



Among-individual variation in the swimming behaviour of the amphipod *Gammarus pulex* under dark and light conditions

Sanne J.P. van den Berg ^{a,b}, Pablo Rodríguez-Sánchez ^c, Jiayu Zhao ^a, Oluwafemi D. Olusoji ^d, Edwin T.H.M. Peeters ^a, Lara M. Schuijt ^{a,*}

^a Aquatic Ecology and Water Quality Management group, Wageningen University and Research, P.O. box 47, 6700 AA Wageningen, the Netherlands

^b Wageningen Environmental Research, Wageningen University and Research, P.O. Box 47, 6700 AA Wageningen, the Netherlands

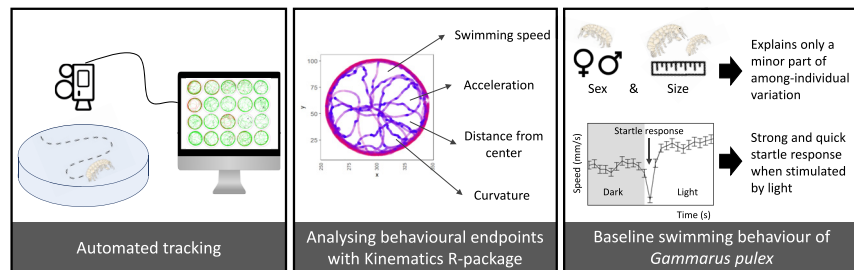
^c Netherlands eScience Centre, Science Park 140, 1098 XG Amsterdam, the Netherlands

^d Hasselt University, Martelarenlaan 42, 3500 Hasselt, Belgium

HIGHLIGHTS

- Baseline behaviour of the freshwater Amphipod *Gammarus pulex* is poorly understood.
- Behavioural analysis by means of a specially made but universal R-package is shown.
- Neglectable differences in behaviour with respect to sex and body length are found.
- *Gammarus* demonstrates a strong and quick startle response when stimulated by light.
- Startle response provides a sensitive endpoint for behavioural studies with *Gammarus*.

GRAPHICAL ABSTRACT



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ABSTRACT

In recent years, considerable computational advancements have been made allowing automated analysis of behavioural endpoints using video cameras. However, the results of such analyses are often confounded by a large variation among individuals, making it problematic to derive endpoints that allow distinguishing treatment effects in behavioural studies. In this study, we quantitatively analysed the effects of light conditions on the swimming behaviour of the freshwater amphipod *Gammarus pulex* by high-throughput tracking, and attempted to unravel among individual variation using size and sex. For this, we developed the R-package Kinematics, allowing for the rapid and reproducible analysis of the swimming behaviour (speed, acceleration, thigmotaxis, curvature and startle response) of *G. pulex*, as well as any other organism. Our results show a considerable amount of variation among individuals (standard deviation ranging between 5 and 115 % of the average swimming behaviour). The factors size and sex and the interaction between the two only explained a minor part of this found variation. Additionally, our study is the first to quantify the startle response in *G. pulex* after the light is switched on, and study the variability of this response between individuals. To analyse this startle response, we established two metrics: 1) startle response magnitude (the drop in swimming velocity directly after the light switches on), and 2) startle response duration (the time it takes to recover from the drop in swimming velocity to average swimming speed). Almost 80 % of the individuals showed a clear startle response and, therefore, these metrics demonstrate a great potential for usage in behavioural studies. The findings of this study are important for the development of appropriate experimental set-ups for behavioural experiments with *G. pulex*.

* Corresponding author.

E-mail address: lara.schuijt@wur.nl (L.M. Schuijt).

1. Introduction

For most aquatic animals, swimming activity is a fundamental behavioural feature and an essential component of organism fitness. Organism swimming behaviour determines encounter rates with abiotic and biotic conditions within the system (Visser and Kjørboe, 2006), effectively influencing processes like predator-prey interactions, reproduction, and feeding. Simultaneously, swimming behaviour can vary and be altered depending on environmental conditions, including exposure to chemicals or other stressors (Gerhardt, 2007).

Swimming behaviour is one of the most important and frequently assessed behavioural endpoint in aquatic ecology (Irschick and Garland, 2001), ecotoxicology (Faimali et al., 2017) and fisheries science (Plaut, 2001). Studies performed in the early 1900s already described altered swimming behaviour in fish after exposure to chemical stressors (e.g., Shelford, 1917; Sollmann, 1906), and many more studies followed, looking for instance at the swimming behaviour of fish (e.g., Gerhardt et al., 2005; Kane et al., 2004; Wiles et al., 2020) and invertebrates (e.g., Bossus et al., 2014; Di Cicco et al., 2021; Nørum et al., 2010). Swimming behaviour is known to have both direct and indirect ecological importance (Brodin et al., 2014). For example, reduced mobility (i.e. swimming activity) is known to have a negative impact on organism's survival through reduced growth and development caused by decreased feeding rates (e.g., Crowl and Covich, 1990; Peckarsky et al., 1993), and increased predation rates (e.g., Faulk et al., 1999). Therefore, swimming behaviour is considered an important and valuable endpoint in eco(-toxico)logical studies.

Gammaridae are frequently used in eco(-toxico)logical studies due to their ubiquitous occurrence in both marine and freshwater systems on the one hand, and due to their high ecological relevance on the other hand. Their high ecological relevance is primarily because Gammaridae play a key role in litter decomposition (Maltby et al., 2002; Piscart et al., 2011), are frequently the dominant macroinvertebrate species present (Peeters et al., 1998), and constitute a valuable food source for fish and other predators (Macneil et al., 1999). Gammaridae, including *Gammarus pulex*, have been successfully used in a variety of behavioural studies including a wide range of swimming behavioural endpoints (De Lange et al., 2006; Kohler et al., 2018b; Nørum et al., 2010; Peeters et al., 2009; Shokri et al., 2021).

In recent years, considerable advancements have been made in tools which allow high-throughput and automated analysis of behaviour using video cameras. Many of those studies use automated stimuli that evoke behaviour such as a startle response. Startle response behaviours have been found for a wide range of animals and usually resemble a strong and rapid reflex after a sudden change, important behaviour for escape and predator avoidance. Stimulation of such behaviour can be done through lighting, electricity and noise/vibration. It has been shown that the startle response (photo motor response, PMR) to a light stimulus affects the swimming response of zebrafish embryos by two phases; (i) directly following the stimulus, animals remain motionless for 1–2 s, (ii) after that, they enter an excitation phase with an increase in swimming speed (Kokel and Peterson, 2011). Although a startle response to a light stimulus is present among a wide range of species, the exact response seem species-specific (Colón-Cruz et al., 2018).

