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Summary

Because earthworms sustain soil functioning and fertility, there is a need to advance the knowledge of their adaptation potential to chemicals in anthropogenic landscapes. Our hypothesis is that there is acclimation to organic chemicals (pesticides) in earthworms that durably persist under conventional farming in anthropogenic landscapes. The adaptation capability of two populations of earthworms *(Aporectodea caliginosa)*having a different chemical exposure history, - one originating from 20 years of organic farming (naïvepopulation) and another from 20 years of conventional farming (pre-exposed population)- to cope with soil organic pollutant (Opus®, epoxiconazole a worldwide used fungicide) were investigated. Several complementary metabolic and energeticendpointswere followed, and cast production was assessed as a behavioral biomarker related to earthworms ecological role for the soil.Basal metabolism reflected by respiration rate was increased in both fungicide-exposed worms compared to controls. Glycogen resources were decreased in the same proportion inthe two populations but more rapidly for the naïve(7 days) than for the pre-exposed population (28 days). Soluble protein and most amino-acidscontents increased in the pre-exposed population only, suggesting a detoxification mechanism. Metabolomic profiles showed a cut-off between fungicide-exposed and control groups in the pre-exposed earthworms only, with an increase in most of the metabolites. Exposure to a low dose of epoxiconazole increased cast production of pre-exposed earthworms, and this resulted in an increase in pesticide disappearance. As far as we know, this is the first study which evidenced there is an acclimation to an agricultural chemical in earthworms derived from conventional farmingthat also relates to a change in their burrowing behaviour, and for which larger consequences for the soil ecosystem need to be addressed. This original finding is of major interest in the frame of ecosystem resilience to global changes. Whether this physiological adaptation is a general pattern of response against fungicides or other pesticides would need to be confirmed with other molecules and agricultural contexts.

Keywords

Land-use, Epoxiconazole, Earthworm adaptation, Energy storage, Metabolomic profile,Soil bioturbation

1. Introduction

Often representing the largest animal biomass, earthworms are present in most terrestrial ecosystems and are considered as efficient ecosystem engineers as they actively modify the physical, chemical and biological properties of the soil (Binet et al., 1998; Bottinelli et al., 2010; Jones et al., 1997; Monard et al., 2008). They sustain several key ecosystem services by enhancing soil structure and nutrient cycling,and play a role in ecosytemic services such as water regulation, pollution remediation and primary production (Blouin et al., 2013). In anthropogeniclandscapes, soil biodiversity and mainly earthwormcommunities have to face disturbances by intensive land-usedue to agricultural practices such as ploughing and tillage, application of fertilizers and chemical pesticides(Paoletti, 1999; Thompson, 1971). The soil compartment is the primary sink for agricultural pesticides, as they are frequently applied several times a year.Concerning fungicides, a large part either do notreach itsplant target,or is washed off from treated foliage, leading to major losses to the soil. Bromilow *et al*(1999a)reported in a field study that only 30% of the fungicide sprays were intercepted by the barley crop.The frequent application of agricultural pesticides and the persistence of some of them eventually leads to increasing amounts of residual compounds in the soil, either as free or bound residues (Gevao et al., 2000; Mordaunt et al., 2005), which can be a threat to lumbricids species. It has been shown that abundance and diversity of earthworm communities are dramatically reduced by chemical and mechanical stress (Decaëns and Jiménez, 2002; Smith et al., 2008). Despite these impacts however, worm populations persist in conventionally (with pesticides usage) cropped fields, albeit in reduced numbers. Assuming that immigration rate is low in earthworm populations(Lavelle and Spain, 2001), this suggests that earthworm populations living in pesticide-polluted soils can cope withchronic chemical stress, either via avoidance behaviour or physiological resistance (Posthuma and Van Straalen, 1993).

A chronic exposure to contaminants for generations may allow adaptation to take place, by favouring individuals that are able to face them.Physiological adaptation, e.g. acclimation, implies that individuals have acquired a degree of tolerance after a pre-exposure to chemicals at some point of their life, which can be lost within a generation. Genetic adaptation to xenobiotics implies constitutive and hereditary mechanisms allowing tolerance such as overproduction of specific compounds, (Brausch and Smith, 2009), or alteration of a target or receptor (Fournier and Mutero, 1994).Such adaptations to metals have been studied in terrestrial invertebrates including earthworms(Donker et al., 1993; Gudbrandsen et al., 2007;

Posthuma, 1990).However to the extent of our knowledge no attempt has been made to evaluate adaptation processes in earthworms against organic pesticides.

