

FOLSOMIA CANDIDA (COLLEMBOLA)
LOCOMOTOR ACTIVITY PATTERN IS CHANGED
BY A NEUROTOXICANT PESTICIDE

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Behavioural traits, including locomotor activity, have become relevant parameters in contemporary ecotoxicology. However, insecticide effects on soil animal behaviour has remained a neglected field until now. The Trebon 30 EC is an expansively used pyrethroid insecticide in many countries, which has a broad spectrum of activity. It is recommended against pests of different vegetables, cereals, fruits, and flowers. The effect of this insecticide on the behaviour of collembola *Folsomia candida* has never been tested. Nevertheless, an effect is likely, because of the neurotoxic nature of the Trebon 30 EC insecticide. This study aimed to determine, (i) whether the Trebon 30 EC insecticide (active ingredient: etofenprox) prevails on the collembola *F. candida* locomotor activity pattern immediately after insecticide application and (ii) if there is any temporal change in the locomotor activity pattern within one hour of insecticide application. Prompt hyperactivity followed by strongly reduced locomotor activity of *F. candida* due to Trebon 30 EC application has been demonstrated in this study. Collembola started to run faster even when a tenfold concentration of the recommended field rate was applied. Moreover, this insecticide causes locomotor irregularity in *F. candida*. Chronic insecticide exposure has a negative impact on all studied locomotor traits of this collembola, resulting in a knockdown effect.

Keywords: *Folsomia candida*, etofenprox insecticide, locomotor activity, neurotoxicant pesticide.

INTRODUCTION

Collembola play a key role in agricultural soils (LARINK 1997). They significantly contribute to vital integrative processes, as decomposition and mineralisation (VERHOEF & BRUSSAARD 1990, BEARE *et al.* 1992, COLEMAN *et al.* 2002). Collembolan populations have an influential position in soil food web networks with strong effects on community structure (HALAJ & WISE 2002, KANEDA & KANEKO 2008). Consequently, any agricultural impact on collembolan populations, including pesticide application, could have a severe outcome on agroecosystem functioning.

Pyrethrins and pyrethroids are commonly used pesticides to control agricultural and household pests. They quickly penetrate and act on the insects' nervous system, causing hyperactivity (ISMAN 2006), then paralysis and loss of movement coordination by prolonging the opening of sodium chan-

nels in nerve cells. This is a relatively quick, so-called knockdown process (SCHLEIER & PETERSON 2011). The knockdown effect has been extensively studied in mosquito control (LIU 2015, LUKWA *et al.* 2012), the housefly (SAWICKI 1962), stored-product beetles (TOEWS *et al.* 2003), locusts (CLEMENTS & MAY 1977) and the bloodsucking bug *Triatoma infestans* (ALZOGARAY & ZERBA 1997). Knockdown resistance can arise after continuous pesticide application. That is why the knockdown effect of the same pesticide may be different in various populations of same species. This problem is especially relevant in mosquito control, where pyrethroids are applied for a long time (LUKWA *et al.* 2012). The knockdown effect is a rapid process, often acting in minutes, sometimes in a few hours. A mosquito is knocked down if it is not able to stand or fly in a coordinated manner after 60 minutes of the insecticide application (WHO 2013). The knockdown outcome is rarely lethal, and recovery occurs in most cases (ALZOGARAY & ZERBA 1997). However, according to BENOIT (1986) and RAJASHEKAR and SHIVANANDAPPA (2017), knockdown usually precedes death.

The Trebon 30 EC (Mitsui Chemicals, Inc.) is an expansively used pyrethroid insecticide in several countries, mainly in Asia. The active ingredient is etofenprox (2-(4-ethoxyphenyl)-2-methylpropyl-3-phenoxybenzyl-ether). This insecticide contains etofenprox in a concentration of 287.5 g/l (30% m/m). Etofenprox acts as contact poison through consumption, and its knockdown effect is also known (BRANCATO *et al.* 2017). This insecticide has 49.1–100 and 27.5 days of half-life under anaerobic and aerobic conditions (at 22 C°), respectively in an agricultural soil (VASQUEZ *et al.* 2011). Absorption of the etofenprox onto soil particles is strong (EU 2013). Etofenprox is a broad-spectrum pyrethroid. It has low toxicity to mammals (YOSHIMOTO *et al.* 1989), but it is very toxic to aquatic animals (ECHA 2012) such as rainbow trout and bluegill sunfish (LC₅₀ 0.032 mg/l, 96 h, for both species), as well as for *Daphnia magna* (LC₅₀ 0.0027 mg/l, 48 h) (CERTIS 2015). It is recommended against pests affecting different vegetables, cereals, fruits, and flowers. In China, etofenprox is one of the most important pesticides for controlling the rice planthopper, the cabbage caterpillar, and the cotton bollworm (ZHANG *et al.* 2013). This active ingredient is also used in the USA (BOINA & BLOOMQUIST 2015), Iran (VATANDOOST *et al.* 2004) and West Africa (MARTIN *et al.* 2002). However, side effects of the etofenprox on soil arthropod behaviour are not known.

