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Effects of imidacloprid exposure on *Chironomus riparius* Meigen larvae: Linking acetylcholinesterase activity to behaviour

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ABSTRACT

Imidacloprid (IMI) is an insecticide that interferes with the transmission of stimuli in the nervous system of insects. It is neurotoxic by mimicking nicotine through its binding to the nicotinic acetylcholine receptor. In this work, experiments comprising 96 h exposure followed by 48 h in clean medium were conducted to evaluate the toxicity of IMI to *Chironomus riparius* and its potential recovery. Behavioural parameters and AChE activity were assessed. After 96 h exposure to IMI, AChE activity, and the behaviour parameters ventilation and locomotion were reduced. There were no signs of recovery after removal to clean water for 48 h.

Ventilation behaviour was the most sensitive parameter and the one with the highest correlation to AChE activity. Despite the possibility that IMI might be having an indirect effect on AChE activity, the behavioural endpoint showed a higher sensitivity than the biochemical response itself.

This work highlights the importance of linking parameters with ecological relevance at individual level (behavioural parameters) with biochemical responses, to unravel xenobiotics mode of action.

1. Introduction

Pesticides used in agriculture are designed to affect target organisms – plagues – but due to their nature they may also affect non-target organisms present in the application site or even in nearby freshwater ecosystems (Crane et al., 1995). To reduce the pesticide's impact in these non-target species, new chemicals have been developed that minimise the compounds resilience time and maintain the pest control effectiveness. One of these innovative and fastest growing groups of pesticides is the neonicotinoids (Tomizawa and Casida, 2003). Imidacloprid [IMI; 1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine], developed by Bayer CropScience® AG, is a very common and worldwide used neonicotinoid employed in the control of sucking insects in crops (Tomizawa and Casida, 2005; Tomlin, 2000). IMI was designed to act as an agonist of the post-synaptic nicotinic acetylcholine receptors (Buckingham et al., 1997; Matsuda et al., 2001), causing their overstimulation and therefore affecting neuronal processes, which may lead to overall impairment and even death. This neurotoxicity is produced through the binding or partial binding to specific subsites or protein subunits of the nicotinic acetylcholine receptor (nAChR), which in turn activates nAChR activity (SERA, 2005). IMI can enter the freshwater bodies by leach or runoff from agricultural fields and can lead to local point-source contaminations (Fossen, 2006; Gupta et al., 2002). Concentrations ranging from 0.13 to 12 μg IMI L^{-1} have been reported for natural field scenarios (CCME, 2007; Phillips and Bode, 2004). Pesticide persistence in aquatic systems is very variable and can occur in short time spans, depending on several abiotic (e.g. light) and biotic (e.g. microbial communities) factors (Liess et al., 1999). Research has been carried out to assess the toxicity of IMI to non-target aquatic macroinvertebrates, focusing not only on ecological relevant pulse exposure scenarios (Stoughton et al., 2008) but also on constant exposure scenarios (Pestana et al., 2009a, 2009b). These studies highlighted the morphophysiological effects (survival, growth, emergence and behaviour) of IMI on the tested species, while few studies have assessed the biochemical/molecular toxic effects of this insecticide on aquatic macroinvertebrates (Jemec et al., 2007). Molecular biomarkers are being increasingly used as early warning tools in laboratory and field experiments (e.g. Domingues et al., 2007; Lemos et al., 2009; Lemos et al., 2010a), since they allow the detection of effects at the subcellular level before they are apparent at higher levels of biological organisation (Lemos et al., 2010b). To more accurately predict the direct consequences, to an organism or population, of the exposure to a known amount of a toxicant, a particular biomarker response should be related to impairment of growth, reproduction or metabolic function directly related to the survival of the organism

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(Depledge and Fossi, 1994). One of the most employed biochemical biomarkers is cholinesterase (ChE) activity. Cholinesterases are nervous system enzymes that have a key role in the maintenance of the normal nerve functions. Acetylcholinesterase (AChE) is the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine that generates post-synaptic potentials. In its absence, acetylcholine continues to stimulate the post-synaptic neuron, leading to uncoordinated movements. Several studies have assessed the inhibition of AChE activity by several toxicants in aquatic organisms (Beauvais et al., 1999; Kallander et al., 1997; Rakotondravelo et al., 2006), linking this biomarker to other end-points such as behaviour, feeding rate or larval emergence (Domingues et al., 2007; García-de la Parra et al., 2006).