While light can be used as stimulus to evoke a startle response, it has also been shown that some species display different baseline behaviour when kept under dark conditions compared to when they are kept under light conditions, and that this baseline behaviour is also species-specific. It is, for instance, known that amphipods have strong day/night activity cycles (Holomuzki and Hoyle, 1990; Navarro-Barranco and Hughes, 2015; Peeters et al., 2009) and that they tend to move away from light (negative phototaxis) (Kohler et al., 2018b). Multiple studies demonstrate that this baseline behaviour differs between species. For instance, the study of Colón-Cruz et al. (2018) demonstrates different responses of two freshwater prawn species to dark-light conditions. In another study, the intertidal amphipod *Echinogammarus marinus* was found to display a stronger behavioural response to light compared to the freshwater amphipod *Gammarus*

pulex, probably due to adaptations to different predation regimes between the freshwater and marine habitat (Kohler et al., 2018b).

Besides the fact that behaviour is species specific, it is also known that behavioural endpoints contain a large variability between individuals belonging to the same species. Swimming behaviour and responses to light can be sex specific and/or may differ between individuals of different age or size. Some studies found differences between male and female behaviour in amphipods, such as differences in locomotion (Ayari et al., 2015), activity level (Peeters et al., 2009) and swimming speed (Cherry et al., 2020). Indeed, such differences in behavioural patterns among individuals from the same population is common throughout the entire animal kingdom (Bell et al., 2009; Moiron et al., 2020), and has been proven to play an important role in e.g. predator-prey interactions (Pettorelli et al., 2015; Pruitt et al., 2012) or dispersal (Cote et al., 2010).

For an accurate interpretation of behavioural results, it is key to understand the baseline behaviour of a species and the causes of variation in behaviour among individuals. Therefore, the aims of this study were twofold: (i) to quantitatively analyse the effects of light conditions and light transition on the swimming behaviour of the amphipod *G. pulex* by high-throughput tracking and (ii) to assess and explain the variability in swimming behaviour among *G. pulex* individuals. With respect to the first aim, we explored differences in swimming behaviour during dark and light conditions. To assess swimming behaviour, we used the endpoints absolute swimming speed, acceleration, curvature, and thigmotaxis (wall-hugging). Next to that, we used light as a stimulus to evoke a startle response of *G. pulex* and we developed two new metrics to analyse this, i.e. 1) startle response magnitude (the drop in swimming velocity directly after the light switches on), and 2) startle response duration (the time it takes to recover from the drop in swimming velocity to normal swimming speed). For the second aim, we analysed how much of the variability in swimming behaviour could be explained by the factors sex and size.

2. Material and methods

2.1. Test organisms and laboratory maintenance

G. pulex were collected in January 2020 from an uncontaminated stream (Heelsumse Beek, The Netherlands, 51°58'40.8"N 5°45'31.6"E) using a hand net. Organisms were transferred to the laboratory and were separated on size by means of 3 sieves (mesh size of 0.5, 2 and 5 mm). Organisms retaining in the 2 and 5 mm sieves were kept (about 600–700 organisms) and were evenly distributed over 3 white plastic buckets filled with water collected from the same stream. The buckets were aerated and kept in a water bath at 19 ± 1 °C with a 12:12 light:dark cycle at a light level of $8 \mu\text{mol s}^{-1} \text{m}^{-2}$ for seven days. Plants and organic material from the field site, in combination with dried leached *Populus* leaves, were provided as food ad libitum.

After 7 days, a total of 400 organisms ranging from 5 mm up to 19 mm were selected and transferred into 4 new aerated buckets containing groundwater (collected at the Sinderhoeve facility, Renkum, the Netherlands, and aerated for 7 days) with a pH of 7.8 and conductivity of 210 $\mu\text{S}/\text{cm}$. The organisms were distributed over the buckets in a way that each bucket contained similar sized individuals by visual estimation. The buckets were kept in the water bath for an additional 7 days under the same conditions as before. As food source and substrate, *Populus* leaves were provided ad libitum and abiotic water properties (dissolved oxygen levels, pH, temperature and conductivity) were measured at the start and at the end of this second 7-day period (Table S4) using a Multi 3630IDS (Multi-parameter portable meter MultiLine®).

2.2. Analysis of behaviour

Swimming behaviour was assessed using a ZebraTower observation cabinet (ViewPoint, France) located in a room which could be completely darkened. The ZebraTower observation cabinet consisted of an infrared

panel with room for 20 observation arenas (glass Petri dishes with a diameter of 9 cm). Above the panel, an infrared sensitive camera connected to a video tracking software, was installed. The infrared panel and camera were situated in a cabinet with walls consisting of a reflective material so that the light intensity was similar at all positions on the infrared panel and the 20 observation arenas.

After the animals were kept for one week in the groundwater buckets in the water bath, 300 of the 400 individuals were randomly selected for behavioural measurements. During each recording, 20 animals, randomly selected from the four different acclimation buckets, were transferred to the observation arenas consisting of 20 glass petri dishes by means of a glass pipet to reduce handling stress. Each arena received one individual and contained groundwater with a depth of 0.3 cm (20 ml) in order to limit vertical movement of the animals.

Swimming behaviour of these individuals was recorded for 8 min, starting with 4 min in the dark, followed by 4 min in the light with a light intensity of $50 \mu\text{mol s}^{-1} \text{m}^{-2}$. After the swimming behaviour was recorded, the organisms were stored individually in the freezer with a small layer of water, to distinguish their sex and measure their body length at a later moment. Each individual was given a unique identifier by combining the number of the recording and the number of the arena that the individual was in during the recording. In total, 15 recordings were performed with 20 individuals in each recording.

We analysed swimming behaviour by means of six different endpoints: absolute swimming speed, acceleration, curvature (the amount by which the swimming curve deviates from being a straight line), thigmotaxis (distance from the centre of the Petri dish), startle response magnitude (i.e. the drop in swimming velocity directly after the light switches on), and startle response duration (i.e. time needed to recover to normal swimming speed). To analyse these endpoints, we used the x- and y-position of each individual over time recorded by the video tracking software EthoVision R XT 11.5 (Noldus). Since the video was recorded at 30 frames per second, the x- and y-position of each individual was recorded every 33 ms. We used

the kinematics package (version 1.0, Rodriguez-Sanchez and Van den Berg, 2021) to calculate absolute swimming speed (Feynman et al., 2011), acceleration (Feynman et al., 2011), curvature (Do Carmo, 2016), and thigmotaxis for each timepoint. Calculation of the other two endpoints is explained later. These four endpoints were analysed in both 10-seconds and 1-second time bins, since a previous study with *G. pulex* showed that the usage of different time bins gives a better understanding of the sensitivity of the data (Kohler et al., 2018b). Fig. 1 shows an example output of the behavioural analysis of one individual for the endpoint swimming speed using 10-seconds time bins. Figs. S1, S2, and S3 show a similar figure for the endpoints acceleration, thigmotaxis, and curvature.