Adaptation, either via physiological or genetically-mediated mechanisms, is an expression of the speciesecological plasticity allowing protection against stresses. However, it isknown to be costly in terms of metabolism and energy usage, especially when it involves over-production of compounds such as protection enzymes (Calow, 1991). Increased metabolic rate or increased energy allocation to detoxification mechanisms can be at the detriment of energy storage, and thus impair otherfunctions such as reproduction and growth (Jansen et al., 2011; Yasmin and D'Souza, 2010).This is particularly important when animals are exposed to low but chronic exposures, such as earthworms in agricultural fields, due to the persistence of residues of pesticides in the soils for several years after application (Gevao et al., 2000).Available energy resources, as measured by main storage compounds, can then reflect energy demands associated with different adaptation strategies. In the earthworm *Dendrobaena octaedra*, Holmstrup*et al*(2011)reported high energetic costs reflected by glycogen depletion of the internal regulation of Al and Ni metals. On a lower level of organization, and possibly on a shorter timescale, metabolomics have recently been used in ecotoxicology studies to investigate the responses of the metabolism to contaminants (Brown et al., 2010; Bundy et al., 2008; Simpson and McKelvie, 2009), and we think they can be a valuable tool to study adaptation to contaminants.

One of the most common cultures in Brittany (France) is winter wheat, with a mean number of 6 pesticides applications per year, the majority of them being fungicides (Agreste, 2006). In particular, members of the triazole family, which act by inhibiting the biosynthesis of ergosterol, have attracted interest because of their high persistency in soils (Bromilow et al., 1999a, 1999b; Passeport et al., 2011), although data available on their toxicity is still scarce.

The toxicity of some fungicides, such as benomyl and carbendazim has been investigated in a few studies in earthworms and enchytraeids, another important member of the soil biota. Avoidance behaviour was reported in Enchytraeids following exposure to benomyl and Carbendazim (Amorim et al., 2005). Holmstrup (2000) also reported a decrease of reproductive rate in the earthworm *Aporrectodea longa* associated with a reduction in the whole earthworm population.

Our aim was to testthe hypothesis that earthworms inhabiting soil under conventional land-use have acquired tolerance to face the regularly applied fungicide epoxiconazole.The response of in situ pre-exposed versus naïve earthworms when exposed to an environmentally relevant dose of the fungicide were thus studied. We addressedwhether an adaptation was

quantifiable in terms of main energy resources storageand metabolism (respiration and metabolites levels)by comparing these two pre-exposed and naïve earthworms, and if these physiological changes were associated with change of their soil bioturbation ability. Our results evidenced that there is a physiological adaptation to fungicide in the earthworms originated from the conventional cropped field, leading to a change in their burrowing behaviourand impacting the fate of pesticide in soil.

2. Materials and Methods

2.1. Earthworm populations, soil and agricultural context

Earthwormsused in this study originated from two agricultural fields, one conventionally croppedand one cropped according to organic agriculture requirements. Both of these fields have been in theseagricultural management strategies for more than 20 years and are located in the same agricultural basin (Vézin-le-Coquet, Britanny, France). Soils are slightly acid silt-clay loams (conventional and organic field, respectively: Clay 14.8 % and 16.6 %; Silt 71.6 % and 71%; Sand 13.6 % and 12.4 %; organic matter 1.67 % and 2.55 %; pH (water suspension) 6.4 and 6.9).The conventional field had been cropped under rotations of wheat/maize/leguminous for 20 years, and annuallytreated with pesticides. The fungicide epoxiconazole was used each year a cereal was planted. Epoxiconazole is a triazole fungicide present in twopesticides formulations (OPUS® and OGAM®)and mainly used on wheat. The organic field has been under rotation with a cereal (2 years) / maize (1year) /lucerne (3years) without any pesticides for 20 years, and was not tilled during the lucerne periods.