At the individual level, behaviour is considered an early warning tool in ecotoxicology since it is one of the most sensitive indicators of chemical stress (Gerhardt et al., 1994) and the first line of defence to environmental stimuli (Beitinger, 1990). Assessing behaviour alterations allows the integration of individual physiological processes and mechanisms with the environmental stimuli that are causing them (Dell'Omo, 2002). Some authors have linked behavioural endpoints (like locomotion) to biochemical biomarkers (such as ACHE), on edaphic (Capowiez et al., 2003; Engenheiro et al., 2005; Jensen et al., 1997) and aquatic organisms (García-de la Parra et al., 2006).

Chironomids are an ecologically diverse group and are one of the most ubiquitous insects within freshwater ecosystems, dominating (in number and biomass) the benthic communities of lotic and lentic environments (Pery et al., 2002), and are being a major food source for other animals (García-Berthou, 1999). They are easily maintained in the laboratory and are commonly used as model organisms for sediment toxicity tests (EPA, 2000; OECD, 2004).

These organisms depend mostly on locomotion and ventilation (whole-body undulation in the water column) to perform all activities that enable them to dislocate, find food, emerge and avoid predators. Due to the above mentioned relation of AChE activity and behaviour, it is especially important to assess the effects of toxicants on AChE activity that might lead to the disruption of vital behavioural parameters. Despite AChE activity being usually used for organophosphate and carbamate exposure, previous studies using IMI and AChE activity have showed that AChE activity can be a sensitive biomarker, but not an early biomarker of stress (Jemec et al., 2007).

The aim of this study was to assess how sublethal exposure to IMI affects both *Chironomus riparius* behaviour and AChE activity, in different periods throughout the exposure and even after the exposure episode.

2. Material and Methods

2.1. Test organism

C. riparius midges were obtained from laboratory cultures established at the University of Aveiro for 3 years. The cultures were maintained in an enclosed transparent acrylic box containing several plastic beakers holding a 2 cm layer of acid-washed and burned commercial sand (<1 mm), and approximately 2.5 L of reconstituted hard water ASTM (ASTM, 2000). A gentle aeration was provided in each beaker. This system permits the occurrence of the whole life cycle of the chironomids, by allowing the swarming and copulation of emerged adults (OECD, 2004). The culture was maintained in standard conditions, at $20\pm2^{\circ}$ C and with a 16–8 h light-dark photoperiod. Freshly laid egg masses are transferred onto crystallising dishes with culture medium until hatching, and the first instar larvae are used either to start a new culture or in bioassays. Water and sediment were renewed every week and the larvae were fed (1 mg animal^{-1} day^{-1}) twice a week with a suspension of ground Tetramin® (Tetrawerke, Germany).

2.2. Imidacloprid

Confidor® 200 SL (Bayer CropScience AG, Monheim, Germany) was used to prepare the stock solution of IMI dissolved in ultra-pure water. The analytical concentration of the stock solution was 0.725 mg L^{-1}. From this stock solution, several nominal concentrations of IMI were prepared: 0.00 (control) 0.50, 1.50 and 3.00 μg of IMI L^{-1} (nominal concentrations). Chemical analyses of the IMI samples from the stock solution and bioassays were conducted by Terracon Laboratorium für Umwelt- und Pestizidanalytik GmbH (Jütterborg, Germany), using a HPLC-PDA-System equipped with 2 HPLC pumps Model LC-10ADvp, Autosampler SIL-10ADvp, column oven CTO-10ASvp and a photodiodenarray-detector (PDA) SPD-M10Avp (Shimadzu, Japan). Procedure consisted in: all samples containing high IMI concentrations (e.g. stock solutions) were diluted with deionised water, while samples with lower concentrations were extracted from 100–200 mL water samples (flow of 0.5 mL min^{-1}) using solid phase extraction (SPE cartridges Supelclean ENVI-18, Supelco, Schnelldorf, Germany) and acetonitrile (1:1 v-v) for elution. 10 μL acetonitrile-extracts were then applied to a chromatography column (LUNA C18, Phenomenex, Aschaffenburg, Germany), at a flow rate of 0.4 mL min^{-1} using water, 0.1% formic acid and acetonitrile as eluents. Detection was carried out at 270 nm with a limit of quantification of 0.1 μg L^{-1}.

