

Impact of agrochemicals on non-target species: *Calathus fuscipes* Goeze 1777 (Coleoptera: Carabidae) as model

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ARTICLE INFO

Keywords:

Immune system
Fungicides
Malpighian tubules
Morphometry
Pesticides

ABSTRACT

Carabid beetles are important in the biological control of arable crop pests. Agricultural practices can produce over time a delayed toxic effect at the organismal and population levels and can compromise the survival on these species. In this research, we quantified the cumulative sublethal effect on body size, Malpighian tubules and immune responses in *Calathus fuscipes* adults living in the potato field and exposed to lambda-cyhalothrin and cymoxanil-based commercial formulations. Reductions of morphological parameters such as body, pronotum and elytron in both males and females from the potato field indicated that the pre-imaginal stages (larvae and pupae) suffer the sublethal effects of exposure to the larvicide control action of lambda-cyhalothrin. Ultrastructural alterations recorded in Malpighian tubules at the level of plasma membrane, mitochondria and nucleus indicated the reduction of the detoxification capability. The basal phenoloxidase and lysozyme-like enzyme activities have measured as markers of immune competence. Spectrophotometric analyses showed that the chronic exposure in field causes an increase of basal phenoloxidase enzyme activity, while the lytic activity of haemolymph was not affected. As a result, the use of larvicides and fungicides have a harmful effect on beneficial species such *C. fuscipes* living in the soil of potato fields. These morphological and physiological results recorded at the organismal level can provide useful information of effects at the population and community levels to preserve the biodiversity of agroecosystem.

1. Introduction

Agricultural management practices – especially the use of agrochemicals against a target pest – affect non-target species and cause a biodiversity decline in agroecosystems (McLaughlin and Mineau, 1995; Desneux et al., 2007). Primary and secondary consumers and decomposers such as nematodes, mites, collembolans, earthworms, isopods, insects and molluscs can severely be affected through topical, residual and dietary exposure. The magnitude of lethal and sub-lethal effects can depend on many variables. Firstly, tolerance, adaptation or sublethal responses of non-target species to agrochemical exposure are closely related to their trophic level (Gall et al., 2015), physiological properties and life stage (Vijver et al., 2004; Morgan et al., 2007). Secondly, effects of agrochemicals depend on the application rate and the level of uptake, persistence, volatilization, metabolization and bioavailability of the active ingredient (Sanchez-Bayo et al., 2013). Finally, all these elements may vary depending on the species tested, the soil type, structure and moisture, crop type and condition, and climate conditions

(Heimbach, 1998; Holland and Luff, 2000; Holland and Reynolds, 2003; Irmeler, 2003; Eyre et al., 2013). Moreover, the time of exposure is also a critical variable in risk assessment of agrochemicals and the lethal or sublethal effects occur on acute or chronic exposure due to the accumulation of residues that can produce over time a delayed toxic effect on organism and population (Desneux et al., 2007; Gill and Garg, 2014).

Carabid beetles are among the most important groups of beneficial arthropods in food chain of all temperate agroecosystems (Kromp, 1999; Holland, 2002). They are of special interest for environmental quality assessment as environmental indicators (Kielty et al., 1996; Lövei and Sunderland, 1996; Rainio and Niemelä, 2003; Avcin and Luff, 2010; Koivula, 2011) and for pest control (Luff, 1983; Kromp, 1999; Symondson et al., 2002). Carabids act predated on invertebrates (Loughridge and Luff, 1983; Asteraki, 1993; Warner et al., 2008; Zaller et al., 2009) and as seed feeders (Honek et al., 2003; Jacob et al., 2006; Gaines and Gratton, 2010; Saska et al., 2014; Kulkarni et al., 2015). The distribution of carabids in agroecosystems can vary

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Table 1

Morphometric parameters measured (mean \pm SE) in male and female bodies of *C. fuscipes* from the potato field and pasture.

| | | body length (mm) | head width (mm) | head length (mm) | pronotum width (mm) | pronotum length (mm) | elytron width (mm) | elytron length (mm) |
|--------------|------------------|---------------------|--------------------|---------------------|------------------------|-------------------------|-----------------------|------------------------|
| potato field | males (n = 32) | 11.06 \pm 0.09 | 2.12 \pm 0.02 | 1.74 \pm 0.03 | 3.38 \pm 0.03 | 2.57 \pm 0.04 | 3.89 \pm 0.04 | 6.95 \pm 0.06 |
| | females (n = 33) | 11.73 \pm 0.11 | 2.20 \pm 0.02 | 1.87 \pm 0.03 | 3.53 \pm 0.03 | 2.56 \pm 0.03 | 4.28 \pm 0.04 | 7.40 \pm 0.07 |
| pasture | males (n = 38) | 11.22 \pm 0.10 | 2.13 \pm 0.02 | 1.76 \pm 0.03 | 3.48 \pm 0.02 | 2.57 \pm 0.04 | 4.07 \pm 0.05 | 6.91 \pm 0.05 |
| | females (n = 40) | 12.10 \pm 0.11 | 2.23 \pm 0.02 | 1.89 \pm 0.03 | 3.70 \pm 0.03 | 2.61 \pm 0.04 | 4.27 \pm 0.06 | 7.65 \pm 0.07 |

