



Eesti Maaülikool
Estonian University of Life Sciences

**THE SUBLETHAL EFFECTS OF NEUROTOXIC
INSECTICIDES ON THE BASIC BEHAVIOURS OF
AGRICULTURALLY IMPORTANT
CARABID BEETLES**

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TOIME PÕLLUMAJANDUSLIKULT OLULISTE
JOOKSIKLASTE PÕHIKÄITUMISTELE**

ENE TOOMING

A Thesis
for applying for the degree of Doctor of Philosophy
in Agriculture

Väitekiri
filosoofiadoktori kraadi taotlemiseks
põllumajanduse erialal

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Estonian University of Life Sciences

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LIST OF ORIGINAL PUBLICATIONS

This thesis is a review of the following papers, which are referred to by Roman numerals in the text. The papers are reproduced by kind permission of the publishers.

- I Tooming, E.**, Merivee, E., Must, A., Sibul, I., Williams I., 2014. Sub-lethal effects of the neurotoxic pyrethroid insecticide Fastac 50EC on the general motor and locomotor activities of the non-targeted beneficial carabid beetle *Platynus assimilis* (Coleoptera: Carabidae). *Pest Management Science*, 70, 959–966.
- II Merivee, E., Tooming, E.**, Must, A., Sibul, I., Williams I., 2015. Low doses of the common alpha-cypermethrin insecticide affect behavioural thermoregulation of the non-targeted beneficial carabid beetle *Platynus assimilis* (Coleoptera:Carabidae). *Ecotoxicology and Environmental Safety*, 120, 286–294.
- III Tooming, E.**, Merivee, E., Must, A., Merivee, M-I., Sibul, I., Nurme, K., Williams, I., 2017. Behavioural effects of the neonicotinoid insecticide thiamethoxam on the predatory insect *Platynus assimilis*. *Ecotoxicology*, 26 (7), 902–913.

Table 1. The contributions of the authors to the papers:

Paper	Idea and study design	Laboratory work	Data analysis	Manuscript preparation
I	ET , EM, AM	EM, ET , AM	EM, ET , AM	All
II	EM	EM, ET , M	EM, ET , AM	All
III	EM, AM, ET	EM, ET , AM, KN, MIM	EM, ET , AM, KN, MIM	All

ET – Ene Tooming; **EM** – Enno Merivee; **AM** – Anne Must; **IS** – Ivar Sibul, **IHW** – Ingrid Helvi Williams; **MIM** – Marten-Ingmar Merivee; **KN** – Karin Nurme; **All** – all authors of the papers.

ABBREVIATIONS

α -cyp	alpha-cypermethrin
CFCR	clean food consumption rate
FSA	food source area
GMA	general motor activity
IPM	integrated pest management
LA	locomotor activity
LD ₅₀	lethal dose (to 50% of test animals)
MFRC	maximum field recommended concentration
RH	relative humidity

1. INTRODUCTION

There is a need in agriculture to achieve more sustainable use of insecticides with the least possible disruption to agro-ecosystems. The application of pyrethroid and neonicotinoid insecticides in agriculture is extensive and due to their high bio-efficacy as well as their broad-spectrum range of action, they are still the best means to control most harmful arthropod pests (Desneux *et al.*, 2007; Garcia, 2011). Pyrethroids with their contact mode of action affect both the peripheral and central nervous systems of an insect, stimulating nerve cells to produce repetitive discharges, causing paralysis (known as insect ‘knockdown’) and possible insect death (Ray, Fry, 2006; Clark, Garcia, 2011; Symington, 2012). Compared to pyrethroids, neonicotinoid insecticides are systemic, which means, after treatment, they distribute through all plant parts and thus are particularly effective against sucking insects. Similarly to pyrethroids, the active ingredients in neurotoxic neonicotinoid insecticides affect the central nervous system of insects, by inducing continuous nervous excitation at low doses, receptor blockage, cell energy exhaustion, paralysis as well as death at higher doses (Goulson, 2013; Simon-Delso *et al.*, 2015).

Unfortunately, non-target, beneficial organisms, such as pollinators, parasitoids and predatory arthropods are inevitably exposed to insecticide treatment. The routes of exposure to pesticides can be topical, residual or even dietary (Kunkel *et al.*, 2001; Mauchline *et al.*, 2004; van Toor, 2006) and depending on pesticide dose/concentration, the effect can be lethal or sublethal. The sublethal effects are defined as “effects on individuals that survive exposure to a pesticide” (Desneux *et al.*, 2007). In addition to direct mortality, the sub-lethal effects of insecticides can cause changes in their physiology and behaviour (Thompson, 2003; Goulson, 2013, Pisa *et al.*, 2015) negatively affecting their population dynamics.

In behavioural ecotoxicology, locomotion reflects the physiological, neurological, anatomical and metabolic status of an individual (Baatrup, Bayley, 1993, 1998; Bayley, 2002; Desneux *et al.*, 2007). Insecticides affect important ecotoxicological biomarkers such as insect locomotion and feeding activities, as well as behavioural thermoregulation, and assessing these parameters gives valuable information about chemical stress in treated insects.

Various studies have been made in recent decades about the sublethal effects of insecticides on beneficial arthropods (Desneux *et al.*, 2007; Prasifka *et al.*, 2008; Pisa *et al.*, 2015). Although ground-dwelling carabids are effective weed seed consumers and predators to many agricultural pests (Kromp, 1999; Honěk *et al.*, 2003; Bohan *et al.*, 2011; Winqvist *et al.*, 2011) the sublethal effects of insecticides have still been little investigated in these insects (Prasifka *et al.*, 2008; Giglio *et al.*, 2011).

Considering the importance of the predatory carabid community in agricultural land, this current thesis was focused on the sub-lethal effects of pyrethroid and neonicotinoid insecticides on basic behaviours such as locomotion, feeding and thermoregulation using *Platynus assimilis* (Coleoptera: Carabidae) as an example. The results of this study should give a better understanding of reduced insecticide use in these non-targeted beneficial insects, and should be taken into account in Integrated Pest Management programs (IPM).

2. REVIEW OF THE LITERATURE

2.1. Neurotoxic insecticide use in agriculture

Along with the growing human population, the use of insecticides has increased from year to year to protect agricultural crop plants from damage by pest insects. Of total world pesticide utilisation, 29.5 % is the share of insecticides (De *et al.*, 2014). Globally, formulations based on organophosphorous, pyrethroid and neonicotinoid chemicals belong to the top three of the most widely used insecticides. By their mode of action, all three are neurotoxic. Up to 40% of global crop yields are lost to pests and diseases every year and for this reason, the application of chemical insecticides remains in the foreseeable future (Field *et al.*, 2017).

Pyrethroids are estimated at 23% of the insecticide world market and are extensively used in agricultural and non-agricultural applications (Schleier, Peterson, 2011; Li *et al.*, 2017). Because of their physiochemical characteristics and contact nature, pyrethroids are highly bio-effective in pest control with relatively low toxicity to mammals (Schleier, Peterson, 2011). Under these circumstances, these insecticides are applied by foliar spraying in large quantities over a wide range of agricultural crops, including oilseed rape, cereals, beans, peas, vegetables etc (Thatheyus, Selvam, 2013). Even though insecticides are designed to kill herbivorous pests, non-target beneficial organisms are still inevitably exposed to the treatment.

Neonicotinoids are registered for use by farmers in more than 120 countries, covering approximately 25% of the global insecticide market (Jeschke *et al.*, 2011; Pollack, 2011). Their use in agriculture, horticulture and forestry can be diverse: seed dressing and pilling, treatment of soil, foliar spraying, seedling dipping etc (Jeschke *et al.* 2011; Simon-Delso *et al.*, 2015). Seed treatments with these insecticides ensure protection of pests for several months (Jeschke *et al.*, 2011) and thus are used in many agricultural crops such as oilseed rape, beets, sunflower, cereals, and potato (Goulson, 2013). As a result, seed and soil treatments have established about 60% of their applications around the world (Jeschke *et al.*, 2011; Goulson, 2013). In most European countries, 100% of neonicotinoid treated sugar beet seeds are used in conventionally cultivated fields (Hauer *et al.*, 2017) and given the systemic nature of these chemicals, additional insecticide applications against pests are not needed during

the vegetation period. The active substance of neonicotinoid insecticide translocates to all plant parts and thus becomes toxic to herbivorous insects (Bonmatin *et al.*, 2014; Simon-Delso *et al.*, 2015). If phytophagous insects feed from the treated plants, they get intoxicated and consequently become easy prey items for predatory arthropods.

Pyrethroids and neonicotinoids are chemically persistent and remain in soil for a long time. There is a remarkable difference between neonicotinoid and pyrethroid insecticide dissipation time in soil. In most cases, the estimated dissipation time for neonicotinoids ranges from 200-1000 days (Goulson, 2013). Existing literature data vary to a great extent, however. For example, the estimated dissipation time for the neonicotinoid insecticide thiamethoxam varies from 7 to 3000 days, depending on soil type, methods used, and publication (Goulson, 2013). For the pyrethroid insecticides however, dissipation time for the active ingredient is less than 55 days, with faster degradation occurring on dry soil (Schleier, Peterson, 2011). Furthermore, it is essential to remember that insecticide application, even at very low dosages, may inevitably lead to widespread environmental contamination. Pyrethroids have a high affinity for strong absorption to soil particles, but there can still be a drift through spray application, wind erosion and heavy rain (Schleier, Peterson, 2011). Sur and Stork (2003) have estimated that in the case of neonicotinoid seed dressings, only 1.6 to 20% of the active substance is absorbed by the crop plant so these chemicals may drift to surface water and adjacent vegetation.

2.2. Deleterious effects of neurotoxic insecticides on non-target beneficial insects

Although insecticides are designed to kill pest insects, they threaten many non-target species as well. Besides causing direct mortality (Douglas *et al.*, 2014; Cutler *et al.*, 2016), exposure to these chemicals at low doses and concentrations may cause a broad variety of physiological and behavioural sub-lethal effects in beneficial insects like pollinators and parasitoids, severely reducing their ecological performance and negatively affecting their population dynamics (Haynes, 1988; Desneux *et al.* 2007; Goulson, 2013; Pisa *et al.* 2015). Pesticide induced alterations in behavioural endpoints of predatory arthropods, including carabids, have received little and only sporadic attention, however (Baatrup, Bayley, 1993; Alzogaray, Zerba, 2001; Prasifka *et al.* 2008; Giglio *et al.* 2011; Douglas *et al.* 2014).

2.3. Basic behaviours as valuable ecotoxicological biomarkers

Almost all animals are able to move. Locomotion is central to many important behavioural patterns such as habitat and microhabitat selection, migration, exploration, predation and escape responses, behavioural thermoregulation, resource searching behaviours, inter- and intraspecific interactions etc. Pesticide induced changes in locomotor activity (LA) mirror an integrated response of the animal to changes in biochemical, physiological and neural processes (Jensen *et al.*, 1997; Baatrup, Bayley, 1998; Bayley, 2002) and consequently, may negatively affect many essential, fitness-related behaviours (Haynes, 1988; Everts *et al.*, 1991; Bayley, 2002). Thus, locomotor activity parameters such as distance moved, time spent moving, velocity and others reflect the physiological and ecological status of the animal and from an ecotoxicological perspective, serve as commonly accepted biomarkers of toxic stress in individuals exposed to sublethal doses of pesticides (Baatrup, Bayley, 1998; Bayley, 2002; Desneux *et al.*, 2007; Prasifka *et al.*, 2008).

The ability to procure enough food is pivotal for the survival of all animals. Feeding is necessary for the maintenance of metabolism, growth and reproductive success and is therefore a second important behavioural biomarker in ecotoxicological studies. By contrast to locomotor activity parameters (Desneux *et al.*, 2007; Prasifka *et al.*, 2008), an easily quantifiable food consumption rate allows direct evaluation of biocontrol efficiency of beneficial predatory arthropods, such as carabids. On pesticide contaminated food items, feeding activity of a number of insects may be inhibited by repellent, antifeedant and reduced olfactory capacity effects caused by these chemicals (Blaney *et al.*, 1990; Desneux *et al.*, 2007; Han *et al.*, 2010a, b; Abouelghar *et al.*, 2013; He *et al.*, 2013). Reduced feeding also occurs in herbivorous insects when they, after exposure to the insecticide azadirachtin (a limonoid present in neem seeds), return to clean food (Mordue Luntz *et al.*, 1985; Rembold, 1989; Simmonds *et al.*, 1990; Koul, Isman, 1991; Timmins, Reynolds, 1992). Abnormalities in clean food consumption rate (CFCR) express toxic stress caused by exposure to pesticides (Sheehan, 1984). So far, no data is available on CFCR for natural enemies of pest insects such as predatory carabids, after exposure to low doses of pesticides.

2.4. The main routes of exposure to pesticides

Predatory carabids, both larvae and adults, are effective natural enemies of many agricultural pests (Kromp, 1999; Lundgren, 2009; Winqvist *et al.*, 2011) and voracious consumers of weed seeds (Honěk *et al.*, 2003; Bohan *et al.*, 2011). They live on the soil surface and run on the ground under the foliage of crop plants. During the whole vegetation period, abundant and species-rich carabids are very active and move considerable distances each day (Thiele, 1977). They may also migrate between field margins and crop fields. Thus, these non-target beneficial arthropods are inevitably exposed to lethal as well as sub-lethal doses of insecticides in treated fields. In carabids, the main routes of potential pesticide exposure are direct hits by spray droplets, contact with contaminated soil particles and plants, as well as by feeding on poisoned prey items and weed seeds (Kunkel *et al.*, 2001; Langan *et al.*, 2001; Prasifka *et al.*, 2008). Carabid communities tend to recover within a month after treatment, but with repeated insecticide application, their numbers and diversity may be severely reduced in agricultural land (Thiele, 1977; Kromp, 1999; Holland, Luff, 2000; Kunkel *et al.*, 2001; van Toor, 2006).

2.5. The model carabid of the study

The test insect of this thesis is the common Euro-Siberian carabid beetle *P. assimilis*. It is a spring-breeder preferring forest habitats and gardens (Thiele, 1977; Lindroth, 1986). Early in the season, it migrates from forest areas into adjacent open fields (Honěk, Kocian, 2003), cereal fields (Purtauf *et al.*, 2005a, b) and grasslands (Grandchamp *et al.*, 2005) to feed. Trophically, *P. assimilis* belongs to the generalist predator guild (Larochelle, 1990; Zalewski *et al.*, 2014). Due to its wide geographical distribution in various ecosystems, this species was considered to be a good representative of carabids for the ecotoxicological studies presented in this thesis.

3. HYPOTHESES AND AIMS OF THE STUDY

In order to use insecticides sustainably, fundamental knowledge about the side-effects of these chemicals on non-target organisms is needed. Data about the sub-lethal effects of pyrethroids and neonicotinoids on beneficial predatory arthropods, including carabids, is still insufficient. In this study, for the first time in insect predators, the sub-lethal effects of pyrethroid and neonicotinoid insecticides over a wide range of administered low doses on the basic behaviours, specifically on the locomotor and feeding activities as well as on behavioural thermoregulation were quantified. The doses and concentrations of the insecticides used were representative of those carabids would encounter in the field.

The hypotheses of the study were:

- Exposure of carabid beetles to neurotoxic pyrethroid and neonicotinoid insecticides over a broad range of sublethal doses and concentrations causes substantial changes in their general motor (GMA) and LA, both in short- and long-term timescales.
- The ability of ground-dwelling carabids for adequate behavioural thermoregulation is negatively affected by sub-lethal doses of neurotoxic pyrethroid insecticides such as alpha-cypermethrin (α -cyp).
- Exposure to sub-lethal doses of neonicotinoid insecticides causes overall chemical stress in predatory carabids, expressed by changes in their feeding rate.

Based on the hypotheses the following aims were formulated:

- 1) To test the potential effects of low doses of α -cyp on the GMA and LA of the common Euro-Siberian carabid *P. assimilis* under controlled laboratory conditions (I).
- 2) To assess the sub-lethal effects of α -cyp on the behavioural thermoregulation of the carabid *P. assimilis* (II).
- 3) To assess short- and long-term effects of thiamethoxam on LA and CFCR of *P. assimilis* (III).

4. MATERIALS AND METHODS

4.1. Test beetles

The *P. assimilis* test beetles were collected from their preferred overwintering sites in brown-rotted tree stumps at a forest margin of Alam-Pedja Nature Reserve (southern Estonia) where no insecticides had been used previously. This guaranteed that they had not been in contact with these chemicals before the experiments. They were collected in April (**I, II, III**) and May (**I, II**) when air temperatures rarely exceeded 10 °C and ground temperatures ranged from 2 to 6 °C. They were kept in a refrigerator at 5-6 °C, in translucent plastic boxes filled with moistened sand and moss (**I**) or brown-rotted wood (**II, III**). 3-5 days prior to the insecticide treatments, they were placed singly in Petri dishes (85 mm diameter) lined with moistened Whatman No.1 filter paper and transferred to a Versatile Environmental Test Chamber MLR-35 1H (SANYO Electric Co., Ltd, Osaka Japan) at 20 °C, 16 h light and 8 h dark (16L: 8D) photoperiod and 70% RH. During their acclimation period, beetles destined for feeding experiments with neonicotinoids were not fed (**III**), but those destined for pyrethroid treatments (**I, II**) were provided daily with clean water and commercial moistened cat food (Friskies Vitality + Nestle Purina, Hungary). After acclimation, the beetles were used in video tracking experiments.

4.2. Insecticide treatments

To ensure that all body parts of the test insects were treated with the pyrethroid insecticide, as might occur during crop plant spraying in the field in spring, a conventional dipping method was used (**I, II**). To simulate probable predation on the poisoned prey items and weed seeds by the carabids after insecticide treatments in the field, food homogenate with a wide range of sub-lethal doses of a neonicotinoid insecticide were administered orally to test insects (**III**).

4.2.1. Fastac® 50EC (BASF)

Commercial insecticide Fastac® 50EC emulsions in water with different concentrations (0.01 to 100 mg L⁻¹) of active substance α -cyp were

prepared immediately before the treatments. Test beetles were dipped in insecticide emulsions for 10 s and, after treatment, were placed singly in Petri dishes (I) or in a special thermal mosaic arena (II) for observations and video-recording of their behaviour. In these experiments, the beetles were exposed to insecticide concentrations 7.5-75 000-fold lower than the maximum field recommended concentration (MFRC) for foliar treatments (750 mg L⁻¹). Control beetles were dipped in distilled water for the same period of time.

4.2.2. Actara® 25WG (Novartis)

Fresh insecticide suspensions and food homogenate from the blue bottle fly larvae (*Calliphora vomitoria*) and tap water were prepared directly before the treatments. For oral administration, 20 µL portions of food homogenate and insecticide mixtures were pipetted into 100 µL hollow plugs cut from 1 mL Eppendorf tubes and presented to the beetles for feeding. The respective thiamethoxam doses used in six insecticide treatment variants, in diminishing order, were 4×10^{-2} , 12×10^{-3} , 4×10^{-3} , 12×10^{-4} , 4×10^{-4} and 4×10^{-5} µg per 20 µL food portion. Considering the mean weight of 37 mg for the beetles (N = 50), the mean doses of thiamethoxam administered to the beetles were 1081.1; 324.3; 108.1; 32.4; 10.8 and 1.1 ng per 1 g of body weight. Thus, the administered insecticide doses were up to about 100-fold lower than the estimated LD₅₀ of thiamethoxam for *P. assimilis* (114.5 ng/g). The same amount (20 µL) of food homogenate with no insecticide content was administered to the control beetles (III).

In the acute toxicity test (LD₅₀), the thiamethoxam treated beetles were kept singly in Petri dishes with moistened filter paper at 20 °C, 16 h light, 8 h dark photoperiod, 100% RH. Dead and live beetles were counted on the fourth day after treatment (III).

4.3. Behavioural experiments

4.3.1. Video tracking

For automated video-tracking, the treated beetles were placed singly into 85–110 mm Petri dishes lined with moistened Whatman filter paper discs (Whatman International Ltd., England) and transferred

into a 50 × 60 × 50 cm lightproof cardboard box (**I**, **III**), or moved to the middle of an experimental thermal mosaic arena placed in a 60 × 60 × 60 cm carton box (**II**). Motor activity of the beetles was recorded by a computer-centered video-tracking system using the computer software Debut Video Capture (NCH Software, USA) and the USB web cameras C120 (Logitech Inc., USA) with the resolution of 640 × 480 pixels at 1 frame s⁻¹ (**I**) and at 15 frames s⁻¹ (**II**), and the USB Logitech HD Pro Webcam C920 (Logitech Inc., USA) with the resolution of 1920 × 1080 pixels at 5 frames s⁻¹ (**III**). Activity of the beetles in Petri dish arenas was video-recorded at 20 °C (**I**, **III**). Temperature on the thermal mosaic arena increased from 20 to 46.6 °C during the experiment (**II**). All Petri dishes with test beetles were numbered to allow recording and analysis of behaviour of individual beetles during the experimental period. The time gap between treatments and video tracking on Day 1 was 20 (**I**), 60 (**II**) and 30 (**III**) minutes, to let the test beetles settle. The number of insecticide treatments and video recording durations varied in different experiments (Table 1).

Table 1. Overview of the video tracking experiments.

Paper	Active ingredient	Number of treatments	Number of replicates	Recording duration (min)		
				Day 1	Day 2	Day 4
I	α-cyp	5+control	10	240	240	-
II	α-cyp	3+control	20	15	15	-
III	thiamethoxam	3+control	24–46*	240+60**	120+60**	120+60**

* During the experiment the number of tested beetles varied because of the partial mortality of the insecticide treated beetles.

**Recordings were made with no food and with clean food source.

4.3.2. CFCR (**III**)

After oral administration of thiamethoxam, CFCR of the treated and control beetles was measured within 1 h on 3 days, 5, 24 and 72 h after the treatments, respectively. Each time, 60 µL of fresh, clean food homogenate from blue bottle fly larvae and tap water was pipetted into 100 µL hollow plugs of Eppendorf tubes and presented to the beetles for feeding. Clean food was weighed immediately before and after feeding, and the difference between the two weights served as the food amount consumed. The tests were carried out in four replications up to 12 beetles in each. The number of test beetles in each replication decreased during the experiment, however, because some of the beetles died due to intoxication.

4.4. Data management and statistical analyses

Several activity parameters were extracted from EthoVision XT Version 9 software (Noldus information Technology, Wageningen, The Netherlands):

- 1) GMA – the number of movements per minute (**I**);
- 2) LA:
 - a) distance moved – start velocity $> 0.20 \text{ cm s}^{-1}$ and stop velocity $< 0.20 \text{ cm s}^{-1}$ (**I**; **II**; **III**);
 - b) the number of entries into the food source area (FSA) (**III**);
 - c) time spent in FSA (**III**);
 - d) time spent in different temperatures, s (**II**);
 - e) the number of hidings in shelters (**II**);
- 3) duration of knockdown (**I**);
- 4) the number of beetles killed by thermoshock (**II**).

Statistical analyses were performed with the statistical software STATISTICA 8.0 (**I**, **II**, StatSoft, USA), STATISTICA 11 (**III**, StatSoft, USA) and R ver 3.1.2 (**III**, R Development Core Team, Austria). The significant effect of α -cyp to the GMA and LA in *P. assimilis* was determined by the analysis of variance (ANOVA) Kruskal-Wallis test (**I**). In behavioural thermoregulation studies with α -cyp General Linear Model and Tukey test (**II**) were used. The LD_{50} of thiamethoxan for *P. assimilis* was found by using probit analysis (**III**). To compare behavioural parameters of *P. assimilis* in various treatments Kruskal-Wallis test was used (**III**). Treatment effects were discussed at the significance level of $P \leq 0.05$ (**I**, **II**, **III**).