2.3. Organism's exposure

The bioassay comprised of 96 h exposure to IMI and subsequent 48 h in clean medium. The experiments were performed in the same temperature and photoperiod conditions as in the cultures, using 200 mL glass beakers (10 replicates per treatment) with five late 3rd instar larvae (ten days old) per replicate. Each beaker contained 40 g of acid-washed inorganic fine sand (<1 mm) and 150 mL of test solution. Organisms were fed with macerated Tetramin® (0.5 mg animal^{-1} day^{-1}) every 48 h until the end of the experiment. Replicates were examined daily for mortality by checking for dead animals on top of the sediment. Mortality was also assessed when animals were transferred to the behavioural tests. Larval stages were checked during the test period. Forty-eight hours after the beginning of the experiment, and before feeding, half of the test solution in each beaker was renewed. After 96 h of exposure, the larvae were removed from the test beakers and transferred to the beakers with clean medium, sediment and food as described above, until the end of the experiment. Acetylcholine activity and behaviour of larvae were measured as response parameters at 48 h exposure, 96 h exposure and 48 h after being removed to clean medium.

2.4. Behaviour

Behavioural patterns were recorded as a function of animal movements by the Multispecies Freshwater Biomonitor (MFB) that was developed by Gerhardt et al. (1994). This technology is based on a quadropole impedance technique, where the organism moves freely inside a chamber that contains two pairs of electrodes attached to the inner walls. One pair of electrode generates a high frequency alternating current, whilst the other pair measures changes of the impedance and its frequency within the chamber, due to the organism movements (Gerhardt, 2000). The data generated is registered and processed with specific MFB software programme. *C. riparius* behavioural patterns in water were summarised in two main types of registered behaviour: locomotion and ventilation [for details, see De Bisthoven et al. (2004) and Azevedo-Pereira and Soares (2010)]. Five larvae from each concentration and control were randomly chosen and placed individually in the MFB chambers with ASTM hard water. Behaviour was recorded during 2 h ($n=12$ recordings per chamber) every 48 h until the end of the test.

2.5. Biochemical analysis

Parallel to behaviour recording, a set of organisms were withdrawn from the experience and frozen in liquid nitrogen (n between 8 and 12). Within two weeks, the entire frozen animal was used to determine AChE activity—preliminary studies (unpublished data) indicated that within the ChE's, acetylcholine was the most relevant substract. Each *C. riparius* was homogenised with 500 μL of phosphate buffer (0.1 M, pH 7.2), using an electrical homogeniser and then centrifuged for 3 min at 5000 rpm and pellet discarded. AChE analyses were performed following Ellmans' method (Ellman et al., 1961) adapted to a microplate reader, and absorbance values read at 404 nm (Guilhermino et al., 1996; Ribeiro et al., 1999) (Labsystems Multiskan EX plate reader–Helsinki, Finland). The enzyme activity was expressed as nmol ml^{-1} mg prot^{-1} min^{-1}. The total quantity of protein was determined following the method of Bradford (1976), adapted to microplate reader (Ribeiro et al., 1999) and absorption measured at 595 nm (Labsystems Multiskan EX plate reader–Helsinki, Finland).

2.6. Statistics

For behavioural experiments, one-way ANOVAs were calculated for each recording period and a two-way ANOVA was used to compare data throughout the experimental period, using IMI concentrations and days as factors for both enzyme activity and behavioural data, testing for IMI concentrations, exposure and recovery periods, and their interactions. Data from behavioural experiments

were arcsine square root transformed to stabilise variances across treatments (Zar, 1996). The Spearman rank correlation was calculated between AChE activity and each behavioural parameter for all sampling periods. Whenever significant differences were observed, a Tukey post-hoc test was used for multiple pairwise comparisons to assess, which treatments were significantly different. For all statistical tests, the significance level was set at $p \leq 0.05$. All calculations were performed with SigmaStat (2006).