For the behavioural endpoints absolute swimming speed, acceleration, curvature and thigmotaxis, the total recording of 480 s was divided into 4 phases based on visual inspection of the swimming behaviour of all recorded individuals. Two phases demonstrate transitory behaviour, and two phases demonstrate stable behaviour. Note that the exact timing of these 4 phases is neglectable, since general trends in the data will be revealed regardless. The first minute of the recording was considered the “acclimatization period” (phase 1) as their behaviour was erratic, and was not included in data analysis. A duration of 1 min acclimatization time is more commonly used in amphipod behavioural assays (e.g. Kohler et al., 2018). This was followed by 3 min of “dark period” (phase 2), during which swimming behaviour is relatively stable under dark conditions (61–240 s). After the light switched on (at $t = 241$), it takes approximately 30 s for swimming behaviour to stabilize to light conditions (241–270). This phase was named the “startle response period” (phase 3). Finally, the remainder of the recording (271–480) is named the “light period” (phase 4), and during this phase swimming velocity is relatively stable under light conditions.

The startle response in phase 3 was evoked by turning on the light and can be characterized by 2 metrics; the startle response magnitude and the startle response duration. Startle response magnitude (SRM) was calculated for each individual by dividing the swimming speed at $t = 241$ (the first

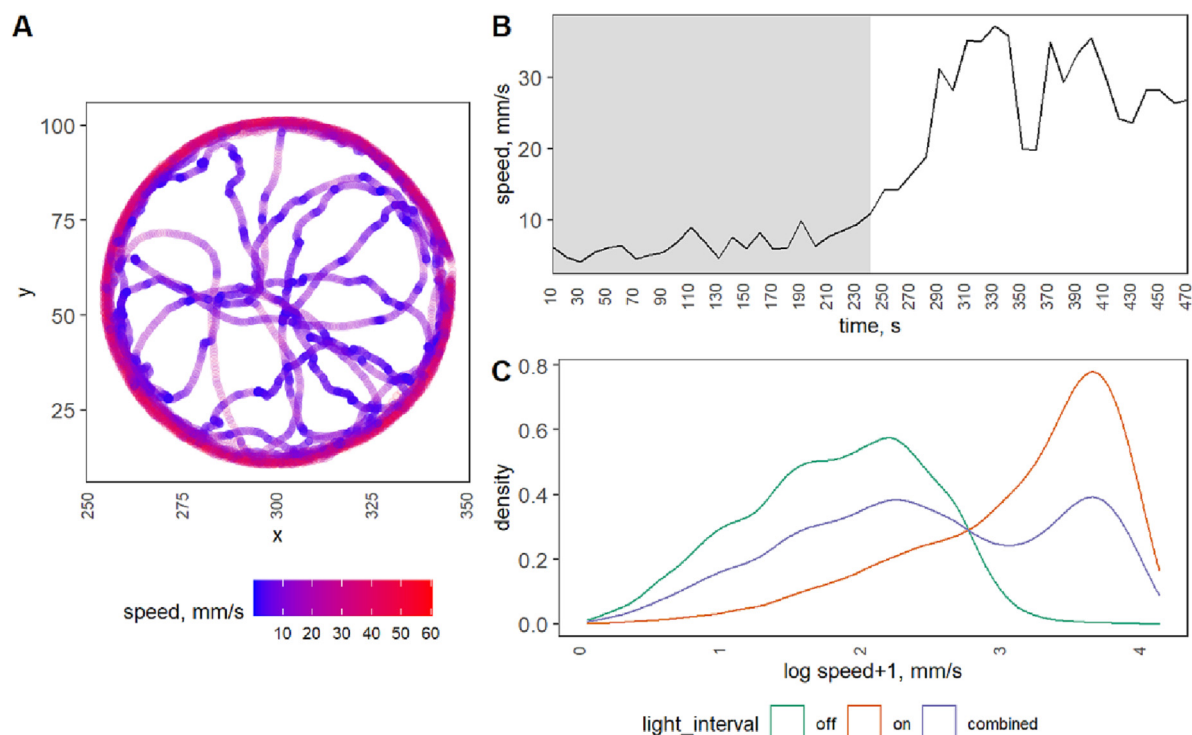


Fig. 1. Behavioural analysis of one individual, showing the path the individual has travelled over the 8-minute measurement period coloured according to absolute swimming speed in mm/s (A), the swimming speed over time (B), and the kernel density distribution of the log transformed swimming speed over time (C) by using 10-seconds time bins. The grey box in fig. B indicates the period that the light was switched off. Fig. C shows a green line for when the light was on, a red line when the light was off, and a blue line for the whole time period combined.

second after switching the light on) by the average swimming speed in the dark period (phase 2) (Eq. (1)).

$$SRM = 1 - \frac{\text{speed at } t = 241}{\text{average speed in the dark}} \quad (1)$$

Startle response duration was calculated per individual by measuring the time between $t = 241$ and the moment that the individual reached 90 % of its average swimming speed during the light period (phase 4) (Pickell et al., 2016).

2.3. Length measurement and sex distinction

The body size of *G. pulex* was measured by photographing each individual and measuring body length using the image analyser software ImageJ (<http://rsbweb.nih.gov/ij/>). The total length was measured as the length from the top of the cephalothorax to the base of the telson (Vellinger et al., 2013).

The sex of the individuals was determined under a stereo microscope (Olympus Corporation SZX2-ILLT®). Sex distinction was done based on 3 different morphological characteristics: the density of the hair on the second antennae, the existence of the female gonad tissue, and the existence of the eggs or shaped foetus. If there were visible dense hairs on the middle back part of the second antennae (Pinkster, 1970), the organism was identified as male. If the female gonad part (Le Roux, 1933), eggs, or foetus was found by dissecting the body, the organism was identified as female.

