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RESEARCH ARTICLE

No effect of insect repellents on the behaviour of *Lymnaea stagnalis* at environmentally relevant concentrations

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Abstract Insect repellents are widely applied to various materials and to both human and animal skin to deter mosquitoes and ticks. The most common deterrent compounds applied are DEET, EBAAP and icaridin (picaridin, Bayrepel). Due to their extensive application, these repellents are frequently detected in surface waters in considerable concentrations. As these compounds are designed to alter invertebrates' behaviour rather than to intoxicate them, we hypothesised that insect repellents have the potential to modify the natural behaviour of non-target invertebrates in natural freshwater bodies. To test this, we used a well-established laboratory assay designed to quantify the odour-mediated foraging behaviour of freshwater gastropods and the great pond snail *Lymnaea stagnalis* (Linnaeus, 1758) as a model organism to test for potential deterrent effects of insect repellents on aquatic snails. Using a wide concentration range from the picogramme per litre to microgramme per litre range (and by far exceeding the range of concentrations reported from natural waters), we found no evidence for a deterrent effect of either of the three repellents on foraging *L. stagnalis*. Our data and other recent studies give no indication for undesirable behavioural alterations by common insect repellents in surface waters.

Keywords DEET · EBAAP · Food searching · Gastropoda · Icaridin · Infochemicals · Semiochemicals

Introduction

In recent decades, we have been experiencing a tremendous range expansion of ectoparasitic arthropods with global climate change, which leads to an increased global demand for insect repellents (Altizer et al. 2013). These compounds are applied to organisms or materials in order to deter insects and ticks from approaching treated surfaces. The most frequently applied repellents are DEET (N,N-diethyl-m-toluamide), EBAAP (IR3535®, 3-[N-butyl-N-acetyl]-aminopropionic acid, ethyl ester) and icaridin (picaridin, Bayrepel, 1-piperidinecarboxylic acid, 2-(2-hydroxyethyl), 1-methylpropyl ester). These compounds are washed off skin and fabrics upon cleaning and subsequently enter the environment indirectly through wastewater and sewage treatment plants and directly via bathing in surface waters (Nendza et al. 2013). Consequently, major formulations containing DEET and icaridin have repeatedly been detected in surface waters in significant concentrations (Knepper 2004a; Nendza et al. 2013). DEET has been detected in ground and surface waters in concentrations up to 3 μg L^{-1} in Europe and even 33 μg L^{-1} in the USA (Nendza et al. 2013). While the use of DEET has strongly declined in Europe in the last decade, it is frequently replaced by icaridin and EBAAP (Büchel et al. 2015). This has led to increased detection of icaridin in low-microgramme per litre concentrations in European lakes and rivers (Bernhard et al. 2006; Knepper 2004b; Nendza et al. 2013), while there are no reports on environmental EBAAP concentrations (Nendza et al. 2013).

Despite this frequent detection of significant repellent concentrations in surface waters, these compounds were not yet given

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full consideration in ecological risk assessment. As they fall under the European Union (EU) biocides legislation, their registration process involves only assays for potential toxicity to non-target organisms (ECHA -European Chemicals Agency 2016). However, insect repellents are not designed to intoxicate the target species, but rather modify their behaviour (Katz et al. 2008). It is thus a plausible hypothesis that these compounds may cause behavioural alterations (such as deterrence) of aquatic non-target organisms, if these compounds inadvertently enter surface water ecosystems. Such possible behavioural effects had not been considered until very recently, when the German Federal Environment Agency initiated a research project to screen for possible effects of repellents on aquatic non-target species. Following a literature survey to identify potentially relevant compounds (Nendza et al. 2013), two initial laboratory studies had been conducted on repellent effects on pelagic (Von Elert et al. 2016) and benthic (Fink et al. 2017) arthropods. We here investigate whether other freshwater invertebrates such as molluscs may be behaviourally affected by insect repellents in naturally relevant concentration ranges. This is achieved through the use of a laboratory-scale behavioural assay we had previously developed to assess the foraging behaviour of freshwater pulmonate gastropods dependent on food-derived chemical cues (Fink et al. 2006a, b; Moelzner and Fink 2014, 2015a). This test system should also be suitable for the assessment of potential deterrent activity of insect repellents on vagile freshwater molluscs. As a suitable model for herbivorous benthic invertebrates, we used the great pond snail *Lymnaea stagnalis*, which is well known both for its ecological importance (Bakker et al. 2013; Elger et al. 2002; Moelzner and Fink 2015b; Nystrom and Perez 1998) and sensitivity to chemical cues from its environment (Dalesman et al. 2006, 2009; Moelzner and Fink 2014, 2015a).