5. RESULTS

5.1. The sublethal effects of α -cyp on the GMA and LA of the carabid beetle *P. assimilis*

At MFRC of α -cyp (750 mg L⁻¹), Fastac[®] caused 100% mortality of the tested carabid beetles within a few hours of treatment. At 0.01–100 mg L⁻¹, the beetles survived brief (10 s) exposure to the insecticide for four days after its application. Thus, the doses received by the beetles were considered as sub-lethal for *P. assimilis* adults. Individual GMA actogram plots demonstrated that α -cyp treated beetles showed remarkable changes in normal GMA rate and pattern, compared to the control group (Fig. 1, **I**). The pronounced rhythmicity of short GMA periods and relatively long-lasting periods of rest, characteristic of beetles not contacted with the insecticide, disappeared for shorter or longer periods of time depending on insecticide concentration. The beetles displayed general motor hyperactivity for several hours after exposure to α -cyp at 0.1 to 10 mg L⁻¹ (Figs. 2C, 3A, **I**). Next day, the beetles recovered from this kind of hyperactivity and at medium concentrations of α -cyp (0.1 and 1 mg L⁻¹), they showed general motor hypoactivity instead (Figs. 2D, 3A, **I**).

A nearly similar effect of α -cyp was observed on the LA of the beetles. At lower concentrations (0.1 and 0.01 mg L⁻¹), α -cyp caused initial short-term (< 2 h) locomotor hyperactivity (Figs. 2E, 3B, **I**) followed by a long-term (>24 h) locomotor hypoactivity of the beetles (Figs. 2F, 3B, **I**). This excitatory effect was short-lived, however, lasting 120 min (ANOVA Kruskal–Wallis test: H=14.66; N=10; P=0.0001) and 90 min (ANOVA Kruskal–Wallis test: H=9.53; N=10; P=0.002) after treatment with Fastac[®] at 0.1 and 0.01 mg L⁻¹ α -cyp, respectively (Fig. 2E, **I**). By contrast, at higher concentrations (10 and 100 mg L⁻¹), a drastic decrease in LA of the beetles occurred after exposure to the insecticide compared to that of the control group (Figs. 2E, 3B, **I**) due to the extremely high percentage time of knockdown effect at these α -cyp concentrations (Fig. 2A, **I**). Next day after α -cyp treatments, all the beetles were in a state of locomotor hypoactivity, independent of the insecticide concentration applied (Fig. 2F, **I**).

5.2. The sublethal effects of α -cyp on the behavioural thermoregulation of *P. assimilis*

5.2.1. The proportion of the insecticide treated beetles killed by overheating on the experimental thermal mosaic arena

Video-tracking experiments on the thermal mosaic arena with beetles of *P. assimilis* after brief (10 s) exposure to α -cyp at 0.1, 1.0 and 10 mg L⁻¹ were conducted on two consecutive days. 100% of beetles exposed to α -cyp at 10 mg L⁻¹ were knocked down. As they were not able to move, they were not used in the experiment on Day 1, but they had fully recovered from knock-down Day 2.

It was found that, after exposure to α -cyp, not all the beetles were able to thermoregulate behaviourally, and died as a result of thermal shock when temperatures on the arena rose to a noxious high level up to 44.6 °C. Several steps of thermal shock were observed. First, the beetles showed uncoordinated movements and partial paralysis of the hind legs. Then, with further temperature increase, total paralysis developed, the beetles stopped moving and died from overheating. The observed thermo-shock response was dependent on the insecticide concentration and time after insecticide application with higher mortality rates at higher α -cyp concentrations. By Day 2, the α -cyp treated beetles had recovered their ability for behavioural thermoregulation to a large extent. For example, 47% of 1 mg L⁻¹ α -cyp treated beetles died on Day 1 but only 9% died on Day 2 (Table 2, **II**). For comparison, none of the beetles in the control group died in the thermal mosaic arena experiment.

5.2.2. The effect of α -cyp exposure on the LA of the beetles

The beetles exposed to α -cyp showed remarkable locomotor hyperactivity on the thermal mosaic arena on Day 1, travelling considerably longer distances (2.6–24-fold in mean) than the control group (Fig. 2A, **II**). The relative difference in LA between the α -cyp treated and control beetles even increased with temperature increase (GLM Tukey test). These results showed that α -cyp negatively affected the ability of the beetles to avoid high temperatures in open areas of the experimental thermal mosaic arena on Day 1. By contrast, the beetles in the control group preferred to stay in shelters with much lower temperatures when temperatures on the open areas of the arena reached dangerously high levels (Fig. 3, **II**). However,

by the day following exposure to 0.1 and 1 mg L⁻¹ α -cyp, the beetles had fully recovered from locomotor hyperactivity (Fig. 2B, **II**). Beetles treated with the insecticide at 10 mg L⁻¹ had fully recovered from knock-down by Day 2. The distances they moved did not differ from those of the control group (Fig. 2B, **II**). Surprisingly, on both days of the experiment, the stimulating effect of α -cyp on the LA of the insects did not depend significantly on its concentration (Fig. 2A, B, **II**).

5.2.3. The effect of α -cyp on the tendency of the beetles to hide in shelters

On Day 1, over the whole range of temperatures from 25 to 44.6 °C, the beetles treated with α -cyp were actively moving and visited shelters considerably more frequently than the control beetles (Fig. 3A, **II**). The difference in the number of runs out of shelters between the α -cyp treated and control beetles even increased with temperature increase (GLM Tukey test, Fig. 3A, **II**) demonstrating that intoxicated beetles were significantly less able to avoid dangerously high temperatures on the open arena. However, surprisingly, the response did not depend on insecticide concentration. Although, some small effects of insecticide exposure on the tendency to hide in shelters were observed on Day 2, the differences were not statistically significant from the control (Fig. 3B, **II**).

5.2.4. The effect of α -cyp exposure on time spent by the beetles at noxious high temperatures

On both days of the experiment, the beetles not treated with insecticide spent 80% of the 900 s heating period in the shelter with the lowest temperature adjusted to 20 °C (Fig. 4A, B, **II**). The time they spent at temperatures above 25 °C drastically decreased with temperature increase (GLM Tukey test, Fig. 4A, B, **II**) demonstrating their ability effectively to avoid noxious heat on the thermal mosaic arena. For example, their exposure to high temperatures in the range of 35.1 to 40 °C was 38.5- and 13.6-fold shorter, on Day 1 and Day 2, respectively, compared to time they spent at the preferred 20 °C (GLM Tukey test, Fig. 4A, B, **II**). By contrast, the time the beetles exposed to α -cyp spent in the 20 °C shelter was shorter by 14.6–51.5% compared to the control depending on the insecticide concentration and time after the treatment (Fig. 4A, B, **II**). They also avoided temperatures above 25 °C (GLM Tukey test, Fig. 4A, B, **II**) but with less success compared to the control group. For example,

on Day 1, the beetles treated with 0.1 and 1 mg L⁻¹ α -cyp were exposed to high temperatures of 25–40 °C 1.9–8.5-fold longer compared to the beetles in the control group (Fig. 4A, **II**). The differences in time exposed to elevated temperatures between the α -cyp treated and negative control beetles even increased with temperature increase (GLM Tukey test, Fig. 4A, B, **II**). However, the time of exposure to high temperatures did not depend on tested α -cyp concentration (Fig. 4A, **II**). The negative effect of α -cyp exposure to heat avoidance efficacy was expressed also on the day following insecticide application, although to a lesser extent (Fig. 4B, **II**).

5.3. The sublethal effects of thiamethoxam on the LA and CFCR

Behavioural experiments were conducted with the carabid beetles *P. assimilis* dietary exposed to the neonicotinoid insecticide thiamethoxam at 1.1 to 108.1 ng g⁻¹. For comparison, the acute toxicity tests showed that with oral administration, the LD₅₀ of thiamethoxam for the adults of this species was 114.5 ng g⁻¹ (Fig. 1, **III**).

5.3.1. Effect of sublethal doses of thiamethoxam on the LA rhythms

LA actograms of individual beetles showed that in control beetles, short LA periods of several minutes duration alternated with long resting periods usually lasting from 10 to 60 min (Fig. 2A–C, **III**). Typically, their LA peaks reached 0.1 to 0.3 m min⁻¹. After dietary administration of thiamethoxam, LA pattern of the beetles changed depending on the insecticide dose and time after its application. Within several hours after the administration of thiamethoxam at the highest dose (108.1 ng g⁻¹), the beetles progressively became hyperactive, their LA peaks (m min⁻¹) increased, resting periods drastically decreased or disappeared (Fig. 2J, **III**). At lower doses (1.1 and 10.8 ng g⁻¹), thiamethoxam had no remarkable effect on the LA pattern of the beetles on Day 1 (Fig. 2D, G, **III**). By contrast, on Day 2, the beetles treated with the insecticide at 10.8 and 108.1 ng g⁻¹ were in a state of locomotor hypoactivity, they moved infrequently and crossed very short distances at a time, usually no more than 0.01 to 0.05 m min⁻¹ (Fig. 2 H, K, **III**). By Day 4, all the beetles, independent of the administered thiamethoxam dose, had fully recovered from the locomotion abnormalities and their LA actograms did not considerably differ from those of the control (Fig. 2F, I, L, **III**).

5.3.2. Effect on the LA, in terms of the distances moved

Distances moved by the beetles depended on both the dose of thiamethoxam administered and the time since treatment (Fig. 3A, **III**). Within several hours of thiamethoxam application at the highest dose (108.1 ng g⁻¹), the beetles became hyperactive. At LA peak, they moved up to 10.6-fold further (3.1–3.2 m per 30 min) than the control beetles (Fig. 3A, **III**). Lower doses of thiamethoxam (1.1 and 10.8 ng g⁻¹) did not affect LA of the beetles on Day 1 (Fig. 3A, **III**). On Day 2, the beetles, independent of the administered insecticide dose, were in a state of locomotor hypoactivity. Within 2 h of observation, their total distances moved were 3.3–11-fold shorter ranging from 0.3 to 1.1 m depending on the insecticide dose, compared to the 3.6 m moved by the control beetles (Fig. 3B, **III**). By Day 4, the beetles in all the three insecticide treatments had fully recovered from locomotor hypoactivity. Their total distance moved varied from 2.5 to 4.9 m per 120 min but significant differences from the control variant (3.6 m/120 min) were not observed (Fig. 3C, **III**).

5.3.3. Effect on the CFCR

Thiamethoxam negatively affected the feeding activity of the insecticide treated beetles. Several hours after insecticide application, a 9.2-fold fall in CFCR occurred in beetles treated with the highest dose (108.1 ng g⁻¹) of thiamethoxam compared to that of the control group (Fig. 4A, **III**). On the contrary, after dietary administration of thiamethoxam at lowered doses of 1.1 and 10.8 ng g⁻¹, no significant differences were found between the CFCRs of the insecticide treated and control insects on Day 1 (Fig. 4A, **III**). Several aspects of LA close to the food source were also measured and analysed. However, no significant differences between the insecticide treated and control insects were found concerning the total distance moved, the number of visits into the FSA and the time spent in the FSA (Fig. 4B–D, **III**). Thus, none of these LA parameters reflected remarkable insecticide dose dependent changes in CFCR on Day 1 (Fig. 4A–D, **III**).

Thiamethoxam suppressed the CFCR of the beetles even more strongly the day following application. All the insecticide treated insects consumed 1.5 to 11.8-fold less clean food homogenate than the control group depending on the administered dose (Fig. 4A, **III**). A significant decline also occurred in all three related LA parameters being in good

accordance with the CFCR response (Fig. 4A–D, **III**). By Day 4, however, the beetles had fully recovered from all the malfunctionings related to locomotion (Fig. 4B–D, **III**). Their normal CFCR had also recovered to a large extent (Fig. 4A, **III**). Despite no difference between the CFCRs in insecticide treated and control insects, thiamethoxam dose had a significant effect on feeding response (Fig. 4A, **III**). Thus, none of the parameters related to mobility mirrored thiamethoxam dose dependent changes in CFCR adequately (Fig. 4A–D, **III**).

6. DISCUSSION

In this thesis, for the first time in predatory insects, sub-lethal effects of α -cyp and thiamethoxam on basic behaviours, such as LA, behavioural thermoregulation and CFCR were quantified in the carabid *P. assimilis* over a broad range of low doses. The results show that carabid beetles are extremely susceptible to both of these neurotoxic insecticides. Concerning α -cyp, significant short- and long-term alterations in normal thermoregulatory behaviour and LA of the beetles occur after brief topical exposure to the insecticide at concentrations up to 7500- and 75 000-fold lower, respectively, than the respective MFRCs. A single dietary exposure to the neonicotinoid insecticide thiamethoxam at doses up to 104-fold lower than LD₅₀ cause considerable short- and long-term changes in both LA and CFCR of the beetles.

The scarce literature data demonstrate, that neurotoxic pyrethroid and organophosphate insecticides, at low doses and through various routes of exposure, cause almost instant elevated locomotor activity in a number of insects, including carabids (Gammon *et al.*, 1978; Benoit *et al.*, 1985; Wood *et al.*, 1993; Jensen *et al.*, 1997; Alzogaray, Zerba, 2001; Salerno *et al.*, 2002; Prasifka *et al.*, 2008) and in some other arthropods (Baatrup, Bayley, 1998). By contrast, at the high end of sublethal doses, these insecticides induce quick paralysis and knock-down response followed by a prolonged locomotor hypoactivity in various arthropods, lasting up to a couple of days or longer (Everts *et al.*, 1991; Baatrup, Bayley, 1993; Bayley, 1995; Jensen *et al.*, 1997; Douglas *et al.* 2014). Our results with the pyrethroid α -cyp and neonicotinoid thiamethoxam confirm these findings in the carabid *P. assimilis* (**I**, **II**, **III**). For example, a brief, single exposure to α -cyp at low sublethal concentrations (0.01 and 0.1 mg L⁻¹) caused immediate short-term locomotor hyperactivity followed by a long-term hypoactivity. At higher sublethal concentrations (10–100 mg L⁻¹) α -cyp evoked quick knockdown and strongly decreases LA of *P. assimilis* beetles within two days after the treatments (**I**). However, we found that, pyrethroid and neonicotinoid insecticides affect locomotion of the beetles differently. By contrast to α -cyp, the knockdown response never occurs after exposure to thiamethoxam (**I**, **III**). Also, the locomotor hyperactivity response develops much more slowly in beetles exposed to thiamethoxam than those exposed to α -cyp (**I**, **III**). Different modes of action of these two neurotoxic insecticides (Ray, Fry, 2006; Clark, Sym-

ington, 2012; Goulson, 2013; Simon-Delso *et al.* 2015), and different routes of exposure in the ecotoxicological experiments (I, III) may be behind these observations.

GMA response may give additional essential information on the intoxication rate of the non-target insects exposed to sublethal doses of insecticides as demonstrated, for the first time, in this study. Our results show that low doses of α -cyp may cause significant increase in GMA even in cases when pyrethroid induced locomotor hyperactivity changes over hypoactivity, and distances moved by the intoxicated and control insects do not differ (I). The GMA response also allows assessment of the intoxication rate in insects at high sublethal doses of neurotoxic insecticides when they are knocked down and locomotion is not possible. Thus, although exposure to sublethal doses of insecticides may cause short- and long-lasting changes in motor activities of the non-targeted arthropods, they have a remarkable ability to metabolize toxic remains and recover from the behavioural abnormalities to a large extent.

Observed significant, concentration- and time-dependent changes in LA and GMA rates and dynamics following exposure to α -cyp at concentrations 7.5 to 75 000-fold lower than the MFRC (http://ec.europa.eu/food/plant/protection/evaluation/exitactive/list_alpha_cypermethrin.pdf, accessed 11 March 2013) suggest the importance of these behavioural endpoints as extremely sensitive and valuable etho-toxicological biomarkers (I). Concerning LA as a good biomarker of toxic stress in arthropods, these results agree with earlier literature data (Jensen *et al.*, 1997; Baatrup, Bayley, 1998; Bayley, 2002). In this study, for the first time, we demonstrate that in addition to LA, GMA may considerably widen the scale of quantifiable motor activity responses to an insecticide (I).

Field studies with carabids have repeatedly revealed a paradoxical increase in pitfall trap catches following various insecticide applications. The number of trapped beetles in treated areas may remain high for several weeks (Coaker, 1966; Chiverton, 1984; Dixon, McKinlay, 1992; Bel'skaya *et al.*, 2002; Lopez *et al.*, 2005; Navntoft *et al.*, 2006). The reason for this phenomenon is unknown. Because pitfall trap catches mirror a combination of population density and LA (Thiele, 1977), some authors suppose that exposure to neurotoxic insecticides leads to a prolonged hyperactivity of the beetles with no impact on population densities in

insecticide-treated areas (Coaker, 1966; Bel'skaya, 2002; Lopez *et al.*, 2005; Navntoft *et al.*, 2006; Prasifka *et al.*, 2008). Our laboratory videotracking experiments with *P. assimilis* do not support this supposition. On the contrary, we found that the α -cyp induced state of elevated LA is a short-term response of the beetles, lasting a couple of hours (I). Thus, locomotor hyperactivity cannot increase pitfall trap catches of carabids in treated fields in a timescale of weeks.

According to the majority of field studies, however, carabids are vulnerable to insecticide control measures but their communities tend to recover within a month through immigration from adjacent untreated buffer areas. As a result of repeated insecticide treatments, the carabid number and diversity still decline (Vickerman, Sunderland, 1977; Chiverton, 1984; Holland, Luff, 2000; Huusela-Veistola, 2000; Wick, Freier, 2000; Goulet, 2003; van Toor, 2006). Both lethal and sublethal effects of insecticide exposure may contribute to low pitfall trap captures. Mortality rates caused by insecticide use may reach up to 81% in small carabids (Vickerman, Sunderland, 1977). Because carabids are highly mobile and intensively migrate between insecticide treated fields and untreated field margins, sublethal effects are of major concern in these insects. The number of beetles encountering low doses may be greater compared to that encountering acutely lethal doses.

Therefore, we suppose that prolonged, toxically induced locomotor hypoactivity of the beetles, as demonstrated for *P. assimilis* with α -cyp in this study (I) may contribute to low trap catches of carabids in the fields where insecticides are applied. The real number of beetles at various levels of intoxication should be larger than estimated by pitfall trap catches.

Although insecticide induced abnormalities in motor activities sensitively reflect intoxication rate, very little is known about their impact on the performance and population dynamics of insects in treated areas (Dempster, 1968; Kunkel *et al.*, 2001). Therefore, collecting quantitative data concerning sublethal effects of pesticides on the behavioural endpoints such as behavioural thermoregulation and CFCR directly affecting survival, populations and predation efficacy of carabids as natural enemies of pest insects, might be more useful.

Insects are able to thermoregulate behaviourally. Through automated videotracking experiments with *P. assimilis* on a thermal mosaic arena,

for the first time in arthropods, we showed that exposure to sub-lethal doses of α -cyp considerably impairs this ability (II). After exposure to the insecticide at high sublethal concentrations (10 mg L⁻¹, 75-fold diluted compared to MFRC), the beetles are quickly knocked down and temporarily lose their ability for locomotion and behavioural thermoregulation completely. At lower application rates of the insecticide (0.1 and 1 mg L⁻¹), the beetles display behavioural abnormalities leading to overheating and death in 9.1 to 47.4 % of the individuals depending on the concentration contacted and time following treatments. Remaining individuals, after contact with α -cyp, show unfavourable, up to 8.5-fold longer exposure to noxious high temperatures from 25 to 40 °C compared to the control group not contacted with the insecticide. The difference in time of exposure to noxious heat even increases in conjunction with temperature increase. Although the beetles are able to recover from deleterious effects of intoxication largely, these harmful sublethal effects in thermoregulation behaviour may last a couple of days. On sunny days when soil surface temperatures reach up to lethal levels, exposure to noxious high temperatures may be a common threat to ground dwelling carabids in open fields (Denlinger, Yocum, 1998; Must *et al.*, 2010). The fall in the tendency of poisoned beetles to hide in thermally favourable shelters (II), increases the probability of prolonged exposure to hot soil surface, IR radiation and rapid overheating.

Almost all life processes and functions of insects worsen at temperatures higher than their thermal optimum. Elevated temperatures have deleterious effects on insect growth, development and reproduction. Excessive temperatures also impair insect metabolism, respiration, thermotolerance, nervous and endocrine systems (Denlinger, Yocum, 1998; Feder, Krebs, 1998; Neven, 2000; Chown, Terblanche, 2007; Huang *et al.*, 2007; Xu *et al.*, 2010). Prolonged exposure to noxious heat causes changes in cell pH and ion concentrations with destructive consequences to vital macromolecules, cell and nuclear membranes, mitochondria and ribosomes (Hochachka, Somero, 1984; Denlinger, Yocum, 1998; Reiber, Birchard, 1993; Neven, 2000).

Thus, our results show that behavioural thermoregulation of carabids is a sensitive, valuable and easily quantifiable ethotoxicological biomarker. α -cyp induced decline in efficacy for avoiding prolonged exposure to noxious heat as demonstrated in a ground dwelling carabid beetle in this study, inevitably leads to negative consequences of heat stress which

directly deteriorate their ecological fitness, performance and population dynamics.

For the first time in predatory insects, we found that exposure to neurotoxic insecticides may also affect their CFCR. A significant reduction in feeding rate may occur after oral administration of low doses of thiamethoxam in carabids as exemplified for *P. assimilis* in this study (III). This response depends on both dose received and time since exposure. The decline in CFCR is a prolonged response of the beetles to the insecticide, which may continue for several days. CFCR is inhibited in both hyper- and hypoactive beetles. We observed that the CFCR response provides useful information about toxic stress also in case when locomotor hyperactivity changes over hypoactivity and the distances moved by the intoxicated beetles and those of the control group do not differ.

In captivity, carabids eagerly predate on neonicotinoid treated seeds and prey animals (Douglas *et al.*, 2014; Cutler *et al.*, 2016). Both larvae and adults are voracious feeders and may ingest close to their own body mass of food each day (Thiele, 1977). Thus, in fields where neonicotinoid insecticides are applied, they could be chronically exposed to these chemicals through feeding on contaminated prey items and plant tissues (Mullin *et al.*, 2005; Moser, Obrycki, 2009; Seagraves, Lundgren, 2012), and they can encounter seed-treated neonicotinoid insecticides through tritrophic food webs (Szczepaniec *et al.*, 2011; Douglas *et al.*, 2014). Both direct mortality (Douglas *et al.*, 2014; Cutler *et al.*, 2016) and insecticide induced, prolonged reduction in feeding (III) may negatively affect efficacy of carabids as natural enemies of phytophagous pest insects in agricultural land where neonicotinoid insecticides are applied.

The observed decline in feeding following exposure to low dose thiamethoxam may also have a detrimental impact on the ecological fitness and populations of carabids. In these predators, feeding rate and its dietary quality during larval development determine body mass and potential fecundity of the reproductively active beetles (Nelemans, 1987; Lövei, Sunderland, 1996; Knapp, Uhnová, 2014). However, the real number of eggs laid depends on adult feeding conditions. At reduced feeding rates, females lay considerably fewer eggs than when unlimited food is available (Sota, 1985; Nelemans *et al.*, 1989; Weseloh, 1993; van Dijk, 1994; Knapp, Uhnová, 2014). Also, a prolonged shortage in food strongly shortens the potential survival period of both carabid adults and larvae

(van Dinther, 1964; Kabacik-Wasylik, Stejgwilllo-Laudanska, 1971; Luff, 1994; Petersen, 1999; Young, 2008).

