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Behavioural repeatability in larval *Limnephilus lunatus* Curtis, 1834 (Trichoptera) in an open-field test

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ABSTRACT

This article investigates inter-individual repeatability in distance moved in an open-field test for larval *Limnephilus lunatus* Curtis, 1834. Repeatability across four trials (two-day trial intervals) was comparable to previous studies on arthropod species (repeatability: $R=0.37$), indicating that *L. lunatus* is a suitable model species in this research field. Two potential nuisance factors were corrected for: (1) progressively declining activity over consecutive trials and (2) case mass:body mass ratio, affecting activity negatively. These factors require consideration in behavioural experiments on larval caddisflies. Pairwise correlations of distance moved among trial days showed that behaviour in the first trial did not correspond well with behaviour in the following trials. Re-analysing the data using only trials 2 to 4 increased the repeatability (repeatability: $R=0.50$), suggesting that future studies should consider not including data derived from initial trials, as the initial trial may constitute a different context than the following ones.

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Animal behaviour;
Limnephilidae;
intra-individual differences;
open-field test; Trichoptera

Introduction

The adaptive significance of consistent individual differences (CIDs) in behaviour is now an integral part of the field of behavioural ecology (Dall, Houston, and McNamara 2004; Bell, Hankison, and Laskowski 2009; Sih et al. 2015). The mechanism behind CID in behaviour is commonly hypothesised (e.g., in the coping-style and pace-of-life syndrome hypotheses) to be CID in physiological traits, such as metabolism or hormone levels (Koolhaas et al. 1999; Biro and Stamps 2010; Réale, Garant, Humphries, Bergeron, Careau, and Montiglio 2010). CIDs are generally hypothesised to be adaptive as they often covary with life-history traits of the individuals (Réale et al. 2010).

Small-bodied arthropods are potentially suitable subjects to investigate the effects of environmental factors on different behavioural types and the effects of different behavioural types on the ecosystem and its communities (Modlmeier, Keiser, Wright,

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Lichtenstein, and Pruitt 2015; Ingram and Burns 2018). Arthropods constitute a major component of the animal biodiversity on Earth and are part of many trophic levels, making them highly relevant for ecological studies (Minelli, Boxshall, and Fusco 2013). Furthermore, they are often convenient experimental models as there are many common, small-bodied, and hardy species which are easy to keep at high numbers for laboratory experiments (Cummins, Smith, Miller, and Fox 1965).

Prior to conducting large-scale studies on ecological relevance of behavioural CIDs, it is beneficial to run trials to determine the strength of CIDs in the target animal population. The aim of this study was to investigate open-field test CIDs (distance moved) in larva of the caddisfly *Limnephilus lunatus* Curtis, 1834 (Trichoptera: Limnephilidae) (Curtis 1834) and thereby get an indication of whether it is a candidate model system for further research. *L. lunatus* is a common European caddisfly which spends its larval stage in still and slow-running freshwaters (Gower 1967; GBIF 2019). It is a relevant model for further behavioural studies as it is the selected species for the pilot Caddisfly Genome Project within the i5K initiative to sequence 5000 arthropod species (Poelchau et al. 2015; HGSC 2018) and a model for limnetic ecotoxicology (e.g., Schulz and Liess 2001).

A few previous studies have used aquatic invertebrates to investigate ecological effects of predator behavioural CIDs on prey (Start and Gilbert 2017; Maskrey, White, Wilson, and Houslay 2018; Start 2018; Siepielski et al. 2020). In contrast to the species used in these studies, *L. lunatus* is an herbivore and could thereby be suitable as a model from a different trophic level. Few limnetic herbivorous arthropods, which are important prey for secondary consumers in freshwater ecosystems (e.g., Klecka and Boukal 2012), have been investigated in relation to behavioural CIDs (but see Bierbach et al. 2016). Furthermore, the case of larval trichopterans constitute an architectural extended phenotype, i.e., an external trait of the animal that does not constitute an actual part of its body (Dawkins 1982; Wiggins 2004; Hansell 2005). Characteristics of this extended phenotype can vary among individuals in terms of specific material used, length, and mass (e.g., Nislow and Molles 1993; personal observation), and such features would be interesting to relate to behavioural types, e.g., by investigating trade-offs between protection value and movement ability among individuals. *L. lunatus* is therefore an interesting candidate species for further experiments on behavioural CIDs in ecological and ethological contexts.

