individuals completing six consecutive broods of offspring. Animals that perished prior to completing six broods of offspring were excluded from analyses. Differences in male offspring production were assessed with one-way ANOVA with Tukey's adjustment for multiple comparisons when variances were equal. 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. Differences in fecundity between daphnids reared under long and short photoperiods were evaluated using Student's *t*-tests. For gene expression experiments, raw C_t 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 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 s.e.m. and the alpha level was 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*. *Daphnia 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 ($P<0.0001$; Fig. 2B). The increase in the percentage of male offspring under the short-day photoperiod was not an artifact associated with a decrease in the total number of offspring produced. Rather, the total number of offspring produced was significantly higher under short-photoperiod conditions for most temperatures assessed (16°C: $P<0.0001$, 18°C: $P<0.0001$, 20°C: $P=0.0124$, 22°C: $P=0.0004$; Fig. 3).

Similar experiments were performed with the often sympatric species *D. magna* (Östman, 2011; US 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 18–22°C under the short-day photoperiod (Fig. 4B). In contrast to *D. pulex*, the proportion of male offspring significantly increased with increasing temperature ($P=0.0004$, 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 that under the short photoperiod (18°C: $P=0.0068$, 22°C: $P<0.0001$; 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*.

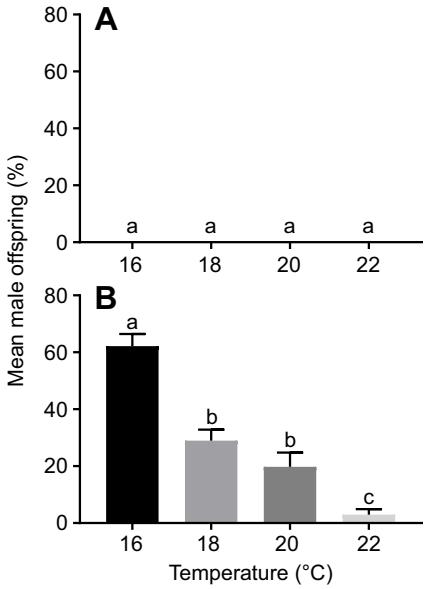

Fig. 2. Mean percentage of male offspring produced by *D. pulex* under different photoperiods and temperatures. (A) Male offspring production under a long-day (16 h:8 h light:dark, L:D) photoperiod (16°C $n=9$, 18°C $n=10$, 20°C $n=10$, 22°C $n=10$). (B) Male offspring production under a short-day (10 h:14 h L:D) photoperiod (16°C $n=9$, 18°C $n=8$, 20°C $n=9$, 22°C $n=8$). Data are presented as means and s.e.m. and different letters denote significant differences among treatments ($P\leq0.05$).

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), although it is unknown whether environmental cues utilize this signaling pathway in orchestrating male sex determination. *Daphnia 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

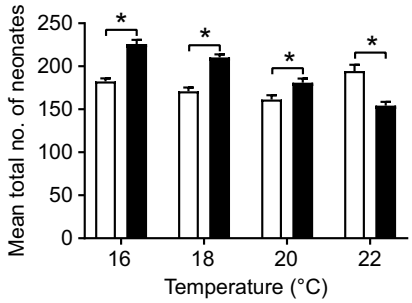

Fig. 3. Fecundity of *D. pulex* under conditions permissive and non-permissive of male production across temperatures. White bars denote the non-permissive long-day photoperiod (16 h:8 h L:D; 16°C $n=9$, 18°C $n=10$, 20°C $n=10$, 22°C $n=10$) and black bars denote the short-day photoperiod (10 h:14 h L:D; 16°C $n=9$, 18°C $n=8$, 20°C $n=9$, 22°C $n=8$). Fecundity was calculated as the mean and s.e.m. of total neonate production over 6 broods of offspring. An asterisk denotes a significant difference between treatments ($P\leq0.05$).

