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Behaviour of damselfly larvae (*Enallagma cyathigerum*) (Insecta, Odonata) after long-term exposure to PFOS

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Long-term laboratory exposure to perfluorooctane sulfonic acid decreases behavioural performance of damselfly larvae (Insecta: Odonata).

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ABSTRACT

Perfluorooctane sulfonic acid (PFOS) is a persistent and ubiquitous environmental contaminant that has been detected in organisms worldwide. Here, we evaluate whether long-term (1 and 4 months) exposure to PFOS contamination affects the behavioural performance of freshwater larvae of the damselfly *Enallagma cyathigerum* (Insecta: Odonata). Our results show reduced behavioural performance with increasing PFOS concentration. In 1 month exposed larvae, no observed effect concentrations (NOECs) were 100 μg/L for general activity. In 4 months exposed larvae, NOECs were 10 μg/L, for each behavioural trait, except swimming acceleration of male larvae where the NOEC was 100 μg/L. When faced with PFOS concentrations above the NOEC, *E. cyathigerum* larvae were less active, less capable to escape a simulated predator attack and less efficient in foraging. Together, our results show that damselfly larvae suffer reduced survival-related behavioural performance.

1. Introduction

Fluorinated organic compounds are a diverse group of chemical compounds used for a variety of specialised consumer and industrial products, such as surfactants, polymers and fire-fighting foams. They have been manufactured for more than 50 years. Recently, it became clear that perfluorinated compounds are accumulating in a great diversity of wildlife throughout the world, from industrialised areas to the Arctic (reviews in Giesy and Kannan, 2001; Beach et al., 2006; Houde et al., 2006; Lau et al., 2007). In response, the United Nations Environmental Program and the Stockholm Convention on persistent organic pollutants (http://www.pops.int/) requested research on these chemicals. In the present study, we examine whether long-term exposure to perfluorooctane sulfonic acid (PFOS) affects the behavioural performance of aquatic larvae of the damselfly *Enallagma cyathigerum* (Insecta, Odonata).

PFOS is the final degradation product of many commercially used perfluorinated products. It is the predominant perfluorinated compound found in animal tissues in the wild. PFOS shows high bioaccumulation potential, resistance to breakdown processes and potential for toxicity. PFOS has been detected in a great diversity of wildlife (e.g. Beach et al., 2006; Houde et al., 2006). In vivo experiments on a wide variety of animals have indicated sometimes dramatic effects on survival, metabolic function and condition (Boudreau et al., 2003; Hoff et al., 2003; MacDonald et al., 2004). Unfortunately, the experimental concentrations of PFOS generally applied are much higher than those detected in natural systems (for this critique, see MacDonald et al., 2004). Furthermore, research was focussed on the detrimental effects of PFOS on vertebrates (e.g. Beach et al., 2006; Lau et al., 2007) and, to a lesser extend, to marine and brackish invertebrates (e.g. Van de Vijver et al., 2003; Cuhna et al., 2005). In contrast, very little is known about the fate of freshwater invertebrates under PFOS exposure.

Behaviour is the cumulative manifestation of genetic, biochemical, physiological and environmental cues, all of which may be affected by pollutants. This makes behaviour a very sensitive measure for pollution (Dell'Omo, 2002). From an applied point of view, details on behavioural performance or dysfunctions may be translated into bioassays to detect or monitor the presence of

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pollutants in the environment. It is noteworthy that very little is known on whether exposure to PFOS affects the behavioural performance of animals. Among the few reports, Rhesus monkeys show hypoactivity in short-term repeat-dose oral toxicity tests with PFOS (OECD, 2002).

Here, we provide a study on long-term exposure of freshwater invertebrates to PFOS. We selected the larval life stage of damselflies (Insecta: Odonata) since earlier work has shown that they are sensitive to various sources of pollution (Hardersen, 2000; Campero et al., 2007). We assessed several aspects of behavioural performance under different PFOS concentrations and exposure times.