Across a wide group of taxa, the forced open-field test is commonly used to measure anxiety-like behaviour and neophobia (e.g., Walsh and Cummins 1976; Roche, Careau, and Binning 2016). It is also used to assess voluntary (stressed-state) activity and novel environment exploration (e.g., Réale, Reader, Sol, McDougall, and Dingemanse 2007; Carlson and Langkilde 2013; Carter, Feeney, Marshall, Cowlshaw, and Heinsohn 2013). In this test, animals are placed ('forced') into an open arena and their movements are tracked over a pre-defined time-interval (Walsh and Cummins 1976). Although the novel environment open-field test is sometimes considered to score exploration tendency specifically (Réale et al. 2007), these obtained scores are likely influenced by several other behavioural traits related to e.g., activity and boldness, especially since novelty is lost after the first trial (Burns 2008; Conrad, Weinersmith, Brodin, Saltz, and Sih 2011; Koski 2014). Hence, open-field activity

scores (e.g., distance moved) would likely be inappropriately labelled as a single specific behaviour, as argued by Roche et al. (2016).

Distance moved in an open-field test was chosen as the target behavioural score for this study. As noted earlier, it is likely related to several behavioural axes, following their characterisation by Réale et al. (2007) (e.g., activity, exploration, and boldness). The measure was chosen for two main reasons. First, the open-field test is easily applicable for a large number of individuals within a short timeframe, using automated tracking software, which makes it suitable as a high-throughput method of screening behavioural types before subjecting the animals to further experimentation. Second, this behavioural trait has been linked to ecologically relevant behaviours in other species (e.g., Gyuris, Hankó, Feró, and Barta 2016).

Previous studies on invertebrates have shown that many species show CIDs (Bell et al. 2009; Mather and Logue 2013; Kralj-Fišer and Schuett 2014). This is further supported by data presented in Figure 1, which summarises 47 studies on 42 species of arthropods (also see Näslund 2020 for more detailed data). There is substantial interspecific variation in repeatability estimates; hence, a general repeatability value for arthropods in open-field tests does not appear to exist. Notably, very few aquatic insect species ($N=4$; Figure 1) have been investigated and none of these has been herbivorous.