Soil used for the exposure experiment was collected from the first 30cm of a permanent (since 1960) organic pasture (17.6% clay, 69.3 % silt, 13.1% sand, 4.0 % organic matter, pH (water suspension) 6.0) located in the same area where no initial epoxiconazole residuals were detected (see method in 2.3). Upon retrieval, it was air-dried until it reached 14% of humidity, then sieved to remove all soil particles larger than 2mm and kept in sealed containers (100 l) until used for the experiment.

Theendogeic species *Aporretodea caliginosa*was chosen for this experiment.Itis an environmentally relevant species for toxicological tests, since it is commonly found in agricultural fieldsand reported as a dominant species(Jordan et al., 2004; Lamandé et al., 2003; Nuutinen, 1992; Söchtig and Larink, 1992). Most standard ecotoxicity tests are conducted on epigeic species mainly with *Eisenia fetida* or *Eisenia andrei*, but these species

lack ecological relevance since they are usually absent from agricultural fields (Dittbrenner et al., 2010).Earthworms were collected by hand-sorting at the beginning of Spring 2012 from the two fields. Adults (presence of a fully developed clitellum) and sub-adults (presence of tubercula pubertatis) were usedand individual weights were recorded (Table 1). Upon collection, they were brought back to the laboratory and maintained in the soil collected from the field at 25% humidity until start of fungicide exposure.Before the experiment, the earthworms were acclimatized for 14 daysin the test soil in the climatic room used for the experiment (Conviron GR96; temperature: 15°C; day/night cycle: 16/8h; humidity: 80 ± 5%).

2.2. Experimental setup

Soil contamination: Epoxiconazole was applied ascommercial formulation OPUS® (125 g active ingredient l^{-1}, obtained from BayerCropScience)diluted in distilled water at 0.1 $\mu g g^{-1}$ soil, which is equivalent to a predicted field concentration calculated for a field application rate of 125 $g.ha^{-1}$ assuming a single application with an homogenous distribution and no crop interception in the top 5 cm of the soil (Dittbrenner et al., 2010). Soil spiking was conducted by manually adding 175 ml of the diluted pesticide solution or distilled water (for the controls) on each 2 kg of soil at 14% water content (1.75 kg dry weight) reaching a final soil water content of 24%. To insure homogeneity of pesticide distribution in the soil, the solution was added in two parts, the soil being thoroughly mixed, resieved using a 2 mm aperture and redisposed as a fine layer.Soil microcosms consisted of polycarbonate boxes (80mm x 50mm, Caubère, Yebles, France) with a lid pierced with tiny holes to ensure sufficient aeration. The microcosms were filled with 100 g of contaminated or control soil, then 0.2 g of dry grass meal was added to the surface of the soil. Then the microcosms were left two days in a cool dark room to ensure aeration of the soil after re-humidification. Water content was checked again in 3 additional control boxes and adjusted to 25% prior to introduction of animals, then checked again each week.

Experimental design: The experimental design is described in table 1. It comprised 11 specimensfrom each population (pre-exposed and naïve) for each treatment (epoxiconazole or control) and sampling time (7and 28 days) plus an initial control group at day 0 (unexposed). Prior start of exposure, each earthworm was rinsed in tap water, gently dried on filter paper, weighed and placed in individual Petri dishes for 48 hours for gut voiding. Then animals were transferred individually to the exposure microcosms (day 0) according to a size-class procedure, insuring a similar mean earthworm weight in each treatment. Soil was spiked with epoxiconazole at 0.15 $\mu g.g^{-1}$ soilor control soil.Exposure lasted for 7 and 28 days, with an

initial control group of 11 individuals for each population (unexposed worms) at day 0. At each sampling date, before use of the microcosms for cast production (see 2.5) and pesticide measurements (see 2.3), earthworms were removed from the soil taking care not to break the casts. 5 of them were used for respirometry assessment (2.4), energy resources (2.6), and metabolites measurements (2.7). The other 6 earthworms were used for enzyme activities measurements in another study and will not be considered here. After the worms have been sampled, 8 microcosms were used for cast production measurements, and 3 for pesticide analysis.

