Aquatic Toxicology 190 (2017) 70–77

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Research Paper

Single and mixture impacts of two pyrethroids on damselfly predatory behavior and physiological biomarkers

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ARTICLE INFO

Keywords:
Pesticides
Combination exposure
behavior
Sub-lethal effects
Aquatic invertebrate
Biomarkers

ABSTRACT

Direct mortality due to toxicity of single pesticide exposure along a concentration gradient, while the most common, is only one important parameter for assessing the effects of pesticide contamination on aquatic ecosystems. Sub-lethal toxicity can induce changes in an organism's behavior and physiology that may have population-level ramifications and consequences for ecosystem health. Additionally, the simultaneous detection of multiple contaminants in monitored watersheds stresses the importance of gaining a greater understanding of the toxicities of combined exposures, particularly at low, environmentally relevant concentrations. Using larvae of the Azure Damselfly (*Coenagrion puella*), we conducted a combined exposure investigation of two widely-used pyrethroid insecticides presumed to share the same neurotoxic mechanism of action, and estimated their effect on predatory ability, mobility and three physiological biomarkers (Glutathione S-transferase; GST, respiratory electron transport system; ETS, and malondialdehyde; MDA). Deltamethrin exposure (0.065 μg/L and 0.13 μg/L) was found to reduce the predatory ability, but it did not affect the larvae's mobility. Esfenvalerate exposure (0.069 μg/L and 0.13 μg/L), on the other hand, induced no significant changes in predatory ability or mobility. The decrease in predatory ability after the combination exposure (0.067 μg/L deltamethrin and 0.12 μg/L esfenvalerate) did not significantly differ from the impact of the single deltamethrin exposures. Glutathione-S-transferase was induced after single esfenvalerate exposure and the lower deltamethrin concentration exposure, but seemingly inhibited after exposure to the higher concentration of deltamethrin as well as the combination of both pyrethroids. Our data indicate that sub-lethal exposure to deltamethrin reduces predatory ability and suggest that sub-lethal combined exposure to deltamethrin and esfenvalerate inhibits the GST detoxification pathway. These effects can eventually result in a lower emergence of adults from contaminated ponds.

1. Introduction

Biodiversity in freshwater environments is on the decline (Ricciardi and Rasmussen, 1999). This is in part due to the rising use of pesticides which are now detected in surface waters globally (Beketov et al., 2013; Kingston, 2011; Stehle and Schulz, 2015). Pesticides are extensively applied in mixtures to most cropping systems as a method to combat growing resistance (Ahmad, 2007). Monitoring efforts reflect this practice by showing that pesticides usually occur together in nature (Ansara-Ross et al., 2012; Gilliom, 2007). As a result, there is a current drive to produce knowledge on the combination effects of sub-lethal pesticide mixture exposures for chemical risk assessment (EC, 2012).

Pyrethroid pesticides are a class of compounds suspected of dose-additive toxicity in mixture exposures on account of their common mode of action (Lydy et al., 2004). The long production history of synthetic pyrethroids and increasing popularity of these products for agricultural plant protection, household pest control and reducing the spread of insect-borne diseases has raised concerns about their potential to come into contact with non-target organisms (Palmquist et al., 2012). Subject to transport via wind, run-off, storm water and waste water, pyrethroids are found in surface waters and sediments around the world where they come into contact with non-target organisms (Amweg et al., 2006; Feo et al., 2010; Graaf et al., 2010; Jabeen et al., 2015; Weston et al., 2013). Monitoring surveys are generally not designed to detect peak concentrations in freshwaters making specific concentrations for testing difficult to determine, however, given their undeniable presence in freshwater environments, the environmentally relevant question is whether or not they affect the organisms living there (Relyea and Hoverman, 2006). The evidence available on combination effects of pyrethroids is determined by in-vitro and acute mortality studies with little attention given to alterations to non-lethal effects such as behavior (Cao et al., 2011; Desneux et al., 2007; Romero

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http://dx.doi.org/10.1016/j.aquatox.2017.06.025
Received 31 January 2017; Received in revised form 18 June 2017; Accepted 25 June 2017
Available online 27 June 2017

et al., 2015). Yet, sublethal effects are important to quantify as these may provide early warning signals and result in ecologically relevant effects, even in the absence of direct lethal effects of contaminants (Relyea and Hoverman, 2006).

Like most insecticides, pyrethroids are neurotoxins and therefore have the potential to impact an organism's behavior. Pyrethroids were developed to kill insect pests by targeting the voltage-gated mechanism that controls the transfer of neural impulses across membranes; a high enough dose induces death by paralysis, but the mechanisms behind the sub-lethal effects of lower doses are less well known (Soderlund and Bloomquist, 1989). Exposure to low concentrations of neurotoxins has been shown to adversely impact non-target aquatic species through sub-lethal toxic effects on their behavior (Baatrup and Bayley, 1993). Sub-lethal neurotoxicity that inhibits an organism's locomotion can impair its ability to search for food, capture prey, eat, reproduce or avoid predators; effects that are well-documented in terrestrial invertebrates (Bredeson et al., 2015; Yao et al., 2015). Disrupting the predator-prey dynamics by either inhibiting predatory or anti-predatory behaviors can alter both the predator and prey life-history traits and fitness with consequences for population dynamics and ecosystem health (Greig-Smith, 1991). Better knowledge of behavioral effects of pyrethroid exposure is therefore important to conserving or restoring ecosystem health in effected watersheds.

