Species-specific behaviours in amphipods highlight the need for understanding baseline behaviours in ecotoxicology

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Abstract

Behaviour in ecotoxicology is expanding with techniques and endpoints used in pharmacology being translated to other vertebrate and invertebrate species. Despite this, data on the baseline behaviours of model organisms, and inter-species variability in behaviour are currently under-studied. This study assessed a range of behaviours associated with anxiety including swimming speed, phototaxis and thigmotaxis in a marine and freshwater amphipod (*Echinogammarus marinus* and *Gammarus pulex*). Differences in sensitivity to these assays were observed between species with *E. marinus* showing a greater sensitivity to the phototaxis assay than *G. pulex*, while in thigmotaxis assays *G. pulex* appeared better suited than *E. marinus* for measuring differences in the use of central zones. Significant inter-species differences were also observed in swimming patterns when breaking the data into ten second time bins but not when data was broken into two-minute time bins. The results of this study provide evidence of phototactic and thigmotactic behaviours in two model crustacean species

with potential for use in behavioural ecotoxicology. Inter-species variability in sensitivity to behavioural assays highlights the importance of systematic assessment of baseline responses for all model species used in behavioural studies. Careful analysis of data is also required when performing behavioural studies so as not to lose sensitivity in your data.

Key words: Amphipod, Behaviour, Phototaxis, Thigmotaxis, Velocity, Ecotoxicology

1. Introduction

Behavioural ecotoxicology is expanding as it provides a link between biochemical and ecological effects of environmental contamination (Scott and Sloman, 2004; Sloman and Mcneil, 2012). However, despite its growing recognition, the use of behavioural endpoints is currently limited by our understanding of the baseline unconditioned behaviours of many organisms, and the relevance of these behaviours to higher level effects (Melvin and Wilson, 2013). In recent years, advancements in computational equipment have provided the means for more sensitive analysis of a wider variety of complex behavioural end points, as well as improving the repeatability of bioassays (Parker, 2016; Pyle and Ford, 2017). Alongside advancements in behavioural automaton has been a rise in calls for the development and optimisation of techniques for behaviour analysis in ecotoxicology, to improve the reliability of assays, and allow for integration of complex behaviours into high-throughput assays.

Behavioural assessment of unconditioned behaviours such as anxiety relies on species-typical, stereotyped responses. For example, some organisms show a natural preference for dark environments when placed in a light/dark choice chamber (Blaser and Rosemberg, 2012). This preference for dark environments has been termed *phototaxis* or *scototaxis* and has been used to assess the effects of neuro-modulating agents on fish

(Maximino, de Brito, Colmanetti, *et al.*, 2010; Maximino, de Brito, de Mattos Dias, *et al.*, 2010; Maximino *et al.*, 2011; Holcombe *et al.*, 2013; Baiamonte *et al.*, 2016) and crustaceans (Guler and Ford, 2010; Fossat *et al.*, 2014; Hamilton *et al.*, 2016). Thigmotaxis or ‘wall hugging’ behaviour is conserved across many species. When placed in a novel environment the organism will stay close to walls or corners. Thigmotaxis has been demonstrated in rodents (Treit and Fundytus, 1988; Simon, Dupuis and Costentin, 1994), zebra fish (Richendrfer *et al.*, 2012; Schnörr *et al.*, 2012; Baiamonte *et al.*, 2016) and crayfish (Tierney *et al.*, 2016) as an anxiety-like behaviour used for the assessment of anxiolytic and anxiogenic compounds. In ecotoxicology, behavioural studies have demonstrated differential inter-species sensitivity to contaminants in activity (Gerhardt *et al.*, 2002), response to predators (Gutierrez, Paggi and Gagneten, 2012), aggression (Tierney *et al.*, 2004), and avoidance (Ward, Simpson and Jolley, 2013) behaviours. However, inter-species variability in the baseline unconditioned behaviours of model organisms is currently understudied. Fisheries research has demonstrated that behavioural responses to light are species specific and that inter-species variation in phototaxis behaviour can be used to reduce bycatch of undesired species (Marchesan *et al.*, 2005; Hannah, Lomeli and Jones, 2015) however, the sensitivity to light between species was not measured. As far as the authors are aware, inter-species variation in the phototactic and thigmotactic behaviours of aquatic invertebrates remains unexplored.