For 252 of the 300 individuals sex could be determined, while the rest of the individuals were too small for sexual characteristics to be visible. Nevertheless, for some of the 252 individuals it remained difficult to determine the sex of small individuals, resulting in a potential monitoring bias in the smaller size range (i.e. more males, because female characteristics were not yet clearly visible). Indeed, we found no females smaller than 8 mm in our sample. Additionally, it is well known for *G. pulex* that males can grow larger than females (e.g. Sutcliffe et al. (1981)). Therefore, we excluded individuals smaller than 8 mm and individuals larger than 14 mm from our analyses. Overall, 55 individuals were removed from the analysis, and data on 197 individuals were kept for further analysis.

2.4. Data wrangling and statistical analysis

Before we performed any statistical analyses, the behavioural endpoints had to be transformed to meet the normality assumption of linear models. The endpoints acceleration and curvature were log transformed using the natural log, whilst speed and thigmotaxis were transformed using $\log(x + 1)$ and square transformations, respectively.

All recordings were done the same day, but the time of the day differed between the recordings. A one-way ANOVA showed no significant relationship between recording and swimming behaviour (Table S1 and Fig. S4-S7), indicating that the time of the day did not affect the behavioural endpoints.

Differences in swimming behaviour between the dark and the light period were analysed by a linear mixed model (LMM) using the lme function in the nlme package (version 3.1–148, Pinheiro et al., 2017), with light interval as fixed effect and individuals and the 1 s and 10s time bins as random effect.

Individual variation was characterized by the coefficient of variation (CV):

$$CV = \frac{SD}{Y} \quad (2)$$

where SD is the standard deviation and Y is the mean of each endpoint in the dark and light phase (phase 2 and 4). Being standardized by its endpoint mean, the CV enables comparison of the degree of among-individual variation between the different swimming behavioural endpoints.

The effect of sex and size on swimming behaviour was analysed with a LMM and a linear model (LM). For the LMM we used size (binned in 1 mm

bins) and sex as fixed effects and analysed the dark and light phase separately, followed by Tukey's honestly significant difference (HSD) multiple comparisons using the gHt function from the "multcomp" package (version 1.4–14, Hothorn et al., 2016). The LM included sex and length of the individuals, as well as the interaction between sex and length. Type III Sums of Squares were used. The interaction term indicated whether the relationship between length and swimming speed differs between males and females.

All of our data handling, transformations, and statistics were performed in R (version 4.0.2, R Core Team, 2020), and all scripts are available on figshare (<https://doi.org/10.6084/m9.figshare.20358942.v1>).

3. Results and discussion

3.1. Effects of lighting conditions on swimming behaviour

Behavioural analyses of 197 gammarids revealed that swimming behaviour varied mainly among the dark and the light periods (Fig. 2), and among individuals (Fig. S4b, S5b, S6b, S7b).

Generally, we found that individuals responded to the light being switched on in all behavioural swimming endpoints (Fig. 2). However, for the endpoints swimming speed and curvature we found the largest and most abrupt changes. For swimming speed, an average increase of around 8 mm/s could be observed during the first 30 s after the light was turned on (from $18 (\pm 7)$ to $26 (\pm 8)$ mm/s; Table 1; Fig. 2A). After this steep increase, the average swimming speed remained stable. Also an increase in acceleration (from $220 (\pm 80)$ to $260 (\pm 69)$ mm/s²; Fig. 2B) and thigmotaxis (distance from centre; from $40 (\pm 3)$ to $42 (\pm 2)$ mm; Fig. 2C) was observed in response to the light being turned on (Table 1). Opposed to the other endpoints, the average curvature showed a steep decline when the light was switched on (from $-0.80 (\pm 0.93)$ to $-1.55 (\pm 0.92)$ mm⁻¹, Fig. 2D), which can be interpreted as individuals swimming more straight with less curves.

Considering behavioural patterns among individuals, we found large differences in behaviour among individuals, as indicated by the standard deviations in Table 1. The coefficients of variation (CV) of thigmotaxis was 0.08 in dark phase and 0.05 in light phase while CVs calculated for the other behavioural measurements were higher (ranging from 0.26 up to 1.15; Table 1), indicating a higher amount of variability for those endpoints. To provide some insight into the actual variation among individuals, we plotted the four behaviour endpoints over time for 20 random individuals, as well as the frequency distributions for the same 20 individuals (Fig. S8-S11). These figures clearly indicate that there are notable differences in swimming behaviour among individuals of *G. pulex* originating from the same field population. This is in agreement with another study (Peeters et al., 2009), in which important differences in locomotion patterns among *G. pulex* individuals were found.

When comparing the swimming behaviour of the dark period (phase 2) with the light period (phase 4), all behavioural endpoints showed a significant difference (LMM, $p < 0.001$; Table 1). Whilst swimming speed, acceleration and thigmotaxis increased during light, curvature significantly decreased (Fig. 2, Table 1). Thus, individuals seemed to swim and accelerate faster during the light phase, with less curves and on the periphery of the arena.

Higher swimming velocities under light conditions have been observed in other studies with amphipods (Bossus et al., 2014; Kohler et al., 2018a), as well as for more distantly related species, such as *Daphnia magna* (Simão et al., 2019). The combination of an increase in swimming speed and a decrease in curvature in the light period could be indicative of a behavioural response to avoid predation. For *Daphnia magna*, increased speed due to higher light intensity is considered an anti-predator defence mechanisms to defend against fish (Rivetti et al., 2016; Simão et al., 2019). *G. pulex* normally lives under relatively dark conditions between macrophytes or under rock and stones in freshwater rivers and streams (Maitland, 1966; Peeters et al., 1998). An increase in light intensity could therefore indicate that they are more exposed to fish predation. The increased swimming speed,