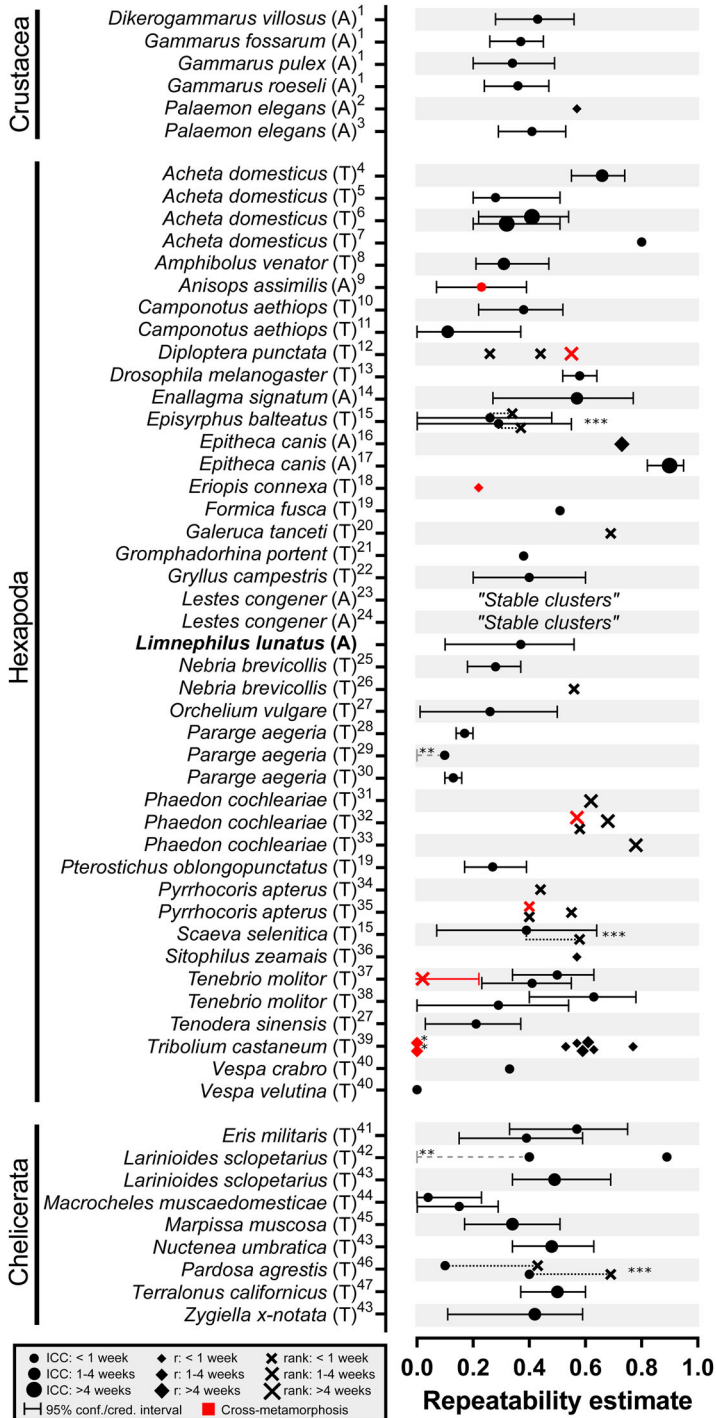
This study aimed at investigating whether *L. lunatus* is suitable as an herbivore model for further investigation into the ecology of behavioural types (animal personality) in limnetic environments. The first step to answer this question is to demonstrate CIDs in behavioural traits in the species and to analyse details of the behaviour and nuisance factors potentially influencing its expression. I hypothesised that the investigated *L. lunatus* population would consist of individuals with different behavioural types, consistent with previous studies of many insect taxa. The prediction that follows, if the hypothesis is correct, is that the behavioural trait is repeatable within individuals over time.

Material and methods

Subject animals

Larvae of *L. lunatus* ($N=22$), a case-bearing caddisfly, were collected in the outlet of Gunnestorps mosse, a vegetated pond in Gothenburg, Sweden (WGS84: 57°44'41.1"N, 11°55'17.1"E) on 19 July 2016. The caddisflies were kept individually in 0.5-L containers filled with 0.25 L of water, under dim light (60 lux). The water (19.1–20.3°C; varying among days, but stable over each trial event) was replaced after each behavioural trial. Coarse sand was provided as bottom substrate and fresh live plant material (*Sparganium* sp.) was provided ad libitum as food (Slack 1936). After the four open-field trials, the larvae were sacrificed in 95% ethanol and the cases were separated from the larvae. Relative case mass of trichopteran can vary substantially across individuals; thus, body- and case mass needs to be weighed separately (Kiffer et al. 2016). Dry-blotted body wet mass and case mass were measured to the nearest 0.0001 g (Precisa XR 205SM-DR; Precisa Gravimetrics AG Dieticon, Switzerland). Species identity was verified using the study by Wallace, Wallace, and Philipson (2003). One individual died during the experiment (on the day before the

final trial day). This individual was dropped from all analyses as behaviour might have been affected by the factor(s) causing its death. The final sample size was consequently 21 caddisfly individuals.



Open-field trials

The open-field trials started on 22 July and were carried out over seven days, with trials every second day; in total, each individual was scored in four trials. The open-field arenas consisted of plastic boxes (bottom area: 15.7×15.7 cm) filled with 2 cm of water. Arena floors were lined with a thin layer of white silicone onto which a layer of light-coloured quartz sand was spread (one sand grain thick) while the silicone hardened. The fixed sand grains provided grip for unrestricted movements of the larva. Temperature in trial arenas was kept at the same level as in the holding containers.