Consequently, the results of our laboratory experiments, carried out within the frame of this thesis, demonstrate that neurotoxic insecticides α -cyp and thiamethoxam, even at extremely low doses and concentrations, probably cause a number of behavioural abnormalities in non-targeted predatory carabids in fields where these chemicals are applied. For the first time, we show that, in addition to locomotion, GMA, behavioural thermoregulation and CFCR are sensitive, valuable and easily quantifiable ecotoxicological biomarkers for predatory arthropods and mirror different aspects of toxic stress. These insecticide induced changes in normal behaviour ultimately may have a deleterious impact on ecological fitness, population dynamics and plant protection performance of these beneficial insects. The results of this study may have importance in IPM programs promoting reduced insecticide use and encouraging natural pest control mechanisms.

7. CONCLUSIONS

The results of this study confirm the hypotheses formulated in this thesis and show that carabids are highly sensitive to low dose neurotoxic insecticide exposure.

It appears that α -cyp and thiamethoxam, over a wide range of low doses and concentrations, may cause a number of substantial short- and long-term abnormalities in many basic behaviours such as LA, GMA, CFCR and behavioural thermoregulation in carabids. The observed sublethal behavioural biases may lead to deterioration of ecological fitness and population dynamics of the beetles in agricultural lands where these chemicals are applied.

For the first time in predatory arthropods, we demonstrate that in addition to locomotion, other important behavioural endpoints such GMA, CFCR and behavioural thermoregulation may serve as sensitive, valuable and easily quantifiable ecotoxicological biomarkers mirroring different rates and aspects of toxic stress.

The observed remarkable declines in CFCR and efficiency of behavioural thermoregulation of the beetles exposed to sublethal doses of α -cyp and thiamethoxam may directly worsen performance of the carabids as natural enemies of pest insects in insecticide treated fields. Thus, the results of this study may have importance in IPM programs promoting reduced insecticide use and encouraging natural pest control mechanisms wherever possible.

Further field studies are needed, however, to explain the exact role of the negative sublethal behavioural effects on the population dynamics and biocontrol efficiency of carabids repeatedly exposed to these chemicals in agricultural land over a timescale of years.

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SUMMARY IN ESTONIAN

Järjest enam pööratakse põllumajanduses tähelepanu jätkusuutlikule taimekaitsevahendite kasutamisele. Neurotoksilised insektitsiidid nagu neonicotinoïdid ja püretroïdid on ühed enim kasutatavad taimekaitsevahendid maailmas, tänu oma laiale toimespektrile ning kõrgele efektiivusele (Desneux *et al.*, 2007; Garcia, 1011). Erinevalt kontaktse toimega püretroïdidest on neonicotinoïdsed insektitsiidid oma toimemehhanismi poolest süsteemsed, st. preparaadid liiguvad töötlemise järgselt üle kogu taime ning on seetõttu eriti tõhusad taimtoiduliste kahjurite tõrjel. Nagu nimigi ütleb, mõjutavad neurotoksilised insektitsiidid putukate kesk-närvisüsteemi, püretroïdide puhul lisaks ka piirde-närvisüsteemi talitlust (Ray, Fry, 2006; Garcia, 2011; Clark, Symington, 2012; Goulson, 2013; Simon-Delso *et al.*, 2015). Mõlemal juhul kutsuvad madalate aktiivainedoosidega töötlemine närvirakkudes esile ebaloomulikke korduvaid närviimpulsside valanguid, mis omakorda tekitavad putukatel paralüüsinähte (püretroïdide puhul „knockdown“ efekt) ning kõrgete dooside korral ka surma.

Kuigi nimetatud insektitsiidid on välja töötatud ennekõike kultuurtaimedest toituvate kahjurputukate tõrjeks, puutuvad nendega tahes tahtmata kokku ka kasulikud mitte-sihtorganismid, kelleks võivad olla näiteks parasitoidid, tolmeldajad ja röövtoidulised putukad. Kokkupuude taimekaitsevahendiga toimub kas pritsimisvedeliku piiskadega, maapinnal ja taimedel kuivanud jääkidega või saastunud toidu söömisel (Kunkel *et al.*, 2001; Mauchline *et al.*, 2004; van Toor, 2006). Taimekaitsevahendi toime putuka käitumisele sõltub doosist ning see võib olla kas letaalne (st. otsesest suremust tekitav) või subletaalne. Viimasel juhul elavad putukad preparaadiga töötlemise üle (Desneux *et al.*, 2007), kuid sellel on otsene mõju isendite füsioloogiale ja käitumisele (Thompson, 2003; Goulson, 2013; Pisa *et al.*, 2015). Populatsiooni seisukohast lähtudes võib kaudne mõju olla aga tihtilugu palju suurem, kui mürgitusest tingitud otsene suremus.

Insektitsiidide subletaalsete dooside kahjulik mõju mitte-sihtorganismidele on jätkuvalt aktuaalne uurimisteema, olles seni keskendunud pigem tolmeldajatele ja parasitoididele (Desneux *et al.*, 2007; Prasifka *et al.*, 2008; Pisa *et al.*, 2015). Röövtoiduliste jooksiklaste fauna on põllumajandusmaastikel liigirikkalt ja arvukalt esindatud ning neil on integreeritud kahjuritõrje programmides tähtis roll mulla umbrohuseemnete varude vähendamisel ning kahjurputukate arvukuse piiramisel (Kromp, 1999; Honék *et al.*, 2003; Bohan *et al.*, 2011; Winqvist *et al.*, 2011). Insek-

titsiidide subletaalseid mõjusid jooksiklaste käitumisele on seni kahjuks ebapiisavalt uuritud (Prasifka *et al.*, 2008; Giglio *et al.*, 2011).

Käitumuslikud ökotoksikoloogilised katsed aitavad hinnata ja kvantitatiivselt mõõta putukate keemilise stressi taset. Lokomotsioon peegeldab taimekaitsevahendi mõju organismide anatoomiale, füsioloogiale, neuroloogiale ja ainevahetustasemele (Baatrup, Bayley, 1993, 1998; Bayley, 2002; Desneux *et al.*, 2007). See on paljude liigisiseste ja liikidevaheliste käitumusmuutuste lahutamatu osa ning on seni enim kasutamist leidnud ökotoksikoloogiline biomarker (Bayley, 2002). Pestitsiidide poolt esile kutsutud muutuste selgitamine teistes põhikäitumistes (näiteks termoregulatsioon ja toitumine) võib anda täiendavat informatsiooni kasulike putukate toksilise stressi tasemest.

Käesoleva doktoritöö hüpoteesid:

- Jooksiklaste kokkupuude püretroidi ja neonikotinoidi erinevate kontsentratsioonide ja doosidega kutsub esile lühi- ja pikajalisi muutusi nende üldmotoorses- ja lokomotoorses aktiivsuses.
- Kokkupuude püretroidi sub-letaalsete kontsentratsioonidega halvendab jooksiklaste loomulikku termoregulatsiooni võimet.
- Neonikotinoidi sub-letaalsete doosidega saastunud toit põhjustab röövtoidualistel jooksiklastel üleüldist toksikoloogilist stressi, mis väljendub muutustena nende tavapärase lokomotoorses- ja toitumiskäitumises.

Nimetatud hüpoteeside testimiseks püstitati järgmised eesmärgid:

- 1) mõõta püretroidse insektitsiidi α -tsüpermetriini (edaspidi α -tsüp) madalate kontsentratsioonide võimalikke lühi- ja pikajalisi mõjusid üldmotoorsele ja lokomotoorsele aktiivsusele süsi-ketasjooksikul (*Platynus assimilis*) (I);
- 2) mõõta α -tsüp nõrkade dooside lühi- ja pikajalisi mõjusid süsi-ketasjooksiku käitumuslikule termoregulatsioonile (II);
- 3) mõõta neonikotinoid tiametoksaami lühi- ja pikajalist mõju süsi-ketasjooksiku lokomotoorsele aktiivsusele ja puhta toidu tarbimismääradele (III).

Laboratoorsed toksikoloogilised käitumiskatsed viidi läbi Eesti Maatülikooli Põllumajandus- ja Keskkonnainstituudi Taimekaitse osakonnas, putukate sensoorse füsioloogia laboratooriumis. Käesoleva doktoritöö uurimisobjektiks valiti Eurosiberi levikuga süsi-ketasjooksik (Lindroth, 1986), kes toitub erinevatest saakloomadest (Larochelle, 1990; Zalewski *et al.*, 2014). Liigi elupaikadeks on jahedad ja niisked metsad (Thiele, 1977; Lindroth, 1986), avamaastikud (Honěk, Kocian, 2003), sealhulgas teraviljapõllud (Purtauf *et al.*, 2005a, 2005b) ja rohumaad (Grandchamp *et al.*, 2005).

Käesolevas doktoritöös töödeldi katsemardikaid laialt kasutatavate neurotoksiliste preparaasidega, milleks on püretroid (Fastac® 50EC, BASF, aktiivaine α -tsüp) ja neonikotinoid (Actara® 25WG, Novartis, aktiivaine tiametoksaam). Putukate töötlemiseks insektitsiidiga kasutati tavapärasest sissekastmismeetodit (**I**; **II**) või suukaudset manustamist (**III**). Mardikate käitumist videofilmilt kõrge resolutsiooniga USB veebikaameratega (Logitech Inc., USA) sagedusega 5 kaadrit sekundis kasutades arvutitarkvara Debut Video Capture (NCH Software, USA). Saadud videosalvestusi analüüsiti programmiga EthoVision XT Version 9 (Noldus Information Technology, Wageningen, Holland). Mõõdeti järgmisi käitumise parameetreid: üldmotoorne aktiivsus (**I**), lokomotoorne aktiivsus väljendatuna läbitud tee pikkusena (**I**; **II**; **III**), toiduallika külastuste arv (**III**), toiduallika juures viibitud aeg (**III**), erinevate temperatuuride mõjualas viibimise aeg (**II**), varjete külastuste arv (**II**), „knockdown“ efekti vältus (**I**) ning termošoki tagajärjel hukkunud mardikate osakaal (**II**). Statistiline andmenalüüs viidi läbi kasutades tarkvara programme STATISTICA (StatSoft, USA) (**I**, **II**, **III**) ja R 3.1.2 (R Development Core Team, Austria) (**III**).

Katsed näitasid, et süsi-ketasjooksiku lühiajaline (10 s) kokkupuude maksimaalse põllul lubatava α -tsüp kontsentratsiooniga (750 mg L^{-1}) kutsus kõigest mõne tunniga esile mardikate 100% suremuse. Madalate kontsentratsioonide korral ($0,01\text{--}100 \text{ mg L}^{-1}$) elasid mardikad töötamise üle, kuid neil täheldati lühiajalist (<2h) kontsentratsioonisõltuvuslikku lokomotoorset hüperaktiivsust, millele järgnes pikaajaline (>24h) lokomotoorne hüpoaktiivsus. Töödeldud mardikatel muutus või kadus ka töötlemata mardikatele iseloomulik üldmotoorse aktiivsuse ja puhkeperioodide vaheldumise muster olenevalt püretroidi kontsentratsioonist ja töötamisele järgnenud ajast. Kõrgemad α -tsüp kontsentratsioonid ($10\text{--}100 \text{ mg L}^{-1}$) põhjustasid mardikatel „knockdown“ efekti, mis väljendus nende

selili kukkumises ja liikumisvõime kadumises. Teiseks päevaks pärast insektitsiidiga töötlust olid mardikad suurel määral nendest nähtudest taastunud, kuid nad jäid võrreldes kontrollmardikatega siiski motoorselt hüpoaktiivseteks (**I**). Sarnaselt püretroidiga põhjustas tiametoksaam mardikate hüper- ja hüpoaktiivsust. Mardikate reaktsioon nendele kahele neurotoksilisele insektitsiidile oli aga mõnevõrra erinev. Kui α -tsüpi kutsus mardikates esile „knockdown“ efekti, siis tiametoksaamil sellist mõju ei olnud. Lisaks sellele kujunes neonicotinoidiga töödeldud mardikatel hüperaktiivsus ajaliselt hiljem, kui seda täheldati püretroidi puhul. Selline tulemus võib olla tingitud nende insektitsiidide erinevast toimemehhanismist (Ray, Fry, 2006; Clarck, Symington, 2012; Goulson, 2013; Simon-Delso et al., 2015).

Mitme nädala vältel peale taimekaitsevahenditega töötlust on põllumajandusmaastikel täheldatud jooksiklaste väljapüükide suurenemist (Bel'skaya et al., 2002, Lopez et al., 2005; Nanoft et al., 2006) kuid kindlat põhjust sellele seni veel leitud ei ole. Arvatakse, et kokkupuude neurotoksiliste insektitsiididega kutsub mardikates esile kauakestva hüperaktiivsuse, mis suurendab kinnipüütud mardikate arvu pinnasepüünistes. Meie katsetulemused seda väidet ei kinnita.

Jooksiklased nagu ka paljud teised putukad reguleerivad oma kehatemperatuuri käitumuslikult. Käesolevas doktoritöös demonstreeriti esmakordselt α -tsüpi kahjulikku mõju jooksiklaste käitumusliku termoregulatsiooni võimele. Termomosaiksel areenil läbiviidud katsed näitasid, et mardikate lühiajaline töötlemine α -tsüpi-ga ($0,1\text{--}10\text{ mg L}^{-1}$) põhjustas olulisi muutusi süsi-ketasjooksiku normaalses käitumises. Vahetult pärast töötlust olid α -tsüpi mardikad võrreldes kontrollrühma mardikatega hüperaktiivsed, liikusid palju areenil ringi ning ei püsinud varjetes, kus temperatuur oli konstantselt $20\text{ }^{\circ}\text{C}$. Sellest tingituna viibisid nad kauem ebasoodsalt kõrgete temperatuuride ($25\text{--}45\text{ }^{\circ}\text{C}$) mõjualas. Kuna mardikate tavapärase võime kõrgeid temperatuure vältida oli halvenenud, hukkus suur osa neist ülekuumenemise tõttu. Püretroidi kahjulikku mõju mardikate termoregulatsiooni võimele täheldati veel 24 h pärast töötlust.

Püretroidiga töödeldud mardikate kalduvus mitte püsida termaalselt turvalistes varjetes (**II**) võib põllumajandusmaastikul väljenduda nende kestvama viibimisega kuuma mullapinnal, kus nad kõrgete temperatuuride ja päikesekiirguse mõjul võivad kergesti ülekuumeneda. Kõrged temperatuurid põhjustavad kahjulikke muutusi nii mardikate biokeemias kui

ka füsioloogias (Denlinger, Yocum, 1998; Feder, Krebs, 1998; Neven, 2000; Chown, Terblanche, 2007; Huang *et al.*, 2007; Xu *et al.*, 2010) mis lõppkokkuvõttes võivad vähendada jooksiklaste ökoloogilist kohasust ja populatsioone ning biotõrje efektiivsust põllumajandusmaastikel.

Esmakordselt demonstreeriti neonikotinoid tiametoksaami mõju röövtoidulise mardika toitumisaktiivsusele. Katsed näitasid, et tiametoksaam (1,1–108,1 ng/g) põhjustas märgatava languse süsi-ketasjooksiku toitumisaktiivsuses, mis sõltus nii insektitsiidi doosist kui ka töötlemisele järgnenud ajast. Tugevaim manustatud doos (108,1 ng/g) kutsus esile drastilise toitumisaktiivsuse languse kahel järjestikusel päeval pärast töötlust. Nõrkade dooside (1,1–10,8 ng/g) puhul oli toitumisaktiivsuse langus märgatav alles teisel päeval pärast töötlust. Katse viimasel, neljandal päeval mardikate toitumisaktiivsus taastus ja saavutas kontrollmardikatega võrdse taseme (III).

Laboritingimustes on täheldatud, et röövtoidulised jooksikud toituvad ahnelt nii neonikotinoidiga töödeldud seemnetest kui ka saakloomadest (Douglas *et al.*, 2014; Cutler *et al.*, 2016). Põldudel, kus kasutatakse taimekaitsevahendeid, tuleb jooksikutel tahes tahtmata kokku puutuda ning toituda insektitsiididega saastunud taimeosadest ja saakloomadest (Mullin *et al.*, 2005; Moser, Obrycki, 2009; Seagraves, Lundgren, 2012). Taimekaitsevahendi poolt põhjustatud mardikate otsene suremus (Douglas *et al.*, 2014; Cutler *et al.*, 2016), nende eluea lühenemine (van Dinther, 1964; Kabacik-Wasylik, Stejgwillo-Laudanska, 1971; Luff, 1994; Petersen, 1999; Young, 2008) ja toitumisaktiivsuse langus (III) võivad oluliselt vähendada röövtoiduliste jooksiklaste biotõrje potentsiaali.

Käesoleva doktoritöö käitumiskatsete tulemused kinnitavad töös püstitatud hüpoteese ning näitavad, et jooksikud on neurotoksiliste insektitsiidide suhtes ülimalt tundlikud. Esmakordselt demonstreeriti, et putukate põhikäitumised nagu lokomotoorne aktiivsus, käitumuslik termoregulatsioon ja puhta toidu tarbimismäär on tundlikud, väärtuslikud ja kergesti mõõdetavad ökotoksikoloogilised biomarkerid, mis aitavad lisaks lokomotsioonile hinnata röövtoiduliste jooksiklaste toksilise stressi erinevaid tasemeid ja aspekte. Edasised teadusuuringud peaksid selgitama püretroididega ja neonikotinoididega korduvtöötlemiste subletaalseid mõjusid jooksiklaste põhikäitumistele ja populatsiooni dünaamikale põllutingimustes aastatepikkuses ajaskaalas. Antud doktoritöös saadud tulemused võivad olla olulised integreeritud kahjuritõrje programmide väljatöötamisel, kus prioriteediks on jätkusuutlik taimekaitsevahendite vähendatud kasutamine ja loodusliku biotõrje potentsiaali ärakasutamine.

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Sub-lethal effects of the neurotoxic pyrethroid insecticide Fastac[®] 50EC on the general motor and locomotor activities of the non-targeted beneficial carabid beetle *Platynus assimilis* (Coleoptera: Carabidae)

Ene Tooming, Enno Merivee,* Anne Must, Ivar Sibul and Ingrid Williams

Abstract

BACKGROUND: Sub-lethal effects of pesticides on behavioural endpoints are poorly studied in carabids (Coleoptera: Carabidae) though changes in behaviour caused by chemical stress may affect populations of these non-targeted beneficial insects. General motor activity and locomotion are inherent in many behavioural patterns, and changes in these activities that result from xenobiotic influence mirror an integrated response of the insect to pesticides. Influence of pyrethroid insecticides over a wide range of sub-lethal doses on the motor activities of carabids still remains unclear.

RESULTS: Video tracking of *Platynus assimilis* showed that brief exposure to alpha-cypermethrin at sub-lethal concentrations ranged from 0.01 to 100 mg L⁻¹ caused initial short-term (< 2 h) locomotor hyperactivity followed by a long-term (> 24 h) locomotor hypo-activity. In addition, significant short- and long-term concentration and time-dependent changes occurred in general motor activity patterns and rates.

CONCLUSION: Conspicuous changes in motor activity of *Platynus assimilis* beetles treated at alpha-cypermethrin concentrations up to 75 000-fold lower than maximum field recommended concentration (MFRC) suggest that many, basic fitness-related behaviours might be severely injured as well. These changes may negatively affect carabid populations in agro-ecosystems. Long-term hypo-activity could directly contribute to decreased trap captures of carabids frequently observed after insecticide application in the field.

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Keywords: alpha-cypermethrin; hyper-activity; hypo-activity; knockdown; Ethovision XT; mobility detection; locomotion detection

1 INTRODUCTION

Pyrethroids have emerged as a major class of highly active neurotoxic insecticides due to their high bio-efficacy and relatively low toxicity to non-target organisms. These chemicals have been extensively used in agriculture, as their application is still the most effective means of controlling pest populations.^{1,2} Wildlife, including beneficial predatory arthropods, in treated fields, as well as in areas surrounding arable land, is almost inevitably exposed to pesticide spray. Routes by which non-target predatory arthropods can be exposed to pesticides include topical, residual and dietary exposure.^{3–5} Even at doses far below the lethal level, this presents a threat to vulnerable species. The unintended, sub-lethal effects of pesticides may severely harm the physiology and behaviour of non-target beneficial arthropods.^{1,6,7} In addition to direct mortality induced by pesticides, these effects must be considered for a complete analysis of their impact in integrated pest management.

Pyrethroids are known for their direct effect on the locomotor apparatus of arthropods, inducing varying degrees of paresis and changes in locomotor activity (LA), depending on concentration.^{1,2,6,8,9} General motor activity and locomotion are fundamental properties of almost all animals, reflecting a variety

of life processes and inherent in many behavioural patterns. They are central in migration, searching behaviour, habitat selection, reproduction, behavioural thermoregulation, and predator avoidance. Thus, quantitative measurements of motor activity patterns reflect many aspects of the animal's interaction with the environment and of the physiological and ecological status of the animal. Changes in locomotion that result from xenobiotic influence express an integrated response of the insect to changes in biochemical and physiological processes^{8,10–12} and may thereby interfere with the normal imperatives of many fitness-related behaviours.^{5,10,13} The relationship between altered LA and fitness-related parameters such as food seeking and predator avoidance,¹⁴ and acetylcholin-esterase inhibition¹² suggest locomotion as an ecologically valuable biomarker.

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Various techniques have been used for quantifying animal movements, but computer-centred video-recording systems are attractive because of their potential for flexibility in application.^{10,15} Recently (2013), Noldus Information Technology company (Wageningen, The Netherlands) has developed new, advanced EthoVision XT 9 software for sophisticated behavioural research. The new EthoVision XT comes with an Activity Detection Option, a new method to detect the changes in pixels within an area between successive video frames, in addition to changes in animal location (<http://www.noldus.com/ethovision-xt/whats-new-ethovision-xt-9>, accessed 15 June 2013). The Activity Detection Option allows the detection of tiny movements of various body parts even in very small animals including insects. This option seems to be useful for detecting motor activity in situations when locomotion does not occur or it is not possible due to the knock-down effect caused by neurotoxic pesticides.¹ In this study, for the first time in arthropods, we try to use the novel Activity Detection Option of Ethovision XT 9 for demonstration of sub-lethal effects of pyrethroid application on the behaviour of carabids.

In spite of the fact that carabids have a great importance in plant protection as effective predators of weed seeds and many agricultural pests,^{16–20} sub-lethal effects of pesticides have been rarely investigated in these insects.^{3,4,16,21,22} In fact, only in a couple of studies,^{12,21} sub-lethal effects of pesticides at few doses on motor activity have been quantified in carabids using automated video-tracking method for the measurements. Although, in general, the influence of pesticides on insect physiology and behaviour could change with time,^{1,6} the dynamics of sub-lethal effects of pesticides at high, intermediate, and low doses on motor activity in short and long timescales is not specifically studied in carabids. Sound knowledge on these effects are required, however, for using various aspects of motor activity as biomarkers of chemical stress.^{11,12,14} Carabids, living on the soil surface, are susceptible to many insecticides used in field crops but populations tend to recover within one month through migration from adjacent areas. However, the long-term repeated insecticide treatments may dramatically depress carabid numbers and diversity in agricultural habitats.^{3,5,16,23,24} At the time of reproduction, ground beetles are very active and cover considerable distances each day.²³ Therefore, a few days after an insecticide spray, there is a mixture of ground beetles consisting of specimens exposed to the spray and newly migrated specimens from marginal sites not exposed to the spray. Thus, during and soon after insecticide application, carabids running on the ground under the foliage of crop plants, through direct exposure to spray droplets as well as through contaminated plants, soil and food items, inevitably contact lethal as well as sub-lethal doses of the used insecticide.