Journal of Experimental Biology (2019) 222, jeb195289. doi:10.1242/jeb.195289

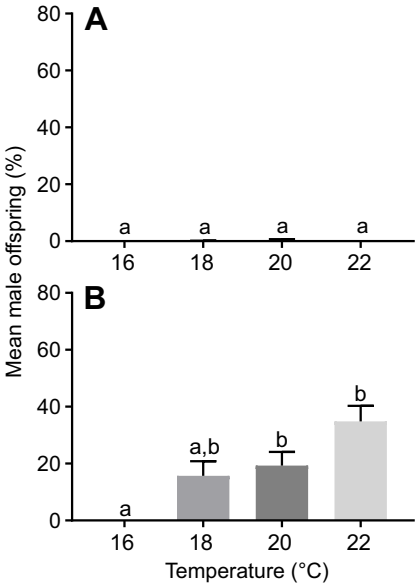

Fig. 4. Mean percentage of male offspring produced by *D. magna* under different photoperiods and temperatures. (A) Male offspring production under a long-day (16 h:8 h L:D) photoperiod (16°C n=10, 18°C n=10, 20°C n=10, 22°C n=10). (B) Male offspring production under a short-day (10 h:14 h L:D) photoperiod (16°C n=9, 18°C n=7, 20°C n=9, 22°C n=9). Data are presented as means and s.e.m. and different letters denote significant differences among treatments (P≤0.05).

with *Met* and *SRC* – the two subunits of the methyl farnesoate receptor.

mRNA levels within the methyl farnesoate signaling cascade varied over the course of the reproductive cycle. *JHAMT* mRNA levels were elevated at 24 h 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 (P=0.0009; Fig. 6A). *FAMT* mRNA levels remained unchanged under both photoperiods throughout the time course of the experiment (Fig. 6B). *Met* mRNA levels also were elevated at 24 h and progressively declined thereafter. *Met* mRNA levels were significantly higher under the short-day photoperiod at 0 and 24 h post-molt (0 h: P=0.0048, 24 h: P=0.0318; Fig. 6C). *SRC* attained maximum mRNA levels at 24 h 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 of the experiment (Fig. 6D).

To summarize, *JHAMT*, *Met* and *SRC* mRNA levels were elevated early in the reproductive cycle, whereas *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.

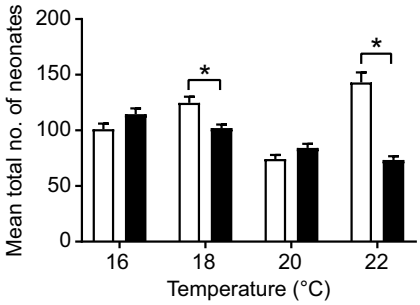

Fig. 5. Fecundity of *D. magna* under conditions permissive and non-permissive of male production across temperatures. White bars denote the non-permissive long-day photoperiod (16 h:8 h L:D; 16°C n=10, 18°C n=10, 20°C n=10, 22°C n=10) and black bars denote the short-day photoperiod (10 h:14 h L:D; 16°C n=9, 18°C n=7, 20°C n=9, 22°C n=9). Fecundity was calculated as the mean and s.e.m. of total neonate production over 6 broods of offspring. An asterisk denotes a significant difference between treatments (P≤0.05).