through time and it is related to many factors such as crop type (cereals, potatoes, grassland) (Holland and Luff, 2000; Honek et al., 2003, 2005, 2006; Saska et al., 2008; Talarico et al., 2016), microclimate condition and prey or seed presence on ground surface (García et al., 2000; Honek and Jarosik, 2000). In addition, agricultural practices such as the use of pesticides (Holland and Luff, 2000) and herbicides (Chiverton and Sotherton, 1991) have a considerable influence on the diversity and abundance of ground beetle species within agricultural areas (Kromp, 1999). Field and laboratory tests have been shown that pesticides have lethal and sub-lethal effects at the organismal level on behaviour (Mauchline et al., 2004; Tooming et al., 2014; Merivee et al., 2015), physiology (White et al., 1990; Çilgi et al., 1996; Römbke and Heimbach, 1996; Desneux et al., 2007) or morphology (Giglio et al., 2011) and at the population and community level (Huusela-Veistola, 1996; Kunkel et al., 2001; Langan et al., 2001, 2004; Bel'skaya et al., 2002; Irmeler and Hoernes, 2003; Miñarro and Dapena, 2003; Thorbek and Bilde, 2004; Koss et al., 2005; Navntoft et al., 2006; Prasifka et al., 2005, 2008).

In this study, we assess the impact of a commercial formulation of lambda-cyhalothrin-based insecticide and cymoxanil-based fungicide on beneficial carabid species living in potato fields, focusing on sub-lethal effects from the organism to the molecular level. In Southern Italy, wide spectrum neurotoxic pyrethroids insecticides such as Ercole® and Karate® (active substance lambda-cyhalothrin) are used in potato growing. Treatments occurred early in the season to control neonate larvae (Elateridae) and later to control larvae and adults of insect pests such as Colorado potato beetles. Fungicide Shelter® (active substance cymoxanil) are selectively used later in the season against Peronosporales that could affect the potato crop.

The model organism chosen for this study was *C. fuscipes*, a generalist predator identified among the most abundant ground beetle species in the arable areas (Traugott, 1998; Holland and Luff, 2000; Holland and Reynolds, 2003; Irmeler, 2003; Cole et al., 2005). Although it seems that soil cultivation has no impact on the survival of this species (Eyre et al., 2013), it may suffer the exposure to residual doses of agrochemicals used in field treatments. Previous studies have been shown that morphological variations in carabids can depend on unfavourable environmental conditions due to urbanisation (Magura et al., 2006; Weller and Ganzhorn, 2004) and exposure to xenobiotics such as heavy metals or pesticides (Holland and Luff, 2000; Maryanski et al., 2002; Magagula, 2003; Giglio et al., 2011). The Malpighian tubules are the most suitable structure to evaluate xenobiotic hazards as biomarkers for risk assessment in environments (Chahine and O'Donnell, 2011; Talarico et al., 2014; Giglio and Brandmayr, 2017) due to their involvement in excretion and osmoregulation (Gillott, 2005; Gullan and Cranston, 2009; Beyenbach et al., 2010). Moreover, insect immune competence involves the expression of cellular and humoral effectors to recognize and immobilize pathogens (Gillespie et al., 1997; Ottaviani, 2005; Siva-Jothy et al., 2005). These effectors are sensitive to xenobiotic exposure such as pesticides that can interact with the immune system decreasing or increasing the immune responses depending on their chemical characteristics (Desneux et al., 2007; Giglio et al., 2011; James and Xu, 2012). Thus, to investigate the sub-lethal effects of the chronic exposure on *C. fuscipes*, we analysed: (a) morphometric parameters to estimate the morphological differentiation

in size of adults inhabiting two sampling area, a pasture as control field and a potato field as treated one; (b) the ultrastructure of Malpighian tubules as marker of detoxification ability by excretion; and (c) the basal phenoloxidase and lysozyme-like enzyme activities as markers of immune competence.

