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Behavioural responses of freshwater planarians after short-term exposure to the insecticide chlorantraniliprole

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ABSTRACT

Recent advances in video tracking technologies provide the tools for a sensitive and reproducible analysis of invertebrate activity under stressful conditions nurturing the field of behavioural ecotoxicology. This study aimed to evaluate behavioural responses of the freshwater planarian *Dugesia subtentaculata* exposed to a model compound, chlorantraniliprole (CAP). This compound is an anthranilic diamide insecticide and due to its neurotoxic action can, at low concentrations, impair behaviour of exposed organisms. Behavioural endpoints measured included feeding and locomotor activities. Feeding responses were based on planarian predatory behaviour using *Chironomus riparius* larvae as prey. Locomotion was measured by the traditional planarian locomotor velocity (*p*LMV) assay and additionally using an automated video tracking system using a Zebrabox® (Viewpoint, France) device. While feeding and *p*LMV were significantly impaired at 131.7 μg/L CAP, the video tracking system showed that total distance covered by planarians was significantly reduced at concentrations as low as 26.2 μg/L CAP. Our results show that more advanced automated video recording systems can be used in the development of sensitive bioassays allowing a reliable, time- and cost-effective quantification of behaviour in aquatic invertebrates. Due to their ecological relevance, behavioural responses should not be disregarded in risk assessment strategies and we advocate the suitability of planarians as suitable organisms for behavioural ecotoxicological studies.

1. Introduction

Behavioural patterns result from the integration of biochemical and physiological processes which in turn can be linked to alterations at upper levels of biological organization (Alonso and Camargo, 2011; Hellou, 2011; Scott and Sloman, 2004). Behaviour has been suggested as a sensitive measure of ecotoxicological effect as it responds earlier than other classical endpoints such as growth and reproduction (Alonso and Camargo, 2011; Pestana et al., 2007). However, these later endpoints are the most often used in ecological risk assessment and regulatory actions since they can easily be objectively quantified. Behavioural endpoints tend to be overlooked in ecotoxicology because their determination is time-consuming and many times measuring behavioural endpoints imply a bias due to the observer's subjectivity limiting their repeatability (Gerhardt, 2007). Therefore, only a few behavioural protocols exist that can be applied to different model species in laboratory assays.

Behavioural assays have been developed based on the activity of freshwater invertebrate species, such as feeding inhibition (Maltby et al., 2002; Pestana et al., 2007), avoidance behaviour (Lopes et al., 2004) and mobility patterns assessed using impedance techniques (Azevedo-Pereira and Soares, 2010; Macedo-Sousa et al., 2007). Moreover, contaminant exposure in aquatic systems often occurs by short-term or pulse exposures (spray drift, surface runoff or drain flow) rather than continuously. Due to this phased exposure, evaluation of post-exposure effects of contaminants, reflecting the capacity of recovery of an organism, can be a more realistic evaluation of the effects at population and ecosystem levels (Reinert et al., 2002). Post-exposure feeding inhibition has been broadly used as a valuable endpoint to determine the effects due to metals and organic compounds in the cladoceran *Daphnia magna* (McWilliam and Baird, 2002) or in the amphipod *Gammarus pulex* (Wilding and Maltby, 2006). Post-exposure effects on locomotor activity has also been used as indicator of stress induced by phthalate esters in *Gammarus pulex* (Thurén and Woin, 1991) and by ammonia in

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the planarian *Polycelis felina* (Alonso and Camargo, 2015). These parameters showed to be robust, sensitive and reliable so they can be used as early warning indicators of stress (Hellou, 2011).

This assessment is of critical importance since changes in behaviour caused by toxicants have the potential to alter ecological functions of individuals reducing their fitness and ultimately conditioning ecosystem function. Therefore, these should be used as an early warning signs of stress induced by environmental pollution (Gerhardt, 2007; Hellou, 2011).

A promising tool for assessment of behaviour, the video tracking analysis, has been developed and used, mainly in terrestrial behaviour research and more recently with aquatic organisms (Augusiak and Van den Brink, 2015; Bossus et al., 2014; Talbot and Schötz, 2011).

Given their amenability to laboratory culturing conditions, freshwater planarians are excellent candidate non-target organisms to study sub-lethal effects of contaminants using a wide array of endpoints (e.g. regeneration, locomotion, feeding, bioaccumulation, biochemical parameters, etc.). Since planarians are small sized predators, commonly found in many freshwater systems (Thorp and Covich, 2009), they can be also excellent model organisms to be used in community ecotoxicology for the assessment of trait and density mediated indirect effects of contamination. Therefore, planarians are ideal model organisms to assess direct and indirect effects of contaminants along trophic chains and thus hold great potential as bioindicators of ecological integrity of freshwaters. Also, freshwater planarians are useful model species on behavioural and biomedical studies as they are easily maintained in laboratory and display specific behavioural responses to psychoactive substances (Pagán et al., 2015; Tallarida et al., 2014). Planarians are well characterized animal models in neurobiology research (Newmark and Alvarado, 2002; Reddien and Alvarado, 2004) and their nervous system shares features with vertebrates in terms of cell morphology and physiology (Buttarelli et al., 2008, 2000).