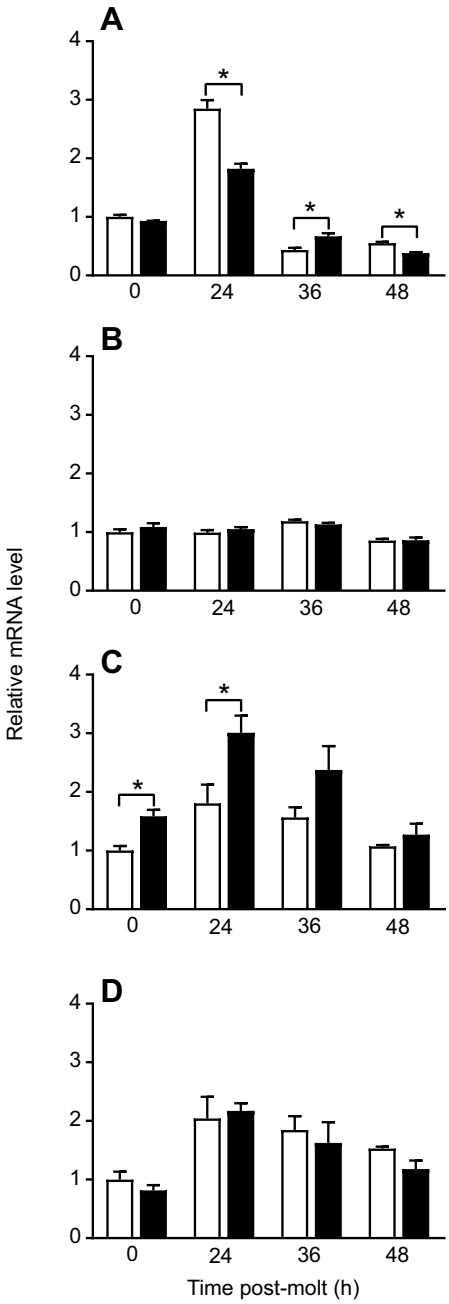

Fig. 6. mRNA expression levels of *JHAMT*, *FAMT*, *Met* and *SRC* under long- and short-day photoperiods at 18°C. White bars denote the long-day photoperiod (16 h:8 h L:D) and black bars denote the short-day photoperiod (10 h:14 h L:D). (A) *JHAMT*, (B) *FAMT*, (C) *Met* and (D) *SRC*. Data are presented as means and s.e.m. of mRNA levels normalized to respective levels under the long-day photoperiod at time zero (n=4 for all treatments and time points). An asterisk denotes a significant difference between treatments (P≤0.05).

Journal of Experimental Biology (2019) 222, jeb195289. doi:10.1242/jeb.195289

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 requirements differed between species. Among the temperatures evaluated, the maximum percentage of male offspring produced by *D. pulex* occurred at 16°C, while the 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, 1969a,b, 1966; 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., 2017, 2015b) and in other closely related processes in crustaceans such as molting (Aiken, 1969; Bliss and Boyer, 1964). The permissive photoperiod used here, 10 h:14 h L:D, occurs in temperate and artic regions and both *D. pulex* and *D. magna* are distributed in these climates (Crease et al., 2012; Ferrari and Hebert, 1982; Mitchell and Lampert, 2000).

In some organisms and systems, stimulus change rather than the magnitude of a stimulus can elicit physiological changes. Our short-day photoperiod organisms experienced an abrupt shift to shorter day-length conditions at the beginning of assays, and it could be argued that the photoperiod change may initiate male sex determination. If this were true, we would expect to observe a corresponding increase in male offspring early in the male sex determination experiments, followed by a tapering as the daphnids continued reproducing under a new, consistent photoperiod. The duration of our experiments allowed us to determine whether the shift in photoperiod was critical to male offspring production. We did not observe a peak in male production early in the experiments; instead, we generally observed that the incidence of male offspring increased over the duration of the six-brood experiment. Thus, the photoperiod itself, rather than the shift in photoperiod, appears to be the critical stimulus. A similar point can be made regarding changes in temperature for these experiments.

Temperature, unlike photoperiod, 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 and may also reflect temperature-related differences in methyl farnesoate production and/or regulation. 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.

Other crustacean groups subject to environmental sex determination have been shown to be influenced by temperature and/or photoperiod (Korpelainen, 1990). For example, in another cladoceran, *Moina micrura*, increasing temperatures resulted in a higher male:female sex ratio, a finding similar to our observations with *D. magna* (Miracle et al., 2011). In the amphipod *Echinogammarus marinus*, longer photoperiods were associated with male-biased sex ratios, while shorter photoperiods were associated with female-biased sex ratios, which is contrary to our results with *D. pulex* and *D. magna* (Guler et al., 2012). In the marine copepod *Tigriopus japonicus*, both photoperiod and temperature have been observed to influence sex ratios (Takeda, 1950). Although it is not surprising that other crustaceans are responsive to temperature and photoperiod cues for environmental sex determination, these other studies, combined with our results, highlight that a species' response to a given stimulus will depend on its specific life history traits.