2. Material and methods

2.1. Animal and study sites

Samples of *C. fuscipes* adults were hand-collected from mid-September early October 2015. We choose this sampling period because it is the major period of activity of this species in the sampling area and is subsequent to the treatments in potato fields. Males and females were collected from two sites on the Sila Mountain. One site was a potato field located at 1200 m a.s.l. (39°21'2.04"N, 16°26'28.80"E; Azienda Rizzuti srl, Guzzolini, Spezzano della Sila, Calabria, southern Italy). In the beginning of May, before potatoes were planted, this field was treated mechanically to avoid weeds and the soil sprayed with Ercole® (active substance lambda-cyhalothrin) against nematodes and insects. In mid-June, depend on local rainy weather, Karate® (active substance lambda-cyhalothrin) was applied as foliar sprays against the potato beetles *Leptinotarsa decemlineata* Say, 1824. In mid-July or beginning August, fungicides Shelter® (active substance cymoxanil) was applied as foliar sprays against potato blight *Phytophthora infestans* Montagne, 1845. All chemical treatments were applied at the recommended field rate (Karate: 105–200 mL per ha of active ingredient in 1000 L of water, from 100 g/L of commercial formulation. Ercole: 10–15 kg per ha of active ingredient, from 0.4 g/100 g of commercial formulation. Shelter: 250 g per ha of active ingredient in 5000 L of water, from 45 g/100 g of commercial formulation). The second site, chosen as control, was a cow pasture with seminatural vegetation, located 9 km south-east of the potato field (39°18'28.69"N, 16°32'4.94" E, 1390 m a.s.l.; San Nicola Silano, Serra Pedace, Calabria, southern Italy).

In the laboratory, the beetles were separated by site, sexed and kept in groups (males and females) in 10 L plastic boxes filled to a depth of 6 cm with moistened humus from the capture areas. The specimens were reared with a light regime of L8:D16, 70% r.h. and at a day/night temperature of 16/13 °C. They were fed on homogenized meat and fruit ad libitum.

2.2. Haemolymph collection

The haemolymph collection was performed 48 h after the adult capture. The haemolymph was collected by puncturing cold anaesthetised adults at the ventral level of the pro-mesothorax articulation with a 29-gauge needle. The first droplet of 5 μ L of haemolymph was collected. Each haemolymph sample was immediately transferred into 95 μ L ice-cold PBS (10 mM sterile phosphate-buffered saline, Sigma-Aldrich) in a 1.5 mL eppendorf tube and centrifuged at 1700g for 5 min at 4 °C. The cell-free haemolymph obtained as supernatant was collected and stored at –20 °C until enzymatic assays.