Open-field arenas were organised in groups of four, with an HD-camera recorder (Sony Handycam HDR- XR155, Sony Corp., Japan) placed centrally above them. Illuminance in the arenas during trials was 260 lux. After the camera was set to record, one animal was put in each of the four arenas and recorded for 10 min; the last nine minutes of the films were used for the analysis of distance moved, using the first minute as a recovery period after handling. All animals were recorded within 2 h, mid-day, every trial day; the location of the test arenas during filming was within a 1 m^2 area of the room. Films were converted to mpeg4 avi format (frame rate: 25 Hz, resolution: 1920×1080 pixels; Total Video Converter 3.71, EffectMatrix Inc.). From the converted videos, each animal was tracked using the animal tracking software idTracker (Pérez-Escudero, Vicente-Page, Hinz, Arganda, and de Polavieja 2014). After a trial, each animal was put back in its holding container.

Figure 1. Estimates of repeatability of open-field activity in previously published articles on arthropods (A: aquatic; T: terrestrial). Y-axis range from 0 (no repeatability) to 1 (perfect repeatability). Symbols in bottom left legend describe length of the period over which the repeatability estimates were estimated and type of statistical analysis ('ICC': intraclass correlation; 'r': Pearson correlation; 'rank': rank-based). Confidence or credibility intervals are given when presented in the original study. See Näslund (2020) for detailed data about experimental settings and analyses in the original studies. References: ¹Bierbach et al. 2016, ²Chapman, Hegg, and Ljungberg 2013, ³Maskrey et al. 2018, ⁴Dochtermann and Nelson 2014, ⁵Royauté, Buddle, and Vincent 2015a, ⁶Royauté and Dochtermann 2017, ⁷Keiser, Ingley, Toscano, Scharf, and Pruitt 2018, ⁸Matsumura, Ito, and Miyatake 2019, ⁹Ingram and Burns 2018, ¹⁰d'Ettoire et al. 2017, ¹¹Udino, Perez, Carere, and d'Ettoire 2017, ¹²Stanley, Mettke-Hofmann, and Preziosi 2017, ¹³Videliere, Rundle, and Careau 2019, ¹⁴Siepielski et al. 2020, ¹⁵Odermatt, Frommen, and Menz 2017, ¹⁶Start and Gilbert 2017, ¹⁷Start 2018, ¹⁸Rodrigues et al. 2016, ¹⁹Carere, Audebrand, Rödel, and d'Ettoire 2018, ²⁰Tremmel and Müller 2014, ²¹McDermott et al. 2014, ²²Santostefano, Wilson, Araya-Ajoy, and Dingemans 2016, ²³Brodin 2009, ²⁴Brodin and Drotz 2011, ²⁵Schuett et al. 2018, ²⁶Labaude, O'Donnell, and Griffin 2018, ²⁷Lichtenstein et al. 2019, ^{28,29,30}Kaiser, Merckx, and Van Dyck 2019a,b, 2020, ³¹Tremmel and Müller 2013, ³²Müller and Müller 2015, ³³Müller and Juškauskas 2018, ³⁴Gyuris, Feró, Tartally, and Barta 2011, ³⁵Gyuris, Feró, and Barta, 2012, ³⁶Morales, Cardoso, Della Lucia, and Guedes 2013, ³⁷Monceau et al. 2017a, ³⁸Monceau et al. 2017b, ³⁹Wexler, Subach, Pruitt, and Scharf 2016, ⁴⁰Monceau, Moreau, Poidatz, Bonnard, and Thiéry 2015, ⁴¹Royauté, Greenlee, Baldwin, and Dochtermann 2015b, ⁴²Kralj-Fišer and Schneider 2012, ⁴³Kralj-Fišer, Hebets, and Kuntner 2017, ⁴⁴Durkin, Roth, and Keiser 2020, ⁴⁵Liedtke, Redekop, Schneider, and Schuett 2015, ⁴⁶Rádai, Kiss, and Barta 2017, ⁴⁷Lichtenstein, Chism, Kamath, and Pruitt, 2017. *Negative correlation coefficients. **Non-significant estimate, hatched line indicates a presumed lower 95% confidence limit reaching 0. ***Data analysed using both parametric and non-parametric analyses, dotted lines connect the estimated repeatabilities from the different analyses.

Distance calculations

Tracks from idTracker (x - and y -coordinates in pixels for each video frame) were smoothed by calculating the average coordinate position for every second. Thereafter, the Euclidean distance was calculated between all consecutive average coordinates. Track lengths in pixels were thereafter recalculated into millimetres, relating the real width of a trial arena in millimetres to the width of the arena on a video frame in pixels, using ImageJ 1.46r (Schneider, Rasband, and Eliceiri 2012).