In addition, eight uncontaminated soil microcosms withoutworms were used as controls for the cast production test to assess the potential formation of non-biogenic aggregates at 7 and 28 days. Another 3 soil microcosms were filled with contaminated soil to track the fate of pesticide without the presence of worms.Humidity control was conducted in 3 additional, non-contaminated soil microcosms, in order to adjust humidity when necessary.

		worms at start	Sampling days and number of worms sampled			treatment
			0	7	28	
Microscosms containing one earthworm	Pre-exposed population (conventional field)	33	11	11	11	CTRL
		22	0	11	11	EPOXI
	"Naïve" population (organic field)	33	11	11	11	CTRL
		22	0	11	11	EPOXI
Microcosms without earthworm	Control microcosms for cast production	0	8	8	8	CTRL
	Control microcosms for pesticide dissipation	0	3	3	3	EPOXI
	Control microcosms for humidity check	0	3	3	3	CTRL

Table 1: Experimental design of the laboratory exposure. Out of each group of 11 microcosms containing one earthworm, 5 randomly picked worms were used for respirometry assessment, energy resources, and metabolite measurements.

2.3. Pesticide concentration in soil

Sub-samples of 2g of soil were retrieved the day of the pollution (day 0), and after 7 and 28 days, from three randomly chosen microscoms out of the 11 replicates.They were dried at 30°C overnight, then kept frozen until pesticide analysis.Epoxiconazole in soil sub-samples was measured by liquid chromatography coupled with mass spectrometer (LC-MS)(Waters alliance 2690, Waters, Saint Quentin en Yvelines, France). 2g of soil sample were extracted in 500 ml of mineral water. After 15 min of ultrasound treatment, the sample was mixed by

rotary shakingfor one hour, and subjected to another 15 min of ultrasound treatment. 0.25 µg of triadimenol was added as internal standard and 1 ml of analytical-grade nitric acid (HNO_3) to acidify the mixture. Then pesticides were extracted from the aqueous mixture in two steps with 25 ml of HPLC-grade dichloromethane followed by 15 min of agitation. The dichloromethane extracts were combined and evaporated to a drop (ca. 10 µl), then 500 µl acetonitrile were added and 0.25 µg of pentabromophenol were added as second internal standard. The extract was evaporated again to a drop and adjusted to 500 µl with 10% acetonitrile acidified (0.1 % formic acid).

This sample was analysed by LC-MS using a high-performance liquid chromatography (Alliance 2695, Waters, Saint Quentin en Yvelines, France) coupled to a quadrupole mass spectrometer model ZQ (Waters-Micromass, Saint Quentin en Yvelines, France) equipped with an electrospray source. Epoxiconazole was separated on a X Terra MS C18 column (150 x 2.1 mm, 3.5 mm particle size, Waters, Saint Quentin en Yvelines, France) at 35 °C. A binary mobile phase gradient (A: ultrapure water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid) was used for pesticide separation. The chromatographic method held the initial mobile phase composition (82% A, 18% B) constant for 10 min, followed by 70% A / 30% B (10min), 50% A / 50% B (15 min), 20% A-80% B (5 min), then again 82% A / 18% B for 10 min. Quantification limit was 2.5 $ng.g^{-1}$ dry soil and extraction yield was 75 ± 7%. A standard curve of epoxiconazole was made with several aliquots of 2 g of dry soil (the same uncontaminated pasture soil used for the microcosms) spiked manually with the purified compound, air-dried for two hours, and extracted the same way as the real samples for quantification, relating to the two internal standards. Pesticides analytical standards were purchased from Dr Ehrenstorfer (Ausburg, Germany).

Recovery of epoxiconazole in spiked soil was initially 80% of the desired concentration (0.1 $µg.g^{-1}$), with a coefficient of variation of 10%, which was considered highly satisfactory, as we used the commercial formulation of the pesticide. Concentrations of epoxiconazole in soil (Table 2)were still two thirds of the initial concentration after 28 days, which is consistent with the long persistencies (half-life > two years) reported in the literature (Bromilow et al., 1999a, 1999b; Liang et al., 2012).

2.4. Respirometry measurements

After 7 and 28 days of exposure, earthworms were removed from the soil microcosm, rinsed, gently blotted dry on filter paper, weighed and left 24 hours in a 250 ml glass jar on a moist

filter paper for gut voiding.Thereafter, the glass jar was hermetically closed for two hours, and CO_2 was measured by a Micro-Gas Chromatograph (3000A, SRA Instruments) equipped with a single capillary column Poraplot U and coupled with a thermal conductivity detector. Then the worm was frozen in liquid nitrogen for further measurements of energy resources (2.6) and metabolomics (2.7).