Amphipods have been used extensively in aquatic ecotoxicology due to their importance in both marine and freshwater environments. They are ubiquitous in almost all aquatic systems, covering a wide trophic range as herbivores, detritovores and predators, as well as providing an important food source for many fish and bird species (Welton, 1979; Múrias *et al.*, 1996; Martins, Maranhão and Marques, 2002; Glazier, 2014). The light/dark choice chamber has been used with the marine amphipod *Echinogammarus marinus* to assess

phototactic behaviours (Guler and Ford, 2010) and swimming velocity has also been shown as a sensitive endpoint in this species for the assessment of effects of antidepressants on behaviours (Bossus *et al.*, 2014). The freshwater amphipod *Gammarus pulex* has been used in numerous studies using behaviour as an endpoint to assess the effects of environmental pollution in freshwater systems (Gerhardt, 1995; Gerhardt *et al.*, 1998; Watts, Pascoe and Carroll, 2001; Lauridsen and Friberg, 2005; De Lange *et al.*, 2006; De Lange, Sperber and Peeters, 2006; Felten *et al.*, 2008; Alonso, De Lange and Peeters, 2009; De Lange, Peeters and Lurling, 2009; Vellinger *et al.*, 2012; Nyman *et al.*, 2013; De Castro-Català *et al.*, 2017)

Our overall aim was to provide information on the baseline unconditioned behaviours of a marine and freshwater amphipod (*Echinogammarus marinus* and *Gammarus pulex*), to aid in the advancement of behavioural assays on complex behaviours in model crustaceans. To meet this aim, our objective in the current study was to compare the phototactic and thigmotactic behaviours, as well as swimming velocity and photosensitivity, of the two species.

2. Methodology

2.1 Specimen Collection and Husbandry

2.1.1 *Echinogammarus marinus*

E. marinus were collected from Langstone Harbour, Portsmouth, UK (50°47'22.8"N, 1°02'35.9"W). The site was characterised by areas of silt or gravel with larger rocks, and colonised predominantly by species of brown algae, namely *Fucoids* and *Ascophyllum nodosum* which form large aggregations. All specimens were collected by hand at low tide by gently shaking them from the algae. Specimens were then transported back to the Institute of Marine Sciences, Portsmouth, UK and acclimated for one week in filtered seawater from

Langstone Harbour at $10 \pm 1^\circ\text{C}$ under a 24 hour dark photoperiod. The acclimation period was determined by preliminary experiments which found 7-days acclimation in the dark sufficient for significant differences in activity due to diurnal rhythms to be lost. Specimens were provided with *Fucus vesiculosus* or *A. nodosum* as a food source and substrate. A water change was performed every three days.

2.1.2 *Gammarus pulex*

G. pulex were collected from the River Ems, Emsworth, UK; ($50^\circ 51' 40.5''\text{N}$, $0^\circ 55' 42.5''\text{W}$). Specimens were caught in a 1mm mesh net using the kick sampling method outlined by the freshwater biological association (FBA, 2015). Organisms were transported back to the Institute of Marine Sciences, Portsmouth, UK and left to acclimate to laboratory conditions for one week in Ems river water at $10 \pm 1^\circ\text{C}$ under a 24 hour dark photoperiod for consistency with *E. marinus* experiments. Leaf litter collected from the sample site acted as a natural substrate and food source.