Statistical analysis

I analysed repeatability using the intraclass-correlation coefficient (R), calculated within a linear mixed model framework using the package rptR (Nakagawa and Schielzeth 2010) in R 3.3.1 (R Core Team, Vienna). Assessment of covariates to include in the models was based on graphical exploration of the obtained data, prior to running the final repeatability analyses. Exploration consisted of visual inspection of plotted confidence intervals in scatter plots and likelihood ratio tests (LRT) of models with different levels of complexity. Covariate selection is presented in the Results section. I also compared the correlation of distance moved among trial days to investigate the pattern of repeatability across trials.

Results

Selection of model covariates

Body-mass effect on distance moved in the open-field test appeared non-linear (assessed by local regression; see Figure 2A). Case mass was positively related to body mass, but with substantial variation around the predicted values (linear regression; see Figure 2B). Individuals with heavier cases in relation to their body mass appeared to move less (linear regression; see Figure 2C). Thus, the ratio between case and body wet mass was added as a covariate (CB). There was also a pattern where distance moved decreased over trial days (Figure 2D). Thus, trial day was also added in the model describing distance moved, as a fixed factor (DAY) with four levels.

The model containing CB and DAY had significantly better fit than the model containing only individual identity (1|ID) as a random factor (LRT: $\chi^2 = 25.6$, $p < 0.001$). Adding DAY led to highly significant improvement of the model fit (LRT: $\chi^2 = 22.4$, $p < 0.001$), showing that the decrease in distance moved from the first to the last trial was significant. Adding CB improved the model fit, but not significantly so (LRT: $\chi^2 = 3.17$, $p = 0.075$). Nevertheless, given that this is a potential nuisance variable that could homogenise intra-individual behaviour and inflate repeatability, I judged it to be more appropriate to adjust repeatability for CB effects, rather than not, following initial evaluation of the data pattern in Figure 2C.

Repeatability of distance moved in an open-field test

Distance moved was significantly repeatable after adjusting for CB and DAY. The estimated repeatability was moderate at $R = 0.37 \pm 0.12$ standard error (SE) ($p = 0.001$)