3. Results

Less than 20% mortality was found for the controls for all the exposure periods, thus validating the experiments (EPA, 2000). No molt was reported between the 96 h exposure and the subsequent 48 h in clean medium.

When test solutions were renewed, a small and rapid increase of the concentration was expected, but not above the nominal concentration. Regardless of this, IMI degraded throughout the 96 h experimental period. At the end of the exposure period there was 40% (0.30 μg of IMI L^{-1}), 63% (0.55 μg of IMI L^{-1}) and 60% (1.20 μg of IMI L^{-1}) less compound in the first, second and third concentrations, respectively, when compared with the nominal concentrations used. For a better understanding, the analytical concentrations detected are used in figures, results and discussion (0.30, 0.55 and 1.20 μg of IMI L^{-1}). The behavioural response patterns of the midges were affected by the presence of the toxicant (Figs. 1 and 2). After 48 h exposure, the larvae exposed to 0.55 μg of IMI L^{-1} showed a statistically significant increase in the locomotory activity, when compared to the control (Tukey's test: $q=4.685$, $p=0.005$; Fig. 1), while animals exposed to the higher two concentrations suffered statistically significant changes in ventilation, when comparing to control [Tukey's test: $q=5.450$, $p<0.001$ (0.55 μg of IMI L^{-1}); $q=7.698$, $p<0.001$ (1.20 μg of IMI L^{-1}); Fig. 2]. After 96 h of exposure, the ventilation activities decreased with increasing concentrations of IMI, with a LOEC of 0.55 μg of IMI L^{-1} [Tukey's test: $q=6.949$, $p<0.001$ (0.55 μg of IMI L^{-1}); $q=18.675$, $p<0.001$ (1.20 μg of IMI L^{-1}); Fig. 2]. For the 96 h exposure period, there was a trend to increase locomotion activity in the lowest concentrations tested although without statistically significant differences to the control [Tukey's test: $q=3.257$, $p=0.097$ (0.30 μg of IMI L^{-1}); $q=3.554$, $p=0.058$ (0.55 μg of IMI L^{-1}); Fig. 1], whereas in animals exposed to 1.20 μg of IMI L^{-1} locomotion was impaired in the midges, being

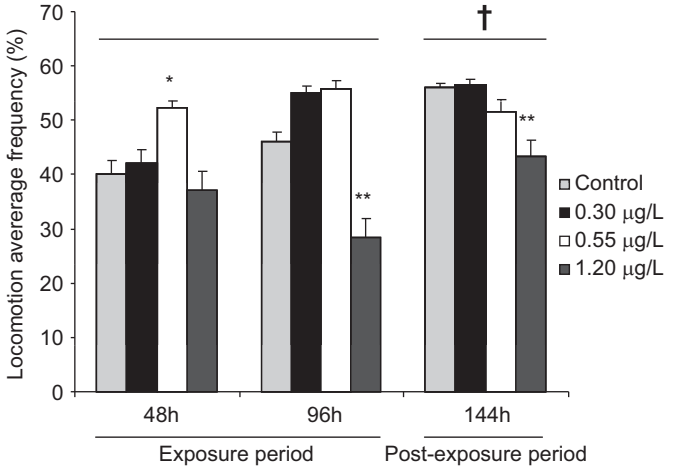

Fig. 1. Average activity frequencies of locomotion (%) of *Chironomus riparius* exposed to imidacloprid for a period of two and four days, followed by a post-exposure period of two days in clean water. (*) represents significance level $p<0.01$ and (**) represents significance level $p<0.001$ in comparison with the control (ANOVA, Tukey's test). (†) represents significance level $p<0.01$ for the comparison between times of exposure (ANOVA, Tukey's test).