2.2 Analysis of Behaviour

Prior to acclimation, specimens were separated by gender and checked under a backlight for internal parasites. Only healthy, unparasitised, adult, males were used in all assays for both species. Following acclimation, the behaviours of both *E. marinus* and *G. pulex* were analysed using a DanioVision™ observation chamber connected to EthoVision®XT 11.5 video tracking software (TrackSys, Nottingham, UK). The observation chamber is comprised of an external hood with an infrared sensitive camera mounted inside, and an internal holder for a multi-well plate or small container. The holder is infrared backlit with an additional cold white light source which can be programmed to operate automatically, providing a controlled environment for behavioural experiments. The experimental set up is outlined in Figure 1. Organisms were transferred from holding tanks, via a plastic spoon to reduce handling stress,

and placed in a behavioural chamber containing tank water to a depth of 15mm. The water depth allowed for free horizontal swimming but limited vertical swimming. The chamber was then placed in the DanioVision™ with the infra red camera mounted directly above and the cold white light plate below. The camera was linked to a PC with tracking software. A total of 480 animals were analysed in the tracking system. For *E. marinus* 240 individuals were analysed with 120 animals per behavioural chamber (i.e. light/dark chamber and thigmotaxis/velocity chamber) under a range of white-light intensities, 100%, 50%, 20% & 5% (4000, 2000, 800 & 200 lux respectively) with 30 individuals used per light intensity. A further 240 replicates were performed for *G. pulex* with 120 animals per behavioural chamber, under the same light intensity range as *E. marinus*

Figure 1: Schematic of experimental design showing position of (A) infra-red camera, (B) PC with EthoVision®XT video tracking software, (C) behavioural chamber containing either *E. marinus* or *G. pulex* with water from their respective holding tanks, (D) cold white light plate.

2.2.1 Phototaxis

The amphipods' preference for light or dark environments was assessed using a novel chamber comprised of a rectangular 90ml (128 x 86mm) well plate placed over a specially designed plate of acrylic creating a light and dark zone in the well (Fig 2a). The plate consisted

of a clear acrylic through which white and infra-red light can pass, and a black acrylic that allows only infra-red light to pass. Light and dark zones were marked in EthoVision®XT, and an exclusion zone was added to prevent organisms being in two zones at one time (Fig 2b). A single organism was placed into the observation chamber and its movements recorded for 8 minutes under a 2-minute dark/2-minute light cycle. During the 2-minute dark cycle the whole chamber was dark. In the 2-minute light cycle, the half of the chamber containing the clear acrylic was lit whilst the half with the black acrylic remained dark.

Figure 2: (A) Light-Dark chamber loaded in the DanioVision™ observation chamber. (B) Dimensions of light, dark and exclusion zones.

2.2.2 Thigmotaxis

Thigmotactic behaviours were measured using a petri dish in the DanioVision™ (figure 3a). The method for measuring thigmotaxis in a 2D arena was adapted from Schnörr *et al* (2012) with larval zebrafish. Briefly, a central and outer zone were measured for the behavioural chamber in the EthoVision®XT software. Dimensions of central and outer zones are shown in figure 3b. The outer zone was 22mm wide and was determined by the size of the larger species, *E. marinus*, which can reach a length of up to 25mm, thus reducing the probability of organisms being in both zones in any one time. Thigmotaxis was then measured as the percentage of time spent in the periphery of the arena i.e. 'wall hugging'. The same 8- minute

light/dark cycle and range of light intensities were used as with the phototaxis experiments. In this instance, the white light cycle was to act as a disturbance to initiate a thigmotactic response.

Figure 3: (A) petri dish loaded into the DanioVision™ observation chamber. (B) Dimensions of central and outer zones.

2.2.3 Velocity

Velocity was measured simultaneously alongside thigmotaxis as described above. The white light cycle was used at a range of intensities to assess photosensitivity as well as to initiate changes in swimming behaviour.