Materials and methods

Test organisms

Juveniles of the freshwater gastropod *L. stagnalis* were hatched and reared from eggs laid by adult individuals originally collected in a pond in Appeldorn, Germany, and kept in a climate chamber at 18 ± 0.5 °C under constant dim light in aged (> 3 day) and filtered tap water. The snails were fed Tetra PlecoMin™ fish food pellets (Tetra, Melle, Germany) ad libitum every second day before and between the behavioural assays.

Repellent assays

The gastropod behavioural assays were conducted as described elsewhere (Fink et al. 2006a, b; Moelzner and Fink 2014). In brief, glass aquaria (320 × 170 mm, 180 mm deep, total volume 10 L), were filled with 1 L filtered and aged tap water with approx. 15 mm depth and kept at constant temperature (21 °C) and lighting. Two point sources at the opposing ends of each aquarium released either the respective repellent at a certain concentration (calculated with respect to the 1 L arena volume) or a solvent control, respectively (for details, see Fink et al. 2006b). This allows the establishment of a clear concentration gradient throughout the aquarium within approx. 20 min (Fink and Roth, unpubl. data). Upon the introduction of five juvenile *L. stagnalis* (shell height 15 ± 5 mm) to the centre of the arena, the experiment was initiated by opening the odour sources, and the relative distance of each gastropod to both point sources was determined after 5, 8, 11, 14, 17 and 20 min. As the five individual *L. stagnalis* within each assay are obviously no independent observations (aquatic gastropods are well known for, e.g. their trail-following behaviour), the mean position of the five experimental animals was calculated for each time point (minute) within each replicate assay as described previously (Fink et al. 2006b).

The system was validated by offering a blend of moderately volatile lipoxygenase products extracted from the benthic diatom *Achnanthes biasolettiana*. The odour bouquet of this and other algae had previously been demonstrated to be highly attractive to pulmonate gastropods (Fink et al. 2006a). In the subsequent repellent assays using the same experimental setup, DEET, EBAAP and icaridin were applied to the arena's odour source compartment in eight nominal, log-scaled concentrations from 5 pg L^{-1} to 50 μg L^{-1}. For each of these eight concentrations of the three repellents, five independent replicate assays were conducted, resulting in a total of 120 behavioural assays with repellents. In addition, we conducted negative (solvent control on both sides of the test arena to exclude a directional bias) and positive (see above) control assays to validate the test system as described previously (Fink et al. 2006a, b; Moelzner and Fink 2014, 2015a). Finally, the snails' behavioural response to the repellents (as their mean position relative to the repellent and control sources) was plotted vs. the effective repellent concentrations (see below) and analysed via linear regressions using R version 3.1.1 (R Core Team 2014).

GC-MS quantification of repellents

In the experiments described above, nominal concentrations of the three repellents were applied. To determine the effective concentrations in the assay, a quantitative re-extraction of the repellents from the assay and a quantitative analysis via gas chromatography and mass spectrometry (GC-MS) were established by using Atrazine as an internal standard and three independent replicates. The repellents were separated in an Agilent 7890 GC system equipped with a HP-5MS capillary column coupled to an Agilent 5975 MSD. All compounds were identified according to their retention time and mass fragmentation patterns in comparison with authentic chemical standards and quantified using compound-specific target ions and calibration functions determined against the internal standard atrazine (Fink et al. 2017). The quantification of the repellents in the behavioural assay arena (in five concentrations) demonstrated that all three repellents were recovered in highly reproducible ratios to the nominal concentrations applied. Effective concentrations were approximately 30–50% below the nominal concentrations, depending on the compound: Mean recovery rates (± 1 SE of n = 3) were 55.8 ± 3.3% for DEET, 70.6 ± 2.3% for EBAAP and 48.6 ± 2.9% for icaridin. Since the applied range of (nominal) concentrations was up to 50 μg L^{-1} and, thus, about tenfold higher than the highest environmentally detected concentrations (Nendza et al. 2013), we are confident that despite the somewhat lower effective concentrations, our system was suitable for a reliable estimation of potential repellent effects in natural surface waters.

Results

Volatile organic compounds extracted from 20 mg dry mass of the benthic diatom *Achnanthes biasolettiana* applied on one side of the arena vs. a solvent control on the opposite side caused a clear attraction of juvenile *L. stagnalis* to these resource-associated odours, while no behavioural response could be observed to control extracts (Fig. 1). In contrast to food-borne chemical cues, none of the tested repellents and concentrations (neither for DEET, EBAAP nor icaridin) caused an alteration in the gastropods' behaviour (determined as mean relative distance to the source of the repellent) as compared to the control assays without repellents. Hence, the linear regressions calculated for the mean position of *L. stagnalis* vs. the applied repellent concentration (in a nominal concentration range from 5 pg L^{-1} to 50 μg L^{-1}) were not significant for any of the three repellent compounds (Fig. 2).

