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

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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.

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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 extent, 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...
null 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 a feeding event, dissolved in the relevant PFOS solution. As a consequence, larvae were reared at every PFOS dose and the control. After 10–14 days, larvae and a blanco control treatment. Thus, for every female, eggs, and subsequently 4

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.

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 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. If the larva moved away and then 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 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 (Alastair Cullem, Croungh University, USA), rendering x-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) 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

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. Post-hoc Tukey tests are only mentioned when significant at the p − 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 ( fireworks, 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

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significant differences for any of the general activity traits. Tukey-tests comparing between larvae exposed for 4 months to the control and 10 µg/L PFOS treatment were also non-significant (Table 1; Fig. 1). Comparing larvae in the 4 and 1 month treatment exposed to 100 µg/L PFOS shows that activity decreased with increasing exposure to PFOS concentrations (Fig. 1). Together, this study shows that the NOEC concentration of general activity in larvae exposed for 1 month is 100 µg/L PFOS while it is 10 µg/L PFOS in larvae exposed for 4 months.

3.2. Swimming performance (4 months of exposure)

For burst swimming characteristics, individual performance decreased with increasing PFOS concentration (swimming speed: \( F_{2,44} = 29.93; p < 0.001 \); swimming acceleration: \( F_{2,45} = 20.90; p < 0.001 \) (Table 2; Fig. 2). Males and females showed a different response (swimming speed: PFOS treatment \( \times \) gender, \( F_{2,44} = 3.48; p = 0.039 \); swimming acceleration: treatment \( \times \) gender, \( F_{4,45} = 5.68; p = 0.006 \)). For both sexes, speed and acceleration decreased with increasing PFOS concentration; but whereas for males the decrease was somewhat uniform, for females little difference was observed between the control and 10 µg/L treatment while a decrease was mainly observed between 10 and 100 µg/L (even dropping below that of males) (Table 2; Fig. 2). NOECs were 10 µg/L for swimming performance, except for swimming acceleration of male larvae the NOEC was 100 µg/L (Table 2; Fig. 2).

3.3. Foraging success (4 months of exposure)

Foraging success decreased in larval performance with increasing exposure to PFOS concentrations (\( F_{2,53} = 64.60; p < 0.001 \)). Males and females differed in feeding success depending on the PFOS treatment (Fig. 3; PFOS treatment \( \times \) gender: \( F_{2,53} = 3.22; p = 0.048 \)). Although we detected an overall gender difference, males and females never differed significantly within concentrations (all Tukey \( p > 0.2 \) (Fig. 3)). The NOECs were 10 µg/L.

4. Discussion

Long-term exposure to PFOS resulted in reduced behavioural performance in larvae of *E. cyathigerum*. In studies where damselfly larvae were not exposed to contaminant but to ecological stress, larvae also moved less, had reduced swimming performance, lowered foraging success, and ultimately lower survivorship and reproductive success (Stoks et al., 2005). Hence, it is expected that

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**Table 1** General activity (means ± SE) in larvae *Enallagma cyathigerum* exposed to different PFOS treatments for periods of 1 and 4 months (male and female showed similar response).

<table>
<thead>
<tr>
<th>Duration</th>
<th>Activity</th>
<th>PFOS µg/L</th>
<th>N</th>
<th>Mean ± SE</th>
<th>Posthoc</th>
<th>NOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 months</td>
<td># Moves</td>
<td>Control</td>
<td>19</td>
<td>16.32 ± 0.81</td>
<td>a</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20</td>
<td>15.60 ± 0.82</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>20</td>
<td>13.10 ± 0.84</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance To</td>
<td>Control</td>
<td>19</td>
<td>121.1 ± 8.4</td>
<td>a</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20</td>
<td>100.4 ± 8.8</td>
<td>a, b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>20</td>
<td>87.8 ± 6.6</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance Av</td>
<td>Control</td>
<td>19</td>
<td>5.75 ± 0.23</td>
<td>a</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20</td>
<td>5.02 ± 0.14</td>
<td>a, b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>20</td>
<td>4.39 ± 0.15</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td>1 month</td>
<td># Moves</td>
<td>Control</td>
<td>15</td>
<td>17.07 ± 0.89</td>
<td>a</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>15</td>
<td>16.13 ± 0.15</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance To</td>
<td>Control</td>
<td>15</td>
<td>12.93 ± 1.09</td>
<td>b</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>14</td>
<td>109.3 ± 11.0</td>
<td>a, b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance Av</td>
<td>Control</td>
<td>15</td>
<td>5.58 ± 0.25</td>
<td>a</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>14</td>
<td>5.36 ± 0.25</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>14</td>
<td>4.24 ± 0.24</td>
<td>b</td>
<td></td>
</tr>
</tbody>
</table>

Moves – number of moves; Distance To – total distance and Distance Av – average distance. Posthoc – Tukey HSD-test; means followed by same letter are not significantly different (\( \alpha = 0.05 \)).