2.3 Statistics

Phototaxis was measured as a comparison of the percent duration of time spent in dark and light zones. Thigmotaxis was measured as a comparison of percent duration of time spent in central and outer zones. Statistical analysis was performed in IBM SPSS Statistics 24. Linear mixed effects models were used for all comparisons. Data was analysed in both 2-minute and 10-second time bins. Total distance was used as a co-variate for the analysis of thigmotactic and phototactic behaviours to correct for animals that did not move during trials (Parker, 2016). Extreme anomalous values generated by the loss of tracking by the EthoVision®XT software were excluded from the data analysis (as defined by values $> \text{median} \pm 3 \times \text{IQR}$) and

never removed more than 3% of data points. Tukey's pairwise comparisons were used for Post Hoc analysis with Bonferroni adjustments to correct for type-II errors. P-values of <0.001 were considered significant.

3. Results

3.1 Phototaxis

Both *G. pulex* and *E. marinus* displayed significantly different behaviours during the phototaxis experiments (Figure 4ab) ($F(1, 266.7) = 52.74$, $p < 0.001$; $F(1, 249.6) = 50.71$, $p < 0.001$). *E. marinus* spent considerably more time (~90) in the dark side of the choice chamber compared to *G. pulex* (~65%) during light on periods ($F(3, 718.9) = 29.82$, $p < 0.001$; S-Table 1; $F(47, 11094.3) = 5.96$, $p < 0.001$; S-Table 2). No significant differences were observed in the two species during the lights off period of the experiment with both species utilising both sides of the choice chamber equally (~50%) (S-Table 1; S-Table 2). Light intensity had no significant effect on phototactic response ($F(3, 229) = 0.79$, $p = 0.501$; $F(3, 235.9) = 0.45$, $p = 0.716$) and data for all light intensities are pooled for Figure 4ab.

Figure 4: Percent duration of time spent in the light side of the well during light/dark phases between *G. pulex* (solid line) and *E. marinus* (dashed line) in (A) 2-minute and (B) 10-second time bins. Shaded areas represent light off. Error bars represent 95% confidence interval. Asterisk indicates significant differences between species for each time point.

3.2 Thigmotaxis

Significantly different behaviours were displayed between *G. pulex* and *E. marinus* during the thigmotaxis experiments (Figure 5ab) ($F(1, 245.7) = 127.62, p < 0.001$; $F(1, 231.3) = 78.61,$

$p < 0.001$). *G. pulex* spent considerably more time (~8-13%) in the centre of the arena compared to *E. marinus* (~3%) for the duration of the trial ($F(3, 726.1) = 7.73$, $p < 0.001$; S-Table 1; $F(47, 11012.4) = 2.77$, $p < 0.001$; S-Table 2). Light intensity had no significant effect on thigmotactic response ($F(3, 241.1) = 0.92$, $p = 0.431$; $F(3, 230.7) = 0.32$, $p = 0.817$) and data for all light intensities are pooled for Figure 5ab.

Figure 5: Percent duration of time spent in the centre zone during light/dark phases between *G. pulex* (solid line) and *E. marinus* (dashed line) in (A) 2-minute and (B) 10-second time bins. Shaded

areas represent light off. Error bars represent 95% confidence intervals. Asterisk indicate significant differences between species for each time point.

3.3 Velocity

Mean velocity data was also recorded during the thigmotaxis trials. Significant differences in velocity were observed between *G. pulex* and *E. marinus* for both 2- minute and 10- second time bins ($F(1, 914.7) = 38.20$, $p < 0.001$; $F(1, 11985.4) = 380.2$, $p < 0.001$). When data was split into 2- minute time bins (figure 6a) both amphipod species had a swimming speed range of ~1-2cm/s and velocity was significantly greater during light on cycles compared to dark ($F(3, 865.2) = 155.25$, $p < 0.001$). There was a significant interaction between species and time ($F(3, 865.2) = 9.53$, $p < 0.001$) whereby *G. pulex* showed a significantly greater mean velocity than *E. marinus* during the second light cycle ($p < 0.001$) no significant differences were observed between species during the other light or dark cycles (S-Table 1). Light intensity had no significant effects on velocity ($F(9, 865.2) = 1.37$, $p = 0.200$) so data was pooled for figure 6a. When data was split into 10- second time bins (figure 6b) amphipod species differed in their velocity range with *E. marinus* having a mean velocity of ~1-3cm/s whereas *G. pulex* remained in the ~1-2cm/s range. A significant interaction was observed between species over time ($F(47, 11874.6) = 12.95$, $p < 0.001$) whereby *E. marinus* showed a peak in velocity (~3cm/s) for first 30- seconds of the first light on cycle followed by a rapid decline in velocity (~1cm/s). *G. pulex* also showed an increase in velocity during 'light on' cycles but the increase in velocity was maintained between (1.6-2.3cm/s). This difference in swimming patterns resulted in *E. marinus* having a significantly greater mean velocity during the first 30- seconds and a significantly smaller velocity during the second 30- seconds of the first light cycle than *G. pulex* (S-Table 2). The second light cycle produced a smaller peak in velocity for *E. marinus* (~2.25cm/s) during the first 30- seconds followed by a rapid decline, mean velocity was significantly less than *G. pulex* in the second half of the light cycle (S-Table 2). Few significant