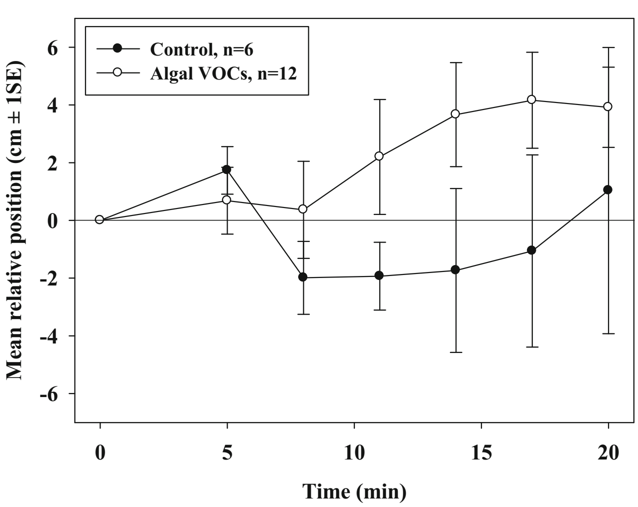

Fig. 1 Mean position (± SE) of juvenile *Lymnaea stagnalis* relative tothe source containers containing either water (control, filled circles, n = 6), or volatile cues extracted from a benthic diatom (open circles, n = 12)

Discussion

We had previously demonstrated extensively that the test system applied here is suitable to quantify the behavioural

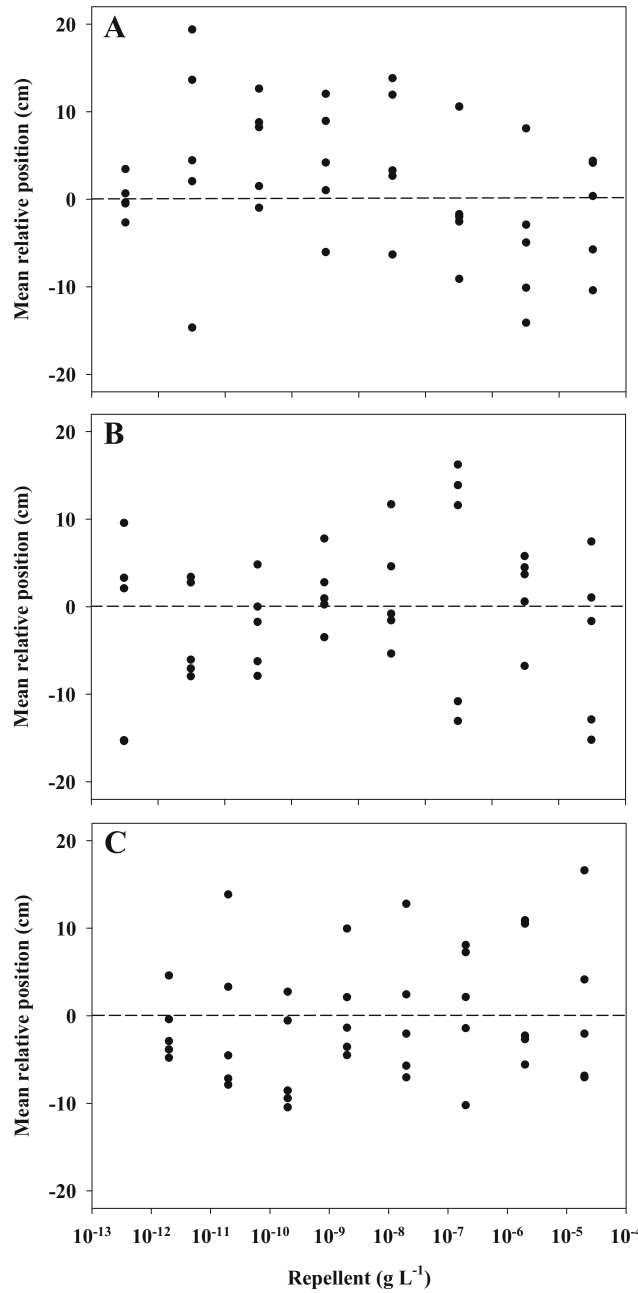

Fig. 2 Mean position of juvenile *Lymnaea stagnalis* relative to the two odour source containers in the behavioural assays (n = 6) with **a** DEET, **b** EBAAP and **c** icaridin vs. the effective repellent concentration; no significant regression models could be fitted to any of the relationships