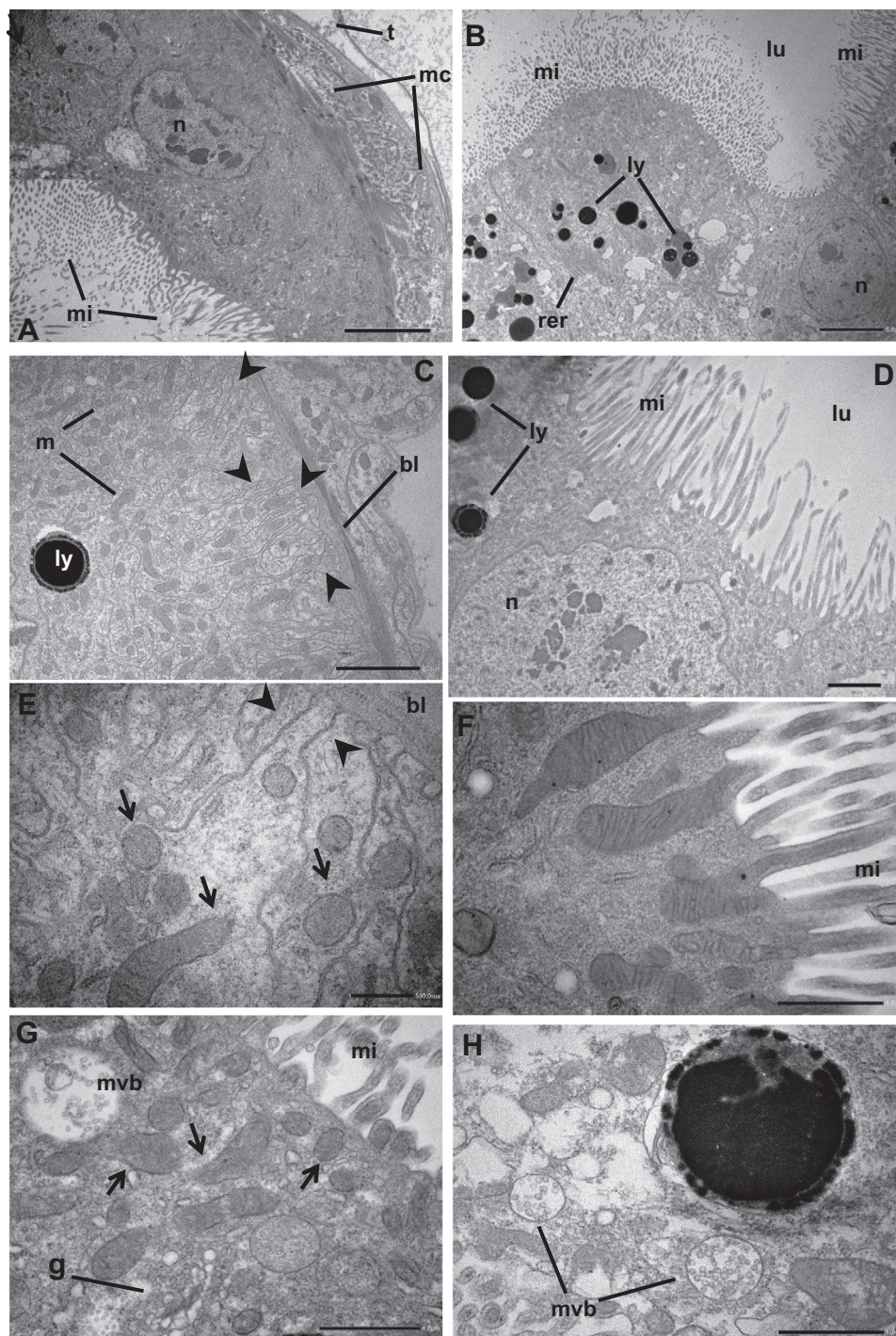


Fig. 1. Transmission electron micrographs of cross sections through the proximal segment of the Malpighian tubules in the control adults of *C. fuscipes* from the pasture. Transversal (A) and longitudinal (B) sections of epithelial cells showing a brush border formed by regular closely packed microvilli (mi). Mitochondria (arrows) occurred adjacent to or within the basal (C and E) and apical (D, F and G) surface of epithelial cells to supply the energy requirements for ionic active transport across the tubule wall. The outer basal surface (arrowheads) of plasma membrane (C and E) is deeply folded forming a basal labyrinth. (H) Detail of epithelial cells showing multivesicular body (mvb) and lysosomes with dense dark amorphous material. bl: basal lamina; g: Golgi complex; lu: lumen; ly: lysosomes; n: nucleus; rer: rough endoplasmic reticulum; t: tracheal cells enveloping tracheoles. Scale bars 5 μ m (A, B); 2 μ m (C, D); 500 nm (E); 1 μ m (F,G,H).

2.3. Morphometric measurements

For morphometric analyses, 144 specimens of *C. fuscipes*, stored in ethanol (70%), were measured (32 males and 33 females from the potato field, treated-group; 39 males and 40 females from the pasture, control-group). Photographs were taken with a stereoscope Zeiss Stemi SV 11Apo and acquired by Matrox PC-VCR software. Body length, elytron length and width, head length and width and pronotum length

and width were measured for each specimen from each site. Measurements were taken using Sigma Scan Pro 5 Software (SPSS[®] Inc.) and expressed as means \pm standard error.

2.4. Transmission electron microscopy

An ultrastructural analysis of Malpighian tubules was performed to evaluate the effect of agrochemicals on the excretion system. Adults