In consequence of its neurotoxic nature, the etofenprox effect was found on the behaviour of the earwig, *Doru luteipes* (CAMPOS *et al.* 2011), honey bee, *Apis mellifera* (MATSUMOTO 2013), bean bug, *Riptortus pedestris* (MAHARJAN & JUNG 2015), and *Aedes aegypti* mosquito (AGRAMONTE *et al.* 2017). As the etofenprox containing insecticides are applied by spraying on fields, collembolan exposure to pesticides is quite likely. Moreover, side effects of etofenprox

(being a neurotoxic agent) on the behaviour of collembolan is also probable; however, such data has been unavailable until now.

Folsomia candida is a representative of soil mesofauna in laboratory studies. A standard avoidance test of polluted soils with this species is available (ISO 2011) and has been used in several studies. The jumping ability of *F. candida* significantly decreased with enhancement of soil Cu and Ni contamination (KIM & AN 2014). A low rate application (one-quarter and one-eighth of the recommended field application rate in Denmark) of the insecticide Dimethoate 400 g/l, EC (active ingredient: dimethoate) enhanced the turning rate and average velocity of *F. candida* during the 36 hours of the study (SØRENSEN *et al.* 1995). The orientation of *F. candida* in the direction of the food source (AUCLERC *et al.* 2010) was also proved.

The side effects of the extensively used insecticide Trebon 30 EC on soil fauna are hardly known. We found (SZABÓ & BAKONYI 2017) that *F. candida* laid smaller eggs in a concentration-dependent manner if Trebon 10 F (active ingredient: etofenprox) was applied. That is why this study aims to determine, whether (i) the Trebon 30 EC insecticide (active ingredient: etofenprox) knock-down effect prevails on the collembola *F. candida* locomotor activity pattern immediately after insecticide application, and (ii) if there is a considerable change in locomotor activity components after etofenprox treatment in different concentrations. Alteration of the locomotor activity pattern within one hour of insecticide application was also determined to check temporary changes.

MATERIAL AND METHODS

Folsomia candida (Willem 1902) is a cosmopolitan soil animal species, which exists almost all over the globe, including North America and Europe. It prefers soils with a high level of organic matter, but is common in various forest and agricultural soils as well. It is a focal laboratory animal and widespread object of different ecotoxicological tests. More information and a detailed description of *F. candida* biology and ecotoxicology can be found in FOUNTAIN and HOPKIN (2005), HOPKIN (1997), and KROGH (2009).

Collembola used in this study was obtained from our stock populations at Szent István University, Department of Zoology and Animal Ecology. This species has been reared continuously in the laboratory for the past 25 years. *F. candida* reared in our laboratory belongs to the lineage B, according to the categories of TULLY *et al.* (2006). Collembolas are kept under standard conditions in Petri dishes with a diameter of 9 cm, according to the method of Goro (1960). The bottom of the Petri dish is filled with plaster of Paris mixed with activated charcoal (10:1 volume ratio). The temperature of the incubation cabinet is $20 \pm 0.2^\circ\text{C}$, with ~100% humidity. The animals are in total darkness. Petri dishes are watered as needed to maintain consistent humidity. The collembolas are fed with dry baker's yeast once per week, ad libitum.