response (attractance vs. repellence) of pulmonate gastropods towards chemical cues (Fink et al. 2006a, b; Moelzner and Fink 2014, 2015a). Similar to previous findings, no preference of *L. stagnalis* for either side of the test arena could be observed when solvent controls were applied to both source containers (i.e. the negative controls), while the pond snails exhibited a strong and predictable odour-mediated preference behaviour for resource-borne (algal) low-molecular compounds. In contrast to this, effective DEET concentrations in a range from 3.3 pg L^{-1} to 33 μg L^{-1} did not cause any statistically significant alteration in the snails' foraging behaviour. Specifically, the gastropods neither appeared to be attracted nor repelled by DEET applied in a concentration range over eight orders of magnitude. Similar to the absence of an effect of DEET on the behaviour of *L. stagnalis*, neither EBAAP nor icaridin had any significantly positive (attracting) or negative (repellent) effect. The maximum repellent concentrations applied here (> 30 μg L^{-1}) were ten times higher than the highest environmental DEET (3 μg L^{-1}) or icaridin (1.4 μg L^{-1}) concentrations reported for European surface waters to date (Nendza et al. 2013). No data is available on environmental EBAAP concentrations to date (Nendza et al. 2013). Therefore, even if higher repellent concentrations than the ones tested here may have affected the gastropods' behaviour, this would be unlikely to have any environmental significance.

Since no effects of either of the repellents could be observed on the gastropods' orientation behaviour at any of the tested concentrations, we assume that insect repellents can either not be perceived by *L. stagnalis* or just have no relevance for the snails' behaviour. We thus decided also not to test for the—originally hypothesised—potential interaction of insect repellents with the snails' behavioural response to algae-borne food-finding cues. Likewise, we did not test for the potential toxicity of the repellents on juvenile *L. stagnalis*, as literature values for acute toxicity (to *Daphnia magna*, as there are no LC_{50} values available for molluscs) are in the 75 to 100 mg L^{-1} range (European Commission 2010), i.e. three orders of magnitude higher than the environmentally relevant concentration range.

The absence of a behaviour-modifying effect of DEET, EBAAP or icaridin on freshwater gastropods in environmentally relevant concentration ranges is in accordance with two other recent studies originating from the same project framework initiated by the German Federal Environment Agency: One of these studies investigated the effects of insect repellents on the freshwater crustacean *Daphnia magna* (Von Elert et al. 2016). Beyond the direct repellent effects, this study also investigated whether the repellents may interfere with the natural predator avoidance behaviour of *D. magna*, which is initiated by semiochemicals (kairomones) released from planktivorous fish (Lampert et al. 2003; Von Elert and Loose 1996). The other study addressed the hypothesis that in running water ecosystems, insect repellents may induce drift behaviour in stream invertebrates such as amphipod crustaceans and mayfly larvae (Fink et al. 2017). Neither of these studies found evidence that any commonly used repellent causes measurable effects on the behaviour of a range of aquatic invertebrates, even beyond the range of concentrations detected in nature (Fink et al. 2017; Von Elert et al. 2016).

In summary, this and further studies give no indications that even higher repellent (DEET, EBAAP or icaridin) concentrations than those observed in surface waters alter the behaviour of various common freshwater invertebrate species as potential non-target organisms. Hence, there is no repellent effect to be expected on the behaviour of lake and stream invertebrates, even though we currently cannot exclude that effects may differ in more complex, natural systems and with multiple stressors. The reason for this striking difference between terrestrial and aquatic invertebrates may lie in differences between the aquatic medium compared to dispersal and action in air (Von Elert et al. 2016).

One factor not yet considered here is the potential interference of anthropogenic chemicals with natural semiochemical-mediated communication, as chemical communication is widespread in aquatic systems (Von Elert 2012). Such 'info-disruption' (Lürling and Scheffer 2007) or 'infochemical effects' (Klaschka 2008, 2009) may cause maladaptive responses that may even result in consequences for entire ecosystems (Lürling 2012; Lürling and Scheffer 2007). However, since *L. stagnalis* was neither attracted nor repelled by any of the commonly applied insect repellent compounds, this is somewhat unlikely, but certainly cannot be ruled out. While it is hence still possible that insect repellents interfere with natural infochemical-mediated interactions, we found no evidence for a direct behaviour-altering effect of insect repellents on a common freshwater benthic herbivore.

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