



Sex Specific Differences Recorded in the Behavior of an Amphipod: Implications for Behavioral Toxicology

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Behavior is a useful endpoint in ecotoxicological research; it links the biochemical effects of contamination with physiology of individuals, which can be applied to higher levels of organization with relevance to ecology. Animals exhibit species-specific and sex specific behaviors. Previous experiments within ecotoxicology using amphipods as models have either not separated by sex or have on the assumption that they may create more variability in the results. Therefore, the objectives of this study were to investigate the effect of time (which controlled light conditions), sex, and the interaction of time and sex on the swimming velocity in males and females of the marine amphipod *Echinogammarus marinus*. *E. marinus* exhibited a phototactic response to light, as is consistent with previous findings. It was determined that females swim significantly faster than males and that this response was enhanced during periods when the lights were switched off. This suggests that a greater understanding of the baseline unconditioned behaviors of experimental organisms is needed to avoid impacts of unknown variables on results. The results of this study reveal significant sexual differences in the baseline behavior of *E. marinus* which has implications for future research in ecotoxicology. It is recommended that in future research specimens be separated by sex prior to experimentation, in order to account for possible behavioral differences such as those observed in the present studies. This study also highlights the need for thorough observation of behavior over shorter time intervals as larger intervals may miss short-term variations.

Keywords: ecotoxicology, behavior, aquatic toxicology, Amphipoda, crustacea

INTRODUCTION

The use of behavior in environmental toxicology has expanded substantially over the past decade (Saaristo et al., 2018). However, there is a need to standardize protocols and understand baseline behaviors to improve the quality and use of these endpoints (Pyle and Ford, 2017). Behavior is defined as an organism-level reaction to a particular stimulus; the result of cumulative interactions between external conditions, biotic and abiotic factors with animal physiology and neurology (Boyd et al., 2002; Gerhardt, 2007; Hellou, 2011; Saaristo et al., 2018). Behavior should be adaptive (although in some situations can be maladaptive) and should enhance an organism's

fitness (Scott and Sloman, 2004). Further, although behavior is typically measured at an organism-level behavior, it is known to have ecological consequences at a population level (Saaristo et al., 2018).

As well as exhibiting species-typical behaviors, individuals will exhibit sex-typical behaviors (also referred to as heterotypical or sexually dimorphic) which can be physical, social and sexual, resulting from genetics, and mediated by endocrine and neural systems (Kelley, 1988; Brain and Haug, 1991; Dulac and Kimchi, 2007). Examples of this include behaviors specific to not only foraging and predator avoidance but also courtship, competition, communication, and care of offspring. Behaviors such as these allow individuals to survive, identify each other, and reproduce successfully which ultimately ensures continuation of the species (Boyd et al., 2002; Dulac and Kimchi, 2007). As a consequence, sexually dimorphic behaviors continue to be selected for (Kelley, 1988).

Behavior is intrinsic to survival and can therefore be a useful endpoint to assess an animal's condition, as it provides links between the biochemical effects of contamination and the physiology of individuals which can be extrapolated to population level effects (Scott and Sloman, 2004; Hellou, 2011). In recent times, the field of behavioral ecotoxicology has greatly expanded, with a growing appreciation of the utility of behavior as a toxicological endpoint, in parallel with advances in technology used to measure behavior (Noldus et al., 2001; Melvin and Wilson, 2013).

Amphipods such as those in the family Gammaridae have been used extensively in ecotoxicological research due to their ecological importance; they are ubiquitous in aquatic environments, cover a wide range of trophic levels (Sutcliffe, 1992; Kunz et al., 2010; Glazier, 2014; Conway, 2015), and provide an important food source for fish and birds (Schneider et al., 1994; McCurdy et al., 2005). They are easy to source and culture, and are sensitive to many pollutants, providing a useful bioindicator species (Kunz et al., 2010; Glazier, 2014). As well as this, cosmopolitan distribution allows for comparison of response to toxicants across environments. They have been used in many ecotoxicological studies using behavioral endpoints (Kunz et al., 2010), especially those looking at the effects of contaminants on phototactic response and mate-guarding (Boyd et al., 2002). Gammarid amphipods have shown sex differences in biochemical and physiological response generally and in response to contaminants or environmental change (see Lebrun et al., 2017 and references within), showing that sex is an important factor to consider in ecotoxicological research. There have been relatively fewer studies looking at sex differences in behavioral response.