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**Table 2** Burst swimming speed and acceleration (means ± SE) in males and females *Enallagma cyathigerum* exposed to different PFOS treatments for a period of 4 months.

<table>
<thead>
<tr>
<th>Swimming</th>
<th>PFOS µg/L</th>
<th>Gender</th>
<th>N</th>
<th>Mean ± SE</th>
<th>Posthoc</th>
<th>NOEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Control</td>
<td>Male</td>
<td>9</td>
<td>16.64 ± 1.38</td>
<td>a</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>13.79 ± 1.07</td>
<td>a, b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Female</td>
<td>9</td>
<td>16.51 ± 0.64</td>
<td>a</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>16.36 ± 0.76</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acceleration</td>
<td>Male</td>
<td>9</td>
<td>6.79 ± 1.13</td>
<td>b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>5.27 ± 0.50</td>
<td>a</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>9</td>
<td>40.45 ± 4.94</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>41.62 ± 5.30</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>66.37 ± 3.17</td>
<td>a</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>57.16 ± 4.54</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>21.89 ± 5.50</td>
<td>b</td>
<td></td>
</tr>
</tbody>
</table>

Posthoc – Tukey HSD-test; means followed by same letter are not significantly different (\( \alpha = 0.05 \)).
exposure to PFOS not only decreases behavioural performance, but also the fitness of damselflies. In addition, PFOS may not only alter the behaviour of the damselfly larvae, but also from their prey and predators, ultimately affecting the success of damselfly larvae in contaminated environments. Considering that behaviour is a highly sensitive and integrated response to internal and external stimuli (Dell’Omo, 2002) and that it can be measured using cheap and simple methods, it may be valuable to use it more often when exploring possible harmful effects of less known contaminants.

We showed that swimming acceleration was affected differently for males (NOEC = 10 μg/L) and females (NOEC = 10 μg/L). Gender differences may be expected when males and females differ in sensitivity to PFOS or in their uptake of PFOS due to their ecology and/or life-history and thus the likelihood of exposure to contaminants. However, all larvae were housed in small cups and food was not contaminated, suggesting it may be possible that males and females of E. cyathigerum differ in uptake/depuration of PFOS from the water they lived in. Clearly, an interesting direction for future research would be to also expose carnivorous damselfly larvae to PFOS through their diet.

Based on current literature, NOECs for PFOS range from 200 to 200, 000 μg/L for aquatic animals (Boudreau et al., 2003; Li, 2008; reviewed in Beach et al., 2006). However, lower NOEC values were observed for some species, such as the aquatic midge Chironomus tentans. Specifically, the NOEC for survival in C. tentans was 94.9 μg/L PFOS and 21.7 μg/L for growth, while for emergence success the NOEC was below the lowest test concentration of 2.3 μg/L (MacDonald et al., 2004). With NOECs of 10 and 100 μg/L PFOS for behavioural traits, it seems that E. cyathigerum may also be regarded as a relatively sensitive species. We want to note that the use of NOEC has been criticised in the past (e.g. Van Der Hoeven, 1997; Van Der Hoeven et al., 1997; Crane and Newman, 2000). Nevertheless, it has also been recommended to continue reporting this summary statistic because it plays an important role in legislation and procedures related to risk assessment and environmental quality (de Bruijn and Hof, 1997). Based on current knowledge, Beach et al. (2006) suggest a conservative freshwater quality value for PFOS of 1.2 μg/L PFOS.

It has been argued that most previous ecotoxicological experiments used unrealistically high PFOS concentrations compared to concentrations measured in natural environment (for this critique, see MacDonald et al., 2004). The highest known PFOS concentration of 8.6 μg/L was measured in Belgium in a large pond in a nature reserve near an industrial plant where PFOS has been manufactured, but which also hosts under the Ramsars convention and the European Bird Directive (ARCADIS, 2006). Concentrations in adjacent channels even reached levels up to 207.5 μg/L PFOS (ARCADIS, 2006). Nevertheless, damselflies were observed at this pond and channels (pers. obs.). The PFOS concentrations reported from Belgium are high compared to those measured in freshwater systems worldwide. Indeed, PFOS concentrations from industrial areas in South Korea maximally reached 0.65 μg/L (Rostkowski et al., 2006). For other studies in Canada and Europe, PFOS concentrations ranged from not observable to 0.069 μg/L (Loos et al., 2007; Stock et al., 2007). Only one study reported values up to 5.9 μg/L PFOS (Skutlarek et al., 2006). This makes that the NOEC values scored during our study are higher then the proposed water quality norm (Beach et al., 2006) and above the concentrations generally observed in natural freshwater ecosystems.

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