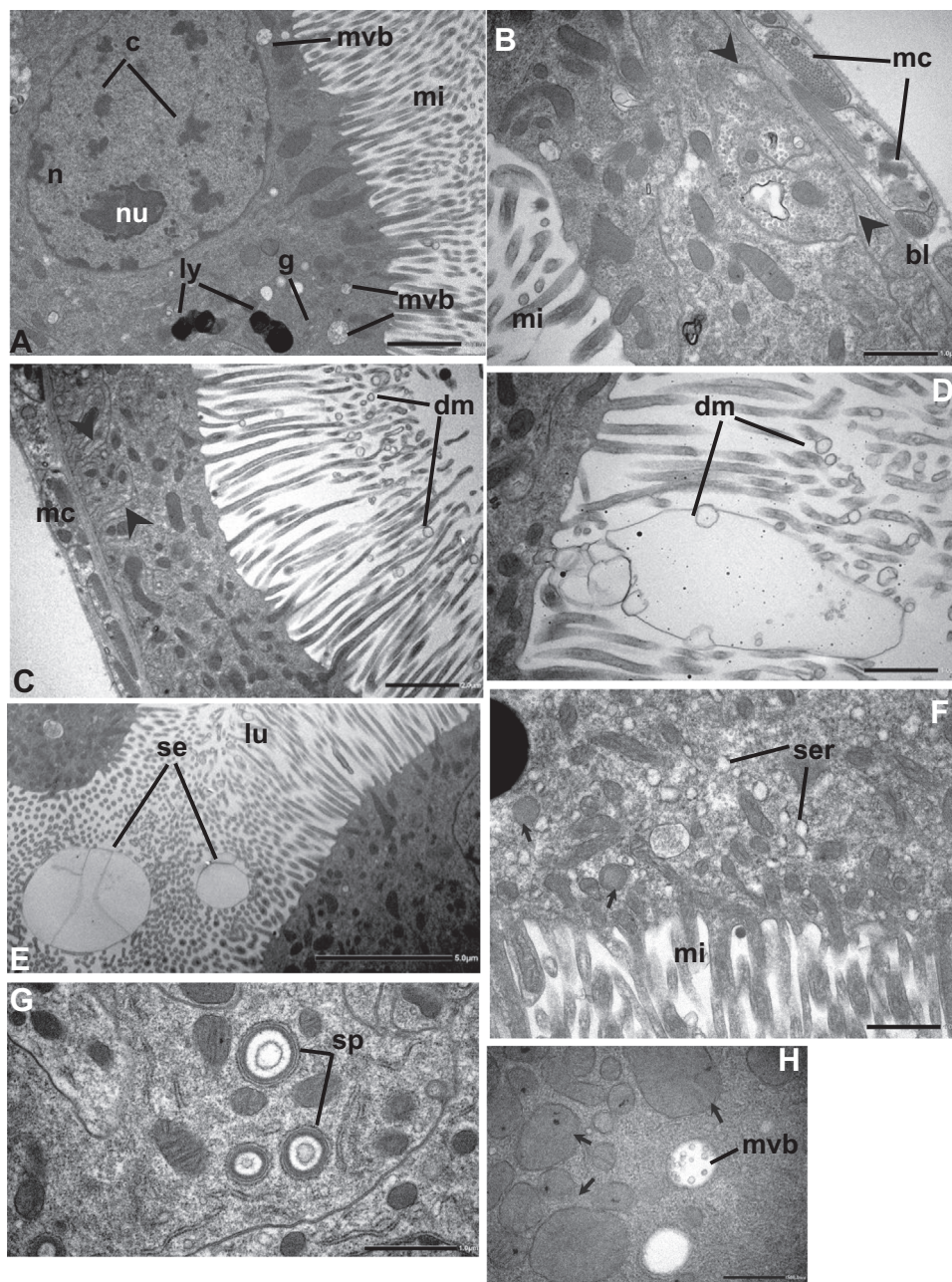


Fig. 2. Transmission electron micrographs of cross sections through the proximal segment of the Malpighian tubules in the adults of *C. fuscipes* from the potato field. (A) Transversal section of epithelial cells showing clumping of nuclear chromatin (c), multivesicular bodies (mvb) and packed microvilli (mi) surrounding the lumen. (B) Detail of the outer basal surface of plasma membrane shows a reduction of invagination (arrowheads). Longitudinal section of inner apical surface of epithelial cells showing dilations of microvilli (dm) at their apical portion (C) or completely swelling (D). Amorphous secretions (se) are released into the tubule lumen (lu) (E) Ultrathin sections of epithelial cells show the smooth endoplasmic reticulum (ser) and mitochondrial swelling with loss cristae (arrows) (F). (G) Laminated concretions surrounded by membrane are evident in the cytoplasm. (H) Detail of mitochondrial swelling with loss cristae (arrows). bl: basal lamina; dm: dilations of microvilli; g: Golgi complex; ly: lysosomes; mc: muscular cell; mi: microvilli; n: nucleus; nu: nucleolus; arrowheads: outer basal surface; ser: smooth endoplasmic reticulum; Scale bars 5 μ m (E); 2 μ m (A, C); 1 μ m (B, D, F, G.); 500 nm (H).

from the pasture and the potato field were cold anaesthetised and Malpighian tubules were removed from the alimentary canal and immediately immersed in 2.5% glutaraldehyde, 1% paraformaldehyde and 7.5% picric acid in 0.1 M phosphate buffer, pH 7.4, with 1.5% sucrose, and were fixed for 2 h at 4 °C. Then they were rinsed in phosphate buffer, post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 2 h at 4 °C and rinsed in the same buffer. Dehydration in a graded acetone series was followed by embedding in Epoxy resin (Sigma Aldrich). Ultrathin sections, cut with a PT-PC PowerTome Ultramicrotome (RMC Boeckeler), were stained with lead citrate and uranyl acetate and then examined with a Jeol JEM 1400 Plus electron microscope (Microscopy and Microanalysis Centre (CM2),

Laboratory of Transmission Electron Microscopy - University of Calabria, Italy) at 80 kV. Measurements of epithelial cells were taken with Image-Pro Plus version 4.5 software (Media Cybernetics) on digitised images and processed as means \pm standard error.

2.5. Phenoloxidase enzyme activity

Phenoloxidase (PO) activity was monitored spectrophotometrically as the formation of dopachrome from 3, 4-dihydroxy-L-phenylalanine (L-DOPA, Sigma-Aldrich). For determination of basal PO, 20 μ L of haemolymph-buffer solution were taken and mixed with 180 μ L of L-DOPA (3 mg/mL in PBS) in a microtiter plate. The enzyme activity at