That said, amphipods have day/night activity cycles (Holomuzki and Hoyle, 1990; Peeters et al., 2009; Navarro-Barranco and Hughes, 2015) and have been shown to exhibit negative phototaxis, or scototaxis, and increase their swimming velocity in response to light (Bossus et al., 2014; Kohler et al., 2018a,b). These are most likely behavioral adaptations for predator avoidance (Newman and Waters, 1984; De Robertis, 2002) and are examples of behavioral endpoints useful in ecotoxicology. As in all sexually dimorphic species they exhibit sex-specific behaviors, an example of which is precopulatory

mate-guarding (or amplexus); males will grasp females dorsally using their modified gnathopods. They remain paired until external fertilization of eggs has occurred, which takes place after the female has molted due to the cuticle being flexible enough to release eggs to the brood pouch (Robinson and Doyle, 1985; Conlan, 1991; Boyd et al., 2002; Hyne, 2011). Borowsky and Aitken (1991) observed sexually dimorphic free-swimming behavior; female *Ampelisca abdita* were suggested to only enter the water column on the nights which they are able to fertilize eggs, in order to avoid predation during their most vulnerable period and to maximize success. Males on the other hand would enter the water column every night as they are readily available to reproduce for the majority of their molt cycle. A similar pattern was also observed in *Corophium* by Lawrie et al. (2000). Female amphipods also exhibit sex specific behaviors during amplexing attempts such as fleeing, remaining defensively curled or straightening the body either to encourage reproduction or when initiating a "kick" to break away from unsuitable mates (Borowsky and Borowsky, 1987; Sutcliffe, 1992, 1993; Hatcher and Dunn, 1997).

Previously, using the intertidal amphipod *Echinogammarus marinus*, Kohler et al., 2018a found species-specific behavioral responses to light, which was suggested to be a result of adaptation to differing predation between the habitats of the species (Kohler et al., 2018a). The authors also found that *E. marinus* presents different behaviors when recorded in different shape and sized recording arenas. For the present study it was speculated that there may also be sex-specific response within species as a result of predation, as predators such as shorebirds and benthic fish preferentially predate on male amphipods (Schneider et al., 1994; McCurdy et al., 2005). Differing predation pressure may be a selective force affecting amphipods, resulting in sexually dimorphic behavior. Sexual dimorphism in behaviors such as free-swimming (Borowsky and Aitken, 1991), aggregation and cover-seeking (Williams et al., 2017) appear to support this hypothesis.

As well as this, sexes may exhibit dimorphic behavioral response due to differences in physiology during their life cycle. For example, female amphipods invest more energy in oogenesis than males in spermatogenesis (Sornom et al., 2012), and undergo more physical changes than males, experiencing more molts in order to sexually mature (Sutcliffe, 1992; Hyne, 2011). Reproductive status may also affect behavior in females. A study by Williams et al. (2017) found that brooding females of *Gammarus pseudolimnaeus* were more likely to aggregate or seek cover in response to predator cues than males and non-brooding females, in order to avoid the greater energy cost of fleeing while carrying young. Cothran (2004) also suggested there may be greater risk for female amphipods when in an amplexing pair, compared to un-paired, to predation by size-selective fish.