2.5. Cast production

Cast production was measured in the soil of 8 microcosms out of the 11 replicates. The cast production test was conducted according to the protocol of Capowiez et al. (2010) but using a sieve of mesh size 2mm. Soil of the microcosms were dried at 40°C overnight, and sieved by shaking the sieve consistently for 10 s.

2.6. Energy resources measurement

Frozen worms were freeze-dried, and ground to a fine powder by multiple 30 sec agitations with inox beads in 2 ml test tubes in a bead-beater (Retsch MM400, Retsch GbmH, Haan, Germany). Each ground sample was separated in several aliquots, 2 mg for total lipids, 5mg for soluble proteins, and 2 x 10 mg for glycogen and metabolites measurements. Lipid aliquots were extracted according to Folch(1957), lipids in the chloroform extract were assayed by the sulfo-vanillin method with a calibration curve of commercial vegetable oil at 525 nm. Proteins aliquots were homogenized in 0.1 M phosphate buffer (pH 6.5), centrifuged and measured according to Bradford (1976) using a calibration curve of bovine serum albumin. Glycogen was measured according to the method of Nicolaï *et al*(2012). Briefly, aliquots were homogenized in 600 µl trichloroacetic acid (4%) and centrifuged at 5000 G (rotor N° 12145, SIGMA, 8000 rpm) for 10 minutes. Then 500 µl of supernatant was recovered and glycogen was precipitated by adding 1.5 ml of ethanol and stirring for 10 minutes.The extract was centrifuged (5000 G) and the ethanol eliminated with a glass pipette. The pellet was washed with 2ml ethanol, then the remaining ethanol was totally evaporated at 70°C.The pellet was redissolved by stirring it overnight in ultrapure water, and centrifuged again. Absorbance was measured at 425 nm with a microplate reader (Fischer Scientific Multiskan FC)after addition of Lugol using a calibration curve of purified glycogen (Oyster type II, Sigma).

2.7. Metabolomics

2.7.1. Sample extraction and derivatization

Metabolite extraction was conducted according to Khodayari *et al*(2013). The freeze-dried and ground sample was homogenized in 600 µL of cold (−20 °C) methanol–chloroform (2:1) using a bead-beating device (Retsch MM301, Retsch GbmH, Haan, Germany). 400 µL of ice-cold ultrapure water was subsequently added, and each sample was stirred. After centrifugation at 4000 G for 10 min at 4 °C, 300 µl of the upper aqueous phase, containing polar metabolites, were transferred to new chromatographic vials and vacuum-dried using a Speed Vac Concentrator (MiVac, Genevac Ltd., Ipswitch, England). The dried extracts were thenredissolved in 15 µL of 20 $mg.mL^{-1}$ methoxyaminehydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in pyridine, incubated under automatic orbital shaking at 40°C for 90 min prior to derivatization. Then, 15 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma) was added, and derivatization was conducted at 40 °C for 45 min under agitation. The derivatization process was automatized using a CTC CombiPal autosampler (GERSTEL GmbH and Co.K.G, Mülheim an der Ruhr, Germany).

2.7.2. GC-MS analyses

Gas chromatography coupled with mass spectrometry (GC–MS) was used to measureup to 58 small metabolites belonging to different classes ofmolecules: amino-acids, polyols, sugars, intermediates of the citric acid cycle and other unclassified biological molecules. The GC–MS system was comprised of a Trace GC Ultra chromatograph, and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA, USA). The injector temperature was set at 250°C. The oven temperature was increased from 70°C to 170°C at 5°C min^{-1}, from 170 to 280°C at 7°C min^{-1}, from 280 to 320°C at 15°C min^{-1}, then the oven remained for 4 min at 320°C. A 30 m fused silica column (TR5 MS, I.D. 2.5 mm, 95% dimethyl siloxane, 5% Phenyl Polysilphenylene–siloxane) was used, with helium as the carrier gas at a rate of 1 $mL.min^{-1}$.One microliter of each sample was injected using the split mode (split ratio: 25:1). We completely randomized the injection of the samples. The temperature of the ion source was set at 250°C and the MS transfer line at 300°C. Detection was achieved using MS detection in electronic impact (EI). We used the selective ion-monitoring mode (SIM) (electron energy: -70 eV), allowing a precise annotation of the detected peaks. The peaks were identified according to both their mass spectra (two ions) and