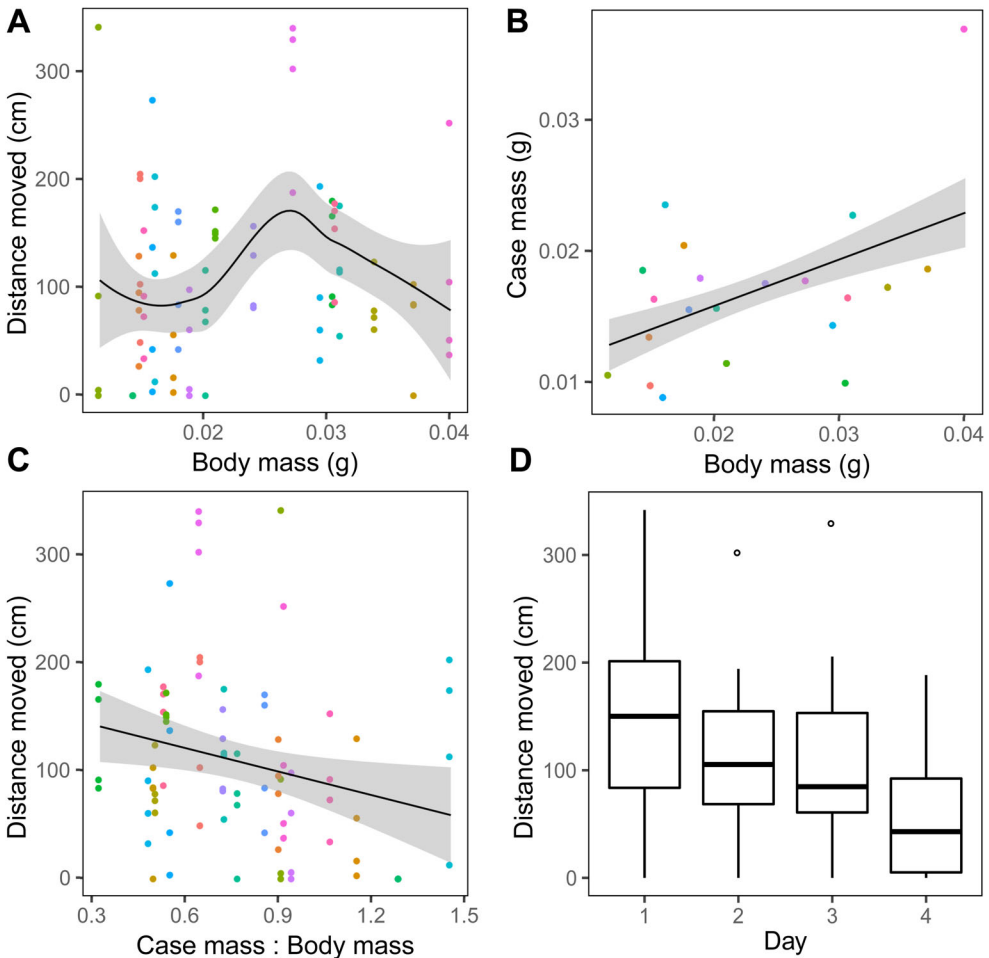


Figure 2. Plots for assessment of covariate inclusion in the linear models of distance moved in the open-field test for *Limnephilus lunatus* Curtis, 1834. (A) Non-linear relationship between body wet mass and distance moved, with loess smoother and 95% confidence limits. (B) Linear relationship between body wet mass and case wet mass, with linear regression and 95% confidence limits. (C) Linear relationship between the ratio case mass:body wet mass and distance moved, with linear regression and 95% confidence limits. (D) Tukey boxplots of distance moved each trial day; all individuals being represented once each day. Whiskers show upper and lower quartile. Horizontal bars in the box show the median. Data points more than 3/2 times of upper quartile are shown as individual outliers. For panel A–C, the data points for each individual are colour-coded in the online version of the manuscript.

(Figure 3A–C). The significance from the permutation test was supported by the bootstrapped confidence interval (Figure 3B). Excluding the marginally significant CB term from the model resulted in a similar estimate of R (0.40 ± 0.12 SE, $p = 0.001$).

Correlation of distance moved across trial days

Pearson correlation analyses across trial days were based on residuals from a linear model including CB as a covariate. Correlation coefficients (r) ranged between