The neurotoxic pyrethroid insecticide Fastac[®] EC (active substance alpha-cypermethrin) is widely used in 24 European countries (http://ec.europa.eu/sanco_pesticides/public/, accessed 11 March 2013) with an application rate per treatment of 0.010–0.015 kg as ha⁻¹ (0.00025–0.00075 kg as L⁻¹ water) (http://ec.europa.eu/food/plant/protection/evaluation/existactive/list_alpha_cypermethrin.pdf, accessed 11 March 2013) for control of many agricultural and forest pest insects, but the behavioural effects of low doses of Fastac[®] are not yet understood in carabids. The specific objective of this study is laboratory quantification of the effect of lowered doses of a commercial formulation of an alpha-cypermethrin-based insecticide, Fastac[®] 50EC, on the motor activities of the common Euro-Siberian carabid *Platynus assimilis* under controlled laboratory conditions, using

computer-centred video tracking. For the first time, we try to explain the behavioural effect of a pyrethroid in carabids over a wide range of concentrations in dynamics, from the maximum field recommended concentration (MFRC) up to very low concentrations with minimal effect. The results of the study should give essential information about sub-lethal effects of Fastac[®] application on carabids essential for population estimations in these non-targeted beneficial insects.

2 MATERIAL AND METHODS

2.1 Test beetles

The predatory beetles of *Platynus assimilis*, 10–13 mm in length, inhabit deciduous forests, damp and shaded sites in parks and gardens.²⁵ In spring and summer, they can spread into fields.²⁶ Adults of *P. assimilis*, which had been pesticide-free for more than 10 years, were collected at a forest margin in southern Estonia in April and May 2012. They were kept in translucent plastic boxes (30 cm × 20 cm × 10 cm) with moistened sand and moss, placed in the Versatile Environmental Test Chamber MLR-35 1H (SANYO Electric Co., Ltd, Osaka, Japan) at 20 °C, 16 h light and 8 h dark (16L:8D) photoperiod and 70% relative humidity (RH). They were fed with commercial moistened cat food (Friskies Vitality + Nestle Purina, Hungary), and provided with clean water daily. During storage, no indications of aggression were observed. After 3–5 days in the laboratory, the beetles were used in video tracking experiments in which their motor activities were monitored for their responses to brief exposure to Fastac[®] 50EC and distilled water (negative control).

2.2 Treatments

Diverse treatment methods have been used to estimate pesticide effects in insects. These include topical application,^{27,28} dipping method,^{29,30} filter paper disc method,³¹ diet bioassay,³² etc. Each of the methods responds to a certain aspect of pesticide application in the field. In this study we used the dipping method^{29,30} which ensures that all the body parts of the test beetle are treated with the pesticide. Commercial Fastac[®] 50EC (BASF) emulsions in water with an alpha-cypermethrin (alpha-cyp) content of 750 (MFRC), 100, 10, 1, 0.1 and 0.01 mg L⁻¹ were prepared immediately before the treatment of the test beetles. A 5 mL glass vial was used to dip test beetles in each alpha-cyp emulsion concentration for 10 s. A negative control group of beetles was dipped in distilled water for the same period of time. All six treatments were performed in 10 replicates. After treatment, each beetle was placed in a separate 110 mm Petri dish with moistened filter paper (Whatman International Ltd, Maidstone, UK) to ensure high RH for the two days of the experiment. Before video-recording, the beetles were given 20 min to calm down from stress caused by the treatment procedure (important for the negative control group of beetles). Mortality was recorded 4, 24, 48 and 96 h after treatment. Beetles were scored dead if no movement was observed after they were prodded with a hot dissecting needle. In a positive control, insects were exposed to a commonly used neonicotinoid insecticide thiamethoxam (the active ingredient in the Actara[®] 25WG, Novartis) at its MFRC (400 mg L⁻¹ or 100 ppm, prepared in distilled water) for 10 s, which resulted in 100% mortality within 4 h after treatment in all cases.

2.3 Video-recordings

The activity of two beetles in separate 110 mm Petri dish arenas, placed in a 50 cm × 50 cm × 50 cm lightproof cartoon box, was

recorded at 1 frame s^{-1} simultaneously by a computer-centred video-tracking system using two USB Webcams C120 (Logitech Inc., USA) fixed at a height of 15 cm above the arenas. The images were digitized into 640×480 pixels frames allowing sufficient spatial resolution to record tiny movements of the head, thorax, legs and antennae of the beetle as well as displacement of the beetle on the arena. The arenas were illuminated from above by a compact fluorescent lamp OSRAM DULUX® S, 11 W (OSRAM, Italy) with the colour temperature of 4000 K. A 30×30 cm cartoon screen was set between the lamp and arenas which caused light to be diffused. Illumination on the arenas (110 lux) was measured by the Digital Light Meter TES-1335 (TES Electrical Electronic Corp., Taipei, Taiwan). All video-recordings were made at 20°C . Activity of the beetles was recorded over a 4 h period on the first day of the experiment, beginning 20 min after treatment, and again 24 h later.

2.4 Analysis of data

Several activity parameters were extracted from EthoVision XT Version 9 software (Noldus Information Technology, Wageningen, The Netherlands): percentage time knockdown (dynamics), general motor activity (dynamics and total), distance moved (dynamics and total). Duration of knockdown was measured visually from playbacks of the recorded video files. General motor activity (GMA) was defined as changes in the body's contour even when the centre of the animal remained in place. The Mobility Detection option with temporal bin widths of 1 min and 30 min was used for extracting patterns and dynamics of GMA of individual beetles, respectively. Both the Highly Mobile Threshold and Immobile Threshold were set at 6% allowing, in addition to distance moved, analysis of tiny movements of various body parts of the knockdown beetles. Distance moved (start velocity $> 0.20 \text{ cm s}^{-1}$; stop velocity $< 0.20 \text{ cm s}^{-1}$) was calculated on individual tracks as the most important parameter of *Platynus assimilis* LA.

2.5 Statistical analysis

Analysis of variance (ANOVA) Kruskal–Wallis test (STATISTICA 8.0, StatSoft, Inc., Tulsa, Oklahoma) was used to determine the significant effect of alpha-cyp to the GMA and LA in *Platynus assimilis*.

3 RESULTS

Our experiments showed that a 10 s treatment with pyrethroid insecticide Fastac® 50EC emulsion at alpha-cyp concentrations of 0.01 – 100 mg L^{-1} did not kill the beetles during the four days after its application. Thus, the doses received by the beetles were considered as sub-lethal *Platynus assimilis* adults. The GMA and LA of the beetles changed, however, compared to that of the negative control beetles, depending on the concentration applied and the time after the insecticide exposure. In contrast, 10 s exposure to Fastac® at alpha-cyp MFRC (750 mg L^{-1}) caused 100% mortality of the beetles within a few hours after treatment.

3.1 Activity rhythms

Individual actogram plots demonstrated that in negative control *Platynus assimilis* beetles, short GMA periods alternated with relatively long-lasting periods of rest (Fig. 1A,B). After insecticide treatment, the GMA rate of the beetles changed, the pronounced rhythmicity in GMA pattern disappearing for a shorter or longer period of time depending on insecticide concentration (Fig. 1C,E,G,I,K). The next day, rhythmicity in GMA recovered to a

lesser or greater extent with more full recovery at lower insecticide concentrations (Fig. 1D,F,H,J,L).

3.2 Knockdown effect

Brief exposure to Fastac® 50EC emulsion at high alpha-cyp concentrations (10 and 100 mg L^{-1}) caused an extremely high percentage time of knockdown (93–100%) in tested beetles over the first 4 h after treatment (Fig. 2A). At 10 mg L^{-1} alpha-cyp, the beetles fully recovered the next day, but at 100 mg L^{-1} alpha-cyp only partial recovery from knockdown was observed 24 h after the treatment (Fig. 2B; ANOVA Kruskal–Wallis test: $H = 31.06$; $N = 10$; $P < 0.001$). By comparison, the proportion of time spent in knockdown was significantly lower in beetles treated with alpha-cyp at 1 mg L^{-1} (Fig. 2A; ANOVA Kruskal–Wallis test: $H = 63.48$; $N = 10$; $P < 0.001$), and the next day, the beetles fully recovered from knockdown (Fig. 2B). No indications of knockdown were observed in negative control beetles and in the beetles treated with the insecticide at 0.01 and 0.1 mg L^{-1} alpha-cyp (Fig. 2A,B).

3.3 General motor activity (GMA)

GMA was expressed as the mean number of movements per minute and was plotted for each 30 min period over the full observation period of 4 h. It was observed that the effect of Fastac® on GMA of the beetles depended on both alpha-cyp concentration and time after insecticide application. For several hours after insecticide application, compared to negative control tests, significant hyperactivity in terms of GMA of the beetles occurred at 0.01 – 10 mg L^{-1} alpha-cyp emulsions (Fig. 3A; ANOVA Kruskal–Wallis test: $H = 5.85$ – 13.17 ; $N = 10$; $P < 0.05$) with striking concentration-dependent variation in time-course of the effect (Figs 1A,E,G,I,K, 2C). At 10 mg L^{-1} , the GMA rate was low immediately after the insecticide exposure but the activity of the beetles significantly increased with time (Fig. 2C; ANOVA Kruskal–Wallis test: $H = 29.52$; $N = 10$; $P < 0.05$). The GMA rate of the beetles treated with 1 mg L^{-1} alpha-cyp remained at a constantly high level over the whole observation period of 4 h (Fig. 2C; ANOVA Kruskal–Wallis test: $H = 4.50$; $N = 10$; $P = 1.0$). By contrast, the extremely high rate of motor hyperactivity observed in the beetles exposed to 0.1 mg L^{-1} alpha-cyp gradually decreased with time (Fig. 2C; ANOVA Kruskal–Wallis test: $H = 54.68$; $N = 10$; $P < 0.05$) but in the range between 150 and 240 min after treatment the decline in activity was statistically not significant (ANOVA Kruskal–Wallis test: $H = 3.65$; $N = 10$; $P > 0.05$). Comparing the GMA rates of consecutive 30 min measuring periods with those of the negative control it became evident that full recovery from general motor hyperactivity occurred 180 to 240 min after the brief insecticide exposure ANOVA Kruskal–Wallis test: $H = 0.006$ – 3.160 ; $N = 10$; $P > 0.05$). The lowest tested concentration of alpha-cyp (0.01 mg L^{-1}) also caused motor hyperactivity but within 120 min after the insecticide exposure the GMA rate of the beetles quickly decreased (Fig. 2C; ANOVA Kruskal–Wallis test: $H = 36.25$; $N = 10$; $P < 0.05$). In the range between 90 and 240 min after alpha-cyp exposure the GMA rates of insecticide treated beetles did not differ from those of negative control (ANOVA Kruskal–Wallis test: $H = 0.006$ – 1.558 ; $N = 10$; $P > 0.05$).

In contrast to the treatments with lower alpha-cyp concentrations (0.01 – 10 mg L^{-1}), constantly very low GMA was observed in the beetles treated with Fastac® emulsion at 100 mg L^{-1} alpha-cyp (Figs 1C, 2C) though the total number of movements per 240 min did not significantly differ from that of

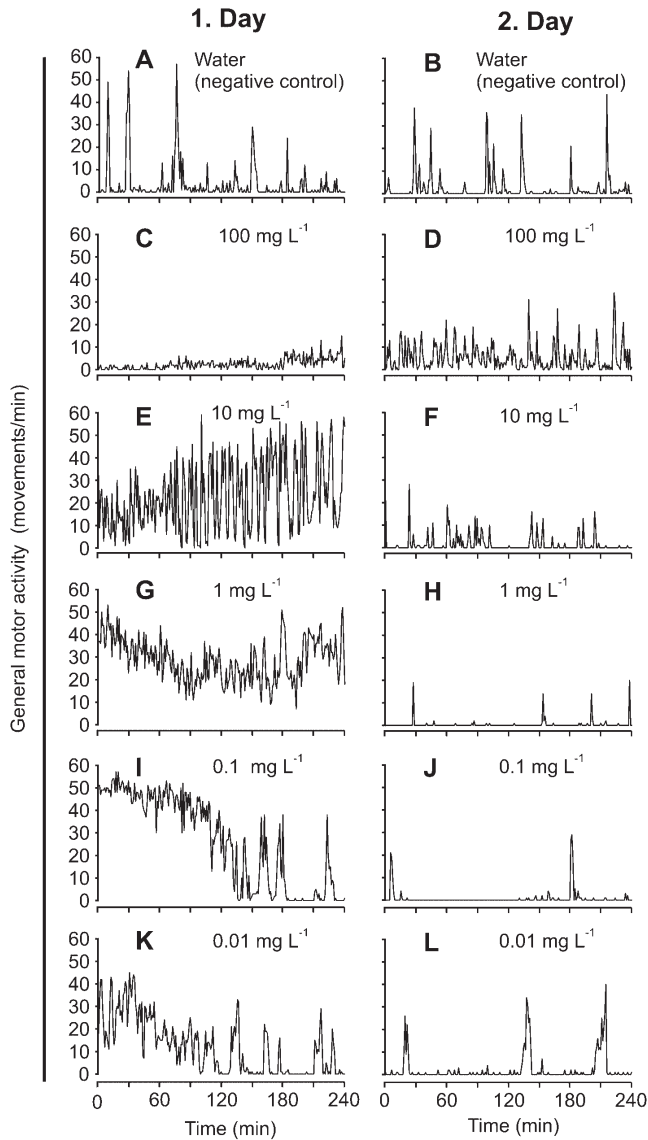


Figure 1. Example actogram plots of individual beetles of *Platynus assimilis* treated with distilled water (negative control) and Fastac[®] EC50 at various concentrations for two consecutive days after the treatment. Concentrations were calculated for alpha-cyp. GMA was measured as the number of movements per 1.0 min period with temporal resolution of 1 s and was plotted over the 240 min period of observation. Thus, GMA may have values up to 60 movements per minute. Note that negative control beetles showed pronounced rhythmicity of relatively long-lasting rest and short activity bouts (A and B). After the treatment with Fastac[®], GMA pattern and rate of the beetles drastically changed depending on the concentration and time elapsed after the treatment (C–L).

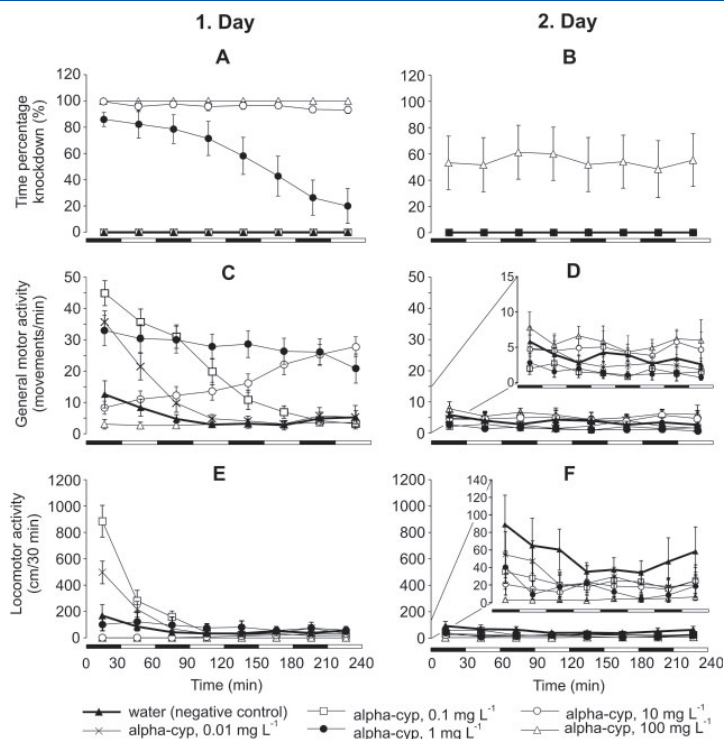


Figure 2. The dynamics of the Fastac® treatment effect on the GMA and LA of the carabid beetle *Platynus assimilis*. Measurements started 20 min after the treatment (Day 1), and continued 24 h later (Day 2). GMA values, expressed as the mean number of movements per 1.0 min, were calculated for every 30 min period during 240 min of the recordings (A, B). LA rate was computed as a distance travelled by the beetles per 30 min period and plotted over the 240 min period of observation (C, D). Inserts (D, F) show the data in a shorter y-axis. Percentage of time spent in knockdown was calculated for 30 min periods and plotted over the full period of observation (E, F). Vertical bars indicate \pm standard error of the means. $N = 10$.

the negative control group (Fig. 3A; ANOVA Kruskal–Wallis test: $H = 1.42$; $N = 10$; $P = 0.23$). Within the first 60 min after insecticide exposure, however, the intoxicated beetles were a little less active than the negative control group but the difference was statistically not proved (ANOVA Kruskal–Wallis test: $H = 0.754$ – 1.988 ; $N = 10$; $P > 0.05$). By the day after application, the impact of Fastac® on GMA of the beetles had almost disappeared (Figs 1B,D,F,H,J,L, 2D) except for emulsions with 1 and 0.01 mg L⁻¹ alpha-cyp where a significant decrease in GMA was noted compared to that of the negative control beetles (Fig. 3A; ANOVA Kruskal–Wallis test: $H = 5.49$ and 4.81, respectively; $N = 10$; $P < 0.05$).

3.4 Locomotor activity (LA)

The curves demonstrating dependence of GMA and LA on alpha-cyp concentration were almost similar in shape (S-curves) for the first day experiments (Fig. 3A,B). A drastic decrease in LA of the beetles occurred after treatment with Fastac® emulsions at high alpha-cyp concentrations (100 and 10 mg L⁻¹) compared to that of the negative control (Figs 2E, 3B; ANOVA

Kruskal–Wallis test: $H = 16.3$; $N = 10$; $P = 0.0001$ and $H = 9.87$; $N = 10$; $P = 0.0017$, respectively) because percentage time of knockdown was extremely high at these alpha-cyp concentrations (Fig. 2A). A considerable hyperactivity in terms of LA was observed at low alpha-cyp concentrations but this excitatory effect was short-lived, however, lasting 120 min (ANOVA Kruskal–Wallis test: $H = 14.66$; $N = 10$; $P = 0.0001$) and 90 min (ANOVA Kruskal–Wallis test: $H = 9.53$; $N = 10$; $P = 0.002$) after treatment with Fastac® at 0.1 and 0.01 mg L⁻¹ alpha-cyp, respectively (Fig. 2E). After these initial hyperactivity periods, LAs of Fastac®-treated and negative control beetles equalized at both 0.1 mg L⁻¹ (ANOVA Kruskal–Wallis test: $H = 0.02$; $N = 10$; $P = 0.88$) and 0.01 mg L⁻¹ (ANOVA Kruskal–Wallis test: $H = 0.01$; $N = 10$; $P = 0.91$) alpha-cyp concentrations. After 240 min, only the beetles treated with Fastac® at 0.1 mg L⁻¹ alpha-cyp travelled significantly longer paths than the negative control beetles (Fig. 3B; ANOVA Kruskal–Wallis test $H = 10.08$; $N = 10$; $P = 0.0015$). The next day, the LA of the beetles treated with Fastac®, independently of concentration, was significantly suppressed compared to that of the negative control beetles (Fig. 3B; ANOVA Kruskal–Wallis test $H = 25.73$; $N = 10$; $P = 0.0001$).

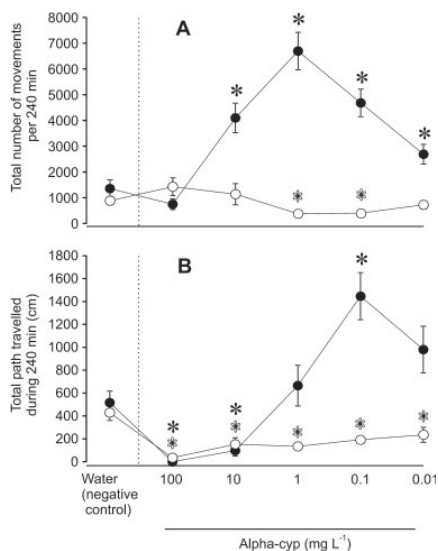


Figure 3. Total mobility responses of *Platynus assimilis* to a brief Fastac[®] exposure at various concentrations. Black and white dots represent the first and second day responses, respectively. Black and white asterisks show significant differences from the negative control (ANOVA Kruskal-Wallis test; $N = 10$; $P < 0.05$) in the first and second day after the treatment, respectively. Vertical bars indicate standard error of the means.

4 DISCUSSION

The first observable indication of intoxication in many insects treated with sub-lethal doses of pyrethroids, including *Platynus assimilis*, is an increase in their LA in terms of time in movement, mean velocity and walked distance.^{21,33–38} The laboratory video tracking technique has demonstrated that brief, sub-lethal exposures to the neurotoxic pyrethroid insecticide Fastac[®] 50EC over a wide range of concentrations (0.01 to 100 mg L⁻¹ as alpha-cyp), may cause significant alterations in the GMA and LA of the carabid beetle *Platynus assimilis*. These insecticide concentrations are up to 75 000-fold lower compared to MFRC (http://ec.europa.eu/food/plant/protection/evaluation/existative/list_alpha_cypermethrin.pdf, accessed 11 March 2013) indicating that carabids are highly susceptible to this pyrethroid even at extremely low doses. Locomotor hyperactivity may be also induced by low doses of a neurotoxic organophosphate insecticide dimethoate in the woodlouse *Porcellio scaber*,¹¹ in the ladybird beetle *Coccinella septempunctata*³⁹ and in the carabid beetle *Pterostichus cupreus*.¹²

In contrast, topical application of the pyrethroid insecticide cypermethrin (4.6 ng per animal) induced an almost instant paralysis of the hind legs and a lack of coordination in movement of the wolf spider *Pardosa amentata*. This phase culminated in total quiescence lasting approximately 12 h in males and 24–48 h in females. Following paresis, the effects of cypermethrin were evident in reduced LA.⁸ Everts *et al.*¹³ also noted a reduction in the walking speed of the erigonid spider *Oedothorax apicatus*, 40 h after receiving an LD50 or higher dose of the pyrethroid deltamethrin.