their retention times. Metabolite levels were quantified, if above their quantification limits, according to calibration curves made with 58 pure reference compounds, including the internal standard. Chromatograms were integrated using XCalibur v2.0.7 software (Thermo Fischer Scientific Inc, Waltham, MA, USA).

2.8. Statistical analyses

For both populations, the effect of epoxiconazole compared to control groups, or mean differences between populations, either on respiration, energy storage or cast production was tested by student-t-tests at each sampling time. The disappearance of pesticide in the soilwas tested by one-way ANOVA with time as factor for soils containing pre-exposed, naïve and no earthworms, followed by post-hoc tests according to the Tukey procedure. On the metabolomics data, two principal component analyses (PCA) were performed on each population as separate datasets on log-transformed and standardized variables. Three axes, explaining 70% of variability, were kept for interpretation. The variables Serine and Threonine were highly correlated (94%) so their arithmetic sum was used in a single variable, so as not to hamper the results of the PCAs. A classification of the metabolites into "functional biochemical groups" was done following Bundy *et al*(2008) and based on biochemical knowledge. Amino-acids were classified either as lipophilic, hydrophilic or neutral. Regardingthe metabolite functional group responses to epoxiconazole, significant differences between control and exposed worms for each metabolite were tested by student-t-tests at 7 and 28 days. Significance level for student-t-tests was set at $p \leq 0.1$.All analyses were conducted using the statistical software of "R 2.12.1" for Macintosh(R Development Core Team, 2008).

3. Results

3.1. Energy dissipation

Fungicide treatment increased CO_2 production in both pre-exposed and naïve populations after 7 and 28 dayscompared to their non-treated controls(Fig 1). Respiration rate in the fungicide-treated group was higher after 7 and 28 days compared to day 0 in the pre-exposed population. Differences became significant between populations at 28 days, where

metabolicrates of the pre-exposed population (both exposed and control groups)werestill higher than at the outset, while it remained constant in the naïve population from day 7 to 28.

3.2. Cast production (CP)

The weight of casts (g of dry cast day^{-1}) correlated linearly and positively to earthworm weights at 7 and 28 days (R^2 =0.34; $p<0.001$ and R^2=0.33; $p<0.05$, respectively, all modalities mixed). The cast production was then calculated as weight difference of non-biogenic aggregates retained in the sieve from the 8 control (without worms) microcosms to the ones with worms and expressed per gram of fresh worm. Worms from both populations displayed similar patterns with constantCP over time in control soil microcosm and significantly changed CP with fungicide treatment (Figure 2). Application of epoxiconazole transitory enhanced CP (7 days) in pre-exposed worms ($p<0.01$), and slightly decreased it, however not significantly, after 28 days in bothworm populations.

3.3. Energy resources

Mean glycogen content was decreased by nearly 20 mg in fungicide-treated compared to control groups after 7 days in the naïve earthworms, and after 28 days in the pre-exposed earthwormsas shown in (Figure 3-A). At the end of the exposure, theworms originating from the conventional-treated field had a slightly lower glycogen tissue level than the naïve ones.
The lipid tissue levels (Figure 3-B) did not show any significant differences between un-exposed and fungicide exposed earthworms or between earthworms originating from the conventional or the organic-treated field. Lipid levels were lower in all treated groups compared to their respective controls, however it did not achieve significance.Protein contents (Figure 3-C) decreased similarly in the 4 groups of worms during the first 7 days.They were then significantly increased by fungicide treatment in both exposed and naïve populations compared to their respective controls after 28 days, with pre-exposed worms having a final protein amount almost twice as high as naïve ones.