2. Materials and methods

2.1. Test organisms

We evaluated the behavioural performance of laboratory reared and field caught damselfly larvae of the species *Enallagma cyathigerum* (Charpentier, 1840). All damselfly larvae were housed in two temperature controlled rooms at 21 °C ± 1.3 and a 16L/8D light regime. Larvae were placed according a random number table with respect to PFOS treatment and family in these rooms and during experiments. Solutions used in our experiments were made through dilution of a standard PFOS solution. Water used in our experiments was dechlorinated tap water. To produce the different PFOS doses we used perfluoro-1-octanesulfonic acid, tetraethylammonium salt, 98%, produced by Aldrich. When the water in rearing cups had evaporated or when cups needed to be cleaned, the water was replaced with the solution of the relevant dose. Damselfly larvae were fed with nauplii of *Artemia salina* on a daily base. *A. salina* were reared in a PFOS-free environment and, prior to a feeding event, dissolved in the relevant PFOS solution. As a consequence, larvae were only exposed to PFOS contamination via the polluted water they lived in and not through the food they consumed. We did not measure PFOS concentrations in the larvae. All PFOS doses reported in this study are nominal concentrations.

To obtain laboratory reared damselflies, mating females ($N = 70$) were caught early July in a natural population (Kalmthout, Belgium, Drielingenven, 51°25′55″ N, 4°26′10″ E) using an insect net. Individuals were transported to the university in small cages. Females were placed for 24 h in oviposition chambers where they laid eggs on wet filter paper (see Cordero, 1990; Van Gossum et al., 2003). Afterwards, females were returned to the field population and released. Egg clutches of 30 females (clutch sizes: 337– 903 eggs) were retained for laboratory rearing. From previous studies, we expected that larvae would need 4–9 months to reach final metamorphosis into adulthood. For each of the 30 females, the filter paper was cut in five parts with approximately equal number of eggs. These five parts of the filter paper were distributed among four PFOS treatments (10, 100, 1 000, 10, 000 μg/L) and a blanco control treatment. Thus, for every female, eggs, and subsequently larvae, were reared at every PFOS dose and the control. After 10–14 days, larvae emerged from the eggs and were kept in identical PFOS treatments. Initially, larvae were maintained per family in groups of ten individuals per cup. After 10 days, larvae were reared individually in cups. Larvae could not see conspecifics in neighbouring cups because the cups were made of non-transparent polypropylene. We monitored survival of larvae for all families and doses. These data will be presented in detail in a different manuscript (Bots et al., manuscript in preparation). In short, all larvae exposed to 10, 000 μg/L PFOS died within 10 days and within 20 days when in the 1 000 μg/L treatment. Mortality in 100 and 10 μg/L PFOS and in the control was much lower. After larvae were exposed for approximately 4 months, behavioural experiments were performed.

Larvae of the field caught animals were collected in the same population in late August. They were transported to the university where they were housed and reared under identical conditions as our laboratory reared larvae. Subsequently, these larvae were exposed for 1 month to different PFOS concentrations (100 and 1 000 μg/L) and a blanco control treatment. To avoid age effects, behavioural experiments with laboratory and field collected animals were done with larvae that had reached the F2 instar.

2.2. Measuring behaviour

Three types of behavioural experiments were conducted with laboratory reared larvae: (1) general activity of larvae; (2) burst swimming performance under simulated predator stress; and (3) foraging success. Behavioural experiments were conducted in the following sequence: first individuals were tested for general activity (day 1), then swimming characteristics were measured (on day 2 and 3) and lastly the foraging experiment was completed (day 4). Field caught larvae were only tested for general activity.

In the first experiment, larvae were placed in plastic containers (15 × 10 × 11 cm) filled with the appropriate PFOS solution (2 cm depth). The experiment lasted 10 h in total. Every half hour the position of each larva was recorded on a 2 cm square grid attached to the bottom of the container. As a measure of general activity, we used the total number of moves and the distance covered by the larvae. For the latter, larvae were assumed to be in the centre of a 2 cm square grid, and the distance covered between two observations was estimated using Pythagoras's Theorem. We acknowledge that the half hourly records of position may underestimate the true mobility of the animals when they moved more than once during this time period or if they moved away and returned to the same position (which is statistically unlikely) (see also Heads, 1985). Therefore, our measure of mobility is conservative.