The main objective of this study was to evaluate behavioural responses of the native freshwater planarian *Dugesia subtentaculata* induced by short-term exposure to chlorantraniliprole (CAP). CAP is an insecticide belonging to the newest anthranilic diamide class and it was selected as a model compound due to its known mode of action and consequent behavioural effects (Hannig et al., 2009; Knight and Flexner, 2007). This compound acts by activation of the ryanodine receptors causing a depletion of intracellular calcium stores, disrupting muscle contraction and feeding behaviour causing paralysis and leading to death <(Casida and Durkin, 2013; Cordova et al., 2006). A key feature of CAP is its low toxicity to mammalians as it has a great selectivity for ryanodine receptors of insects (Lahm et al., 2007; Sattelle et al., 2008). Furthermore, CAP is persistent in soils and natural sediments (EFSA, 2013; USEPA, 2008) and its harmful effects to non-target freshwater invertebrates have been reported at predicted environmentally relevant concentrations (EFSA, 2013; Rodrigues et al., 2015).

Effects of sub-lethal concentrations of CAP were thus evaluated using planarians' locomotor activity assessed through the traditional locomotor velocity (*p*LMV) assay and by video tracking using an automated system. These responses were further compared to a simple post-exposure feeding bioassay.

2. Material and methods

2.1. Test organisms

D. subtentacula were collected in a non-polluted small stream, Ribeira de Boialvo (40°30′ 13.6″N; 8°20′ 33.7″W), central Portugal. In the laboratory, animals were allowed to acclimate to laboratory conditions: they were gradually cultivated in ASTM hard water (ASTM, 1980) medium, at 20 ± 1 °C, in the dark, for at least one week prior to bioassays and were fed with *Chironomus riparius* larvae. Organisms showing no signs of injuries and active when exposed to light were used in the ecotoxicological assays.

2.2. Chlorantraniliprole

The insecticide chlorantraniliprole (analytical standard, CAS No. 500008-45-7) was purchased from Sigma–Aldrich (UK). Analytical grade acetone was used as solvent in stock solution, which was protected from light to avoid degradation of CAP. Experimental solutions were prepared by dilution of the stock solution in ASTM medium. A concentration of 0.0002 v/v% of acetone was used for solvent control treatments.

2.3. Experimental design for the assessment of behavioural responses

Dugesia subtentaculata (0.6 ± 0.1 cm, total length) were exposed for 96 h to a gradient of CAP concentrations: 7.7 ± 0.4; 26.2 ± 2.3 and 131.7 ± 5.1 μg/L (measured concentrations, mean ± SD) plus solvent control treatment. Exposure was performed with groups of 10 organisms with 5 replicates per treatment, in crystallizing dishes containing 100 mL of experimental solution, at 20 ± 1 °C, in complete darkness and with no food added. After 96 h, individuals were randomly collected from each replicate and different behavioural endpoints were assessed, as described below.

2.3.1. Feeding activity assay

Post-exposure feeding activity was assessed using 12 planarians per treatment individually placed into crystallizing dishes with 20 mL of ASTM medium. Fifteen live *C. riparius* larvae (6 days old) were added as food in each replicate. Feeding rate was measured as the number of larvae ingested per planarian over a 24 h period.

2.3.2. Locomotion

2.3.2.1. Locomotor velocity (pLMV) assay. Fifteen planarians per treatment were individually placed in the center of a clear acrylic box (50.0 cm × 50.0 cm) positioned over graph paper (grid lines spaced 0.5 cm apart) and with ASTM medium covering the bottom. Post-exposure locomotor velocity was thus measured as the number of grid lines that each planarian crossed or re-crossed per min, during 2 min of observation, after a 30 s adaptation period.

2.3.2.2. Automated video tracking system. After exposure, twenty planarians per treatment were allocated to 24-multiwell plates with 0.5 mL ASTM medium in each well. Planarian locomotion was video tracked using a ZebraBox^{TM} apparatus and the ZebraLab® v3 software (Viewpoint, France). Video tracking continuously monitored planarian behaviour over a 10 min period (+2 min of acclimation to light conditions −550 lx). Locomotion was determined by calculating the distance covered (cm) per min during a 10 min observation period. A background correction of 20 was used to properly detect individuals.

2.4. Chemical analysis

Nominal CAP concentrations in experimental solutions used (sampled after 96 h of exposure) were verified by chemical analysis. Three water samples (100 mL per sample) per treatment were analyzed. All samples were frozen at −20 °C.

2.4.1. Chemicals

Liquid chromatography–mass spectrometry (LC–MS), grade methanol and acetonitrile (Li Chrosolv Hypergrade) were purchased from Merck (Darmstadt, Germany). Formic acid to acidify