Other studies have indicated there may be a difference between male and female behavioral response in amphipods, such as differences in locomotion (Ayari et al., 2015), and activity level (Peeters et al., 2009). Previous ecotoxicology experiments utilizing amphipods have been inconsistent with regard to separating by gender. Animals have either been assessed as a mixed gender sample or have been separated on the presumption

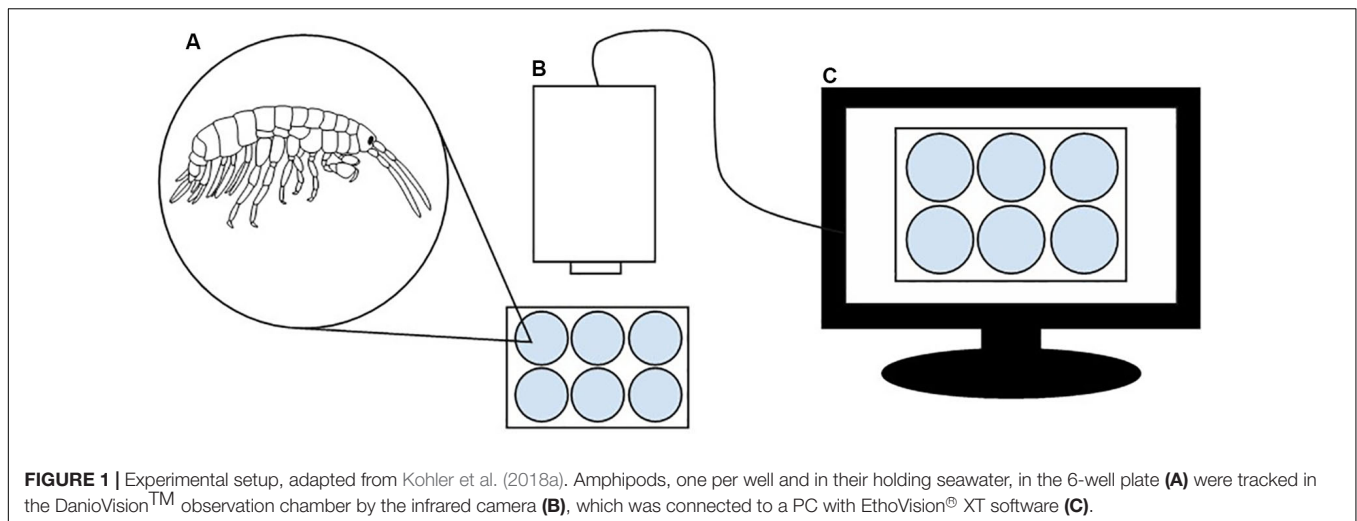


FIGURE 1 | Experimental setup, adapted from Kohler et al. (2018a). Amphipods, one per well and in their holding seawater, in the 6-well plate (A) were tracked in the DanioVision™ observation chamber by the infrared camera (B), which was connected to a PC with EthoVision® XT software (C).

that they would differ in behavior, and/or responses to stimuli. The implications of which are important for ecotoxicological research especially as variability in innate responses by separate sexes may add to the underlying variation in overall datasets. This is particularly important when behavioral data can have a high degree of intraspecific variability. Conversely, sorting sexes in small invertebrates can be time consuming. When trying to develop high-throughput testing protocols anything that reduces experiment time will minimize costs. Therefore, the objectives of this study were to compare the swimming velocity of the intertidal amphipod *E. marinus* stimulated by altering periods of light and dark.

METHODOLOGY

Specimen Collection and Husbandry

Echinogammarus marinus were collected in late November 2018 from Lock Lake, Langstone Harbor, Portsmouth, United Kingdom (50°47′22.8″N and 1°02′35.9″W), at low tide by searching through and under algae (*Fucus vesiculosus* and *Ascophyllum nodosum*). They were then transported to the Institute of Marine Sciences, Portsmouth, United Kingdom, separated by sex and placed in separate buckets.