Our temporal assessment of mRNA levels within the methyl farnesoate signaling pathway revealed that *JHAMT* mRNA levels were maximally expressed at 24 h 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. (2015a). 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 h post molt. This difference in our studies may reflect differences in the time points analyzed. Toyota et al. (2015a) noted significantly elevated *JHAMT* mRNA levels under a short day photoperiod at 30 h post-ovulation. We noted elevated but significantly lower levels under the short-day photoperiod, as compared with the long-day photoperiod, at 24 h post-molt. At 36 h, levels were no longer elevated above 0 h levels under either photoperiod in our study, although levels were now significantly elevated under the short-day photoperiod as compared with the long day photoperiod. We may have missed the peak in *JHAMT* mRNA levels under the short-day photoperiod that was noted by Toyota et al. (2015a).

Met mRNA levels were elevated under the permissive short-day photoperiod, as compared with the non-permissive long-day photoperiod, early in the time course. The Met protein is in the basic 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 found here in *D. pulex* agrees with these previous findings, wherein maximum *Met* mRNA levels are attained early in the reproductive cycle. Further, we observed a significant increase in *Met* mRNA levels at both 0 and 24 h 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, was highest early in the molt/reproductive cycle (0–24 h), 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., 2015a) and suggests that JHAMT is primarily responsible for the induction of methyl farnesoate synthesis. mRNA levels of the MfR subunits Met and SRC displayed increases 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, providing insight into phenological differences between species or between clones and their response to thermal cues. Further, we 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.

Acknowledgements
We would like to thank the members of the LeBlanc laboratory for their assistance.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: G.A.L.; Formal analysis: A.A.C.; Investigation: A.A.C., M.H.H.; Resources: G.A.L.; Writing - original draft: A.A.C.; Writing - review & editing: G.A.L.; Visualization: A.A.C.; Supervision: G.A.L.; Project administration: G.A.L.; Funding acquisition: G.A.L.

Funding
The work was funded by the National Science Foundation grant IOS-1350998 to G.A.L. and a fellowship from the Scholar Rescue Fund, Institute of International Education to M.H.H.

References
Aiken, D. E. (1969). Photoperiod, endocrinology and the crustacean molt cycle. *Science* **164**, 149-155.
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.
Bliss, D. E. and Boyer, J. R. (1964). Environmental regulation of growth in the decapod crustacean *Gecarcinus lateralis* . *Gen. Comp. Endocrinol.* **4**, 15-41.
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.
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. Taylor, D. J. (2012). Transcontinental Phylogeography of the *Daphnia pulex* Species Complex. *PLoS One* **7**, e46620.
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. F. and Dodson, S. I. (2000). Latitudinal patterns in the size distribution and seasonal dynamics of new world, freshwater cladocerans. *Limnol. Oceanogr.* **45**, 22-30.
Guler, Y., Short, S., Kile, P. and Ford, A. T. (2012). Integrating field and laboratory evidence for environmental sex determination in the amphipod, *Echinogammarus marinus*. *Mar. Biol.* **159**, 2885-2890.
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.
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.
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.
LeBlanc, G. A., Wang, Y. H., Holmes, C. N., Kwon, G. and Medlock, E. K. (2013). A transgenerational endocrine signaling pathway in crustacea. *PLoS One* **8**, e61715.
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.
Miracle, M. R., Nandini, S., Sarma, S. S. S. and Vicente, E. (2011). Endocrine disrupting effects, at different temperatures, on *Moina micrura* (Cladocera: Crustacea) induced by carbendazim, a fungicide. *Hydrobiologia* **668**, 155-170.
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., 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.
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. (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. (2000). Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna*. *Environ. Toxicol. Chem.* **19**, 2107-2113.
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. (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. (1966). Light and temperature requirements for diapause development and release in *Daphnia*. *Ecology* **47**, 368-374.
Stross, R. G. and Hill, J. C. (1968). Photoperiod control of winter diapause in the fresh-water crustacean, *Daphnia*. *Biol. Bull.* **134**, 176-198.
Takeda, N. (1950). Experimental studies on the effect of external agencies on the sexuality of a marine copepod. *Physiol. Zool.* **23**, 288-301.
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.
US EPA (2002). *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, 821-R-02-012. Washington, DC: EPA.

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.