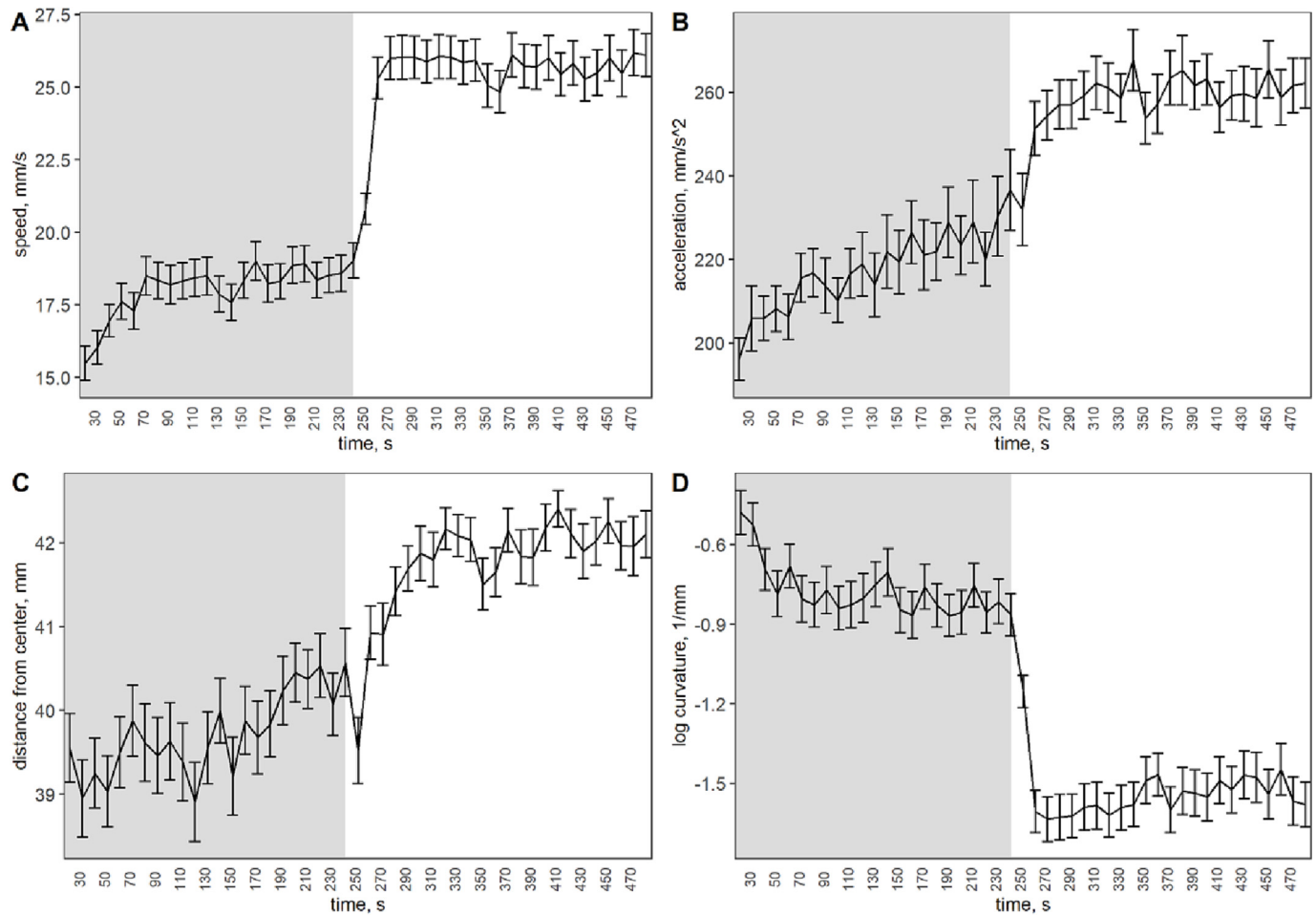


Fig. 2. Average swimming behaviour of all 197 recorded *G. pulex* individuals over 10s time bins, with swimming behaviour described as swimming speed (A), acceleration (B), distance from centre (thigmotaxis, C), and curvature (D). The grey area in the graph indicates the first 4 min (10–240 s) during which the light was switched off, whilst the white area indicates the last 4 min (241–480 s) during which the light was switched on. Error bars represent $2 \times$ the standard error (SE).

acceleration and lower curvature that we find during high light intensity could indicate that the gammarids are swimming fast and straight, with less curves, to reach a safe place (usually with darker conditions) as fast as possible. Hence, the changes in swimming behaviour during the light period could be an adaptive response to avoid fish predation, as also has been suggested by Kohler et al. (2018b).

In general, *G. pulex* showed a preference for the periphery of the Petri dish over the more central zones, since the average thigmotaxis was between 40 and 42 mm and the maximum possible thigmotaxis of the Petri dish was 45 mm. Under light conditions, *G. pulex* increased the distance to the centre of the Petri dish, as the periphery offers shelter. A preference for the periphery of the swimming environment compared to the more

central zone can be observed by a wide variety of organisms and is also called wall-hugging behaviour. This wall-hugging behaviour has been proven a viable endpoint for behavioural studies in crayfish (Tierney et al., 2016) and zebrafish (Bownik and Wlodkowic, 2021; Schnörr et al., 2012) and is used as an index of anxiety in animals (Schnörr et al., 2012). Our study shows that *G. pulex* seems to engage in increased thigmotaxis behaviour when exposed to a stimuli (such as light). A similar phenomenon was observed for *G. pulex* by Kohler et al. (2018a). However, thigmotaxis is heavily influenced by the experimental set-up that is used, since the dimensions of the petri dish (i.e. size and shape of the arena) determine the potential thigmotaxis that could arise during the measurement. Therefore, thigmotaxis results should be interpreted carefully.

Table 1

Average swimming behaviour of all individuals (\pm standard deviation (SD)) and coefficient of variation (CV = SD/mean) for four behavioural endpoints in both the dark and the light phase, and the results of the linear mixed model (LMM) that tested whether the behavioural endpoints significantly differed between the dark and light phase. For both analyses, 10-s time bins were used, and only data from the dark and the light phase were included (phase 2 and 4).

	Lightning conditions				LMM	
	Dark phase		Light phase		Light-dark phase (light interval, df = 1)	
	Average (\pm SD)	Coefficient of variation (CV)	Average (\pm SD)	Coefficient of variation (CV)	F-value	p-value
Speed (mm/s)	18 (\pm 7)	0.39	26 (\pm 8)	0.32	307	p < 0.001
Acceleration (mm/s ²)	220 (\pm 80)	0.36	260 (\pm 69)	0.26	147	p < 0.001
Thigmotaxis (mm)	40 (\pm 3)	0.08	42 (\pm 2)	0.05	113	p < 0.001
Curvature (mm ⁻¹)	-0.80 (\pm 0.93)	1.15	-1.55 (\pm 0.92)	0.59	320	p < 0.001

3.2. Startle response to light stimulus

The startle response of *G. pulex* is imperceptible when using 10s time bins of the average swimming speed of all individuals over time (Fig. 2A). However, when 1 s time bins are used, a decline in swimming speed can be observed for the majority of individuals during the first second(s) after the light switches on (Fig. 3A). This startle response becomes more clear when focusing on the average swimming speed of all individuals using 1 s time bins (Fig. 3B). Additionally, variation in swimming speed was lowest

in the first second after the light was switched on ($t = 241$, Fig. 3B). This is the first indicator that the startle response is a potentially good metric for comparing the behaviour of multiple individuals.