The sex of individuals was determined by looking at specimens under a dissecting microscope, specifically for differences in secondary sex characteristics as detailed by Sexton and Spooner (1940). Males are generally larger than females and have enlarged gnathopods. Females have brood plates (oostigites) whilst males have genital papillae. Any specimens which appeared to be parasitized with behavioral altering trematodes were excluded from the analysis (Guler et al., 2015).

Specimens were acclimated for 1 week in a growth room set at $10 \pm 1^\circ\text{C}$ and a 24 h dark photoperiod in order to reduce any stress or physiological changes resulting from collection and transportation (Oberner and Baldwin, 2006) and to remove any variations in behavior that may result from biological rhythms. Amphipods are known to have both

circadian and circatidal rhythms (Harris and Morgan, 1984; Holomuzki and Hoyle, 1990; Kohler et al., 2018a,b). Seawater was changed midway through the week. Animals were supplied with the algae *F. vesiculosus* and *A. nodosum* as a food source and substrate.

Analysis of Behavior

Animals were transferred from their acclimation tanks to standard six-well plates. A plastic spoon was used to transport animals in order to minimize any effect on behavior as a result of handling stress (Tran et al., 2016). Each well in the multi-well plate (diameter = 36 mm) contained seawater at a depth of 1.5 cm to limit vertical movement but still allow for free horizontal swimming. The sexes of individuals to be placed in each well were organized using a random number generator. Before trials started, it was checked and noted whether females were gravid, as their behavior may differ due to carrying eggs or offspring. As only one female was gravid, its results were omitted from the final analysis as this did not provide enough replicates to statistically test against males and non-gravid females.

Behavior of *E. marinus* was analyzed using a DanioVision™ observation chamber following methods by Kohler et al. (2018a). The hardware consists of an infrared camera positioned above an observation chamber with a cold white light source that can be controlled via programming. There is a holder in which a petri dish or multi well plate can be secured. An external hood covers the chamber. This was connected to EthoVision® XT 11.5 video tracking software (Noldus, Netherlands) on a PC, an example of software which tracks animals using pixels resulting from frame by frame digitisation of the analog video signal, a more dependable method of recording behavior than manually (Spink et al., 2001). Experimental setup is illustrated in **Figure 1**. Each trial lasted 8 min, during which the light [at 50% (2000 lux)] was controlled in a dark/light cycle; off during minutes 0–2 and 4–6 and on during minutes 2–4 and 6–8. A total of 34 females and 32 males were recorded.