As demonstrated in *Platynus assimilis*, changes in LA of the beetles depend on both insecticide concentration and time remaining after its brief application. At higher concentrations (10–100 mg L⁻¹ alpha-cyp), Fastac[®] strongly decreases LA of the beetles in terms of the distances moved due to quick knockdown. Compared to negative control, a remarkable short-term increase in LA, lasting 90 to 120 min after brief exposure to Fastac[®], occurs at low concentrations (0.01 and 0.1 mg L⁻¹ alpha-cyp), however. After that period, no indications of locomotor hyperactivity occur, though, except for close to the MFRC (100 mg L⁻¹ alpha-cyp) GMA of the Fastac[®]-treated beetles is significantly higher than in negative control beetles. Thus, GMA may give some additional valuable information on sub-lethal effects of pesticides in beneficial arthropods; it allows evaluation of the intoxication rate of a beetle exposed to higher pyrethroid insecticide doses when the beetle is knocked down and locomotion is not possible. Our results with *P. assimilis* also showed that 24 h after Fastac[®] application the LA of the beetles was much lower than that of the negative control beetles independent of Fastac[®] concentration. Almost similar concentration- and time-dependent effects of pyrethroids on carabid LA have been observed by Prasifka *et al.*²¹ They found that brief exposure of *Scarites quadriceps* (Coleoptera, Carabidae) to tefluthrin and lambda-cyhalothrin at two concentrations which correspond to one-quarter and one-half of the concentration of these pyrethroids in soil at the maximum label rate to control corn rootworm larvae (*Diabrotica* spp) and cutworm larvae (Lepidoptera, Noctuidae), respectively, at planting, induced locomotor hyperactivity in the beetles with higher rate of activity at higher concentration. Because the effect of these pyrethroids on LA of the beetles was tested at two concentrations only, the exact shape of the dose/response curve still remains unclear for *S. quadriceps*. Prasifka *et al.*²¹ also found that locomotor hyperactivity caused by tefluthrin and lambda-cyhalothrin in *S. quadriceps* is a short-term response lasting no more than 60 min after exposure, and that it is followed by locomotor hypo-activity of the beetles. Using a video-tracking technique, both lethal and sub-lethal concentrations of all eight tested pyrethroids, applied as films on filter paper at concentrations 0.69–690 µg cm⁻², have been shown to produce considerable hyperactivity in nymphs of *Triatoma infestans* (Hemiptera: Reduviidae) with higher rates of hyperactivity at higher pyrethroid concentrations. Unfortunately, in this study, recordings were made for a short period (30 min) only after exposure without analysing the dynamics of the effect.³⁶

Changes in locomotor behaviour can remain long after exposure to the insecticide has ceased and the toxic residues have been metabolized by the animal. In the carabid *Pterostichus cupreus*, Jensen *et al.*¹² measured several LA parameters of the beetles 24 h after topical application of dimethoate at doses of 0.64, 1.35, and 2.32 mg g⁻¹. They found that the beetles responded to increasing dimethoate application rates by decreasing the time spent in LA, reducing average walking velocity and consequently covering shorter distances. Unfortunately, it remains unclear how the changes in LA caused by dimethoate develop in *Pterostichus cupreus* with time. In a laboratory experiment, the locomotor behaviour of male woodlice was measured before and after a 48 h exposure to dimethoate-contaminated soil.⁴⁰ Over the period of exposure, this acetylcholinesterase inhibitor induced gradually increasing activity in terms of time in movement, mean velocity and walked distance when compared with a control group. This excited state of LA was maintained in the exposed group even after 21 days of recovery on uncontaminated soil. Our

results with *Platynus assimilis* showed that, 24 h after Fastac® application, initial short-term locomotor hyperactivity of the beetles is replaced with long-lasting hypoactivity independent of the Fastac® concentration applied.

Striking, concentration-dependent changes in the rates and dynamics of GMA and LA caused by alpha-cyp over five orders of magnitude of sub-lethal concentrations in *Platynus assimilis* suggest that locomotion in conjunction with GMA is an extremely sensitive and valuable toxicological biomarker. Thus, concerning locomotion as a good biomarker, our results are in agreement with earlier literature data,^{10–12} and for the first time, we demonstrate that measurements of GMA may give some additional valuable information on the intoxication rate of non-targeted beneficial insects considerably widening the scale of quantifiable motor activity responses to an insecticide. Our results also demonstrate that EthoVision XT 9 software provided with Activity Detection Option (Noldus Information Technology, Wageningen, The Netherlands) is an appropriate and cost-effective tool for automated quantitative etotoxicological measurements of chemical stress.

Frequently, in field studies with carabids, pitfall trap captures increase following pyrethroid and some other insecticide applications. After exposure to these chemicals, the numbers of trapped carabids may remain high for several weeks.^{41–47} It is unclear however, why more carabids have been collected in the insecticide treatments, but because pitfall traps measure a combination of population density and activity,²³ several authors suppose that neurotoxic insecticide applications may simply increase LA of the beetles without impacting population densities in insecticide-treated plots.^{21,41,45–47} Our results on *Platynus assimilis* do not support the idea that in long-term field experiments, in insecticide treated plots, increases in the numbers of trapped carabids are caused by elevated LA. On the contrary, we found that pyrethroid-induced locomotor hyperactivity is a short-term effect lasting no more than a couple of hours after the pyrethroid application. An alternate explanation is that carabid beetles may accumulate in the treated areas owing to the abundance of easily accessible prey, killed and paralyzed insects.^{45,46} Chiverton⁴² explained high pitfall trap catches in another way, noting that the insecticidal elimination of prey likely increases carabid hunger, leading to increased activity and capture in pitfall traps.

Most authors, however, found that carabids are susceptible to many insecticides, including pyrethroids, used in field crops but that populations tend to recover within one month through re-invasion from adjacent areas. However, in the long-term, repeated insecticide treatments may decrease carabid number and diversity in agricultural habitats.^{5,24,42,48–52} Both lethal and sub-lethal effects of insecticide application may cause low pitfall trap catches in fields. Vickerman and Sunderland⁴⁸ demonstrated mortality rates of up to 81% of small adult carabids due to insecticide use. Due to high mobility and migrations between insecticide treatments and adjacent areas, sub-lethal effects are of major concern in carabids because the number of individuals encountering sub-lethal concentrations is frequently greater than the number encountering acutely lethal doses. We suppose that long-term locomotor hypo-activity caused by insecticides over a wide range of concentrations, as demonstrated in *Platynus assimilis* with alpha-cyp, may directly contribute to low numbers of carabids trapped in agricultural fields.

Even though dose dependent changes in motor activity caused by various insecticides could serve as valuable biomarkers of chemical stress in non-targeted beneficial insects, there is still

little scientific evidence that alterations in animal motor activity cause a direct impact on its population dynamics. Therefore, more subtle behavioural endpoints that provide quantitative data might be more useful.¹ Changes in motor activity suggest, however, that many other fitness-related behaviours of an insect such as searching behaviour, predator avoidance, habitat and microhabitat selection, behavioural thermoregulation, etc., directly affecting its survival and populations might be changed as well. Further studies are needed to shed more light on the issue.

5 CONCLUSIONS

The EthoVision XT 9 software (Noldus Information Technology) supplied with a novel Activity Detection Option is a sensitive and cost-effective tool for automated quantitative measurements of concentration dependent changes in insect motor activities caused by neurotoxic insecticides over a wide range of sub-lethal concentrations (five orders of magnitude tested for alpha-cyp). Dynamics of the activity showed that locomotor hyperactivity observed at the lowest tested concentrations of alpha-cyp (0.1 and 0.01 mg L⁻¹) is a short-term effect lasting no more than a couple of hours after treatment. Thus, our results on *Platynus assimilis* do not agree with the hypothesis that in some long-term field experiments, in insecticide treated plots, paradoxical increases in the numbers of trapped carabids might be caused by elevated LA. Locomotor hypo-activity following locomotor hyperactivity is a long-term effect of alpha-cyp application in *P. assimilis* at all tested sub-lethal concentrations ranged from 0.01 to 100 mg L⁻¹, and may directly contribute to low numbers of carabids trapped after pyrethroid treatments in agricultural fields. Conspicuous changes in LA rate and pattern of *P. assimilis* beetles treated at alpha-cyp concentrations up to 75 000 times lower than MFRC suggest that many other fitness-related behaviours might be severely injured as well.

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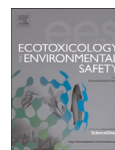
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Low doses of the common alpha-cypermethrin insecticide affect behavioural thermoregulation of the non-targeted beneficial carabid beetle *Platynus assimilis* (Coleoptera: Carabidae)

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ABSTRACT

Sub-lethal effects of pesticides on behavioural endpoints are poorly investigated in non-targeted beneficial carabids. Conspicuous changes in locomotor activity of carabids exposed to sub-lethal doses of neurotoxic insecticides suggest that many other behaviours of these insects might be severely injured as well. We hypothesize that behavioural thermoregulation of carabids may be affected by low doses of neurotoxic pyrethroid insecticide alpha-cypermethrin which may have direct deleterious consequences for the fitness and populations of the beetles in the field. Automated video tracking of the carabid beetle *Platynus assimilis* Paykull (Coleoptera: Carabidae) on an experimental thermal mosaic arena using EthoVision XT Version 9 software (Noldus Information Technology, Wageningen, The Netherlands) showed that brief exposure to alpha-cypermethrin at sub-lethal concentrations ($0.1\text{--}10\text{ mg L}^{-1}$) drastically reduces the ability of the beetles for behavioural thermoregulation. At noxious high temperature, a considerable number of the beetles died due to thermo-shock. Other intoxicated beetles that survived exposure to high temperature displayed behavioural abnormalities. During heating of the arena from 25 to 45 °C, insecticide treated beetles showed a significant fall in tendency to hide in a cool shelter (20 °C) and prolonged exposure to noxious high temperatures, accompanied by changes in locomotor activity. Next day after insecticide treatment the beetles recovered from behavioural abnormalities to a large extent but they still were considerably longer exposed to noxious high temperatures compared to the negative control beetles. Our results demonstrated that behavioural thermoregulation is a sensitive and important etho-toxicological biomarker in ground-dwelling carabids. Prolonged exposure to unfavourably high temperatures has an array of negative effects decreasing fitness and survival of these insects at elevated thermal conditions with deep temperature gradients, typical of agricultural habitats. These results may have importance in IPM programs promoting reduced insecticide use.

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1. Introduction

The sub-lethal effects of pesticides on the behavioural endpoints of various non-targeted beneficial insect groups have been the subject of an increasing number of studies over recent decades (Desneux et al., 2007; Garcia, 2011; Haynes, 1988). These effects are defined “as effects on individuals that survive exposure to a pesticide (the pesticide dose/concentration can be sublethal or lethal). A sublethal dose/concentration is defined as inducing no apparent mortality in the experimental population” (Desneux et al., 2007). Changes in fitness-related behavioural patterns caused by exposure to pesticides may, in addition to direct

mortality, significantly affect survival and populations of natural enemies of pest arthropods. However, although an abundant and diverse ground-dwelling carabid fauna may have a great importance in integrated pest management (IPM) practices as effective predators of weed seeds and agricultural pests (Bohan et al., 2011; Honěk et al., 2003; Kromp, 1999; Lundgren, 2009; Winqvist et al., 2011), the sub-lethal effects of pesticides have been little investigated in these insects (Giglio et al., 2011; Kunkel et al., 2001; Mauchline et al., 2004; Prasifka et al., 2008; Tooming et al., 2014).

Nontarget carabids are susceptible to many insecticides used in agricultural crops. Application of organophosphorus (Thiele, 1977; Vickerman and Sunderland, 1977; Kromp, 1999; van Toor, 2006; Giglio et al., 2011), organochlorine (Edwards and Thompson, 1973; Thiele, 1977), carbamate (Thiele, 1977; Kunkel et al., 2001; van Toor, 2006), pyrethroid (Pullen et al., 1992; Wick and Freier, 2000;

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Langmaack et al., 2001; Bel'skaya et al., 2002; van Toor, 2006) and neonicotinoid (Kunkel et al., 2001; Mullin et al., 2005; van Toor, 2006; Pisa et al., 2015) chemicals in pest control drastically reduces the numbers of carabids in the field. Although, carabid communities tend to recover within a month through immigration from adjacent areas, repeated insecticide treatments may dramatically depress their populations in agricultural habitats (Holland and Luff, 2000; Kromp, 1999; Kunkel et al., 2001; Thiele, 1977; van Toor, 2006). Thus, during and soon after insecticide treatments, carabids running on the ground under the foliage of crop plants, through direct exposure to spray droplets as well as through contaminated soil, prey items and plants, inevitably contact lethal as well as different sub-lethal doses of the used insecticide. At the field margins immigration of untreated beetles from adjacent areas into the pesticide treated field takes place. Since the pyrethroids are relatively persistent with detectable residues still present many days after treatment (Harris et al., 1978; Erstfeld, 1999) in the field, the immigrant beetles may also encounter over a wide range of sub-lethal doses of these insecticides long after their application.

Widely used neurotoxic pyrethroid insecticides (http://ec.europa.eu/sanco_pesticides/public/, accessed 11.09.13) affect both the peripheral and central nervous system of insects, by acting on the voltage-gated sodium channel proteins located in nerve cell membranes. By prolonging the opening of these channels, pyrethroids stimulate nerve cells to produce repetitive discharges, causing paralysis (known as insect 'knockdown') and possible insect death (Clark and Symington, 2012; Garcia, 2011; Ray and Fry, 2006). The neurotoxic pyrethroid insecticide alpha-cypermethrin is widely used in 24 European countries with an application rate per treatment of 0.010–0.015 kg as ha⁻¹ (0.00025–0.00075 kg as L⁻¹ water) (http://ec.europa.eu/food/plant/protection/evaluation/ex_istactive/list_alpha_cypermethrin.pdf, accessed 11 March 2013) for control of a number of agricultural and forest pest insects, but the behavioural effects of sublethal doses of alpha-cypermethrin are insufficiently understood in carabids.

In behavioural toxicological studies, various quantitative locomotor activity parameters of an insect have been frequently measured and analysed to demonstrate sub-lethal effects of low doses of pesticides at an individual level. Using an automated computer-centred video-tracking technique, pyrethroid insecticides were found to have a direct, concentration dependent effect on the locomotor apparatus of arthropods, inducing varying degrees of paralysis and changes in locomotion (Baatrup and Bayley, 1993; Desneux et al., 2007; Garcia, 2011; Haynes, 1988; Khambay and Jewess, 2010; Prasiřka et al., 2008; Tooming et al., 2014). Locomotion is a fundamental property of animals, reflecting a variety of life processes and inherent in many behavioural patterns such as migration, searching behaviour, habitat and micro-habitat selection, behavioural thermoregulation, etc., important for survival and reproduction. Thus, quantitative measurements of locomotion reflect many aspects of an animal's interaction with the environment and of the physiological, ecological, and the toxicological status of the animal (Baatrup and Bayley, 1993, 1998; Bayley, 2002; Desneux et al., 2007). Conspicuous changes in locomotion of the carabid beetle *Platynus assimilis* treated at alpha-cypermethrin concentrations up to 75,000-fold lower than the maximum field recommended concentration (MFRC) (Tooming et al., 2014) suggest that many other fitness-related behaviours of these insects might be severely injured as well, negatively affecting their survival and populations in agro-ecosystems. Even though, dose dependent changes in locomotion parameters (mean velocity, distance moved, time moving, meander and others) caused by various insecticides could serve as sensitive and valuable biomarkers of chemical stress, there is still little scientific evidence that alterations in locomotion cause a direct impact on

an animal's survival and population dynamics. Therefore, more subtle behavioural endpoints that provide quantitative data might be more useful (Desneux et al., 2007).

Carabids thermoregulate behaviourally (Must et al., 2010). Steep temperature gradients are common near the ground both above and below the surface, and the microclimate can be greatly modified, especially when vegetation is present. On sunny summer days (air temperature 22 °C), in the open habitats of the carabid *P. assimilis*, the subject of this study, maximum soil surface temperatures measured in sunlit areas can reach 55 °C while minimum soil surface temperatures in shaded areas are 30–40 degrees lower (Must et al., 2006a). Therefore, the species needs to get quick and adequate information about ambient temperatures, especially dangerously high temperatures, as it is crucial to avoid them to survive. Indeed, tiny, dome-shaped sensilla innervated by a temperature sensitive (cold) neuron have been found on the antennae of *P. assimilis* (Must et al., 2006b, 2010) and several other carabids (Merivee et al., 2000, 2001, 2002; Must et al., 2006a). At 44.4 ± 0.6 °C, the first indications of partial paralysis (of the hind legs) have been observed in this species (Must et al., 2010). Brief forays into high temperature zones are readily tolerated, however, as long as the insect has the option of retreating frequently to a more moderate environment to prevent overheating (Chown and Nicolson, 2004; Denlinger and Yocum, 1998; Must et al., 2010). The Euro-Siberian stenothermic *P. assimilis* reproducing in the spring is rather common in its preferred cool, wet and shaded forest habitats (Thiele, 1977; Lindroth, 1986). However, in spring and summer, this night-active species can spread into open fields (Honšak and Kocian, 2003). Also the species inhabits wheat fields (Purtauf et al., 2005a, 2005b) and grasslands (Grandchamp et al., 2005) in small numbers. Tropically, it belongs to the generalist predator guild (Larochele, 1990; Zalewski et al., 2014). Thus, the common and wide spread *P. assimilis* is important in various ecosystems and is a good representative for all carabids. Temperature preference depends on the physiological condition of the beetles but, in most cases, lies close to 10 °C (Thiele, 1977). We hypothesize that the ability of ground-dwelling carabid beetles for adequate behavioural thermoregulation may be negatively affected by sub-lethal doses of neurotoxic pyrethroid insecticides such as alpha-cypermethrin which may have direct deleterious consequences for the fitness and populations of the beetles in the field.

In this study, sub-lethal exposures to the widely used neurotoxic pyrethroid insecticide alpha-cypermethrin were used to assess the effects of pyrethroids on behavioural thermoregulation and fitness of carabids. Specifically, experiments were designed to assess changes in locomotion parameters of *P. assimilis* on a special thermal mosaic arena using an automated video tracking system. The insecticide dose range (0.1–10 mg L⁻¹) used in the study is representative of what carabids would encounter in the field.

2. Material and methods

2.1. Test beetles

Adult *P. assimilis* from a population which had not contacted pesticides for more than 10 years were collected from their preferred overwintering sites in brown-rotted tree stumps in forests of Alam-Pedja Nature Reserve (southern Estonia) in late April and early May 2013. At that period of time, in forest biotopes, the air temperatures only rarely exceeded 10 °C and the temperatures on the ground ranged from 2 to 6 °C. The beetles were kept in plastic boxes (30 × 20 × 10 cm³; 20 beetles in each) filled with small pieces of brown-rotted wood in a refrigerator at 5–6 °C. Three to four days prior to the experiments, the beetles were placed singly in Petri dishes (85 mm diameter) lined with moistened Whatman

No. 1 filter paper and transferred to a Versatile Environmental Test Chamber MLR-35 1H (SANYO Electric Co., Ltd., Japan) at 20 °C, L16: D8 photoperiod and 70% RH. They were fed with commercial moistened cat food (Friskies Vitality+Nestlé Purina, Hungary), and provided with clean water daily. After that acclimation period, locomotor activity of the beetles, on a special thermal mosaic arena, was monitored for their responses to a brief exposure to low doses of alpha-cypermethrin and distilled water (negative control) using an automated video tracking system.

2.2. Treatments

Diverse treatment methods have been used to estimate sublethal effects of pesticide application in insects. In this study, the conventional dipping method (Azimi et al., 2009; Muljar et al., 2012; van der Steen, 2001) was used; this ensures that all body parts of the test beetle are treated with the pesticide including dome-shaped thermoreceptors on their antennae (Must et al., 2006, 2010). The dipping method also guaranteed that all the beetles in a test series were treated with an equal dose of the pesticide. Commercial Fastac[®] 50EC (BASF) (50 g L⁻¹ alpha-cypermethrin) emulsions in water with alpha-cypermethrin content of 10, 1 and 0.1 mg L⁻¹ were prepared immediately before treatment of the test beetles. Thus, the used alpha-cypermethrin emulsions were 75–7500-fold diluted compared to the maximum field recommended concentration (750 mg L⁻¹) (<http://ec.europa>.

[eu/food/plant/protection/evaluation/existactive/list_alpha_cypermethrin.pdf](http://ec.europa.eu/food/plant/protection/evaluation/existactive/list_alpha_cypermethrin.pdf), accessed 11 March 2013). The range of alpha-cypermethrin concentrations from 0.1 to 10 mg L⁻¹ was chosen because earlier video tracking of *P. assimilis* had shown that 10 s exposure to alpha-cypermethrin at these concentrations caused significant changes in locomotor activity of the beetles (Tooming et al., 2014) suggesting that these concentrations could affect thermoregulation of the beetles as well. A 5 mL glass vial was used to dip test beetles individually in each alpha-cypermethrin concentration for 10 s. The beetles of the negative control group were dipped in distilled water for the same period of time. Individual beetles served as replicates. All four treatments were initially performed in 20 replicates. During the experiments, it was observed, that some of the beetles died due to thermoshock. Therefore, the number of replicates was increased until within each treatment-day the number of survived beetles for video-tracking analyses was always 20. After treatment, the beetles were placed singly in a thermal mosaic arena (Fig. 1) for observations and video-recordings of their locomotion at various thermal conditions. The beetles exposed to alpha-cypermethrin at 10 mg L⁻¹ were quickly knocked down and did not recover within 60 min of the treatment. Because these beetles were not able to walk, they were not used in the first day video-tracking experiments. However, the next day, they had fully recovered from knockdown, and were used in the video-tracking experiments in order to test their ability for thermoregulation. Brief exposure to alpha-cypermethrin at

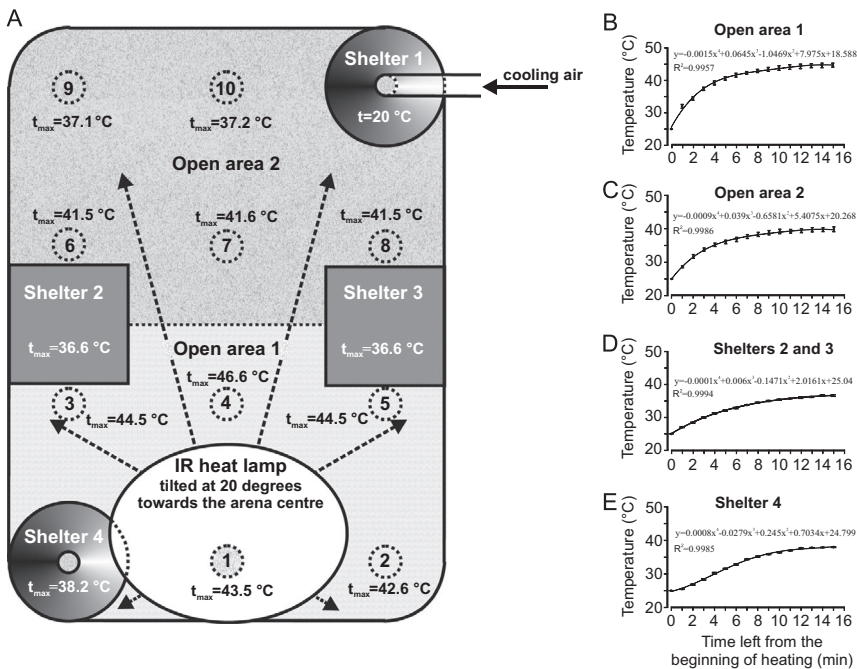


Fig. 1. (A) The experimental thermal mosaic arena, made of a white plastic box with dimensions 30 × 20 × 4 cm³ and containing a 1 cm layer of moistened sand at the bottom, was divided into two notional parts (pale and dark shading) regarding their distance from the IR heat lamp: Open area 1 and Open area 2, respectively. (B) and (C) characterize thermal conditions of Open area 1 and Open area 2 as quantified by average of sand surface temperatures measured by thermocouple circuits at five different points: see dotted circles 1–5 and 6–10, respectively. (D) and (E) show sand surface temperature curves of the Shelters 2–4. Temperature in Shelter 1 was held constant at 20 °C by a cooling air stream. t_{max} indicate maximum temperatures on the sand surface at various measuring points 15 min after the IR lamp was turned on. Two inverted glass funnels (6 cm diameter) covered with black paper and raised 0.5 cm above the sand surface served as Shelters 1 and 4. Two 6 × 6 cm² beige carton pieces mounted 0.5 cm above the sand surface served as Shelters 2 and 3. Temperature curves (B–E) were calculated using fourth degree polynomial functions.