Almost 80 % of the individuals showed a clear startle response (a lower swimming speed at $t = 241$ s compared to its average swimming speed in the dark phase) after the light was switched on. This was equally distributed over males and females, of which respectively 78 and 81 % showed a clear startle response. For those individuals, we found an average drop of 51 % (\pm SD 25 %) in swimming speed (startle response magnitude), and it took

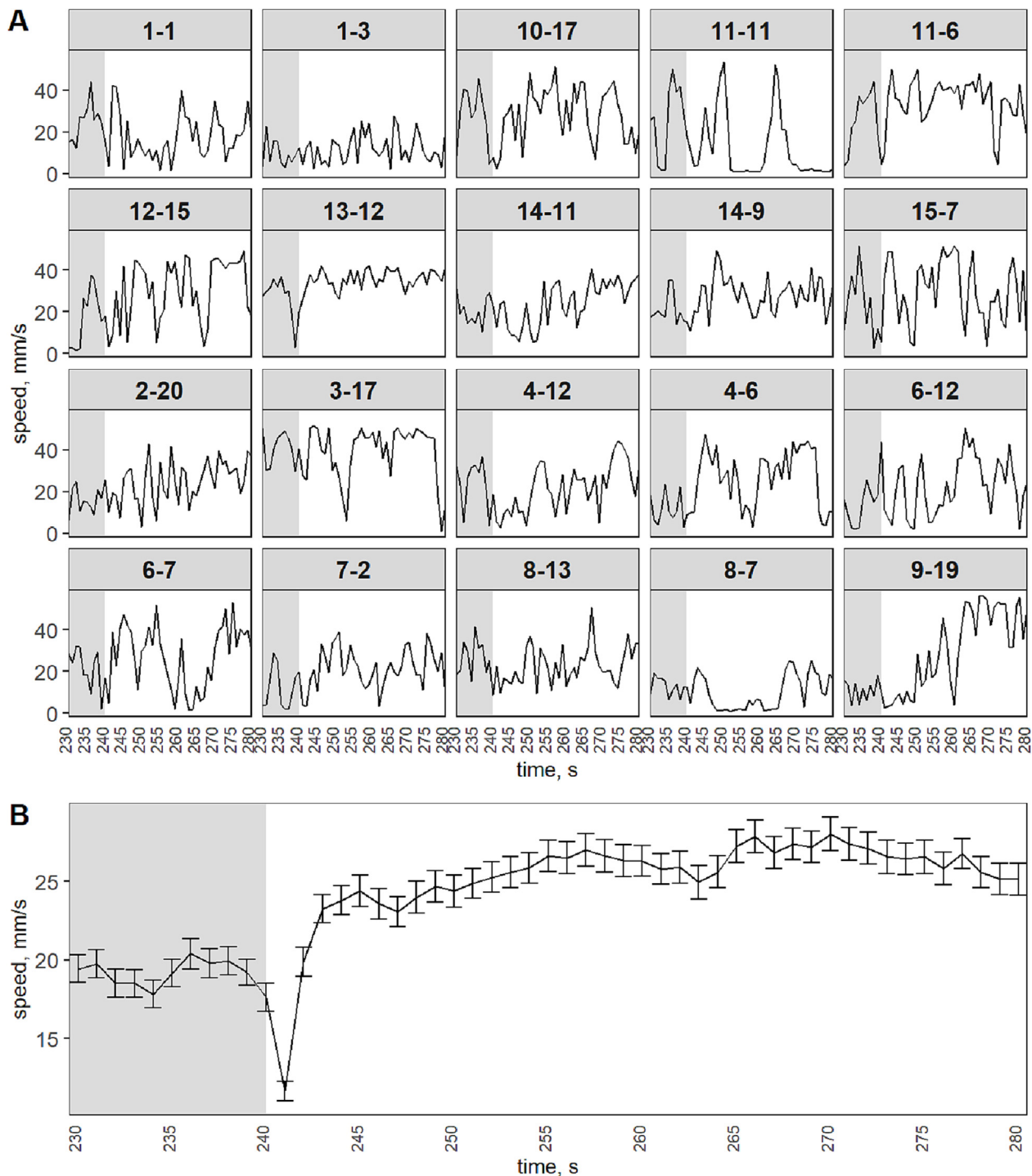


Fig. 3. A closer look into the change in swimming speed during the light-transition period (230–280 s) using 1 s time bins. (A) shows the swimming speed of 20 randomly selected *G. pulex* individuals (the numbers above the panels indicate the unique identifier of each individual, consisting of the number of the recording and the number of the arena that the individual was in during the recording), whilst (B) shows the average swimming speed of all 197 *G. pulex* individuals. The grey area in the graph indicates the last 10 s of the dark phase (230–240 s), whilst the white area indicates the first 40 s of the light phase (240–280). Error bars represent $2 \times$ SE.

on average 3.9 s (\pm SD 4.2 s) to recover and reach their average light swimming speed (startle response duration, see Fig. 3A for some example individuals). This results in a CV of 0.49 for the startle response magnitude, and a CV of 1.05 for the startle response duration (result not shown). The CV for the startle response magnitude is comparable to the CV of swimming speed and acceleration (Table 1), indicating a comparable amount of variability for those endpoints.

Most of the tested *G. pulex* individuals showed a clear and quick startle response when light was switched on, i.e. a steep drop in swimming speed (startle response magnitude), followed by a recovery period (startle response duration). To our knowledge, this is the first study to quantify the startle response to a light stimulus of *G. pulex*. Startle responses have been observed in several aquatic invertebrates, including hermit crabs (Briffa et al., 2013; Nanninga et al., 2020), marine bivalves (Clements et al., 2021), planaria species (Martinez et al., 2021) and *Daphnia magna* (Bedrossiantz et al., 2020). However, startle response behaviour has primarily been studied in larval stages of zebrafish, mostly because the mechanisms controlling the startle response of zebrafish are known to be conserved in higher invertebrates (Wolman and Granato, 2012), including humans. A recent study found, for instance, that gene mutations causing a deviation in the startle response of zebrafish were also associated with causing deviations in the locomotor system of humans (Meserve et al., 2021).

The startle response is often defined as a coordinated, stereotypic and genetically encoded defence reflex to an unexpected and sudden stimulus (Bownik and Wlodkovic, 2021), and is thought to be an escape response to predation (Clements et al., 2021). Our results, together with the results of other studies (e.g., Clements et al., 2021), suggest that startle response is a behaviour that is highly repeatable. This makes startle responses a useful endpoint for behavioural studies.