The experiment was performed in 9 cm diameter Petri dishes filled with a 0.5 cm layer of plaster of Paris and powdered graphite (9:1) mixture. It was kept moist during the experiment to allow for 100% moisture content in the dish. Three concentrations were

applied as follows: control, 1.3 and 13 ml Trebon 30 EC/l. 1.3 ml Trebon 30 EC/l is a recommended field concentration of this insecticide against pests in potatoes. After the Trebon 30 EC application, Petri dishes were aerated for one hour to allow the naphthalene, one of the components of this insecticide, to evaporate. According to our previous experiments, one hour is enough for naphthalene evaporation under our experimental circumstances. *F. candida* young adults (2.22 ± 0.17 mm) were used in the experiment.

Collembolan behaviour was studied with the aid of video records. One plastic ring (15 mm in diameter) was placed in the middle of the Petri dish and one *F. candida* individual was put into it. Two series of experiments were performed. In the first experiment, individuals were introduced into the plastic ring immediately after naphthalene evaporation and video record was started prompt (3 treatments, 12 replicate per treatment). With the aid of this experimental set-up, we can answer the following question: which method alters *F. candida* activity when it comes in contact with the pesticide immediately after spraying? In the second experiment, collembola were left for one hour in the plastic ring after naphthalene evaporation, then the video recording started (3 treatments, 4 replicate per treatment). Some collembola died during this hour, but others survived. With the aid of this experimental set-up, we can answer the following question: compared to the control, is there any change in the activity of the collembola which survived?

The covered Petri dishes were placed under an STM9-TRI binocular microscope. The illumination was homogeneous with the aid of a fiber-optic light source placed around the objective lens. The video record was performed with a 3.2 MP MicroQ-W (widefield) PRO camera and the processing speed was 12 frames s^{-1} . Records were analysed with the BORIS v. 3.60 software (FRIARD & GAMBA 2016). The movement of each collembola within the arena was recorded for two minutes. The total times of locomotion activity and path length, i.e. total walked distance, time spent standing in two minutes and average velocity during locomotion activity were determined. Moreover, path lengths of the locomotion activity were also assessed. The total video length (2 minutes) was divided into 0.5-second parts (PL0.5). The distance moved during all 0.5 seconds was also determined. Distribution of these distances was plotted in histograms.

Statistical analyses were performed with the PAST statistical software package (HAMMER *et al.* 2001). The Trebon 30 EC effect was determined by the generalized linear model (GLM). Uniformity of path length distribution was checked by the Mann-Whitney test. The accepted level of significance was $p < 0.05$ in all cases.

RESULTS AND DISCUSSION

Immediately after insecticide contact (in the first two minutes after putting collembolans into the contaminated Petri dish), hyperactivity of *F. candida* was detected. Applied concentrations of the Trebon 30 EC were not related to locomotion activity and time spent standing (Table 1). However, longer path length and average velocity during locomotion activity were observed at higher insecticide concentration (Table 1). A positive relationship between path length and average velocity during locomotion activity was observed ($y = 0.0087x$; $r = 0.95$; $p < 0.001$) (Fig. 1). This relationship was independent of the treatments. We found in other unpublished experiments that the longer path length, the quicker the collembola *F. candida* run. Similar results have

Table 1. Relationship of the measured locomotor activity traits of *F. candida* and Trebon 30 EC concentrations in the first two minutes after putting collembolans into the contaminated Petri dish. NS: non significant. Concentration unit is ml Trebon 30 EC/l.

Behaviour	Relationship with pesticide concentration	Significance (p)
Locomotion activity (LA) (sec)	$y = 0.39x + 104.4$	0.57 (NS)
Standing (S) (sec)	$y = -0.39x + 15.6$	0.57 (NS)
Path length (PL) (mm)	$y = 6.94x + 224.0$	0.024
Velocity (V) (mm/sec)	$y = 0.06x + 2.1$	0.007

been found in studies with carabid beetles *Pterostichus cupreus* (JENSEN 1997) and *Scarites quadriceps* (PRASIFKA *et al.* 2008).