236 differences were observed in velocity between species during dark cycles (S-Table 2). A
 237 significant interaction was observed in velocity between species, time, and light intensity
 238 ($F(141, 11874.6) = 2.16, p < 0.001$) *E. marinus* showed a greater increase in velocity during light
 239 cycles with increasing light intensity (Figure 7).

Figure 6: Mean velocity during light/dark phases between *G. pulex* (solid line) and *E. marinus* (dashed line) in (a) 2-minute and (b) 10-second time bins. Error bars represent 95% confidence intervals. Shaded areas represent light off. Asterisk indicates significant differences between species for each time point.

Figure 7: Heatmap representation of mean velocity over time in 10-second time bins for *G. pulex* and *E. marinus* for each light intensity. Shaded areas of the time scale represent 'lights off'.

4. Discussion

In this study, the phototactic, thigmotactic and swimming behaviours of a marine and freshwater amphipod were analysed. Both amphipod species exhibited negative phototaxis but *E. marinus* showed a stronger aversion to the light than *G. pulex*. Both *E. marinus* and *G. pulex* exhibited positive thigmotaxis, however *G. pulex* showed a weaker thigmotactic response than *E. marinus* by spending more time in the central zone of the arena overall. Light on and off was also able to initiate changes in thigmotactic behaviour in *G. pulex* but not in *E. marinus*. Both species of amphipod showed an increase in swimming speed during light disturbances when looking at the data in 2-minute time bins, no significant differences were observed between species. However, splitting the data into 10 second time bins produced significant differences in swimming behaviour between species. *E. marinus* showed an initial peak in velocity during light disturbances followed by a steady decrease. *G. pulex* responded

to light with an increase in velocity that remained constant for the duration of the entire light cycle.

Previous work has shown that *E. marinus* exhibit negative phototaxis when placed in a light/dark choice chamber (Guler and Ford, 2010). The same study reported that the phototactic response in *E. marinus* could be altered by exposure to the antidepressant fluoxetine. However, this assay was unable to account for changes in activity as organisms in the dark zone were not visible. Our assay allows for video tracking of organisms in both light and dark zones allowing us to correct for activity. The results of our study support the work by Guler and Ford (2010) in that amphipod species were negatively phototactic and provides evidence that phototaxis in amphipods may be a useful assay in ecotoxicology testing for the assessment of behavioural modifying compounds on behaviour. Swimming speed and activity have been demonstrated as a sensitive endpoint for use in ecotoxicology with amphipods (Wallace and Estephan, 2004; De Lange *et al.*, 2006; Bossus *et al.*, 2014; Cartlidge, Nugegoda and Wlodkovic, 2017) with both increased and decreased activity reported depending on the toxicant. The results of this study support that velocity is a sensitive behavioural endpoint that can be altered by exposure to a light disturbance in the form of increased activity as an escape response. The results of this study support work by Bossus *et al* (2014) who found increased velocity in *E. marinus* when exposed to a light disturbance. The study also reported impacts on swimming speed and reaction to light disturbance when exposed to anxiolytic compounds, further supporting that our assay could be a useful addition to standard toxicology testing.