3.3. Effects of sex and size on swimming behaviour

To determine to what degree sex and size explain the underlying among-individual variability in swimming behaviour of *G. pulex*, we explored the single and interaction effects of sex and size on the different swimming behavioural endpoints. We found a significant interaction between size, sex and swimming speed and between size, sex and curvature during both the dark and the light period (phase 2 and 4, Table 2). Male swimming speed slightly increased with size, whilst female swimming speed showed the opposite pattern (Fig. 4A). For curvature we found the opposite relationship, with a decreasing trend for males and an increasing trend for females (Fig. 4D). Nevertheless, even though a significant interaction effect was found for these two behavioural endpoints, the low R^2 of the model indicates that only a small amount of the variation in swimming behaviour can be accounted for by sex and size (adj. R^2 0.03 and 0.04; Table 2).

To confirm, we tested the relationship between size and the behavioural endpoints using linear regression on the male and female data separately. This showed that the regression slopes were close to zero, and size only explained a minor fraction of the found variation (Table S5). Nevertheless,

largest differences in swimming behaviour between males and females were found for the larger sizes (Fig. 4). While the confidence intervals of males and females of size ranges 8–12 mm are overlapping for all behavioural endpoints, this overlap disappears for individuals above 12 mm length for the endpoints swimming speed and curvature (Fig. 4), especially during the dark phase.

We found similar results using a LMM after categorizing all individuals in different size bins of each 1 mm (Table S2). Multiple comparisons revealed a significant difference between males and females for the largest size bin for swimming speed (Fig. S12), acceleration (Fig. S13) and curvature (Fig. S14) in the dark phase, with females swimming and accelerating slower compared to males, but with a higher curvature. This can partially be explained due to the fact that females grow less large than males (Crane, 1994). Therefore, when comparing males and females larger than 12 mm, it is likely that the females are actually older than the males, and therefore may have a reduced fitness. Also, there were only 4 females larger than 12 mm present in our data, resulting in an unbalanced dataset that can potentially influence results. Nevertheless, we can conclude that the baseline behaviour of individuals ranging between 8 and 12 mm is similar, regardless of their sex, whilst there are potential differences in the swimming behaviour between larger males and females.

Comparing our results to the literature, we found that Peeters et al. (2009) also demonstrated that *G. pulex* males were more active than females after having recorded the swimming behaviour of individuals ranging from 8 to 15 mm, although they make no statements on the extend of this difference between sex. In contrast, a study very similar to ours, but performed with the marine amphipod *Echinogammarus marinus*, found clear sex-related differences in swimming speed, with females swimming significantly faster than males when comparing their baseline behaviour (Cherry et al., 2020). The difference between our results and those of Cherry et al. (2020) can be due to interspecies differences, and thereby emphasize the need to understand the baseline behaviour of your test species well. Ayari et al. (2015) also found variations in behavioural patterns between individuals belonging to the marine amphipod species *Orchestia gammarellus*, but could not explain this variation by differences in locomotion between males and females.

Considering the startle response magnitude and startle response duration, we did not find any significant main or interaction effects with sex and/or size (Table 2, Fig. 4). Since there are no studies available in the literature that studied the startle response in amphipods in similar detail as this one, we could only compare our findings with a study performed on mussels. Clements et al. (2021) found that the startle response duration was on average three times longer in smaller mussels compared to larger mussels. However, besides inter-species differences, this difference can be due to the fact that the size range used in the study of Clements et al. (2021) was substantially larger than the size range used in our study.

Generally, we found that sex and size could not explain much of the among-individual variability in the swimming behaviour of *G. pulex* for individuals ranging between 8 and 12 mm (all models have an R^2 lower than 0.05). This is in agreement with Alonso et al. (2009), who also did not find

Table 2

Results of the linear model to test whether there was a significant interaction between size, sex and swimming behaviour. The p-values are bold when indicating significance ($p < 0.05$).

		Sex		Size		Interaction: sex x size		p-value model	Adj. R^2 model
		t-value	p-value	t-value	p-value	t-value	p-value		
Dark phase	Speed	2.88	0.004	1.87	0.063	-3.09	0.002	0.012	0.04
	Acceleration	1.91	0.057	1.14	0.257	-2.15	0.033	0.081	0.02
	Thigmotaxis	1.27	0.205	0.91	0.364	-1.52	0.131	0.19	0.01
	Curvature	-3.09	0.002	-1.97	0.050	3.29	0.001	0.008	0.04
Light phase	Speed	2.28	0.024	1.90	0.060	-2.49	0.014	0.045	0.03
	Acceleration	2.10	0.038	1.61	0.110	-2.30	0.022	0.073	0.02
	Thigmotaxis	0.81	0.421	0.78	0.438	-0.98	0.326	0.493	-0.003
	Curvature	-2.31	0.022	-1.74	0.083	2.53	0.012	0.040	0.03
Startle response phase	Startle response magnitude	1.16	0.247	-0.274	0.785	-1.37	0.171	0.140	0.01
	Startle response duration	-1.72	0.087	-0.46	0.644	1.88	0.061	0.164	0.01