Hyperactivity was detected when three different strains of the bug *Triatoma infestans* were treated with deltamethrin, lambda-cyhalothrin and tetramethrin (SFARA *et al.* 2006). Rapid horizontal movement of the agricultural pest stink bug *Halyomorpha halys* was observed immediately after exposure to nine pyrethroid insecticides by LEE *et al.* (2013). Dimethoate, an organophosphate compound, induced hyperactivity at sublethal doses in the woodlice, *Porcellio scaber* (BAYLEY & BAATRUP 1996). However, the predatory bug, *Supputius cincticeps* showed hypoactivity after chlorantraniliprole application (DE CASTRO *et al.* 2013). In our experiment, the insecticide Trebon 30 EC immediately enhanced the locomotor velocity and path length of *F. candida*, which are signs of hyperactivity. This reaction and lack of the knockdown effect im-

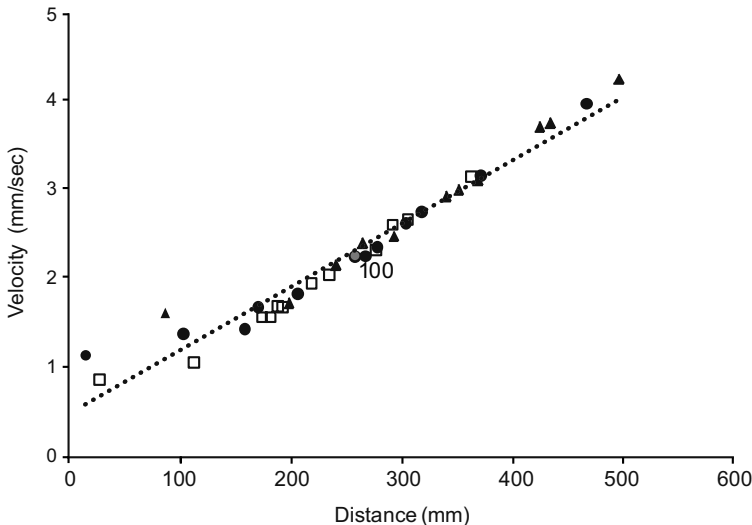


Fig. 1. Relationship between walked distance and velocity of *F. candida*. Open square: control, filled circle: treatment 1.3 ml Trebon 30 EC/l, filled triangle: treatment 13 ml Trebon 30 EC/l

Table 2. Skewness and kurtosis of the path lengths in 0.5 second during the locomotion activity of *F. candida* in the relation of Trebon 30 EC concentration.

Trebon 30 EC (ml/l)	0	1.3	13
Skewness	1.615	1.233	0.88
Kurtosis	1.398	-0.032	-0.849

mediately after insecticide application may be an escape reaction, which helps collembola survive on the field.

PL0.5 distribution changed with insecticide concentration (Fig. 2). In the control treatment, the frequency of short distances dominated, i.e., collembola usually moved short distances. Higher concentrations enhanced the frequency of longer path lengths. The proportion of the 0-5 mm long path were 35.7, 26.9 and 21.1 percent of the total in the control, 1.3 and 13 ml Trebon 30 EC/l treatment, respectively. The PL0.5 distribution in the control and 13 ml Trebon 30 EC/l treatments differed significantly ($p = 0.038$). Therefore, the distribution mean was altered. Skewness values are positive in all cases, showing that data are skewed right. However, skewness became lower as the concentration was raised (Table 2). Kurtosis values decreased as the concentration increased (Table 2). This figure indicates that outliers, e.g., extreme path lengths, become more frequent with the increase of the concentration (WESTFALL 2014). Lower skewness and kurtosis values parallel with enhanced concentration may be a sign of the locomotor irregularity due to insecticide. Pyrethroid insecticides cause locomotor irregularity in rats (WEINER *et al.* 2009), but this occurrence is still unexplored.

The length of the initial hyperactivity is different depending on the species tested, mode of the action of pesticide, and applied concentrations. No comparative study is available about the most decisive factors, which determine the hyperactivity length of the insects. Concentration proved to be a significant feature when carabids, *Platynus assimilis* (TOOMING *et al.* 2014, 2017) and *Scarites quadriceps* (PRASIFKA *et al.* 2008) were tested.

Table 3. Relationship of the measured locomotor activity traits of *F. candida* and Trebon 30 EC concentrations when collembolans spent one hour in the contaminated Petri dish. Concentration unit is ml Trebon 30 EC/l.