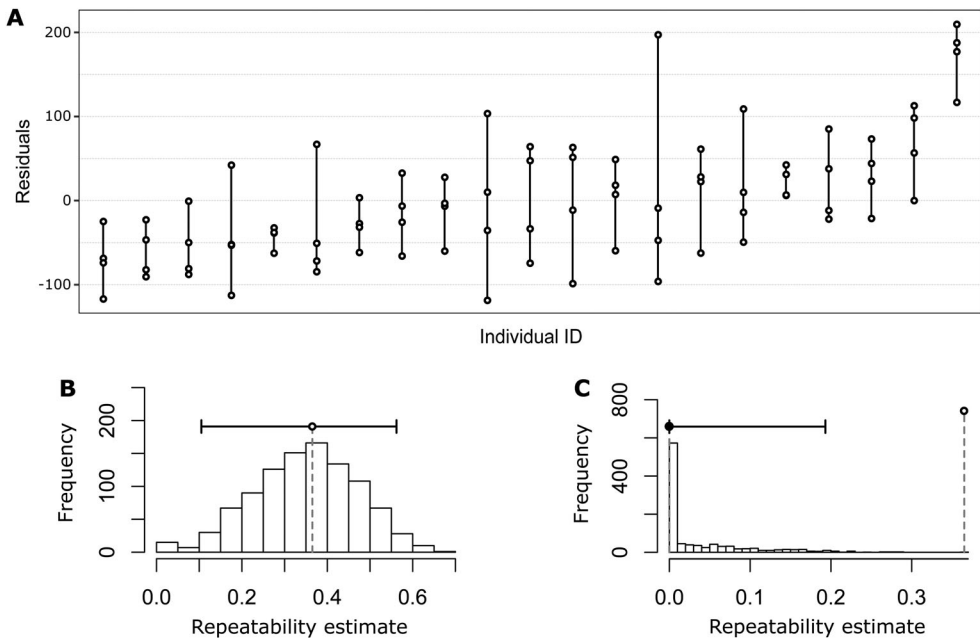


Figure 3. Repeatability of distance moved in open-field tests over four trials. (A) Residuals from the linear model of distance moved (in mm) for *Limnephilus lunatus* Curtis, 1834, without individual identity (ID) as random factor, sorted from left to right based on mean values for the individuals. Factors included in model: ratio between case and body wet mass and trial day. (B) Summary histogram of bootstrap repeatabilities ($N = 1000$) for ID with estimated repeatability (\circ) and 95% confidence interval (whiskers). (C) Summary histogram of permuted repeatabilities ($N = 1000$) along with permutation median (\bullet) and 95% percentiles (whiskers), and estimated repeatability (\circ).

Table 1. Correlation matrix showing Pearson's product moment correlation coefficients (r), with 95% confidence interval within parenthesis, and their significance (p).

	Day 2	Day 3	Day 4
Day 1	$r = 0.18$ (–0.27 to 0.57) $p = 0.44$	$r = 0.48$ (0.07–0.76) $p = 0.03$	$r = 0.14$ (–0.31 to 0.54) $p = 0.55$
Day 2		$r = 0.45$ (0.02–0.74) $p = 0.04$	$r = 0.60$ (0.23–0.82) $p < 0.01$
Day 3			$r = 0.45$ (0.03–0.74) $p = 0.04$

$r = 0.14$ and $r = 0.60$, with the correlation between trial days 1 and 2 ($r = 0.14$) and between trial days 1 and 4 ($r = 0.125$) being particularly low and non-significant ($p > 0.4$), as compared to other trial day-comparisons (Table 1). Mean (\pm standard deviation) distance moved during a trial was 1046 ± 562 mm across all four trials (trial 1: 1518 ± 921 mm; trial 2: 1008 ± 760 mm; trial 3: 1052 ± 740 mm; trial 4: 607 ± 574 mm).

Re-analysing repeatability for only trials 2–4

Running the repeatability analysis for only trials 2–4 increased the estimated repeatability to $R = 0.50 \pm 0.13$ SE ($p = 0.003$) with CB included in the model, and $R = 0.54 \pm 0.12$ SE ($p < 0.001$) without CB.

Discussion

*Distance moved in an open-field test is repeatable in *Limnephilus lunatus**

This experiment shows that distance moved by larval *L. lunatus* in a standardised open-field test is moderately repeatable after correcting the scores for confounding covariates. Overall, the estimated repeatability value, incorporating all four trials, falls within the typical range of previously published results from arthropods (Figure 1), and it is very similar to other aquatic non-predatory (*Gammarus* spp.) and omnivorous (*Palaemon* sp.) species. When excluding the first trial, an increase in the estimated repeatability is observed. Hence, *L. lunatus* is deemed to be a potentially useable model for further studies, particularly if the animals are put through an initial familiarisation trial prior to the start of the experimental trials.

Consistent patterns of behaviours within individuals is the definition of animal personality (e.g., Gosling 2001; Wolf and Weissing 2012), and hence, the population of larval caddisflies investigated here could be considered to consist of different personality types. Although the measure of distance moved may be a representation of a quite simple behaviour, variation among individuals in this behavioural trait could have wide-ranging consequences for their mortality- and growth rates, as it could affect both predator exposure and food intake (Werner and Anholt 1993). This opens this species to usage in the investigations of effects of individual variation in personality (scored in open-field tests) on population, community, and ecosystem dynamics, as called for by several authors (Boukal 2014; Modlmeier et al. 2015; Toscano, Gownaris, Heerhartz, and Monaco 2016; Start and Gilbert 2017). Investigations of differential selection pressures on animals with different behavioural types could be an interesting and important area of research. However, the observed intra-individual variance suggests that several scores should be taken of each individual to reduce the risk of mis-classifying the behavioural type of an individual.