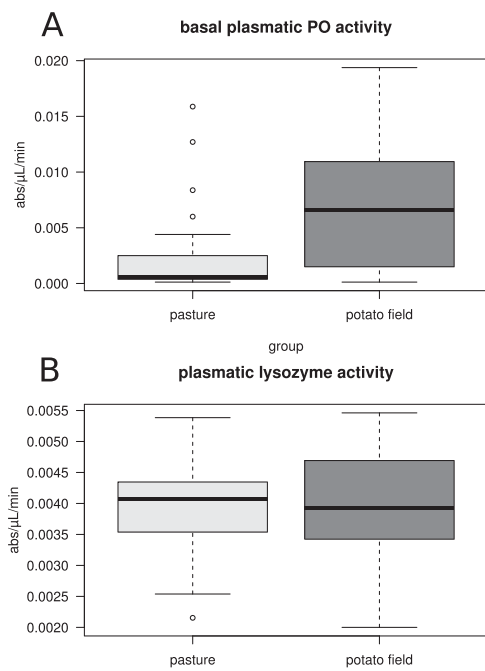


Fig. 3. (A) Basal phenoloxidase activity in *C. fuscipes* from pasture and potato field measured as the slope of the reaction curve at V_{max} . (B) Lysozyme-like enzyme activity in *C. fuscipes* measured as the slope of the reaction curve at V_{max} . The enzymatic activities in specimens from sampled sites were recorded as absorbance units for μL of haemolymph per min and reported as mean \pm SE (for statistic analyses see the text). The boxplot represents the interquartile range (IQR = $Q_3 - Q_1$) and bars represent first (Q_1 , top) and third quartiles (Q_3 , bottom) of enzyme activity values in adults from pasture and potato field. The central horizontal black line indicates the median. The ends of dashed lines (ends of the whiskers) represent the lowest datum and the highest datum.

20 °C was recorded at 492 nm for 30 min in 1 min intervals using a plate reader (Sirio S, SEAC). All samples were assayed in duplicate. The enzyme activity was measured as the slope (absorbance vs time) of the reaction curve during the linear phase of the reaction (V_{max} value; between 3 and 8 min after the reaction began). The slope of the reaction curve at V_{max} was plotted as absorbance per μL of haemolymph per min for specimens from each sites.

2.6. Lysozyme-like enzyme activity

A turbidometric assay was used to measure lysozyme-like activity in the haemocyte-free plasma. The assay is based upon the lyses of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus*. 10 μL of haemolymph-PBS mixture (described above) was loaded into the well of a 96-well microplate followed by 190 μL of a *Micrococcus lysodeikticus* (strain ATCC 7468, DSMZ) cell wall suspension (1.6×10^8 cell/mL of cold PBS). The turbidity reduction in the wells were read on a plate reader (Sirio S, SEAC) at 25 °C for 45 min in 5 min intervals at 450 nm. All samples were assayed in duplicate. The enzyme activity was reported as the change in absorbance (absorbance vs time) of the reaction curve during the linear phase of the reaction (V_{max} value; between 5 and 15 min after the reaction began). The slope of the reaction curve at V_{max} was plotted as absorbance per μL of haemolymph per min, for adults from the pasture and potato field. Standards of enzyme activity were made using lysozyme from chicken egg whites (Sigma) and a suspension of *M. lysodeikticus* as substrate. The standards were incubated and recorded simultaneously with the haemolymph samples to confirm that the assay was progressing as expected (i.e. absorbance values decreasing).

2.7. Statistical analyses

Statistical analyses were performed using R version 3.0.1 software

(R Development Core Team, 2013). For morphometric data, a two-way ANOVA was carried out on measurement by sex and sampling site. Normality Shapiro checks and Bartlett test were carried out and the assumptions were met. Basal PO and lysozyme-like enzyme activities were measured and compared among females and males from the pasture and the potato field. The differences among specimens from both sites were assessed by non-parametric statistics, i.e. Wilcoxon rank sum test, since the null hypothesis of the Bartlett test could not be rejected.

3. Results

3.1. Morphometric analyses

A two-way ANOVA was carried out on measurement by sex and sampling site. Measurements of morphometric parameters are shown in Table 1. There was a statistically significant interaction between the effects of sex and sampling site on elytron length [$F(1, 139) = 5.043$, $p = 0.026$], whilst for all others measurements interactions were not recorded [$F(1, 139) < 3.334$, $p > 0.05$]. The test for the main effect of sex revealed a highly significant difference between females and males for all measurements ($p < 0.001$), being the females always bigger than males. For the main effect of the sampling site, the test revealed that the animals from potato field are significantly smaller than those from the pasture for body length [$F(1, 139) < 6.659$, $p = 0.011$] and highly significant smaller for pronotum width [$F(1, 139) < 16.651$, $p = 7.54e-5$].