Behavior	Relationship with pesticide concentration	Significance (p)
Locomotion activity (LA) (sec)	$y = -7.1x + 114.6$	0.000
Standing (S) (sec)	$y = 7.1x + 5.3$	0.000
Path length (PL) (mm)	$y = -19.1x + 261.9$	0.000
Velocity (V) (mm/sec)	$y = -0.13x + 2.3$	0.000

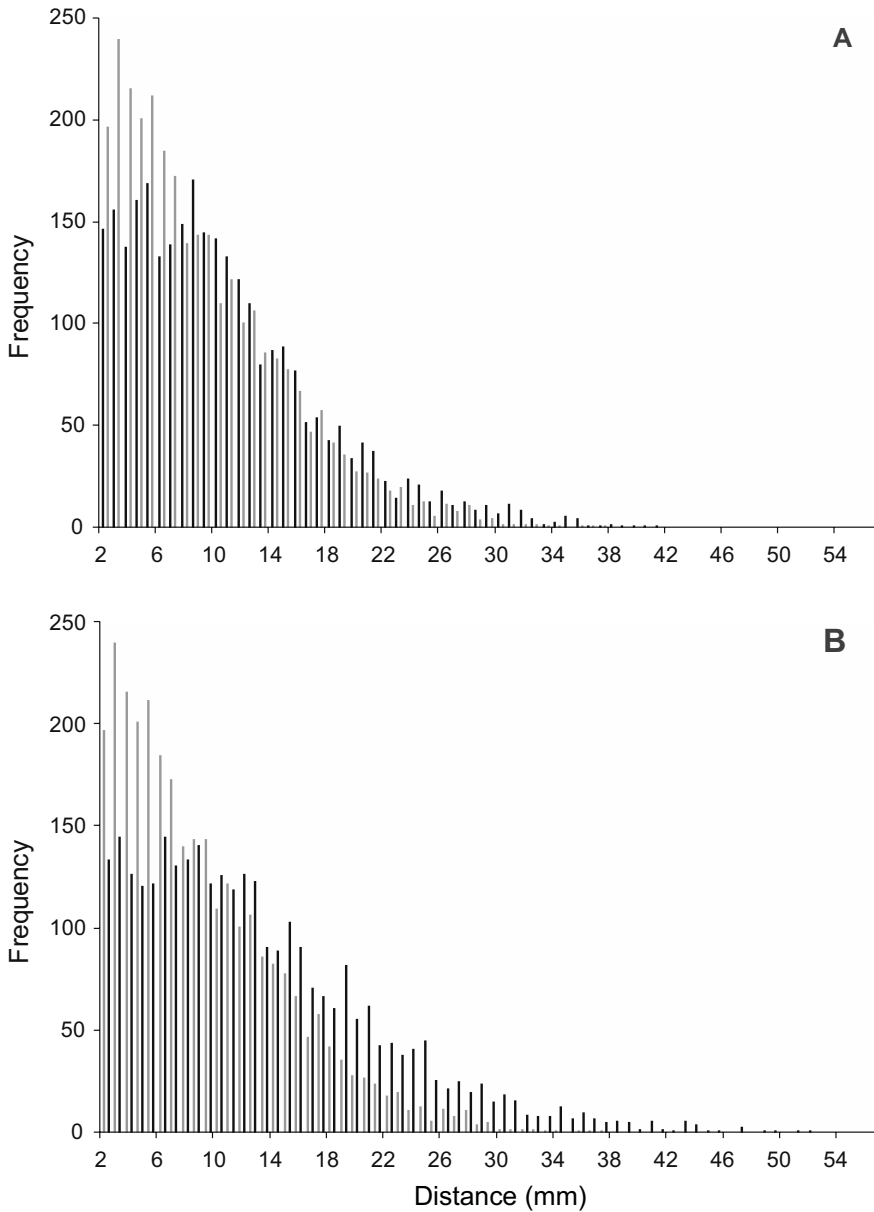


Fig. 2. Histogram of the path lengths in 0.5 second during the locomotion activity of *F. candida*. Histograms are created with 5-mm path length distance class-interval grouping. Frequency shows the number of times each class-interval occurs. A: control (grey) and 1.3 ml Trebon 30 EC/l (black), B: control (grey) and 13 ml Trebon 30 EC/l (black). The number of observations was 2989, 2878 and 3078 for the control, 1.3 ml Trebon 30 EC/l and, 13 ml Trebon 30 treatment, respectively

Pesticide application can have opposite effects in insect locomotor behaviour during the time-course (BENOIT 1986). This was the case in our study, where remarkably different results were found if the *F. candida* remained in contact with the insecticide for one hour before video recording. In this case, all parameters (locomotion activity, time spent standing, path length, average velocity during locomotion activity) were significantly related to the concentration increase (Table 3). Nonetheless, time spent standing has a positive, but all locomotion activity, path length and average velocity during locomotion activity have a negative correlation with the insecticide concentration. All these results unequivocally show that one hour of insecticide exposure has a knockdown effect on all studied locomotor traits of the *F. candida*. Etofenprox application to *Anopheles gambiae* mosquito females resulted in a one hundred percent knockdown effect from 30–55 minutes, depending on the population location (LUKWA *et al.* 2012). This data is compatible with our results, suggesting a similar mode of action of etofenprox on *F. candida* and *A. gambiae*.