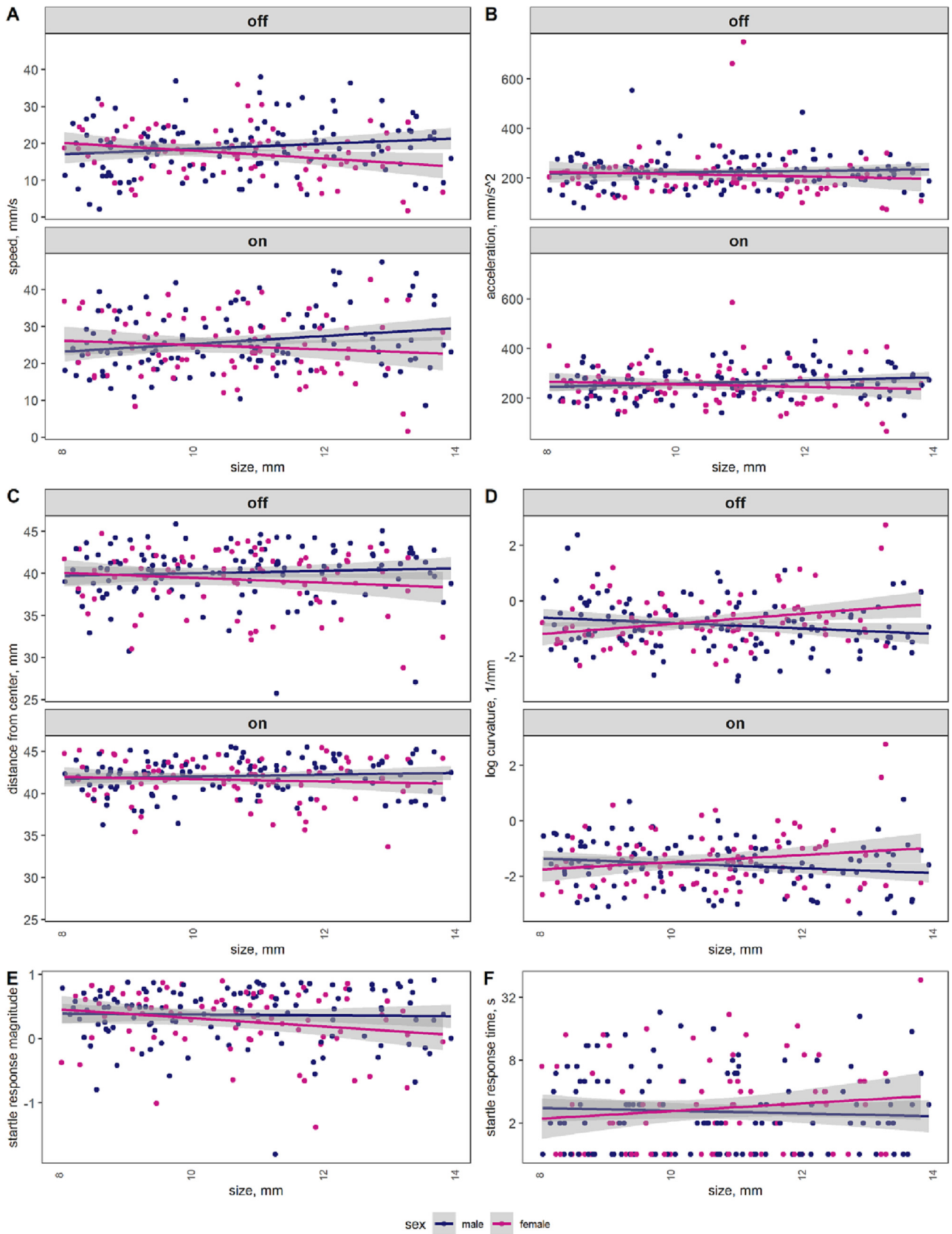


Fig. 4. The relationship between size and swimming behaviour for males and females for the endpoints swimming speed (A), acceleration (B), thigmotaxis (C), curvature (D), startle response time (E), and startle response magnitude (F). Males are depicted in blue, whilst females are purple. Grey areas in the graphs are 95 % confidence intervals. Filled circles show actual data and lines show predictions from the linear model for males (blue) and females (purple).

an effect of length and sex on the swimming activity of *G. pulex*. Practically, this means that when individuals ranging between 8 and 12 mm are used in an behavioural assay, they are assumed to demonstrate the same baseline behaviour. This means that males and females of this size range can be pooled in the control treatment data, increasing the statistical power of potential behavioural studies.

Nevertheless, when using behavioural endpoints in, for instance, eco (– toxicological) experiments, it is important to consider that even though we did not observe differences in baseline behaviour between *G. pulex* males and females ranging between 8 and 12 mm, sex-specific responses towards (chemical) stressors or other experimental factors remains possible. For zebrafish, for instance, evidence indicates important sex differences in behavioural and neuropharmacological responses to chemicals (Genario et al., 2020). Similarly, sex-specific responses towards (chemical) stressors have also been observed in amphipods (Alonso et al., 2010; Dong et al., 2020; Gismondi et al., 2013; Gismondi et al., 2015; Huang et al., 2022; Sornom et al., 2010). Therefore, by only using males or females in behavioural research, extrapolation to potential population impacts might be problematic by over- or underestimating population-level effects. The same principles apply to other experimental factors that can cause a potential bias in population-level effects. Consider, for instance, the use of a limited size range. Often, size is related to age and/or life stage, and the use of a limited size range can therefore result in the selection of a specific age group or life stage in an experiment, whilst individuals of different life stages might differ in their response to experimental factors.

4. Conclusions

This study provides novel insights into the behaviour of the amphipod *G. pulex* by showing that light conditions affect swimming behaviour, a light stimulus evokes a strong startle response, and that sex and size explain only a minor part of the large variation in these behavioural endpoints. During light conditions, individuals seemed to swim more on the periphery of the arena with a higher speed, less curves and higher acceleration, probably in search of shelter. Additionally, switching on the light evoked a startle response in 80 % of the recorded individuals, resulting in a steep drop in swimming speed (startle response magnitude), followed by a recovery period (startle response duration). These two novel metrics for evaluating the startle response of *G. pulex* have promising potential for use in behavioural studies, since the measured startle response appears to be highly repeatable.

We found that sex and size could not explain a large part of the among-individual variability in the swimming behaviour of *G. pulex* for individuals ranging between 8 and 14 mm (all models have a R^2 lower than 0.05). Nevertheless, even though we did not observe differences in baseline behaviour between males and females, future experiments looking into different treatments (e.g. ecotoxicological studies) should still include both sexes in experiments due to possible sex-specific differences in treatment response (e.g. sex-specific differences in sensitivity towards (chemical) stressors).

Additionally, standardization of behavioural tests is crucial to obtain reliable and reproducible results, since many factors can potentially influence behavioural endpoints. Therefore, details like sex and size of the tested animals should always be reported, even if a direct relationship between these factors and behaviour has not yet been proven. We recommend always conducting baseline studies with the experimental setup and species of interest before studying behavioural effects on the treatment you would like to test. This will allow adjusting your experimental setup to match the baseline behaviour of the test organisms, ensuring that your experimental setup allows the observation of potential changes in behaviour caused by your treatment.

We developed the kinematics package as a side project during the present research. This package was designed as a reusable package for conducting similar surveys on motion-related behaviour, and is freely available in CRAN (<https://cran.r-project.org/web/packages/kinematics>), the official repository for R packages. Also, we made all raw data and R scripts used to process this data publicly available on figshare (<https://doi.org/10.6084/m9.figshare.20358942.v1>), hoping to stimulate transparent and reusable science.

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CRediT authorship contribution statement

Sanne van den Berg: Conceptualization, Investigation, Methodology, Software, Statistical analysis, Writing - original draft. Pablo Rodríguez-Sánchez: Software. Jiayu Zhao: Methodology. Oluwafemi Olusoji: Statistical analysis. Edwin Peeters: Statistical analysis, Writing - review & editing. Lara Schuijt: Conceptualization, Investigation, Statistical analysis, Writing - original draft.

Data availability

We made all raw data and R scripts used to process this data publicly available on figshare (<https://doi.org/10.6084/m9.figshare.20358942.v1>)

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2023.162177>.

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