Overall, the details on the causation of CID in insects and other invertebrates are largely unknown. In some species, genetic factors determine consistency in behaviours (e.g., Osborne et al. 1997; Nakayama, Sasaki, Matsumura, Lewis and Miyatake 2012). Environmental factors during ontogeny may also influence CIDs (Royauté and Dochtermann 2017). While carry-over effects from the wild may be possible, all animals were captured within a very restricted area, reducing the risk that the individuals had substantially different environmental experiences. Furthermore, food was constantly available, and all individuals were socially isolated during the duration of the experiment, which standardises the environment during the trials. In general, the investigation of the causation of CIDs in insects requires more research focus. In addition, the ecological relevance of the measured trait, in this case distance moved in an open-field test, needs to be followed up in realistic experimental settings (Koski 2014).

The fact that animals were collected in a small area could possibly contribute to a reduced repeatability, if this behavioural trait is heritable, as individuals from the same area may be genetically related. To mitigate such effects, future studies could use animals from several geographically distinct areas. This, however, would also require more details on the environmental factors that may differ between collection sites, to account for possible carry-over effects.

Effects of case- and body mass

Body mass was not clearly positively associated with distance moved, as would be expected if stride length was the main determining factor influencing the movement data collected (Arnold, Cassey, and White 2017). Instead, there was a general tendency for a decrease in distance moved with increasing case mass:body mass ratio, albeit with substantial variation around the estimated regression line. The cases of *L. lunatus* are constructed of negatively buoyant plant material and, hence, it is possible that more energy is needed to move around a relatively heavier or bulkier case, affecting activity negatively. There is general agreement that the case is, at least partly, an anti-predation related construction providing shelter and/or cryptic appearance (Wiggins 2004). For instance, extra stones added to the case of *Silo pallipes* (Fabricius, 1781) (a stone-house building caddisfly) can reduce the risk of predation by fish (Otto and Johansson 1995). Hence, a trade-off between predation risk (strength of the case) and movement ability could be possible. Since the observed effect was not significant here, the negative effect of a heavier case merits further investigation. Relative case mass should, however, be considered in future studies of case-bearing caddisflies subjected to open-field tests.

Differences among trial days

The first trial did not compare well with other trials when looking at the correlation matrix of distance moved across days. However, the three subsequent trials showed relatively consistent positive correlations and excluding the first trial increased the repeatability estimate substantially in a re-analysis of the data. This pattern indicates that unique factors present during the first trial day are triggering a behavioural reaction that is different from the following trials. A potential factor is the novelty aspect of the test situation, which is only present in the first trial. Similar ‘first trial’-effects, with lower individual consistency with subsequent trials are often not investigated, but have been found in both mammals and fish assayed in open-field tests (Rudeck, Vogl, Banneke, Schönfelder, and Lewejohann 2020; Thomson, Lamb, Besson, and Johnson 2020), which, together with results presented here, suggests that such investigations are generally needed for all taxa.

There were also significant trial day effects in the repeatability model, indicating declining activity over trial days. This could potentially be associated with learning, if the larvae recognised the trial situation and associate it to something that makes them less active (e.g., a threatening situation, or the lack of food in the trial arena). Invertebrates are today recognised as more behaviourally complex than previously thought (Dukas 2008), and a novelty-reaction the first day, followed by progressive habituation, could be possible. Similar trial day effects were detected in some freshwater amphipod species, but other species showed no or opposite patterns over consecutive trial days (Bierbach et al. 2016). Accumulating stress effects of the testing, or trial fatigue, is another candidate factor that could lead to the trial-day effect (Roche et al. 2016). These hypothesised effects could be tested in future studies, e.g., by applying different inter-trial intervals in different experimental groups; the use of *L.*

lunatus in open-field tests could be a simple and convenient candidate experimental system for these types of studies.

Conclusions

The experiment presented here shows that *L. lunatus* is a potentially suitable insect herbivore model for investigating the ecological relevance of CID in limnetic environments. Furthermore, the open-field test is a potentially suitable tool to assess CID in movement behaviour in this species, particularly after statistically correcting for potential nuisance factors and after discarding data from the initial trial round. Thus, future studies could use this model system to address more elaborate ecological and ethological questions.

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Disclosure statement

No potential conflict of interest was reported by the author.

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Data availability

Data and R code used for the analyses are available at the figshare database, doi:10.6084/m9.figshare.8066690.v2.

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