3.4. Metabolomics

3.4.1. Metabolic profiles of fungicide-exposed and control populations

Twenty eight metabolites were detected and quantified in the earthworm tissues. From this dataset, 22 were kept for interpretation (table 1). For the conventional population (Figure 4A),

scores plots on axis 2 and 3 showed that the worms exposed for 28 days formed a separate cluster from the 28 days control worms along axis 2 and 3. This pattern was not observed in the organicpopulation(Figure 4B) along any of the three axes, as it seems the time effect is greater than the fungicide effect. Indeed, the exposed and the control groups move in the same way with time, but are not clearly separated.

3.4.2. Metabolic changes in the worms populations

In the loadings plots of the PCAs (figure 4C and 4D), several coordinated responses were identifiable. In the conventional population (Figure 4C), the 7variables that have loadings lower than -0.5 on axis 2form a first cluster containing mostly lipophilic amino-acids.Another clusteris in the lower half of the plot corresponding to variables having low loadings (roughly, <-0.2) on axis 3, composed of the three sugars, succinate, ornithine, the amino-acids glycine and proline and putrescine.On the other hand in the organic population (Figure 4D), we see two clear clusters that could correspond to the incubation time: the top right corner for seven days and the top left corner for 28 days. Day zero would correspond to the sugars Glu and Rib (lower half). The induced changes in metabolite concentrations after exposure was then measured as normalized concentrations in percentage of the control value and compared according to functional groups (Figure 5). In the conventional worms, several metabolites increased after 28 days of epoxiconazole exposure in all four biochemical groups (amino-acids, sugars, organic acids and ornithine-putrescine), up to 7-fold for aspartate, 4-fold for aminobutyrate, and 2-fold for ornithine and putrescine.This general increase in metabolite levels was not observed in the naïve population, which displayed different trends. Indeed, in Figure 5E, certain amino-acids (Asn, Asp, Phe and Val) increased slightly, whereas othersdropped below 100% or stayed stable at 28 days.Not much variation was observed in organic acidsof the organic earthwormsat 28 days, except for aminobutyrate which dropped to 50% of the control value, contrasting to its 4-fold increase in the pre-exposed animals. Putrescine level increased by 2-fold after 28 days in both populations whereas ornithine decreased to below 50% of the control value in the organic population only.

4. Discussion

4.1. Acclimation in energetic processes and metabolism

Animals can tolerate pollutants via biotransformation, excretion or scavenging of free radicals (when pollutants induce oxidative stress). For example, detoxification of several pollutants, e.g atrazine or paraquat, is mediated via the enzyme Glutathione-S-Transferase. This enzyme, belonging to phase II detoxification mechanisms, acts through binding xenobiotics to glutathione and facilitating its excretion (Anderson and Gronwald, 1991; 2007, 2000). Another common detoxification pathway to xenobiotics is the group of cytochrome p450 oxidases. This family of enzymes transform the structure of organic chemicals, hence greatly altering their toxicity (Ribera et al., 2001; Rodríguez-Castellanos and Sanchez-Hernandez, 2007). However, no data on epoxiconazole tolerance pathways e.g. detoxification mechanisms was available in the literature.

On the other hand, the literature suggests that there are energetic costs in organisms for coping with pollutants (Fisker et al., 2011; Holmstrup et al., 2011; Wiegand et al., 2007). The way organisms handle energetic processes can therefore inform us on adaptation mechanisms. As an example by measuring the main energy resources, Pook et al (2009) showed that a metal-resistant population of marine harbour ragworm (*N. diversicolor)* had a lower scope for growth than a reference (non-resistant) population and demonstrated a metabolic cost, or tradeoff, of resistance.Other tradeoffs can be the co-selection of traits along with tolerance to xenobiotics, such as life history traits. Interestingly, in the conventionally cropped field, the worms sampled had a lower initial mean weight but with constant tissue composition compared to the organic worms (Table 1). This could be a result of the selection of smaller individuals, by the global agricultural management of the field, as a possible combined effect of fertilizers, pesticides and tillage (tillage was less frequent in the organic field due to the lucernecropping). This assumption is supported by the fact that the bigger anecic species such as *L. terrestris*or *A. giardi*are usually the most impacted in cropped soil(Edwards and Bohlen, 1996).