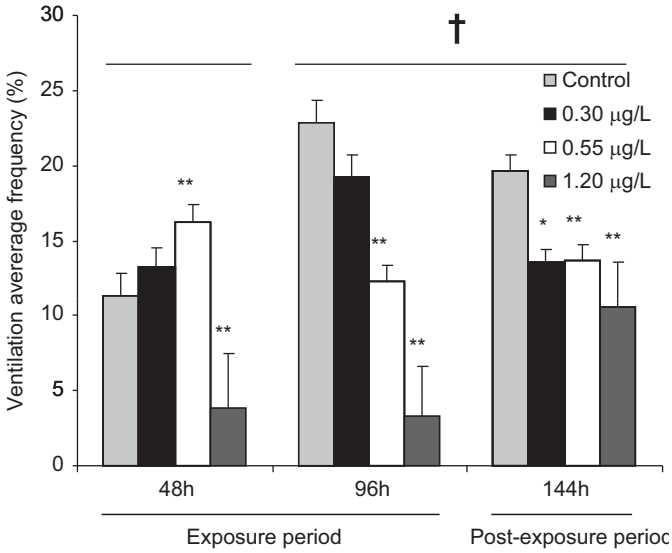

Fig. 2. Average activity frequencies of ventilation (%), of *Chironomus riparius* exposed to imidacloprid for a period of two and four days, followed by a post-exposure period of two days in clean water. (*) represents significance level $p<0.01$ and (**) represents significance level $p<0.001$ in comparison with the control (ANOVA, Tukey's test). (†) represents significance level $p<0.01$ for the comparison between times of exposure (ANOVA, Tukey's test).

statistically significant when compared to the control (Tukey's test: $q=9.244$, $p<0.001$; Fig. 1).

After the 48 h post-exposure period (144 h of total time), behavioural activities demonstrated that the organisms where still affected by the stress agent. Locomotory activity at 1.20 μg of IMI L^{-1} was still significantly reduced when compared to the control (Tukey's test: $q=6.200$, $p<0.001$; Fig. 1). *C. riparius* ventilation activity was still impaired after this post-exposure period, with statistically significant differences for all concentrations tested when compared to the control [Tukey's test: $q=4.580$, $p=0.007$ (0.30 μg of IMI L^{-1}); $q=5.333$, $p<0.001$ (0.55 μg of IMI L^{-1}); $q=8.744$, $p<0.001$ (1.20 μg of IMI L^{-1}); Fig. 2].

No differences were found in the control animals between the ventilation activity at the end of the exposure period and the post-exposure recording period (Tukey's test: $q=1.254$, $p=0.649$). Nevertheless, although still being statistically different from control, animals exposed to 1.20 μg of IMI L^{-1} had an increase of ventilation activities comparing the exposure and post-exposure periods (Tukey's test: $q=8.678$, $p<0.001$).

When comparing the post-exposure with the exposure period, an increase of locomotion frequencies was found being statistically significantly different [Tukey's test: $q=7.113$, $p<0.001$ (48 h); $q=4.467$, $p=0.005$ (96 h); Fig. 1]. No statistically significant differences for ventilation frequencies were found when comparing exposure and post-exposure periods between 96 and 144 h (Tukey's test: $q=1.933$, $p=0.358$; Fig. 2).

Acetylcholinresterase activity in chironomids exposed for 96 h to IMI was reduced, being statistically significant at the highest concentration tested (Tukey's test: $q=4.607$, $p=0.008$; Fig. 3). After the 48 h post-exposure period, a decrease of AChE activity was also observed, being statistically significant for all treatments tested when compared to the control group [Tukey's test: $q=8.281$, $p<0.001$ (0.30 μg of IMI L^{-1}); $q=9.098$, $p<0.001$ (0.55 μg of IMI L^{-1}); and $q=12.445$, $p<0.001$ (1.20 μg of IMI L^{-1}); Fig. 3]. Statistically significant differences for AChE activity during exposure (48 and 96 h) were found (Tukey's test: $q=5.169$, $p<0.001$; Fig. 3), while no statistically significant differences for AChE activity were found when comparing