Second, to estimate swimming performance (speed and acceleration), larvae were placed in a plastic tank (50 × 30 cm filled with the appropriate PFOS solution (2 cm depth)) with a calibration rod. Larvae were induced to swim by gently prodding them with a needle, simulating a predation attempt. Several swimming bursts of each larva were videotaped (JVC colour video camera KY-F50, at 25 images /s). For each larva, three swimming bouts interspersed by 3–5 min were recorded. Successive positions of the centre of the thorax of the individual on these videos were digitised using Didge (Alistair Cullum, Creighton University, USA), rendering xy-coordinates for 2 s of film (=100 frames), starting two frames prior to the onset of the tactile stimulus. In order to remove digitisation noise, the x and y coordinates were filtered with a fourth-order, zero phase-shift butterworth filter (low-pass cut-off frequency = 3 Hz). Next, the distance travelled between two video frames (S_t) at a given time t was calculated using Pythagoras's theorem, and instantaneous velocities (v_t) and accelerations (a_t) were calculated as first-order numerical derivatives by

$$v_t = \frac{S_{t+1} - S_{t-1}}{2\,\Delta t}$$

$$a_t = \frac{v_{t+1} - v_{t-1}}{2\,\Delta t},$$

where Δt equals the time step between two video frames. Since maximal escape velocity and acceleration reflect an individual's performance when faced with a predatory attack, the highest instantaneous velocity and acceleration out of the three trials were used as a measure of escape performance.

Third, foraging success was determined by placing larvae in Petri dishes with 20 *Daphnia* of selected size (food items of about 2 mm in size) and the appropriate PFOS solution. Remaining *Daphnia* were counted after 2 h. *Daphnia* that were no longer present were considered eaten by the larva.

2.3. Statistical analyses

All data were analysed with (generalised) ANOVA's. In each analysis, explanatory variables are PFOS concentration (categorical), gender and their interaction. Dependent variables were, total distance covered (experiment 1), swimming speed (experiment 2) and swimming acceleration (experiment 2), for which we used normal error structure. For the number of moves (experiment 1) we used a Poisson error structure and the log-link function. For feeding success (experiment 3) we used a binomial distribution and the logit link function. To assess differences in the average distance covered between observation bouts (experiment 1) we used a repeated measurements approach (autoregressive covariance structure) since subsequent measurements taken on the same individual are not independent.

All analyses were performed using proc MIXED (normal error), proc GENMOD (Poisson and binomial error) and proc GLIMMIX (repeated measures) in SAS 9.1. In the repeated measurement analysis, correct degrees of freedom for the fixed effects F-tests were adjusted for statistical dependence using the Kenward–Roger method (Kenward and Roger, 1997). Descriptive statistics are presented as means ± SE. Posthoc Tukey tests are only mentioned when significant at the $p \leq 0.05$ level. In addition we determined NOECs for each of our behavioural experiments, and when applicable, for males and females separately.

3. Results

3.1. General activity (1 and 4 months of exposure)

The number of moves made during the 10 h experiment decreased significantly in larvae exposed for 4 months ($\chi^2 = 7.63$; df = 2; $p = 0.022$) and 1 month ($\chi^2 = 7.45$; df = 2; $p = 0.024$) (Table 1; Fig. 1). Similarly, total distances and average distances decreased in larvae exposed for 4 months (total distance: $F_{2,56} = 4.44$; $p = 0.016$ and average distance: $F_{2,245} = 4.55$; $p = 0.011$) and 1 month (total distance: $F_{2,41} = 3.47$; $p = 0.041$ and average distance: $F_{2,199} = 8.76$; $p < 0.001$) (Table 1). Among larvae exposed for 4 months, males and females differed in average distance covered for all PFOS concentrations, but not in the number of moves. Male larvae on average moved longer distances compared to females (males moved on average 0.81 ± 0.36 cm more than females; $F_{1,245} = 5.18$; $p = 0.024$). In contrast, no sex differences were observed in larvae exposed for 1 month ($F_{1,196} = 0.28$, $p = 0.595$). Tukey-tests comparing between larvae exposed for 1 month to 10 μg/L, 100 μg/L and the control treatment revealed no