Detection of the knockdown effect (SCHLEIER & PETERSON 2011) in any experiment depends on several factors, such as, e.g., insect species, insecticide category, the application method and rate, and observation time. TOEWS *et al.* (2003) tested the knockdown effect of the insecticide spinosad on eight species of stored-product beetles and found significant differences among the species. Similarly, CHEN *et al.* (1985) found differences in the effects of 10 pyrethroids, tested on the diamondback moth *Plutella xylostella*. *Rachiplusia nu*, a soybean pest moth, showed distinct knockdown behaviour on four cyanopyrethroids insecticides and permethrin (Russo 2012). Moreover, permethrin was more toxic if it was applied topically, compared to the situation where larvae were exposed to pyrethroid film on filter paper. The extent of the Fastac 50 EC (active ingredient: alpha-cypermethrin) insecticide knockdown effect on the predatory carabid beetle showed positive concentration dependence, being most effective at the highest alpha-cypermethrin concentration (TOOMING *et al.* 2014).

Prompt hyperactivity followed by reduced locomotor activity of *F. candida* due to Trebon 30 EC application has been demonstrated in this study. Coinciding with our results, LAVTIŽAR *et al.* (2016) found a quick avoidance reaction of *F. candida* to the insecticide chlorantraniliprole, which is a ryanodine receptor (calcium-channel) agonist. No hyperactivity was detected if dimethoate, an organophosphate insecticide, was applied in a laboratory experiment. However, dimethoate reduced *F. candida* velocity and shortened path length during the 36 hours of the study (SØRENSEN *et al.* 1995). In another study, this collembola species was not able to avoid dimethoate-contaminated soil. The reason was probably that dimethoate inhibits acetylcholinesterase activity (PEREIRA *et al.* 2013). All these pieces of evidence suggest the high

diversity of *F. candida* locomotor activity responses to different kind of pesticides and their active ingredients.

Direct, short-term and long-term effects of any insecticide may be considerably different (FITCHES & GATEHOUSE 1998, JANSEN & GOMEZ 2014). BENOIT (1986) presented a generalized sequence of symptoms of how pyrethroids influence the locomotor activity of the croachcroach *Periplaneta americana*. It is verified that hyperactivity (excitation phase) is followed by incoordination and then a knockdown (tetanisation) stage. This sequence of symptoms is demonstrated in the present study as well. The time window is an important point. We found in this study that Trebon 30 EC application provokes instant hyperactivity in *F. candida*. This reaction may help the collembola to escape from further insecticide contact for at least a few minutes. The maximum walking speed of *F. candida* was 0.97 mm/sec in a laboratory experiment (SØRENSEN *et al.* 1995) and 4.3 mm/sec in our study. With this walking speed, the first few minutes after insecticide spraying would be enough to find pore space in the soil, which is a safe place from insecticide droplets. However, in order to test this hypothesis, the repellent or attractant effect of Trebon 30 EC on *F. candida* should be tested.

The effects of the formulated pesticide and its active ingredient may substantially differ, as was demonstrated in the case of the glyphosate herbicide. It was found that the formulating adjuvant polyethoxylated tallowamine (POEA) is much more toxic to the water flea *Daphnia magna* than the formulated pesticide Roundup (SZÉKÁCS *et al.* 2014). TOOMING *et al.* (2014) found that locomotor activity of the carabid *Platynus assimilis* changed at 75 000-fold lower alpha-cypermethrin concentration than the recommended maximum field concentration. However, TOOMING *et al.* (2014) tested the insecticide Fastac 50EC, its active ingredient alpha-cypermethrin. It is not clear whether the active ingredient or some formulating adjuvant of the insecticide used in the experiment provoked the observed effect. Possible different effects of the formulated pesticide and its active ingredients has been an open question in *F. candida* locomotor activity studies until now.

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