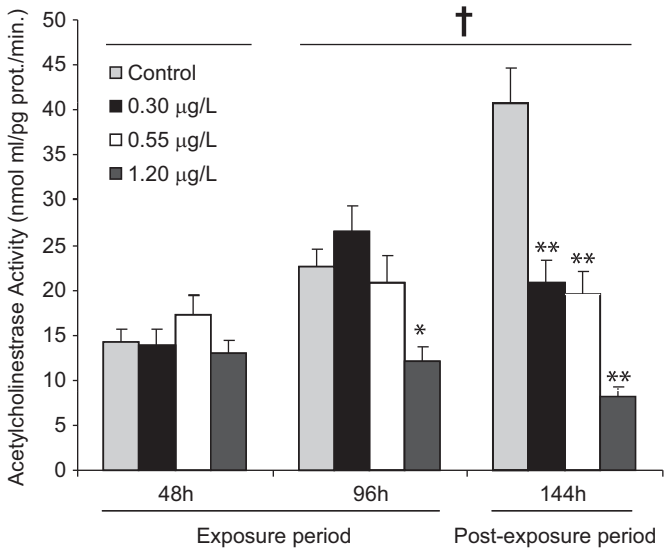

Fig. 3. Average acetylcholinesterase activity, of *Chironomus riparius* exposed to imidacloprid for a period of two and four days, followed by a post-exposure period of two days in clean water. (*) represents significance level $p<0.01$ and (**) represents significance level $p<0.001$ in comparison with the control (ANOVA, Tukey's test). (†) represents significance level $p<0.01$ for the comparison between times of exposure (ANOVA, Tukey's test).

exposure and post-exposure periods between 96 and 144 h (Tukey's test: $q=1.562$, $p=0.514$; Fig. 3).

The relation between AChE activity and the behavioural parameters were also assessed. Despite AChE activity did not show any significant relation with locomotion [$r=-0.034$, $P=0.889$ (48 h); $r=0.105$, $P=0.662$ (96 h); $r=0.196$, $P=0.403$ (144 h)], a strong and significant correlation with ventilation was found for 96 and 144 h [$r=-0.164$, $P=0.508$ (48 h); $r=0.546$, $P=0.016$ (96 h); $r=0.553$, $P=0.011$ (144 h)].

4. Discussion

In the present study laboratory ecotoxicology tests are encouraged as aquatic organisms can be subjected to exposure to contaminants in levels depending not only on natural conditions but also on pesticide persistence in the environment, in order to apply an environmentally relevant procedure for pesticides testing. One should bear in mind that contamination of aquatic environments by pesticides is often due to agricultural fields' runoff, spray drift or even ground water flows (Liess et al., 1999); and that these inflows of pesticide to aquatic systems can be variable according to their application in farming soils and might be followed by periods of long-term exposure to low concentrations of the pesticides (Naddy et al., 2000). Previous acute tests (Azevedo-Pereira et al., in press), showed a 48 h LC50 (95.0% confidence interval) of 19.90 µg of IMI L^{-1} (14.64–27.16 µg L^{-1}), and Pestana et al. (2009b) also reports a 96 h LC50 (95% CI) for *C. riparius* of 12.94 µg of IMI L^{-1} (9.74–18.22) under same exposure conditions. The values here obtained are in accordance with the previous studies. The lower concentrations of IMI when compared with the nominal concentrations, detected at the end experimental period, were probably due to bacterial growth in the beakers due to food addition, which can increase the degradation rate of imidacloprid (CCME, 2007).

The chironomids behaviour was affected by exposure to sublethal doses of IMI. During the exposure period, animals exposed to the highest concentration exhibited a decrease in locomotory activity, while those exposed to the lower concentrations exhibited a small increase in locomotion in the first 48 h of exposure (Fig. 1). This non-monotonic dose-response behaviour is probably due to the lower toxicity in lower concentrations not impairing the larvae's attempt to escape from the contaminated medium. The increased mobility thus probably reflects avoidance behaviour, as stated by De Bisthoven et al. (2004), while in higher concentrations the compounds' toxicity weakens the larvae's ability to respond and escape.

Similarly to locomotion, ventilation involves characteristic swimming movements (Brackenbury, 2000) that allows the larvae to travel considerable distances by drifting (e.g. to escape from contaminated sites), thus the same non-monotonic trend observed in the ventilatory activities may be explained by the same rationale. This increased mobility, to avoid toxicant in the lowest concentration was then reduced as toxicity increased, i.e., longer exposure period.