We here investigate the effects of a combined pyrethroid exposure on both behavior and suborganismal biomarkers of a non-target aquatic invertebrate. We exposed the larvae of the Azure Damselfy (*Coenagrion puella*) to environmentally relevant concentrations of deltamethrin and esfenvalerate, two common pyrethroids that have been detected in surface waters alone and in combination (Lindström and Kreuger, 2015). The Azure Damselfly occurs in the agricultural landscape where pyrethroids are likely to contaminate its habitat. It is a mid-trophic, non-target organism with an aquatic larval stage making it ideal to study the effects of aquatic contamination on predatory behaviors. While the Azure Damselfly is a common species in Europe, other coenagrionids are red-listed; perhaps due to pesticide exposure as recent evidence suggests (Kasai et al., 2016). Damselflies have also previously been used successfully to study sub-lethal effects of pesticides in laboratory settings (Campero et al., 2007; Chang et al., 2009, 2007; Hardersen and Wratten, 1998).

The behavioral assay tested for alterations in predatory behavior was scored as four hunting behaviors and general activity. Hunting behaviors are an assessment of prey capture abilities which are important for an organism's growth and development and general activity is an assessment that informs whether or not changes in predation are a result of paralysis. For example, if the damselfly's ability to walk or swim is unimpaired then any decrease in predation observed would not likely be due to paralysis, but rather a mechanism specific to predation.

In addition to the behavior assay, three suborganismal biomarkers that are commonly used as indicators of toxic exposure and suspected of causing negative fitness effects (Hyne and Maher, 2003; Maltby, 1999; Monaghan et al., 2009), were also investigated. The physiological biomarker assays that were conducted measured changes in glutathione S-transferase activity (GST), metabolic rate (activity of the electron transport system, ETS) and oxidative damage (formation of malondialdehyde, MDA). GST is a family of endogenous enzymes that serve as a physiological defense against toxicity. Specifically, GST offers a passive protection toward pyrethroid insecticides by binding to their molecule (Kostaropoulos et al., 2001). Studies have shown upregulation of GST activity is an organismal response to toxic chemical exposure in invertebrates (Antognelli et al., 2006; Janssens and Stoks, 2013) and enhanced tolerance to deltamethrin in *Anopheles stephensi* has been attributed to increased GST activity (Ganesh et al., 2003). Activity of the electron transport system is strongly correlated with an organism's energy expenditure in basal metabolism; an expenditure shown to increase under sub-lethal toxic stress with negative consequences for an organism's growth and reproduction (De Coen and Janssen, 2003; Packard, 1971). Reactive oxygen species are a natural by-product of mitochondrial activity kept in check by an organism's antioxidant defense enzymes. Toxic contaminants have been shown to induce reactive oxygen species in exposed organisms and cause oxidative stress by overwhelming the physiological defenses resulting in costly oxidative damage to biomolecules such as fat (Barzilai and Yamamoto, 2004; Kingston, 2011).

2. Methods

2.1. Behavioral assay

In an effort to avoid prior pesticide exposure, the larvae in the study were hatched from eggs oviposited in the laboratory. To receive eggs from females, tandem pairs of *Coenagrion puella* were captured at two ponds in Uppsala, Sweden. One pond was located in a pastoral setting near a cemetery and the other was situated between a residential area, floodplain and forest. While neither location is adjacent to agricultural activity, we cannot fully exclude the possibility the ponds received low levels of pesticide residues via run-off or spray drift. The males were released and the females were taken to the lab and kept individually in plastic jars for oviposition. The inside of the jars was covered with filter paper, wherein the females laid their eggs. Eight egg clutches were transferred to 0.9 L plastic containers of reconstituted water for hatching. All water used in this study was reconstituted soft water (RSW) comprised of $NaHCO_3$ (48 mg/L), $CaSO_4{\cdot}2H_2O$ (30 mg/L), $MgSO_4{\cdot}7H_2O$ (61.37 mg/L) and KCl (2 mg/L) (Greenberg et al., 1985). Newly hatched larvae were maintained together at 20 ± 1 °C under a 16 h light: 8 h dark photoperiod in aerated 3 L plastic containers of RSW and grass clippings for structure.

Pesticide exposure was conducted when the larvae reached 5 weeks old. The deltamethrin and esfenvalerate stock solutions were 100,000 μg/L in acetone and diluted to test concentrations with RSW. Acetone concentrations across all solutions, including the controls, were adjusted for uniformity. All control vessels were solvent controls. Larvae were exposed individually in 40 mL glass vessels to 20 mL

Table 1
Pesticide exposure concentrations: nominal and measured values, and sample sizes of the behavioral assays and molecular toxicity biomarker analyses.

Pesticide	Nominal Concentration (μg/L)	Measured Concentration (μg/L)	Uncertainty (95% confidence, μg/L)	Sample Size			
				Behavior	GST	ETS	MDA
Control	–	–	–	14	9	9	8
Deltamethrin	0.23	0.065	± 0.018	15	7	6	9
Deltamethrin	0.45	0.130	± 0.038	14	6	7	8
Esfenvalerate	0.23	0.069	± 0.021	16	8	6	10
Esfenvalerate	0.45	0.130	± 0.038	15	8	7	10
Mixture							
Deltamethrin	0.23	0.067	± 0.020	15	5	5	6
Esfenvalerate	0.23	0.120	± 0.063				