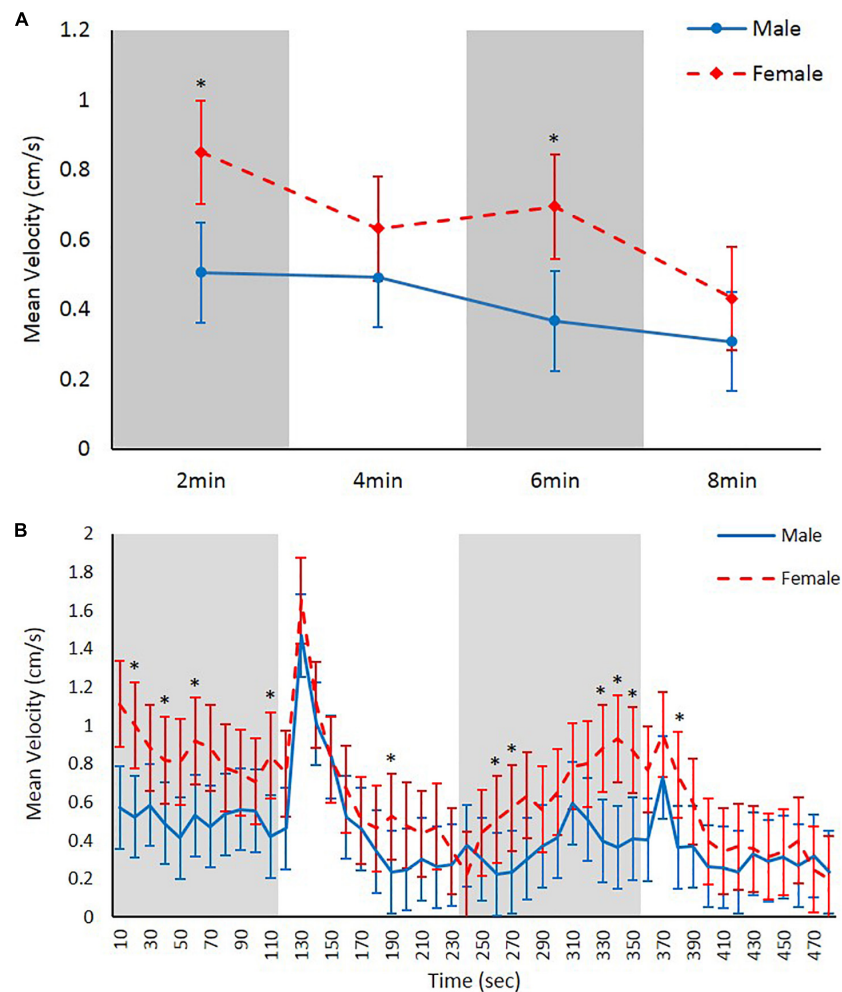


FIGURE 2 | Mean swimming velocity (cm/s) in male and female *Echinogammarus marinus* recording over **(A)** 2 min time and **(B)** 10 s time bins. Error bars represent $2 \times \text{SE}$. * ≤ 0.00075 .

Statistics

Statistical analysis was performed in IBM SPSS Statistics 25. Data were analyzed in 2-min time bins and 10-s time bins after being found to be normally distributed by Kolmogorov–Smirnov and Shapiro–Wilk tests. A linear mixed effects model was used for both the 2-min data and 10-s data in order to compare differences in amphipod swimming velocity for each time bin and sex and the interaction between times and sexes. Linear mixed effects models are useful in analysis of behavioral data as they are able to analyze categorical and hierarchical data with fixed and random effects and their interactions while correcting for repeated measures (Koerner and Zhang, 2017). In this experiment, fixed effects, or factors, were time, sex, and the interaction of time and sex (time*sex). For Post Hoc analysis, independent samples *t*-tests were used for comparing differences in velocity between sexes at each time bin, for both the 2-min, and 10-s data, with manual Bonferroni correction to correct for type II error. One-way ANOVAs were used for both data sets to compare velocity of one sex between all the time bins, with

Bonferroni tests. Data were considered significant where *p*-values were <0.05 , except in the manual Bonferroni correction for the independent samples *t*-tests, where the critical *P*-value of 0.05 was divided by the number of replicates, 66, to give the new critical *p*-value of 0.00075.

RESULTS

Females were significantly faster swimmers than males both when analyzing the 2 min and 10 s binned data ($F = 20.738$, $df = 1$, and $p < 0.001$ and $F = 83.519$, $df = 1$, $p < 0.001$, respectively; **Figures 2A,B**). The variance ($\pm \text{SE}$) for the random factor (specimen) was (0.007584 ± 0.006482) 2 min bins and (0.013064 ± 0.006752) for 10 s bins. Time was a significant factor in the velocity of the amphipods for 2 min and 10 s dataset with greater mean velocities typically occurring during periods of darkness ($F = 7.080$, $df = 1$, and $p < 0.001$ and $F = 10.289$, $df = 1$, $p < 0.001$, respectively; **Figure 2**). The overall patterns of

swimming behavior can be interpreted differently when looking at the data binned into different time intervals (Figures 2A,B). Both males and females reacted to the lights coming on reaching their maximum speeds during the experiment before settling down. The extent to which females swam faster was greater during dark periods (~65–85% faster) than light periods (~25–35% faster) although no statistically significant interactions were observed between sex and time ($p > 0.05$).