Forty-eight hours after the exposure period, the animals did not show signs of recovery. Although in clean water, it is possible that the IMI presence (or its metabolites) within the midge is still sufficient to be mimicking acetylcholine at the post-synaptic nicotinic acetylcholine receptors (nAChR), thus affecting the larvae, but no assessment of internal IMI body burdens were made to fully understand this. Nevertheless, in another study (Azevedo-Pereira et al., in press), midges subjected to the same exposure period revealed a behavioural recovery after 6 days of post-exposure. This period might be what it takes for the excretion of residual IMI and its metabolites. Although in some insects (e.g. houseflies) IMI may readily be excreted, it can also be metabolised and its main metabolites can also present insecticidal activity and therefore extend the toxicity of the pesticide (Nishiwaki et al., 2004; Suchail et al., 2004). The chironomids exposed to the highest concentration, although not fully recovering to values compared to the control, have a significant increase of AChE activity after transferring to clean water. A longer post-exposure period could allow for the organism to fully recover, as seen with the behaviour recovery after 6 days of post-exposure in clean water (Azevedo-Pereira et al., in press).

In this study, the AChE activity of the larvae decreased with increasing concentrations of IMI from the 96 h exposure onward (including after the short post-exposure period; Fig. 3). To our knowledge, few data about AChE activity related with IMI are available in the literature: acute testing with the earthworms *Aporrectodea nocturna* and *Allolobophora icterica* (Capowiez et al., 2003) showed no effects on AChE activity. On the other hand, chronic testing with the daphnid *Daphnia magna* (Jemec et al., 2007) reported a clear impairment of the enzyme activity with increasing concentrations of IMI–LOEC of 10 mg L^{-1} for a 21 d exposure period.

In uncontamined conditions acetylcholine (ACh) binds its receptor (AChR), leading to the activation of the ion channel, and afterwards it is hydrolysed by AChE (Tomizawa and Casida, 2003). The neonicotinoid-binding site in AChR is the same as or closely coupled to that of ACh, and displays saturable and reversible binding with fast kinetics (Tomizawa and Casida, 2003). This way it is possible that the binding of the neonicotinoid to the receptor in the AChR and subsequent non-connection of the neurotransmitter ACh to the nicotinic receptor will cause the inhibition of AChEs' activity, as seen here.

This constant stimulation of the nicotinic AChR receptors by this agonist (IMI) incites the general physiological impairment of endpoints related to the nervous function, thus leading to a decrease of both ventilation and locomotion as well as AChE activities. In this work, a high correlation between AChE and ventilation activity was found, which would strength the reasoning of the link of this enzyme activity and behavioural patterns and thus with more ecological relevant levels. This correlation between biomarker activity and a measurable behavioural endpoint

can be considered as being sensitive, because although in this case enzyme activities are probably not an early and sensitive biomarker of stress, they may reflect a general impaired physiological state of the organism. Nevertheless, this decrease of AChE activity is most probably a consequence of the agonistic activity of IMI to the receptor, and the ventilation impairment is probably due to the continuous stimulation of the receptor, conferring it an independent relation.

Changes in behaviour due to the continuous stimulation of the nervous system by xenobiotics provokes uncontrolled muscular tremors that by reducing locomotion will most probably interfere with foraging activities, reducing the input for the high energy demanding ventilation activities (Penttinen and Holopainen, 1995), as well as the energy budget needed for the overall physiological processes such as growth and emergence (Alexander et al., 2008). Moreover, the ability to drift or escape from predators, which in turn are due to important impacts at the population and community level will also be affected (Alexander et al., 2008; Engenheiro et al., 2005; Pestana et al., 2009b).

5. Conclusion

Our results suggest that ventilation is a more sensitive endpoint than locomotion. Furthermore, this work highlights the understanding of the behaviour responses (as an early warning system) in relation with biochemical responses, giving a sensitive representation of the organism physiological response to environmental factors. Despite AChE activities give us a picture of IMI's mode of action in the organism, this work suggests that behaviour, and more specifically ventilation, is a more sensitive parameter than biochemical responses. Added to its higher ecological relevance, ventilation can be used as a relevant endpoint for ecotoxicology testing.

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