1 mg L⁻¹ caused only a short-term knockdown effect and the beetles had recovered fully 60 min after the contact with the insecticide. No indications of knockdown were observed in the beetles exposed to alpha-cypermethrin at 0.1 mg L⁻¹.

Our preliminary experiments at 20–25 °C and 70% RH showed that a 10 s treatment with alpha-cypermethrin at concentrations of 0.1–10 mg L⁻¹ did not kill the beetles during the four days after their application, in agreement with Tooming et al. (2014). Thus, the doses received by the beetles were considered as sub-lethal for *P. assimilis* adults. In a positive control, insects were exposed to the commonly used neonicotinoid insecticide thiamethoxam (the active ingredient in the Actara[®] 25WG, Novartis) at its maximum field recommended concentration (400 mg L⁻¹ or 100 ppm, prepared in distilled water) for 10 s, which resulted in 100% mortality within 4 h of treatment in all cases.

2.3. Video-tracking

The locomotor activity of the test beetles at various thermal conditions, ranging from 20 to 46.6 °C, was recorded at 15 frames s⁻¹ on a special thermal mosaic arena (Fig. 1) by a computer-centred video-tracking system using an USB Webcam C120 (Logitech Inc., USA) fixed at a height of 30 cm above the arena. A red IR heat lamp (BR125 IR 250 W 230–250 V E27 ES RED, Philips), placed 22 cm above the arena, was used for heating and creating a thermal mosaic gradient on the moistened sand surface of the arena. Time ranges corresponding to respective temperature ranges on the experimental thermal mosaic arena within 900 s heating/cooling period used in video-data analysis for calculating temperature are presented in Table 1. To block out unwanted visual stimuli from outside the arena, we placed it in a 60 × 60 × 60 cm³ lightproof carton box. The arena was illuminated from above by a compact fluorescent lamp OSRAM DULUX[®] S, 11 W (OSRAM, Italy) with the colour temperature of 4000 K. A 30 × 30 cm² carton screen was set between the lamp and the arena to diffuse the light. Illumination on the arena (110 lx) was measured by the Digital Light Metre TES-1335 (TES Electrical Electronic Corp., Taiwan). Prior to each trial, the initial temperature on the arena was adjusted to 25 °C. Test beetles were released individually in the middle of the arena. A cooling air flow, the IR heat lamp and the recording webcam were turned on for 15 min

Table 1

Time ranges corresponding to respective temperature ranges on the experimental thermal mosaic arena within 900 s heating/cooling period. Data averaged from 10 measurements.

Area of the arena	Temperature ranges (°C)	Time ranges (s)
Open area 1	25–30	0–45
	30.1–35	46–123
	35.1–40	124–261
	40.1–45	262–900
Open area 2	25–30	0–86
	30.1–35	87–234
	35.1–40	235–900
Shelter 1	20	0–900
Shelters 2 and 3	25–30	0–267
	30.1–35	268–483
	35.1–40	484–900
Shelter 4	25–30	0–208
	30.1–35	209–564
	35.1–40	565–900

(900 s) simultaneously 60 min after treatment, and again 24 h later. The images were digitized into 640 × 480 pixels frames allowing sufficient spatial resolution to record movements of various body parts of the beetle as well as displacement of the beetle on the arena. We predicted that with previous exposure to increasing concentrations of alpha-cypermethrin, beetles released onto the arena under high temperatures (1) would show significant alterations in locomotor activity (hyper- or hypoactivity) as biases from normal thermoregulation behaviour; (2) would be less able to move to shelters and avoid noxious high temperatures and IR radiation; (3) would be less able to discriminate differences in temperature on the open arena leading to prolonged exposure to unfavourably high temperatures.

To evaluate differences in ability for thermoregulation between beetles treated with alpha-cypermethrin and the negative control group, several locomotor activity parameters were extracted from the recorded video files using EthoVision XT Version 9 software (Noldus Information Technology, Wageningen, The Netherlands): distance moved on the open arena, the number of hidings in shelters, time spent at different temperatures and the number of beetles killed by thermoshock. Distance moved (start velocity > 0.20 cm s⁻¹; stop velocity < 0.20 cm s⁻¹) was calculated on individual tracks as one of the most important parameters of locomotion. To characterize locomotion parameters at various phases of heating, the 900 s heating/cooling period was divided into three equal sub-periods: 0–300 s, 301–600 s and 601–900 s. To simplify automatic calculation of time spent by the beetles at different thermal conditions, temperatures on the thermal mosaic arena over the 900 s heating/cooling period were divided into five ranges: constant 20 °C, 25–30 °C, 30.1–35 °C, 35.1–40 °C and 40.1–45 °C. Calculated temperature curves for certain areas of the arena (Fig. 1) allowed determination of average temperature for each point on the arena at each instant of time. Then, time ranges were calculated for corresponding temperature ranges in each area of the arena (Table 1) and used in the EthoVision XT program to estimate thermal conditions of test beetles in various areas of the arena from playbacks of the recorded video files.

2.4. Statistical analysis

General Linear Model and Tukey test (STATISTICA 8.0, StatSoft, Inc., Tulsa, Oklahoma) was used to determine the significant effect of alpha-cypermethrin to the various aspects of behavioural thermoregulation in *P. assimilis*. Treatment effects were discussed at the significance level of $P \leq 0.05$.

3. Results

3.1. The proportion of test beetles killed by overheating on the experimental thermal mosaic arena within 900 s of the heating/cooling period

Our video-tracking experiments on the special thermal mosaic arena with *P. assimilis* exposed to low doses (0.1, 1.0 and 10 mg L⁻¹) of alpha-cypermethrin demonstrated that not all the beetles were able to thermoregulate behaviourally, and died as a result of thermal shock when temperatures on the arena rose to a dangerously high level. Several steps of thermal shock were observed. First, the beetles showed uncoordinated movements and partial paralysis of the hind legs. Then, with further temperature increase, total paralysis developed, the beetles stopped moving and died from overheating. All the deaths took place in open areas of the arena exposed to IR radiation from the IR heat lamp. This thermo-shock response was concentration dependent with a higher proportion of insects killed at higher alpha-cypermethrin

Table 2

Percentage of the beetles that were killed by thermoshock during the 900 s heating experiments on the experimental thermal mosaic arena. The number of tested beetles within each treatment-day is indicated in parentheses.

Day of the experiment	Day 1			Day 2		
	Fastac [®] 50EC	Water (negative control)		Fastac [®] 50EC	Water (negative control)	
Concentration of alpha-cyp (mg L ⁻¹)	1	0.1		10	1	0.1
Percentage of killed beetles	47.4 (38)	16.7 (24)	0 (20)	28.6 (28)	9.1 (22)	0 (20)

concentrations (Table 2). The beetles which survived thermal mosaic arena experiments on Day 1 were used in the experiments again on Day 2. The number of insects that died on Day 2 was considerably smaller than on Day 1 indicating that the beetles had recovered their ability for behavioural thermoregulation to a large extent. For example, 47% of 1 mg L⁻¹ alpha-cypermethrin treated beetles died on Day 1 but only 9% died on Day 2. By contrast, none of the beetles in the negative control group died. In further analysis of several important aspects of behavioural thermoregulation we used only video-trackings of beetles that survived the 900 s heating/cooling period on Day 1.

3.2. The sub-lethal effect of alpha-cypermethrin exposure on the locomotor activity of *P. assimilis* under various thermal conditions

The locomotor activity of the beetles of the negative control group, on open areas of the thermal mosaic arena, drastically decreased with temperature increase compared to that observed at lower temperatures within the first 300 s period of heating (GLM Tukey test, Fig. 2). During the initial 300 s period of heating, the beetles diligently moved around the arena, 246 ± 49 and 257 ± 43 cm on average on Day 1 and Day 2, respectively, and quickly found the shelter with the lowest temperature (20 °C). In the 20 °C shelter, they hid for a total of 81.8% and 79.8% of the 900 s heating period on Day 1 and Day 2, respectively (Fig. 3). A 5.0–18.7-fold decline in locomotion (GLM Tukey test, Fig. 2A, B) and conspicuous tendency to hide in the 20 °C shelter (Fig. 3A, B) when temperatures on the open arena rose above 36–40 °C (300–900 s of heating) on both days of the experiment, demonstrates that beetles not exposed to insecticide were effectively able to avoid elevated temperatures by natural behavioural thermoregulation. Compared to the negative control, respective high temperature induced decreases in locomotor activity of the beetles exposed to 0.1 and 1 mg L⁻¹ alpha-cypermethrin, within 900 s heating period on Day 1, were relatively small, 1.8–3.9-fold in average (GLM Tukey test, Fig. 2A).

During the whole 900 s period of observation on Day 1, the alpha-cypermethrin treated beetles showed remarkable locomotor hyperactivity, travelling considerably longer distances (2.6–24-fold in mean) than those in the negative control group (Fig. 2A). The relative difference in locomotor activity between the alpha-cypermethrin treated and negative control beetles increased with temperature increase (GLM Tukey test). On Day 2, the beetles exposed to 0.1 and 1 mg L⁻¹ alpha-cypermethrin fully recovered from locomotor hyperactivity so as distances moved by the insecticide treated beetles and negative control did not differ (Fig. 2B). The beetles treated with 10 mg L⁻¹ alpha-cypermethrin were knocked down by 100% and therefore not used in the experiment on Day 1, but they fully recovered from knock-down on Day 2. Their locomotion on Day 2 did not differ from that of negative control group (Fig. 2B). However, both on Day 1 and Day 2, the stimulating effect of alpha-cypermethrin on the locomotor activity of the beetles did not significantly depend on its concentration (Fig. 2A, B). These results showed that alpha-cypermethrin negatively affected on the ability of the beetles to avoid high temperatures in open areas of the experimental thermal

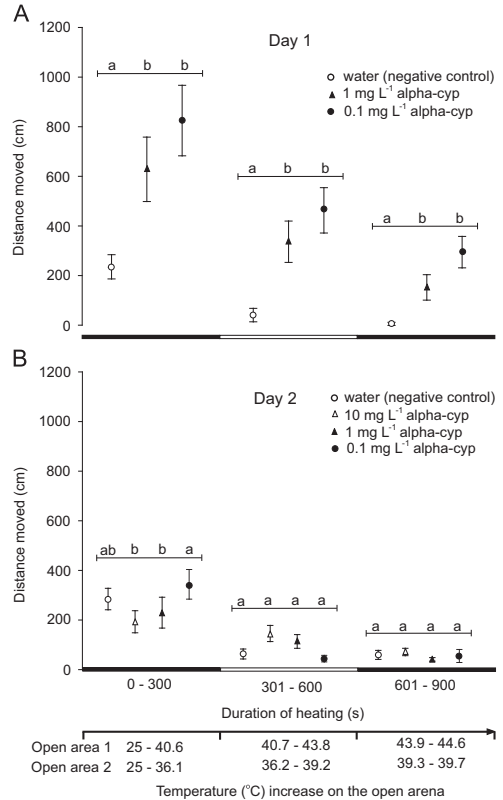


Fig. 2. Distance moved in open areas of the thermal mosaic arena by the variously treated beetles of *P. assimilis* during 900 s of heating from 25 to 45 °C. Because the beetles treated with 10 mg L⁻¹ alpha-cypermethrin did not recover from knock-down within a 60 min waiting period this group of insects was not used for video-tracking on the first day of the experiments. Note that on Day 1, the beetles treated with alpha-cypermethrin travelled significantly longer distances on the open arena with noxious high temperatures compared to the negative control group. Vertical bars show standard errors (SE) of the means; different small letters indicate significant differences between the treatments at particular time/temperature intervals (GLM Tukey test; $N=20$; $P<0.05$).

mosaic arena on Day 1. By contrast, this insecticide had no effect on the locomotion of the beetles on Day 2 (Fig. 2B).

3.3. The effect of low doses of alpha-cypermethrin on the tendency of the beetles to hide in shelters at various thermal conditions

Within the initial 300 s period of heating, when sand surface

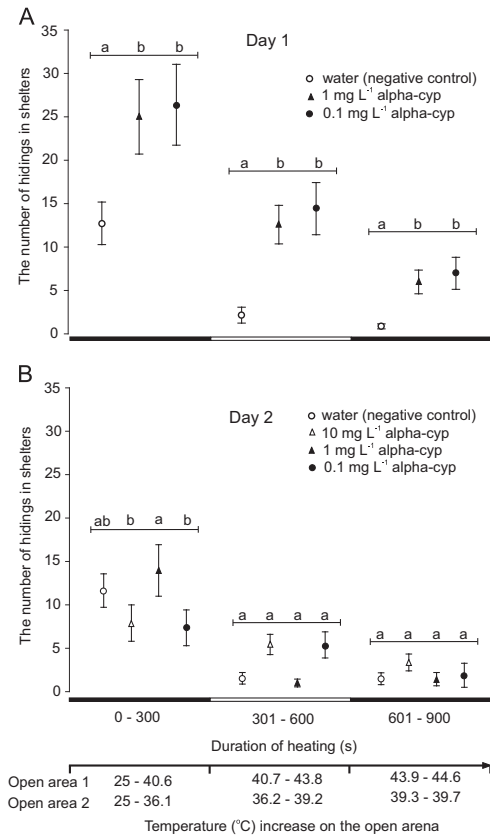


Fig. 3. The number of hidings in shelters displayed by the beetles treated with alpha-cypermethrin and water (negative control) on the thermal mosaic arena. Note that on Day 1, the beetles treated with alpha-cypermethrin tend to run out of shelters, which provides protection from direct IR radiation from the IR heat lamp, considerably more frequently than the beetles of the negative control group. Vertical bars show SE of the means; significant differences between the treatments at particular time/temperature intervals are marked with different small letters (GLM Tukey test: $N=20$; $P < 0.05$).

temperatures in the open arena increased from 25 to 40.6 °C, the beetles of the negative control displayed high locomotor activity (Fig. 2A, B) and 12.7 and 11.5 times on average on Day 1 and Day 2, respectively, entered into and left from the shelters mounted on the thermal mosaic arena (Fig. 3). On both days of the experiment, during the next 600 s of heating when temperature increased further the beetles preferred to spend more time in shelters and the number of runs out of shelters into the open arena drastically decreased to an average of 6.4–18.1 times compared to the initial 300 s period of heating (GLM Tukey test, Fig. 3A, B). By contrast, the number of runs out of shelters also decreased with temperature increase in the beetles exposed to low doses of alpha-cypermethrin on Day 1 but to a considerably lower extent, a mean of 1.8–4.2 times, compared to the negative control group (GLM Tukey test, Fig. 3A). At the same time, within the whole 900 s heating period, the insecticide treated beetles hid in and left from shelters 2.0–9.9 times more frequently than the beetles of the negative

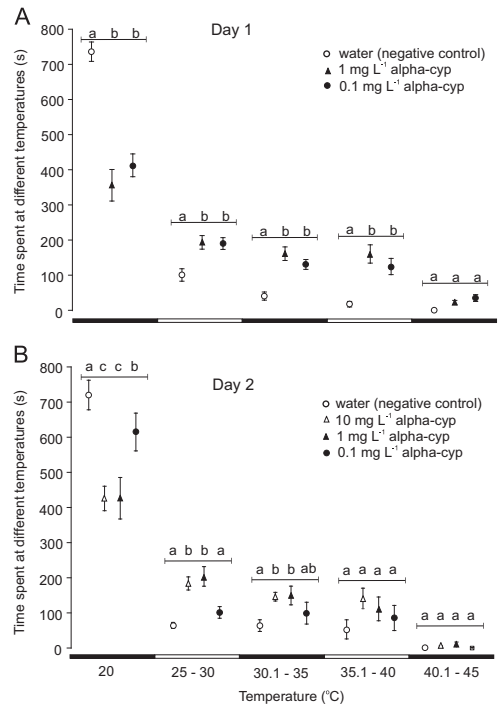


Fig. 4. Time spent by the beetles exposed to sub-lethal doses of alpha-cypermethrin and water (negative control) at different temperature ranges within 900 s heating period. The beetles treated with alpha-cypermethrin stayed significantly longer at unfavourably high temperatures above 25 °C compared with the negative control group. Vertical bars indicate SE to the means. Different small letters show significant differences between the treatments, including negative control, at particular temperature intervals (GLM Tukey test: $N=20$; $P < 0.05$).

control (Fig. 3A). The difference in the number of hidings in shelters between the alpha-cypermethrin treated and negative control beetles even increased with temperature increase (GLM Tukey test, Fig. 3A) demonstrating that intoxicated beetles were significantly less able to avoid dangerously high temperatures on the arena. Although, some little effects of alpha-cypermethrin treatment on the tendency of the beetles to hide in shelters were observed on Day 2, the differences from negative control were not statistically significant (Fig. 3B). Even though, low doses of alpha-cypermethrin significantly affected the tendency of the beetles to hide in shelters on Day 1, the effect did not depend on the insecticide concentration (Fig. 3A).

3.4. The effect of alpha-cypermethrin exposure on time spent by the beetles at noxious high temperatures

The beetles of the negative control group 81.8% and 79.8% of the 900 s heating period, on Day 1 and Day 2, respectively, stayed in the shelter with the lowest temperature on the thermal mosaic arena equal to 20 °C (Fig. 4A, B). Compared to the negative control, time spent in the 20 °C shelter by the beetles exposed to alpha-cypermethrin was shorter by 14.6–51.5% depending on insecticide concentration and day of the experiment (Fig. 4A, B).

The time spent by the negative control beetles at elevated

temperatures above 25 °C drastically decreased with temperature increase (GLM Tukey test, Fig. 4A, B) demonstrating that they were able effectively to avoid noxious high temperatures on the arena. For example, exposure to high temperatures ranging from 35.1 to 40 °C was 38.5- and 13.6-fold shorter, on Day 1 and Day 2, respectively, compared to that at preferred 20 °C (GLM Tukey test, Fig. 4A, B). The beetles of the negative control entered areas with temperatures above 40 °C only occasionally for less than a mean of 1 s (Fig. 4A, B).

The beetles treated with alpha-cypermethrin also avoided elevated temperatures above 25 °C (GLM Tukey test, Fig. 4A, B) but with less success than the negative control group. On Day 1, the intoxicated beetles (0.1 and 1 mg L⁻¹ alpha-cypermethrin) were exposed to high temperatures of 25–40 °C 1.9–8.5-fold longer compared to the negative control beetles (Fig. 4A). The differences in time exposed to elevated temperatures between the alpha-cypermethrin treated and negative control beetles increased with temperature increase (GLM Tukey test, Fig. 4A, B). However, the time of exposure to noxious high temperatures did not depend on tested alpha-cypermethrin concentration (Fig. 4A). By contrast, on Day 2, the effect of treatments on the behaviour of the beetles depended on alpha-cypermethrin concentration (Fig. 4B). Exposure to the lowest concentration of alpha-cypermethrin (0.1 mg L⁻¹) only slightly prolonged the time spent by the beetles at temperatures above 25 °C but the differences from the negative control were statistically not significant (Fig. 4B). Intoxication by 1 and 10 mg L⁻¹ alpha-cypermethrin 2.1–3.2-fold increased the time the beetles were exposed to high temperatures of 25–40 °C compared to that of the negative control but in the range of 35.1–40 °C the difference was statistically not significant (Fig. 4B). Areas with high temperatures above 40 °C were seldom visited by the insecticide treated beetles, for 0.5–36.6 s in mean, depending on insecticide concentration and day of the experiment but the differences from the negative control were statistically not significant (Fig. 4A, B).

4. Discussion

Insects inhabit a wide range of thermal environments, but there is very little variability at the maximum temperature (40–50 °C) in which they can survive (Heinrich, 1981). In the carabid beetle *P. assimilis*, the first indications of paralysis occur at 44.4 ± 0.6 °C (Must et al., 2010). Thermoregulation enables these poikilothermous animals maintain a relatively stable, optimal body temperature or temperature range, either above or below ambient temperature, by behavioural or physiological means (Heinrich, 1981, 1993). Our video-tracking experiments on the thermal mosaic arena with *P. assimilis* demonstrated that behavioural thermoregulation via temperature dependent locomotion and proper microhabitat selection functions extremely effectively in these ground-dwelling insects allowing safe protection from high temperature injury.

This is the first attempt in insects to demonstrate that low doses of pesticides may have a detrimental effect on behavioural thermoregulation, an important, fitness-related behavioural endpoint. Our results show that brief exposure to sub-lethal doses of alpha-cypermethrin, 75–7500-fold diluted compared to the MFRC, drastically decreases the ability of *P. assimilis* beetles for behavioural thermoregulation. At noxious high temperatures, a considerable number of the beetles die due to thermo-shock. Other intoxicated beetles which survive exposure to high temperatures display behavioural abnormalities. Immediately after treatment, they show considerable locomotor hyperactivity and a remarkable decrease in their tendency to hide at elevated temperatures in cool (20 °C) shelters compared to those not contacted with the

insecticide. These concentration and temperature dependent changes in behaviour of the insecticide treated beetles lead to an undesirable, up to 8.5-fold longer exposure to noxious high temperatures than is characteristic for normal beetles. The harmful impact of low doses of alpha-cypermethrin on thermoregulatory behaviour lasts up to 24 h after the insecticide treatment. Although, the beetles have recovered from behavioural abnormalities to a large extent, the distances moved and the tendency to hide in shelters do not differ from those of the negative control group, but they are considerably longer exposed to noxious high temperatures compared to the negative control beetles.

Insects are extremely vulnerable to high temperature injury. Brief exposures to temperature extremes can cause immediate thermal shock and death. Lethality is a function of both temperature and time. At higher temperatures shorter exposures are needed to kill the insect. At moderately high temperatures the insect is capable of surviving a series of non-lethal lesions, but at a certain point, the lesions accumulate to a critical level and cause death (Denlinger and Yocum, 1998). Solar radiation can quickly elevate body temperature to lethal levels in these small-bodied poikilotherms. The challenge of maintaining water balance at high temperature makes the problem even more formidable (Hadley, 1994; Wharton, 1985). The fall in tendency to hide in shelters in *P. assimilis* beetles contacted with low doses of alpha-cypermethrin, at elevated temperatures, increases the probability for exposure to IR radiation and rapid overheating. Our experiments showed that, on the preheated to 44 °C sand surface, due to IR radiation from the IR heat lamp, the body temperature of the beetle quickly, within 15 s, rose above the sand surface temperature which may be lethal for the beetles (Must et al., 2010). Similar results are also available for other insects (Heinrich, 1993; Denlinger and Yocum, 1998). When exposed to solar irradiation (54 mW/cm²), wings of the papilionid butterfly *Pachliopta aristolochiae* may heat by more than 20 °C above ambient temperature within 20 s, while the more massive thorax heats much more slowly: 10 °C within 150 s (Schmitz and Wasserthal, 1993). These findings explain why alpha-cypermethrin induced disturbances in behavioural thermoregulation, at high temperature extremes, may frequently lead to overheating and lethal consequences in ground-dwelling carabid beetles. In addition to direct mortality caused by prolonged exposure to lethal high temperatures, a complex of abnormalities may occur at the cellular and higher levels of biological organization in response to heat stress at noxious high temperatures as exemplified in the pyrethroid induced deleterious changes in behavioural thermoregulation of *P. assimilis*.