DISCUSSION

To aid the development of amphipods as a model for studying behavioral toxicology, this study was conducted to determine whether male and female *E. marinus* have detectable differences in swimming behaviors in response to a light stimulus. Ultimately there is a trade-off between increased “noise” in a dataset if variability does exist between gender vs increased resources in the form of time by separating individuals by sex. The aim of this study was to determine whether separation by gender is useful or not in behavioral experiments with *E. marinus*.

Both male and female *E. marinus* increased their swimming velocity rapidly in response to light the light switching on but then settled down as shown previously (Bossus et al., 2014; Kohler et al., 2018a,b). However, greater mean velocities were recorded during the periods when the lights were off. Females were significantly faster than males in our experiments although looking at the 2-min and 10-s time bin data, it is possible to arrive at different conclusions. The 10-s time bins give a more detailed picture of amphipod activity throughout the trials and show there is a large range in swimming velocities. While both sets of data support our hypotheses that swimming speeds differ between the sexes the extent to which they differed appeared to vary between light stimuli, with a greater differentiation of speeds during dark vs light periods. The 10-s data demonstrated a better resolution in behavioral phenotype with reaction to the light stimulus clearly visible with both sexes but lost when pooling the datasets into 2 min bins. This highlights the importance of how the data is processed and analyzed in forming conclusions as pointed out by Melvin et al. (2017) and Kohler et al. (2018a).

Our data are in contrast to another study by Williams et al. (2017) who found males had 18% higher maximum velocity than females, as well as 44% higher average velocity in the freshwater amphipod *G. pseudolimnaeus*. Interestingly, this study also observed that non-brooding females had 33% higher maximum velocity than brooding females. Kohler et al. (2018a) recently found species specific behaviors in amphipods suggesting this may arise from different predatory pressures. They highlighted the caution required with cross-species extrapolations, especially in behavioral ecotoxicology when “no observable effects” may arise from low or different sensitivities in some species rather than to the stimulus.

The results of our study could also have arisen through the experimental design. Another study by Kohler et al. (2018b) found that arena size and shape had an important bearing on a variety of behavioral endpoints. Interestingly, the larger the arena

the greater the velocity recorded for male freshwater *Gammarus pulex* suggesting for some endpoints the organisms needed “space to behave.” Females (max ~10 mm) of amphipods are typically smaller than males (max ~15 mm; Ford et al., 2004), whether this had a bearing on the overall speeds within the 6 well plates is currently unknown. Peeters et al. (2009) observed sexual dimorphism in activity levels of *G. pulex*; during the first night of a 7-day, 12:12 h (light:dark) regime, whereby males were more active than females. The authors of the study emphasized that inter-individual variation in activity must have been partially linked to sex of specimens. Ayari et al. (2015) found no significant difference in locomotor rhythm patterns of male and female amphipods (*Orchestia gammarellus*), a semi-terrestrial species, but also noted inter-individual variations in activity patterns. Boates et al., 1995, however, found no significant difference in crawling behavior of male and female *C. volutator*, an intertidal species, in response to predation by birds.

This study has established that *E. marinus* exhibits some differential responses to light based on sex. Females were found to have greater swimming velocity than males, which is the opposite of what has been observed in other species. Irrespective of whether this represents an interspecies difference or a difference in experimental design, the consequences in generating more variation within the datasets remains the same when considering to separate sexes prior to experiments. Therefore, going forward we recommend researchers conduct baseline studies of their model species and statistically determine intra-species variation. This could be particularly important when variation between sexes maybe greater than the variation that could be induced through e.g., exposure to contaminants leaving the observer to conclude their null hypotheses.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

ETHICS STATEMENT

This study was conducted and authorized through the University of Portsmouth Ethics Committee.

AUTHOR CONTRIBUTIONS

AF led the project. T-RC and SK conducted the experiments. T-RC, SK, and AF helped writing and editing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00370/full#supplementary-material>

TABLE S1 | Data and statistics for **Figure 2**.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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