3.2. Malpighian tubules ultrastructure

3.2.1. Control adults from the pasture

The Malpighian tubules of *C. fuscipes* adults open in the posterior end of the midgut and are about 60 μm in diameter. The tubule wall consists of a single layer of cuboid cells surrounding a central lumen (Fig. 1A, B, D); the cells have a large nucleus (Fig. 1A, B, D) about 10 μm in diameter resting on a thin basal lamina (Fig. 1C, E). Outside the basal lamina, well developed tracheoles and muscle fibres are present (Fig. 1A). The cells of Malpighian tubules have numerous well-developed labyrinth-like invagination of the plasma membrane at the outer basal surface (Fig. 1A, C, E) and a brush border at the lumen surface consisting of regular closely packed microvilli (Fig. 1A, B, D, F, G). Basal invagination and apical microvilli (about 0.22 μm in diameter) are in close association with many elongated mitochondria (about 4 μm in length) (Fig. 1C, E-G) to supply the energy requirements for ionic active transport across the tubule wall. An extensive network of endomembrane, including numerous Golgi complexes (Fig. 1G), endoplasmic reticulum (Fig. 1F) and multivesicular bodies (Fig. 1H), is localized in the perinuclear area. Large lysosomes (1.8 \pm 0.05 μm in diameter, $n = 49$) with dense dark amorphous material are present in the cytoplasm indicating an autophagic activity of a cytoplasmic turnover process (Fig. 1B, D H).

3.2.2. Treated adults from the potato field

In adults from the potato field, some epithelial cells of Malpighian tubules show ultrastructural alterations (Fig. 2) compared with the cells in control adults (Fig. 1). A large amount of nuclei appears with an increased electron density of chromatin (Fig. 2A). At the basal labyrinth level, the most prominent cellular modification observed is a reduction of invagination in the outer surface of plasma membrane that become disorganized (Fig. 2B,C). In the cytoplasm, mitochondrial swelling with loss of cristae (Fig. 2F, H), and spherites with well-defined and distinct lamellar structures are evident (Fig. 2G). Microvilli are dilated at their apical portion (Fig. 2C, D) and release amorphous secretions into the lumen (Fig. 2E).

3.3. Phenoloxidase and lysozyme-like enzyme activities

Basal PO activity was significantly higher in *C. fuscipes* adults from the potato field (0.007 ± 0.001 Abs/ μ L/min; $n=31$) than in adults from pasture (0.003 ± 0.0006 Abs/ μ L/min; $n=25$; Wilcoxon rank sum test, $p=0.0036$) (Fig. 3A).

The baseline lytic activity of haemolymph was not significantly higher in adults from the potato field (Wilcoxon rank sum test, $p=1$; $n=16$) compared with adults from the pasture ($n=16$) (Fig. 3B).

4. Discussion

Although carabid assemblages in agroecosystems represent a set of species capable of withstanding agricultural practices, our results showed that carabids active on and inside the ground such as *C. fuscipes* unavoidably contact persistent sublethal doses of pollutants and can be damaged at different levels of the biological organization (morphological, physiological and biochemical).

Morphological characteristics in insects are considered adaptive of their ecological niche. In carabid beetles, morphological studies have shown that variation in body size and related morphological parameters of larvae and adults are related to the diet and habitat of species (Forsythe, 1987, 1991; Bauer and Kredler, 1993; Evans, 1994; Bauer et al., 1998; Talarico et al., 2007, 2011; De La Fuente et al., 2010; Barton et al., 2011; Brygadyrenko and Reshetniak, 2014) or to the geographical variation of populations (Alibert et al., 2001; Laparie et al., 2010; Benítez, 2013; Bravi and Benitez, 2013). In our study, morphometric analyses showed that the difference in habitat characteristic between the sampled sites have no effect on sexual dimorphism in both populations of *C. fuscipes* from the pasture and the potato field. However, significant reductions were recorded in the body, pronotum and elytra length of females and in the elytra width and pronotum of males from the potato field. *C. fuscipes* larvae reached their highest feeding activity from early November to April and adults first emerged in May, with a maximum of activity at the beginning of September pointed out the time of reproduction (Traugott, 1998). As a result, the first treatment in the potato field performed in May affects the third larval stages and pupae of *C. fuscipes* being in the ground. Thus, decrease in body size, recorded in adults caught in September and October, might be a response to a reduction of food availability for third larval stage due to the prey mortality and to the disturbance that occur during morphogenesis on the pupal stage in the pesticide treated potato field.

The Malpighian tubules are the most suitable structure to evaluate xenobiotic hazards as biomarkers for risk assessment in environments (Chahine and O'Donnell, 2011; Talarico et al., 2014; Giglio and Brandmayr, 2017) due to their involvement in excretion and osmoregulation (Gillott, 2005; Gullan and Cranston, 2009; Beyenbach et al., 2010). Our ultrastructural analyses on cell tubules of *C. fuscipes* adults exposed to pollution from the potato field have shown ultrastructural variations occurring in plasma membrane, mitochondria and nuclei. Ultrastructural modification due to exposure to nicotinoid have been recorded in Malpighian tubules of Hymenoptera and Diptera. Laboratory assay have been shown that alterations in tubule cells depend on the exposure time length and the pesticide chemical characteristic, besides the pesticide concentration (De Almeida Rossi et al., 2013; Decio et al., 2013; Catae et al., 2014; Ferreira et al., 2013). The reduction of invagination at the basal labyrinth level, the dilatation of microvilli at the apical portion of epithelial cells and the release of amorphous secretions into the tubule's lumen indicated tissue degeneration in specimens of *C. fuscipes* from the potato field. These ultrastructural alterations at the cell membrane level may be due to the effect of lambda-cyhalothrin exposure on the active transport canals of plasma membrane (Jones and Lee, 1986; Ali, 2012), which can potentially compromise absorption and excretion of tubule cells. Accordingly, mitochondrial swelling reflects the entry of solutes and