In this study, significant differences between species were observed for all three behavioural parameters. These differences may be explained by differences in the life history

of the two amphipod species. *E. marinus* is a marine amphipod that lives in the intertidal zone in association with species of algae that are used as both habitat and food source (Martins, Leite and Constantino, 2014). Strong positive thigmotaxis and negative phototaxis behaviours would keep organisms in association with the algae during circatidal rhythms and strong currents. *G. pulex* lives in freshwater rivers and streams in association with rocks and stones (Maitland, 1966), negative phototactic and positive thigmotactic behaviour would keep them under stones and hidden from predators. However, *G. pulex* and other freshwater invertebrates engage in drift when searching for food or more suitable habitat (J M Elliott, 2002a; J. M. Elliott, 2002b; Humphries and Ruxton, 2003). During drift, organisms disassociate from the benthos and are transported via the current. *G. pulex* also does not live in association with its food source like *E. marinus* so would have to spend more time unattached to stones and in the open to forage for food. Differences in swimming behaviours between species may be an adaptive response to predation. *E. marinus* are predated predominantly by birds during low tide (Múrias *et al.*, 1996; Martins, Maranhão and Marques, 2002). Birds hunt by eye and are sensitive to movement of prey species (Mouritsen, 1994; Dierschke *et al.*, 1999). For example, the ringed plover uses foot vibrations to stimulate the movement of small crustacea to make them more visible (Pienkowski, 1983) so the initial burst of speed followed by freezing could be an adaptive response to avoid predation. *G. pulex* are predated on predominantly by fish (Bakker, Mazzi and Zala, 1997), so continuous swimming until reaching a place of safety could be an adaptive response to avoid fish predation.

Results were the same when splitting data into 2- minute or 10- second time bins for phototaxis and thigmotaxis. When analysing velocity data, 2- minute time bins showed no significant differences between species, however, significant differences in velocity and swimming patterns were observed when splitting data into 10- second time bins. This

highlights the importance of carefully designing experiments so that sensitivity of data is not lost during analysis. *E. marinus* exhibited very strong thigmotactic behaviour so differences could not be observed between light and dark cycles suggesting that this species is less sensitive to this assay than *G. pulex*. The opposite was observed for phototactic behaviour, whereby *E. marinus* showed a stronger difference in phototactic response than *G. pulex* suggesting that this species is more sensitive to this assay. These inter-species differences in phototaxis, thigmotaxis and swimming behaviour highlight the importance of collecting baseline data on your test species. It has been demonstrated that behavioural ecotoxicology studies show varied results in the literature (Sumpter, Donnachie and Johnson, 2014) which is thought to be due to a lack of standardisation and optimisation of assays. Results of this study suggest that it is possible that results of behavioural assays may also vary as a result of inter-species variations in baseline behaviours rather than sensitivity to the compound tested. Collection of baseline behaviours of your chosen species is necessary for accurate interpretation of results by determining whether your species is unaffected by your chosen contaminant or rather they are simply not sensitive to the behavioural assay.

5. Conclusions

This study has demonstrated that amphipods exhibit phototactic and thigmotactic behaviours which can be measured successfully in laboratory assays alongside changes in swimming speed. *E. marinus* and *G. pulex* showed significant inter-species variability in their sensitivity to these assays which is thought to be a result of differences in their life histories including habitat type and predation. Significant differences between the two species were the same between 2-minute and 10-second time bins for phototaxis and thigmotaxis assays, however,

when measuring velocity data, significant differences between species were only observed for the 10-second time bins. The behaviours outlined in this study show potential for use in ecotoxicological study however care should be taken to ensure that your test species is first sensitive to your assay, and that data analysis is sufficiently sensitive to avoid false negative results. This study also demonstrates a scope for the use of amphipods in the assessment of complex behaviours in ecotoxicology.

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