Growth, development, reproduction, and other fitness-related physiological processes occur most successfully within a fairly broad range of optimal temperatures; at higher temperatures, these functions deteriorate in direct proportion to the excess in temperature. Elevated temperatures have deleterious effects on insect metabolism, respiration, nervous and endocrine systems (Chown and Terblanche, 2007; Denlinger and Yocum, 1998; Feder and Krebs, 1998; Neven, 2000). In response to high temperatures, nearly all organisms express heat-shock proteins, highly-conserved proteins that contribute to thermo-tolerance by functioning as molecular chaperons (Chown and Terblanche, 2007; Denlinger and Yocum, 1998; Neven, 2000; Xu et al., 2010; Zhao et al., 2010; Zhao and Jones, 2012). Heat-shock response invokes a cost, however, expressed as a reduction in survival and fecundity, an inhibition of growth, changes in developmental time (Feder, 1999; Feder and Krebs, 1998; Huang et al., 2007; Krebs and Loeschcke, 1994) and impaired locomotion (Robertson, 2004). Heat stress may cause alterations in cell pH and ion concentrations which have dramatic effects on macromolecules such as proteins, DNA, RNA, lipids, and carbohydrates, and on cellular structures, such as cell and nuclear membranes, mitochondria and ribosomes

(Denlinger and Yocum, 1998; Hochachka and Somero, 1984; Neven, 2000; Reiber and Birchard, 1993). In carabids, egg production is strongly influenced by temperature (Ernsting and Huyer, 1984; Ernsting and Isaaks, 2000; Heessen, 1980). Eggs increase in number and decrease in size with temperature increase.

Low doses of neurotoxic insecticides may cause high incidence of sub-lethal effects in insect motor activities including indications of paralysis and 'knock-down' effect, impaired walking, locomotor hyper- and hypo-activity, escape from shelters, and excessive grooming (Bradbury and Coates, 1989; Desneux et al., 2007; Haynes, 1988; Jensen et al., 1997; Kunkel et al., 2001; Prasifka et al., 2008; Tooming et al., 2014; Wiles and Jepson, 1994). Intoxicated carabid beetles usually recover within a few days in the laboratory (Kunkel et al., 2001; Prasifka et al., 2008; Tooming et al., 2014), but in the field, they are shown to be highly vulnerable to predation by ants (Kunkel et al., 2001). However, there is still little scientific evidence that pesticide induced changes in carabid behaviour may cause a direct impact on fitness and population dynamics; exceptions include alterations in foraging behaviour of *Harpalus rufipes* (Dempster, 1968) and behavioural thermoregulation, a fitness-related, quantifiable behavioural endpoint, as demonstrated in *P. assimilis* in this study.

5. Conclusions

This is the first attempt to demonstrate negative sub-lethal effects of pesticides on thermoregulation behaviour in beneficial arthropods. The results derived in the automated video-tracking experiments with *P. assimilis* on the thermal mosaic arena with temperature extremes from 20 to 45 °C support our hypothesis that brief exposure to low doses of alpha-cypermethrin (0.1–10 mg L⁻¹) drastically reduces the ability of the beetles for behavioural thermoregulation. Concentration dependent long-term changes in several aspects of thermoregulation behaviour last even 24 h after the insecticide treatment. As a result, prolonged exposure of the pyrethroid treated beetles to unfavourably high temperatures has an array of negative effects decreasing fitness and survival of these beneficial insects at elevated thermal conditions with deep temperature gradients, typical for agricultural habitats. Thus, behavioural thermoregulation is a sensitive and important fitness-related etho-toxicological biomarker in ground-dwelling carabids. These results may have importance in IPM programs promoting reduced insecticide use.

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Behavioural effects of the neonicotinoid insecticide thiamethoxam on the predatory insect *Platynus assimilis*

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Abstract Little information is available regarding sublethal effects of neonicotinoids on insect predators, many of which perform important roles in ecosystem functioning and bio-control. In this study, dose-dependent sublethal effects of a dietary administered neonicotinoid insecticide thiamethoxam on two basic behaviours, locomotion and feeding, were quantified in the carabid *Platynus assimilis* (Coleoptera, Carabidae) using automated video-tracking and weighing of consumed food, respectively. Acute toxicity tests showed that, when orally administered, the LD₅₀ of thiamethoxam for *P. assimilis* beetles was 114.5 ng/g. Thiamethoxam at 108.1 ng/g caused a short-term locomotor hyperactivity within several hours of treatment. Next day after exposure to the insecticide, all the beetles were in a state of locomotor hypoactivity independent of the administered dose ranging from 1.1 to 108.1 ng/g. Reduction in clean food consumption rate (CFCR) is another altered behavioural endpoint of poisoned insect predators as first demonstrated in this study. On the first day of thiamethoxam administration, a remarkable reduction in feeding only occurred in beetles treated at 108.1 ng/g but on the next day, this negative effect appeared even at doses ten to a hundred-fold lower. Recovery from locomotion abnormalities and reduced feeding took several days. Both locomotor

activity and CFR are sensitive and valuable ecotoxicological biomarkers of carabids which should be taken into account in Integrated Pest Management programs where optimal combination of reduced insecticide use and biological control by predatory insects is crucial to achieve best results.

Keywords Ecotoxicology · Acute toxicity · Sublethal effects · Locomotor activity · Food consumption rate · Carabids

Introduction

Predatory carabids, both larvae and adults, as effective natural enemies of many agricultural pests (Kromp 1999; Lundgren 2009; Winqvist et al. 2011) and voracious consumers of weed seeds (Honěk et al. 2003; Bohan et al. 2011), alongside with diminished use of pesticides, are important component of Integrated Pest Management (IPM) programs (Zhang and Swinton 2009). Nontarget, ground-dwelling carabids are inevitably exposed to lethal as well as sublethal doses of pesticides in treated fields. Conventional application of organophosphorus (Vickerman and Sunderland 1977; Van Toor 2006; Giglio et al. 2011), organochlorine (Edwards and Thompson 1973; Thiele 1977), carbamate (Kunkel et al. 2001; Van Toor 2006), pyrethroid (Wick and Freier 2000; Langmaack et al. 2001; Bel'skaya et al. 2002; Van Toor 2006) and neonicotinoid (Kunkel et al. 2001; Mullin et al. 2005; Van Toor 2006; Pisa et al. 2015) insecticides drastically reduce the numbers of carabids in treated fields. Today, according to European Union (EU) directive 2009/128/EC, there is a necessity in

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agriculture to achieve more sustainable use of pesticides with the least possible disruption to agro-ecosystems, and to encourage natural pest control mechanisms through collecting accurate and balanced information on the potential side effects for non-target beneficial organisms arising from the use of these chemicals (<http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:02009L0128-20091125>, accessed 30.08.16).

In carabids, the main routes of exposure to pesticides include direct contact with spray droplets, contaminated soil and plants, and feeding on prey items and seeds exposed to insecticides. In addition to direct mortality, pesticides may cause a broad variety of physiological and behavioural sublethal effects in non-target beneficial insects, severely reducing their performance and negatively affecting their population dynamics, as most frequently demonstrated in pollinators and parasitoids (Haynes 1988; Desneux et al. 2007; Goulson 2013; Pisa et al. 2015). In this study, pesticide sublethal effects are defined "as effects on individuals that survive exposure to a pesticide (the pesticide dose/concentration can be sublethal or lethal)" (Desneux et al. 2007). These effects may severely reduce the efficacy of parasitoids as biological control agents (Elzen et al. 1989; Roger et al. 1995; Stapel et al. 2000; Desneux et al. 2007; Pisa et al. 2015). Similarly, pesticide induced alterations in behaviour can also decrease pest control efficacy in carabids. Unfortunately, the sublethal effects of pesticides on behaviour in these insects have received little and only sporadic attention (Prasifka et al. 2008; Giglio et al. 2011; Douglas et al. 2014; Tooming et al. 2014; Merivee et al. 2015).

Neurotoxic neonicotinoid insecticides are registered for use in more than 120 countries. They comprise approximately one quarter of the global insecticide market (Jeschke et al. 2011; Pollack 2011) and this rate is increasing (Simon-Delso et al. 2015). These substances act as nicotinic acetylcholine receptor (nAChRs) agonists opening cation channels in the central nervous system of insects. They induce continuous nervous excitation at low doses, but receptor blockage, cell energy exhaustion, paralysis and death at higher doses (Goulson 2013; Simon-Delso et al. 2015). Due to structural differences between arthropods and mammalian nAChRs neonicotinoids bind more strongly to insect nAChRs than to those of vertebrates and thus, they are selectively more toxic to insects (Tomizawa and Casida 2005; Goulson 2013; Simon-Delso et al. 2015). In agriculture, horticulture, and forestry, neonicotinoids are used in many different ways like seed dressing and seed pilling, foliar spraying, soil treatment, dipping of seedlings, trunk injections in trees and application with a brush on the stems of fruit trees (Jeschke et al. 2011; Simon-Delso et al. 2015). Due to their physicochemical characteristics and systemic nature, they are absorbed by the roots or leaves and

translocated to all parts of the plant, which, in turn, makes them effectively toxic to herbivorous insects (Bonmatin et al. 2014; Simon-Delso et al. 2015). Seed and soil treatments constitute about 60% of their applications worldwide (Jeschke et al. 2011; Goulson 2013). In developed countries, seed treatments with neonicotinoids are widely used for a broad variety of crops such as oilseed rape, sunflower, cereals, beets and potatoes (Goulson 2013). Seed dressing and seed pilling with these insecticides protect all parts of the crop plant against pest insects for several months after sowing (Jeschke et al. 2011). Phytophagous insects become intoxicated by feeding on treated plants and fall to the soil surface where they may be consumed by predatory arthropods, including carabids.

The large-scale application of neonicotinoids even at very low dosages, inevitably leads to a widespread environmental contamination with these insecticides posing serious risks to harm nontarget beneficial organisms. It is estimated that when neonicotinoid seed dressings are used, only 1.6 to 20% of the active ingredient is absorbed by the target crop (Sur and Stork 2003). This is a considerably smaller proportion of the accurately targeted active ingredient than is accessible by traditional spray applications to foliage, which commonly exceed 50% efficiency (Graham-Bryce 1977). In both cases, the remainder of the active ingredients enter the soil where they persist for a long time. The estimated dissipation times (DT50) in soil of the most commonly used neonicotinoids typically range from 200 to 1000 days (Goulson 2013). Existing data vary to a great extent, however. For example, the DT50 for thiamethoxam varies between 7 and 3000 days, depending on soil type, conditions of the study, and publication (Goulson 2013). Neonicotinoid insecticides are highly toxic to a wide range of nontarget invertebrates and field recommended concentrations and doses are likely to cause both lethal and a broad range of important sublethal impacts (Goulson 2013; Pisa et al. 2015). Very little information is available regarding sublethal effects of neonicotinoids on insect predators, many of which perform important roles in ecosystem functioning and biocontrol.

In behavioural ecotoxicology of insects, including carabids, various locomotion parameters (mean velocity, distance moved, time moving, meander and others) are generally accepted as sensitive and valuable biomarkers of chemical stress caused by sublethal doses of pesticides at an individual level (Baatrup and Bayley 1998; Bayley 2002; Desneux et al. 2007; Prasifka et al. 2008; Tooming et al. 2014). Of these, locomotor activity (LA) parameters, path length and movement velocity express the level of intoxication best (Bayley 2002; Tooming et al. 2014). Locomotion mirrors the physiological, metabolic, neurological and anatomical status of an animal. At the same time, it is inherent in patterns of many basic behaviours such as

migration, foraging, habitat and micro-habitat selection, behavioural thermoregulation, inter- and intraspecific interactions etc., which are important for survival and reproduction. Thus, pesticide induced changes in insect locomotion inevitably interfere with the normal imperatives of many fitness-related behaviours (Haynes 1988; Everts et al. 1991; Bayley 2002; Merivee et al. 2015). Nevertheless, there is still little scientific evidence that pesticide induced alterations in locomotion affect performance, population dynamics and biocontrol efficacy in predatory arthropods. One example is the investigation on the carabid beetle *Harpalus pennsylvanicus*, where perturbations in locomotion caused by exposure to several insecticides, including the neonicotinoid imidacloprid, increase its vulnerability to predation by ants in the field (Kunkel et al. 2001) which, undoubtedly, negatively affects ecological fitness of the beetles on treated areas. Therefore, quantitative data on behavioural endpoints, including feeding, that directly affect ecological fitness, survival and biocontrol efficacy of natural enemies might be useful (Desneux et al. 2007; Prasifka et al. 2008; Merivee et al. 2015).

Feeding is essential for the maintenance of all life processes and is, therefore of special interest in ecotoxicological studies. Food consumption rate is principally an easily quantifiable behavioural endpoint that allows direct evaluation of biocontrol efficiency of natural enemies, such as predatory carabids. On food sources contaminated with sublethal doses of pesticides, including neonicotinoids, feeding behaviour of many insects may be interfered with by repellent, antifeedant, or reduced olfactory capacity effects (Blaney et al. 1990; Desneux et al. 2007; Han et al. 2010a, b; Abouelghar et al. 2013; He et al. 2013). In several phytophagous insects it has been demonstrated that reduced feeding may also occur when they, after exposure to pesticides (azadirachtin), return to clean food (Mordue Luntz et al. 1985; Rembold 1989; Simmonds et al. 1990; Koul and Isman 1991; Timmins and Reynolds 1992). Abnormalities in clean food consumption rate (CFCR) serve as an indication of physiological stress caused by exposure to pesticides (Sheehan 1984). Unfortunately, no data exist on CFRC in predatory arthropods, including carabids, intoxicated by sublethal doses of pesticides.

In the present study we focused on these lacks of knowledge on the effects of neonicotinoids on the locomotor and feeding activities with the example of *Platynus assimilis* Paykull (Coleoptera, Carabidae), which may have direct deleterious consequences for the biocontrol efficacy of these predatory beetles in the field. A wide range of sublethal doses of the widely used neonicotinoid insecticide thiamethoxam were administered orally to the insects to assess changes in LA parameters for 4 days after treatment, using a computer-centered automated video tracking system. Changes in CFRC were measured directly by weighing

the presented food before and after feeding, and calculating the difference during the same period of time after the treatments. Lethal dose (LD₅₀) of thiamethoxam to adult *P. assimilis* was determined before the experiments by acute oral toxicity testing because literature data were not available. The thiamethoxam dose range (1.1, 10.8 and 108.1 ng/g) used in the study is representative of what carabids would encounter in the field.

Materials and methods

Test beetles

The medium-sized Euro-Siberian carabid *P. assimilis* was the test insect of this study; it is 10–13 mm in length, reproduces in the spring and is common in its preferred deciduous forest habitats, parks and gardens (Thiele 1977; Lindroth 1986). In spring and summer it can migrate into open fields (Honěk and Kocian 2003). It also inhabits cereal fields (Purtauf et al. 2005a, b) and grasslands (Grandchamp et al. 2005) in small numbers. Tropically, it belongs to the generalist predator guild (Larochelle 1990; Zalewski et al. 2014). Thus, the widespread *P. assimilis* is essential in various ecosystems, and in previous ecotoxicological studies (Tooming et al. 2014; Merivee et al. 2015) it is considered as a good representative for all carabids.

The test beetles of *P. assimilis* were taken from the forests of Alam-Pedja Nature Reserve (southern Estonia) where no insecticides have been used for more than 20 years. They were collected from their preferred overwintering sites in brown-rotted tree stumps in April 2016. They were kept in 3 L plastic containers (50 beetles in each) filled with moist pieces of brown-rotted wood in a refrigerator at 5 °C. Five days prior to the insecticide treatments, the beetles were placed individually in transparent Petri dishes (85 mm diameter) lined with 80 mm moistened filter paper disks and transferred to a Versatile Environmental Test Chamber MLR-351H (SANYO Electric Co., Ltd., Japan) at 20 °C, L16: D8 photoperiod and 70% RH for acclimation to the experimental conditions. During this short acclimation period, the beetles were not fed.

Oral administration of thiamethoxam to the beetles

Six water suspensions of the commercial insecticide Actara® 25 WG (Syngenta) were prepared with thiamethoxam (active ingredient) concentrations of 8×10^{-4} , 24×10^{-5} , 8×10^{-5} , 24×10^{-6} , 8×10^{-6} and $8 \times 10^{-7}\%$, respectively. Food for the test beetles was prepared by homogenising together equal amounts (3 g) of the blue bottle fly larvae (*Calliphora vomitoria*) and tap water using the Benchtop Homogenizer Polytron® PT-MR 1600 E

(Kinematica AG, Switzerland) to obtain a semifluid homogenate of a suitable consistency for pipetting. Then, 3 mL of the food homogenate and 1 mL of the respective Actara® suspension were pipetted into a 5 mL Eppendorf tube, and carefully mixed. For oral administration, 20 µL portions of these food homogenate and insecticide mixtures were pipetted into 100 µL hollow plugs cut from 1 mL Eppendorf tubes and presented to the beetles for feeding. Thus, the respective thiamethoxam doses used in six insecticide treatment variants, in diminishing order, were 4×10^{-2} , 12×10^{-3} , 4×10^{-3} , 12×10^{-4} , 4×10^{-4} and 4×10^{-5} µg per 20 µL food portion. Considering the mean weight of 37 mg for the beetles ($N = 50$), the mean doses of thiamethoxam administered to the beetles were 1081.1; 324.3; 108.1; 32.4; 10.8 and 1.1 ng of body weight (g). The same amount (20 µL) of food homogenate with no insecticide content was administered to the control beetles. All the beetles used in the experiments fully consumed their food portion within 30 min. For treatments, fresh insecticide suspensions and food homogenates were used prepared directly before their administration to the beetles.

Determination of lethal dose (LD₅₀) in acute toxicity test

LD₅₀ (the dose that kills 50% of test animals) was determined by oral administration of thiamethoxam at six environmentally relevant doses of 1.1; 10.8; 32.4; 108.1; 324.3 and 1081.1 ng/g, respectively, mixed in 20 µL food homogenate. The number of beetles (N) tested in the different treatments varied from 23 to 72. In total, 234 beetles were used in these acute toxicity tests. After insecticide administration, the beetles were kept individually in 85 mm Petri dishes (RH ≈ 100%) with 80 mm moistened filter paper discs at the bottom, at 20 °C and L16:D8 photoperiod. Dead and live beetles were counted on the fourth day after treatment with insecticide. The beetles were scored dead when they did not respond to touch with a preparing needle.

Video-recording of the beetles' LA

For video-tracking, the beetles were placed singly into 85 mm Petri dishes lined with 80 mm moistened Whatman® filter paper discs (Whatman International Ltd., England) and transferred into a 50 × 60 × 50 cm lightproof cardboard box, up to 12 insects at a time, where they were allowed to settle for 20 min. On the first day (DAY 1), video-recordings of 4 h duration was started 30 min after insecticide administration. On the second (DAY 2) and fourth day (DAY 4) after the treatment, LA of the beetles was video-recorded within 2 h. The activity of up to 12 beetles was recorded with the resolution of 1920 × 1080 pixels at 5 frames/s simultaneously by a computer-centred video-tracking system using the USB Logitech HD Pro Webcam

C920 (Logitech Inc., USA) fixed at a height of 40 cm above the Petri dishes, and the computer software Debut Video Capture (NCH Software, USA). On each day, video-recording started at 11.00 a.m. The Petri dishes were illuminated from above by four LED lamps MR 16 (12 V, 6 W, 400 lm, 3000 K). Illumination at the level of Petri dishes with test beetles (3000 lux) was measured by the Digital Light Meter TES-1335 (TES Electrical Electronic Corp., Taipei, Taiwan). All video-recordings were made at 20 °C. To shorten the duration of the experiments, two identical computer-centred video-tracking systems were prepared which allowed video-recordings of LA of up to 24 insects simultaneously. The number of tested beetles (N) varied from 24 to 46 depending on the insecticide dose used, and the time elapsed from insecticide administration. The number of insecticide treated beetles during the period of the experiments decreased because some of the beetles died due to intoxication. All Petri dishes with test beetles were numbered to allow recording and analysis of behaviour of individual beetles during the experimental period. Immediately after video-recordings of the beetles' LA, the same beetles, without any additional treatment, were used in the feeding experiment.

CFCR

CFCR of the insecticide treated and control beetles was measured within 1 h on 3 days, 5, 24 and 72 h after the treatments, respectively. Each time, 60 µL of fresh, clean food homogenate was pipetted into twelve 100 µL hollow plugs of the Eppendorf tubes and presented to the beetles for feeding. For measurement of the amount of consumed food, two weighings were carried out using analytical balances AS 220/X (RADWAG Wagi Elektroniczne, Poland). First, the plugs with food were weighed immediately before feeding, up to 12 portions simultaneously. Then, after feeding, the plugs with remaining food were weighed again. Difference between the two weighings served as the food amount consumed. Since, the beetles were fed in Petri dishes at saturated air humidity conditions (RH = 100%; condensed water droplets on the inner surfaces of the dishes) no weight losses due to evaporation were observed. All treatments were carried out in four replications up to 12 beetles in each. On DAY 1, 5 to 6 h after treatment the number of beetles in each replication varied from 10 to 12. Over subsequent days, the number of insecticide treated beetles per replication decreased because some died due to intoxication. Thus, on the DAY 2, the number of insecticide treated beetles per replication varied from 7 to 12, and on the DAY 4 from 6 to 12 depending on thiamethoxam dose. Behavioural activity of the beetles close to the food source was video-recorded by the computer-centred video-tracking system described above allowing recording

and analysis of their activity individually during the experimental period.

Analysis of video data

Distance moved (start velocity > 0.20 cm/s; stop velocity < 0.20 cm/s) with the temporal resolutions of 1 and 30 min, and total, as the most important LA parameter was extracted offline from the recorded video files using EthoVision XT Version 9 software (Noldus Information Technology, Wageningen, The Netherlands). Temporal bin widths of 1 and 30 min were used for constructing locomotor activity actograms and dynamics of individual beetles, respectively. In the feeding experiments, the following activity parameters were extracted from the recorded video files using EthoVision XT software: total distance moved (TDM), the number of entries into the food source area (FSA) and time spent in the FSA. The circles (20 mm diameter) around the plugs of the Eppendorf tubes were defined as the 'FSAs'.

Statistical analysis

All statistical analysis were carried out using programs STATISTICA 11 (StatSoft, USA) and R ver 3.1.2 (R Development Core Team, Austria). Probit analysis was used to find the LD₅₀ of thiamethoxam for *P. assimilis*. To compare behavioural parameters of *P. assimilis* in various treatments Kruskal-Wallis test was used. All the test results were considered statistically significant at $p < 0.05$.

Results

Median lethal dose (LD₅₀) of thiamethoxam for *P. assimilis* adults with oral administration

The acute toxicity tests showed that with oral administration, the LD₅₀ of thiamethoxam for *P. assimilis* beetles was 114.5 ng/g (95% confidence interval 73.6–192.5 ng/g) (Fig. 1). The susceptibility of the beetles to this insecticide varied to a large extent, however. For example, when the lowest dose (1.1 ng/g) was used, two of 46 tested beetles died due to intoxication. At the highest dose (1081.1 ng/g), 19 of 24 tested insects died, which means that 5 beetles survived the dose.