water into the matrix due to agrochemical action on mitochondrial membrane transport. The chromatin clumping in nuclei suggests progressive inactivation of the nuclear component and an increased electron density of chromatin resulting in a lowered transcriptional activity. Laminate concretions, observed in carabids from the potato field, have been also reported for Malpighian tubules in other insects under pollution conditions (Sorour, 2001; Ferreira et al., 2013). However, the function of spherites is not yet totally understood. Actually, they are related to many factors such as the increase (Ferreira et al., 2013) or the decrease of excretion activity (Sorour, 2001) and the exposition to heavy metals (Wu et al., 2009; Talarico et al., 2014). Our results highlight that Malpighian tubules are good candidate for monitoring studies as biomarkers of sublethal effects induced by toxicants. However, further laboratory investigation are needed to clarify if the ultrastructural modifications are due to lambda-cyhalothrin, cymoxanil or both.

Analyses performed on immune responses in *C. fuscipes* adults have shown that while the chronic exposure to commercial formulation of lambda-cyhalothrin-based insecticide and cymoxanil-based fungicide causes an increase of basal PO enzyme activity, the lytic activity of haemolymph is not affected. We assume that these results are related to the different role of the analysed effectors within the immune response patterns. Insect immune competence involves the expression of cellular and humoral effectors to recognize and immobilize pathogens (Gillespie et al., 1997; Ottaviani, 2005; Siva-Jothy et al., 2005). These effectors are sensitive to xenobiotic exposure such as pesticides that can interact with the immune system decreasing or increasing the immune responses depending on their chemical characteristics (Desneux et al., 2007; James and Xu, 2012). Antimicrobial peptides such as lysozymes are present constitutively at a very low level in the haemolymph and perform a hydrolytic action against the peptidoglycan of Gram-positive cell walls (Ratcliffe et al., 1985; Gillespie et al., 1997; Nappi and Ottaviani, 2000), Gram-negative bacteria (Yu et al., 2002) and have also fungistatic propriety (Fiolka et al., 2005). However, the lytic activity in haemolymph increases only upon challenge, whereas we measured only the basal activity in *C. fuscipes* adults from the pasture and potato field. Therefore, this issue requests further investigation to test if the agrochemical exposure have an effect on disease resistance performed by lysozyme-like enzyme activity. The prophenoloxidase enzymatic cascade (proPO) is a humoral defence that comprises a complex cascade of serine proteases allowing the conversion of prophenoloxidase to PO (Marmaras et al., 1996; Gillespie et al., 1997; Nappi and Ottaviani, 2000; Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2005; Siva-Jothy et al., 2005). It is involved in physiological processes such as the cuticular melanization and sclerotization, besides the defence reactions including wounding, clotting, melanotic incapsulation, production of cytotoxic molecules (Marmaras et al., 1996; González-Santoyo and Córdoba-Aguilar, 2012; Moreno-García et al., 2012). Recent advances have documented a relation among PO levels in haemolymph, cuticular thickening and resistance to pyrethroids in mosquitoes (Wood et al., 2010; Cornet et al., 2013; Kasai et al., 2014). Because of the lipophilic nature of pyrethroids, the cuticle is the major route for penetration (Antwi and Reddy, 2015). Pyrethroids dissolve in the outer wax layer, penetrate the cuticle both through the network of the wax canals and spiracles and they are transported by haemolymph in all tissue (Lin et al., 2012; Kasai et al., 2014). Thus, higher PO levels, recorded in *C. fuscipes* adults exposed to field treatments, enhance the cuticular melanisation and sclerotization (though not necessarily the result is in cuticle thickening) to slow down insecticide penetration across the cuticle as an enzyme-mediated detoxification mechanism of resistance as observed in dipteran species (Lin et al., 2012; Kasai et al., 2014).

5. Conclusions

This is the first report that consider the sublethal effects of

pyrethroids and fungicides on carabid beetles. Our results indicated that though *C. fuscipes* adults tend to recover migrating in adjacent areas of treated fields (Traugott, 1998; Holland and Reynolds, 2003; Eyre et al., 2013), the long-term repeated treatments against potato pests have subtle effects on morphometric parameters, ultrastructure of Malpighian tubules and immune competence of this beneficial species. These effects recorded at different levels of the biological organization (molecular, cellular, tissue, organism) may provide more accurate information to predict the effect of exposure at higher levels such as population and community.

Acknowledgements

This research was supported by the Ministero dell'Ambiente e della Tutela del Territorio e del Mare - Ente Parco Nazionale della Sila (grant. no. 2678, XIII/4), assigned to P. Brandmayr.

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