Effect of sublethal doses of thiamethoxam on the LA rhythms

Plots of distances moved vs. time with temporal resolution of 1 min represent actograms allowing visualization peculiarities of the LA pattern of individual beetles (Fig. 2). In control beetles, short LA periods of several minutes

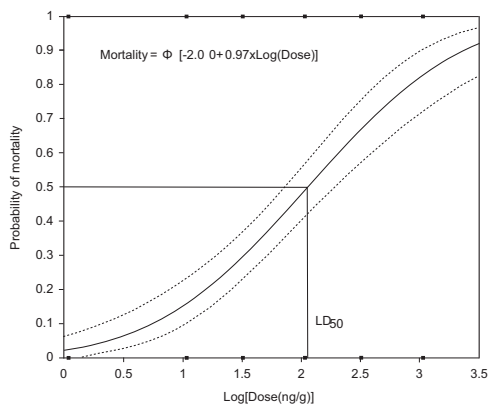


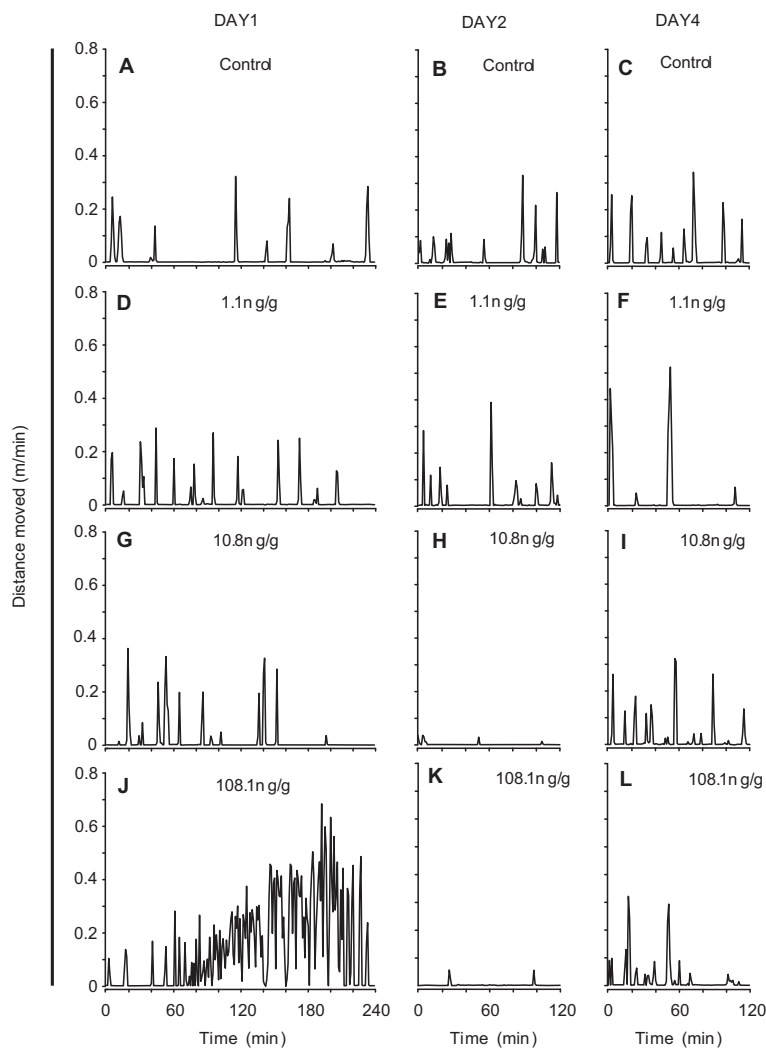
Fig. 1 Mortality probability of *P. assimilis* adults at different doses of thiamethoxam within four days after dietary exposure. *Solid and dotted black lines* indicate the probit regression curve and its 95% confidence interval, respectively. The LD₅₀ represents the dose (114.46 ng/g) that causes a lethal response in 50% of test insects. The *small squares* on the horizontal axis show the real doses tested

duration alternated with long resting periods usually lasting from 10 to 60 min (Fig. 2 a–c). Typically, LA peaks reached 0.1 to 0.3 m/min. LA actograms of the insects treated with 1.1 ng/g thiamethoxam were very similar to those of the control on all 4 days of observation (Fig. 2d and f). When the administered thiamethoxam dose was increased to 10.8 ng/g, the LA pattern of the beetles did not differ from control on DAY 1 (Fig. 2g) but 24 h later, on DAY 2, the beetles became hypoactive (Fig. 2h). They ran infrequently and crossed very short distances at a time, in most cases, 0.01 to 0.05 m/min. By DAY 4, however, the intoxicated beetles had recovered their normal LA pattern (Fig. 2i). LA actograms of the beetles treated with the highest dose of thiamethoxam (108.1 ng/g) drastically changed with time. On DAY 1, about 60 min after the beginning of the 4 h observation period, (i.e. about 90 min after thiamethoxam administration) the beetles progressively became hyperactive (Fig. 2j). The state of hyperactivity lasted for several hours, after which the LA of the beetles gradually decreased. On DAY 2, the beetles were in a state of hypoactivity, they changed their position seldom and usually moved no more than 0.05 m/min at a time (Fig. 2k). By DAY 4, the beetles had fully recovered from the locomotion abnormalities and their LA actograms did not considerably differ from those of the control (Fig. 2l).

LA, in terms of the distances moved

LA of the beetles depended on both the dose of thiamethoxam administered and the time following treatment (Fig. 3a). On DAY 1, the control beetles moved a mean of

Fig. 2 Example LA actograms of *P. assimilis* dietary exposed to various sublethal doses of thiamethoxam on DAY 1, DAY 2 and DAY 4. Locomotion of the beetles was video-tracked within 4 h (DAY 1) or within 2 h (DAY 2 and DAY 4) of treatment. Each LA actogram is a representative of one beetle



0.29 to 0.95 m per 30 min. Within the first 30 min of observation, the LA of the beetles treated with the highest dose (108.1 ng/g) of insecticide did not differ from that of the control. Thereafter, the beetles became hyperactive, and within the next 30 min, they moved 1.8-fold further than the control. In addition, locomotor hyperactivity of the insecticide treated beetles even increased achieving its maximum values (3.1–3.2 m/30 min) at 90 to 180 min. Thus, hyperactive insects moved up to 10.6-fold further than the control beetles. After that hyperactivity maximum with duration of 90 min, LA of the insecticide treated beetles began to

slowly decrease to the level of 1.4 m/30 min at the end of the 4 h observation period (210 to 240 min), which was still 4.9-fold higher than the control. LA of the beetles treated with 1.1 and 10.8 ng/g thiamethoxam did not differ from that of the control beetles on DAY 1. At the end of the observation period (150–240 min), the distances moved by the beetles treated with thiamethoxam at a dose of 10.8 ng/g were 58 to 117% longer than control but the differences were statistically not significant.

On DAY 2, the beetles of all three insecticide treatments were in a state of locomotor hypoactivity. Within the 2 h of

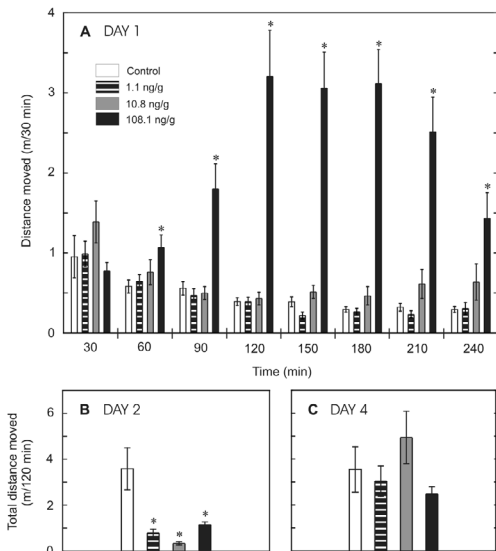


Fig. 3 The effect of sublethal doses of thiamethoxam on the LA of *P. assimilis*. LA was expressed by the distance moved with temporal resolution of 30 min to demonstrate locomotor activity dynamics within 240 min after treatment (a). TDM were calculated for DAY 2 and DAY 4 to show LA changes in a longer time-scale (b and c, respectively). Asterisks show significant differences from the control (Kruskal-Wallis test; $P < 0.05$; $N = 43\text{--}46$ (DAY 1), $N = 25\text{--}46$ (DAY 2), $N = 24\text{--}44$ (DAY 4)). The decline in the number of tested insects in DAY 2 and DAY 4 was caused by partial mortality of the insecticide treated beetles during the experiment

observation, they moved 3.3–11-fold shorter distances ranging from 0.3 to 1.1 m depending on the insecticide dose, compared to the 3.6 m moved by the control beetles (Fig. 3b). By DAY 4, all the beetles had fully recovered from locomotor hypoactivity independent of the administered thiamethoxam dose. Their total distance moved (TDM) varied from 2.5 to 4.9 m/120 min but significant differences from the control variant (3.6 m/120 min) were not observed (Fig. 3c).

Effect of sublethal doses of thiamethoxam on the CFCR

Our experiments showed that thiamethoxam considerably affected feeding activity of the treated beetles depending on dose and time after insecticide administration. On DAY 1, 5–6 h after treatment, the control beetles consumed a mean of 12.9 mg/h clean food (Fig. 4a). Compared to the control, a 9.2-fold fall in CFCR was observed in beetles treated with the highest dose (108.1 ng/g) of thiamethoxam. Also, at administration of the insecticide at a dose of 10.8 ng/g, the insects consumed considerably less than the control group,

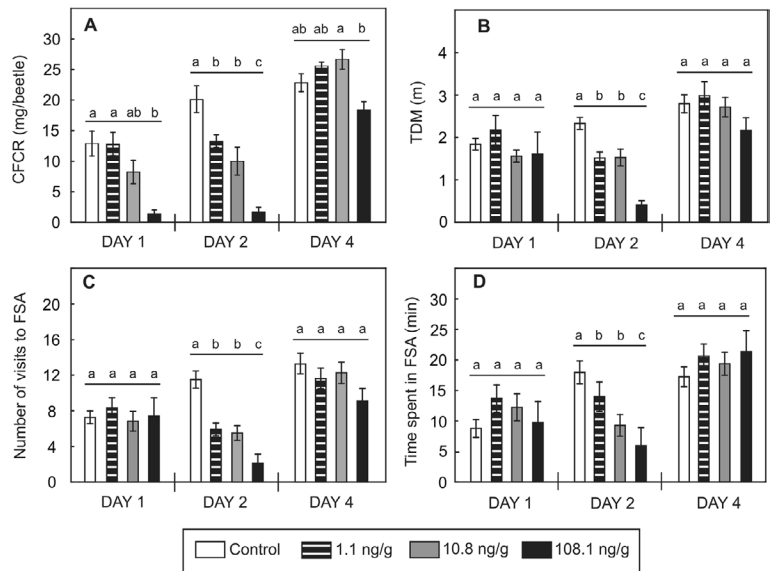
but the difference was statistically not significant. By contrast, the lowest administered dose (1.1 ng/g) did not affect CFCR of the beetles on DAY 1. We also compared several other aspects of behavioural activity close to the food source between thiamethoxam treated beetles and control. However, no differences between the treatments, including control, were found concerning the TDM, the number of visits into FSA and the time stayed in FSA (Fig. 4b–d). Thus, 5 to 6 h after treatment, none of the three measured LA parameters mirrored essential insecticide dose dependent changes in CFCR (Fig. 4a–d).

On DAY 2, the restricting effect of thiamethoxam on the CFCR of the beetles was even stronger. In all the three insecticide treatments, the beetles consumed 1.5 to 11.8-fold less insecticide-free food than the control depending on the administered dose (Fig. 4a). All the three LA parameters, TDM, the number of visits into FSA and the time stayed in FSA remarkably decreased after exposure to the insecticide independent of the administered dose, being in a good accordance with the CFCR response (Fig. 4a–d).

By DAY 4, however, insecticide treated beetles had recovered normal feeding activity to a large extent (Fig. 4a). A little increase in CFCR of the beetles treated with the two lowest doses of the insecticide (1.1 and 10.8 ng/g) was observed but the difference from control was statistically not significant. By contrast, the highest administered dose of thiamethoxam (108.1 ng/g) caused a 10% decrease in food consumption rates of the beetles, but again, the difference from that of the control insects was statistically not significant. Significant difference in CFCR was detected between the two insecticide treatments at 10.8 and 108.1 ng/g, however, indicating that thiamethoxam dose had an effect on the feeding response. By DAY 4, the beetles had fully recovered from behavioural malfunctionings related to mobility and no difference was detected between the treatments, including control, were observed (Fig. 4b and c). Again, none of the LA parameters reflected the insecticide dose dependent changes in CFCR adequately (Fig. 4a–d).

Further, we compared behavioural activity at the FSA in beetles that survived the insecticide treatments with that of the beetles that died henceforth during the experiment. Since the numbers of dead insects by DAY 2 were 0 of 44, 0 of 46, 8 of 48 and 20 of 44 at 0 (control), 1.1, 10.8 and 108.1 ng/g of thiamethoxam, respectively, only the two last treatments were taken into account in this analysis for DAY 1. Surprisingly, no significant differences in TDM, the number of visits into FSA and the time stayed in FSA between these two categories of test beetles were observed (Wilcoxon test, $p > 0.05$). Due to comparatively low mortality from DAY 2 to DAY 4 ranging between 0 and 3 individuals per treatment, respective comparisons of the behavioural activities between the two categories of insects were not done for DAY 2.

Fig. 4 Behavioural activity of *P. assimilis* adults after dietary exposure to sublethal doses of thiamethoxam on Petri dish arenas supplied with clean food. The measurements were made during 60 min observation periods, on DAY 1. Different letters show statistically significant differences between the treatments (Kruskal-Wallis test; $P < 0.05$; in feeding experiment, $N = 4$, the number of tested beetles in each replicate varied from 6 to 12 depending on treatment and DAY (a); in video-tracking experiment, $N = 23\text{--}46$ depending on treatment and DAY (b–d)). Vertical lines indicate SE of the means



Discussion

In this study, for the first time in predatory carabids, dose-dependent sublethal effects of a neonicotinoid insecticide on two basic behaviours, locomotion and feeding, were quantified. The results, obtained by using video-tracking technologies, show that the neonicotinoid thiamethoxam administered orally at three tested environmental doses (1.1; 10.8 and 108.1 ng/g) considerably affects the LA of the carabid beetle *Platynus assimilis*. Two measured behavioural endpoints associated with mobility, the LA pattern and distance moved, change after a single insecticide treatment depending on the insecticide dose and time after application. About 90 min after administration of thiamethoxam at the highest dose (108.1 ng/g) close to its LD_{50} (114.5 ng/g) the beetles become hyperactive but none of them die due to intoxication within 6 h after ingestion. The normal alternation of long rest periods and short LA periods disappears and is replaced by almost continuous elevated LA lasting for several hours. By contrast, dietary exposure to ten and a hundred-fold lower doses of thiamethoxam, 1.1 and 10.8 ng/g, respectively, do not have any noticeable effect on LA of the beetles within several hours after insecticide administration. Short-term locomotor hyperactivity also occurs in insect predators, including carabids, exposed to low doses of other classes of neurotoxic insecticides, for example pyrethroids (Alzogaray and Zerba 2001; Sfara et al. 2006; Prasifka et al. 2008; Tooming et al. 2014) and organophosphates (Jensen et al. 1997; Singh et al. 2001).

So far, only few quantitative data exist on the dynamics of LA in predatory insects exposed to neurotoxic insecticides other than the neonicotinoid thiamethoxam described in this study. Tooming et al. (2014) showed, however, that in contrast to thiamethoxam, the pyrethroid insecticide alpha-cypermethrin causes remarkable changes in the LA of *P. assimilis* at a low range of sublethal doses, and the changes develop much more quickly compared to those caused by the neonicotinoid. The beetles display a short-term (60 to 90 min) locomotor hyperactivity starting a few minutes after their brief exposure to this pyrethroid at concentrations of 0.01 and 0.1 mg/L, while higher sublethal concentrations (1–100 mg/L) cause a quick knock-down effect with disability to move and lasting from several hours up to a couple of days depending on the insecticide concentration. Our findings also showed that the day after thiamethoxam administration, at all dietary administered doses (1.1–108.1 ng/g), the beetles of *P. assimilis* are in a state of long-term locomotor hypoactivity. Thus, our results confirm that LA is a highly sensitive ecotoxicological biomarker for assessing long-term toxic stress caused by neonicotinoid insecticides. The observed changes in distances moved appear even at approximately 1% of the LD_{50} consistent with the few existing comparable data for terrestrial arthropods exposed to low doses of organophosphorous insecticide dimethoate (Bayley 1995; Sørensen et al. 1995) and pyrethroid alpha-cypermethrin (Tooming et al. 2014).

Recovery from thiamethoxam induced locomotion anomalies in *P. assimilis* takes several days consistent with earlier literature data obtained for the predatory carabid

beetle *Chlaenius tricolor* (Douglas et al. 2014). Similar alternation of antagonistic changes in LA have also been observed in *P. assimilis* exposed to a wide range of sublethal doses of the pyrethroid alpha-cypermethrin (Tooming et al. 2014) and in the carabid *Scarites quadriceps* exposed to the pyrethroid insecticides lambda-cyhalothrin and tefluthrin (Prasifka et al. 2008). These abnormalities in arthropod locomotion may, in some cases, last long after contact with neurotoxic insecticides. For example, changes in locomotor behaviour of the woodlouse *P. scaber* caused by intoxication with the organophosphorus insecticide dimethoate have been observed 21 days after insecticide exposure (Bayley 1995). Both locomotor hyperactivity and locomotor hypoactivity reflect overall toxic stress of an insect and strongly indicate a reduction in performance (Bayley 2002; Kunkel et al. 2001; Merivee et al. 2015).

In the field, neonicotinoid seed treatment decreases carabid activity-density (Leslie et al. 2010; Douglas et al. 2014) but the reasons for this are not well understood. Both lethal and sub-lethal effects of insecticide use may cause low pitfall trap catches. Our results show that in addition to direct mortality caused by neurotoxic insecticide application (Vickerman and Sunderland 1977; Mullin et al. 2005; Pisa et al. 2015) long-term locomotor hypoactivity may also contribute to low numbers of carabids trapped in agricultural fields where neonicotinoids are used. The real number of live beetles in various levels of toxic stress should be larger than pitfall trap catches show.

Thiamethoxam-induced reduction in CFCR is another altered behavioural endpoint of poisoned predatory insects as first demonstrated for *P. assimilis* in this study. This effect depends on both thiamethoxam dose and time from exposure. The observed decline in CFCR of the beetles develops relatively slowly and it may continue for a couple of days depending on the administered insecticide dose. Our results show that on the first day of thiamethoxam administration, a remarkable reduction in feeding only occurs in beetles treated with a high sublethal dose (108.1 ng/g) close to the LD₅₀, but on the next day, this negative effect appears even at doses ten to a hundred-fold lower than that. Recovery from reduced feeding takes several days.

In the laboratory, carabid beetles readily attack prey items and seeds poisoned with neonicotinoid insecticides (Douglas et al. 2014; Cutler et al. 2016). Rates of carabid food consumption in the field are not well quantified, but both carabid larvae and adults are voracious feeders and many species consume close to their own body mass of food each day (Thiele 1977), suggesting that they could be chronically exposed to neonicotinoids in treated fields when plant seeds and prey items, poisoned with these insecticides, are abundant. In the field, predatory insects, including carabids, can encounter seed-treated neonicotinoid insecticides through zoophytophagy (Mullin et al. 2005; Moser and

Obrycki 2009; Seagraves and Lundgren 2012), and they can be exposed via poisoned herbivorous prey (Szczepaniec et al. 2011; Douglas et al. 2014). Thus, it is likely that in addition to direct mortality (Douglas et al. 2014; Cutler et al. 2016) long-term reduction in feeding may diminish carabid performance as natural enemies of pests in the fields where neonicotinoid insecticides are applied.

Long-term reduction in feeding caused by intoxication with sublethal doses of thiamethoxam may also negatively affect ecological fitness of the beetles, for example, fecundity and longevity. Insect predators have to expend energy constantly, and if a suitable diet is not available, they must live on reserves accumulated in periods of food abundance. Food is used to build fat reserves, especially before reproduction and hibernation (Thiele 1977; Lövei and Sunderland 1996; Arrese and Soulages 2010). In carabids, food consumption rate and its quality during larval development determine adult body size, which is a major determinant of their potential fecundity (Nelemans 1987; Lövei and Sunderland 1996; Knapp and Uhnová 2014). Realized fecundity depends on adult feeding conditions, however. When food consumption is limited, females lay far below their possible maximum egg capacity (Sota 1985; Nelemans et al. 1989; Weseloh 1993; Van Dijk 1994; Knapp and Uhnová 2014). In addition, continuous starvation drastically shortens the potential survival period in both carabid adults and larvae (Van Dintner 1964; Kabacik-Wasylik and Stejgwill-Laudanska 1971; Luff 1994; Petersen 1999; Young 2008).

Both the changes in LA and decline in CFCR express toxic stress. Therefore, certain synchronism of abnormalities in LA and CFCR of carabid beetles' diet exposed to sublethal doses of thiamethoxam is not surprising. The doses that affect LA concurrently have an effect on CFCR. Feeding is considerably inhibited in both hyperactive and hypoactive beetles. The effect of thiamethoxam on feeding is largest on DAY 2 when the beetles are sluggish and largely immobile. By the DAY 4, the normal LA of the poisoned insects is recovered simultaneously with the recovery of their normal CFCR. Our results show that both of these two ecotoxicological biomarkers are essential to assess toxic stress adequately in insect predators exposed to sublethal doses of neonicotinoids. In the period when locomotor hyperactivity changes over locomotor hypoactivity, important LA parameters such as distance moved do not reflect depth of toxic stress properly. In this case, CFCR data may give valuable additional information on physiological condition of an insect contacted with these insecticides.

Even though, LA is a precise and relevant ecotoxicological biomarker of toxic stress in nontargeted predatory insects there exist few data demonstrating that changes in LA have a direct effect on fitness and biocontrol

performance. For a rare positive example, in laboratory conditions, carabids exposed to sublethal doses of insecticides usually recover from locomotion abnormalities within several days (Kunkel et al. 2001; Prasifka et al. 2008; Tooming et al. 2014), but in the field they are easily predated by ants (Kunkel et al. 2001). Therefore, fitness-related behavioural endpoints such as foraging (Dempster 1968), behavioural thermoregulation (Merivee et al. 2015) and food consumption rate might be more useful for assessing the impact of sublethal doses of insecticides on the performance and efficiency of carabids as biocontrol agents. To conclude, for the first time in predatory arthropods, our results show that low-dose neonicotinoid insecticide thiamethoxam induced short- and long-term negative effects on insect locomotion, as well as long-term reduction in CFCR. Both LA and CFCR are sensitive and valuable ecotoxicological biomarkers of carabids which should be taken into account in IPM programs where optimal combination of reduced insecticide use and biological control by predatory insects is crucial to achieve best results.

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LIST OF PUBLICATIONS

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- Tooming, E.**, Merivee, E., Must, A., Merivee, M-I., Sibul, I., Nurme, K., Williams, I.H. 2017. Behavioural effects of the neonicotinoid insecticide thiamethoxam on the predatory insect *Platynus assimilis*. *Ecotoxicology*, 26 (7), 902–913.
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