To investigate such energetic costs, we measured the worm's metabolic rate (µg CO2 g^{-1} worm (fresh weight) $hour^{-1}$)as a proxy ofenergy dissipation, and the main energy storage compounds which are glycogen (main sugar resource), total proteinsand lipids.Metabolic ratewas increased in both populations after 7 days of exposure, showing that the fungicide increased metabolic rate, but to a greater extent in the pre-exposed earthworms. Energy dissipation in both control and exposed groups were indeed higher than the naïve earthworms after 28 days. The increase in putrescine and alanine observed in the twopopulations when exposed suggests a stress response in both groups of earthworms, these two metabolites have previously been suggested as universal biomarkers in metabolomics studies (Groppa and

Benavides, 2008; Rhee et al., 2007; Simpson and McKelvie, 2009). However, the increase in most amino-acids contents in the pre-exposed earthworms after 28 days, which is not observed in the naïve animals, indicates a particular metabolic response. Several studies reported that the available resources in amino-acids were at the centre of metabolic activity during stress responses (Krasensky and Jonak, 2012; Lankadurai et al., 2013; Simpson and McKelvie, 2009). Moreover, the increase in alanine, aspartate, aminobutyrate and succinate observed in the pre-exposed populations could indicate an activation of the alanine, aspartate and glutamate pathway (Kanehisa and Goto, 2000).

The increase in both metabolic rates was reflected by depletion in the lipid and the glycogen resource. However a temporal delay appeared in the glycogen usage, indicating a differential mobilisation of this sugar resource between naïve and pre-exposed earthworms. The naïve earthworms seem to consume glycogen earlier than the pre-exposed group. Glycogen breakdown is reflected by the slight increase of glucose in fungicide-exposed groups of earthworms after 28 days. Soluble proteins were also significantly higher after 28 days in the pre-exposed worms only, which could indicate the higher synthesis of detoxificationenzymes such as cytochrome p450(Lukkari et al., 2004). Metabolic profiles of control and exposed groups in pre-exposed earthworms became distinct after 28 days, indicating that metabolic networks have been rearranged to maintain internal homeostasis and performance of the organisms.

Overall, all these findings show that pre-exposition of earthworms over generations in the conventional farming system has led to physiological adaptation, as evidenced by their higher reaction to the fungicide. Other studies have shown that separation of metabolic signatures (PCA analyses) increase with higher doses of the pollutants, e.g DDT, endosulfan or copper (Bundy et al., 2008; Simpson and McKelvie, 2009). It is likely that, in our study, fungicide recommended application rate corresponds to a low sublethal dose and the differences in metabolic signatures would become clearer with higher concentrations of the pesticide. Nevertheless, the differences observed demonstrate an impact even at this environmentally realistic level. As both populations were selected from fields under long term (20 to 25 years) conventional and organic farming, an adaptation mechanism on the genetic level could be assumed, but would need to be proven.

4.2. The link with earthworm burrowing and the pesticide fate in soil

Several studies aimed to correlate biochemical or cellular responses in earthworms to pollutants with ecologically important endpoints. For example, Maboeta et al. (2001) showed that there was a link between decrease in the abundance of field populations of the earthworm *Microchaetus sp*. and decrease in the animals' neutral red retention time (a biomarker of cellular damage). By showing a strong reduction of earthworm growth by pesticides, (1992) postulated that these contaminants were likely to cause a delay in sexual maturity in juveniles and have eventually have an impact on earthworm abundance in the field. However, pesticide impacts on burrowing behavior have only poorly been studied because of the difficulty to visualize or estimate burrowing activity, and few studies have tried to link pollutant impacts at low levels of organisation (cellular, biochemical) with earthworm burrowing (Capowiez et al., 2010; Gupta and Sundararaman, 1991). With regard to the ecological importance of earthworms through the burrowing of the soil, it is likely that, when attempting to assess the ecosystem services rendered by earthworms to the soil, earthworm burrowing behaviour is as important as population numbers as it can have drastic impacts for soil functioning (Capowiez & Bérard 2006).

Here, the low dose application of fungicideresulted in an increase in cast production after seven days, which is consistent with the recent results of Dittbrenner et al(2010), where cast production was increased only at the lowest concentration of the pesticide, but decreased at higher doses. The impacts of pesticides on soil bioturbation have been investigated in a few articles using 2D and 3D (X-ray tomography) terraria, cast production method and the avoidance behaviour test. Most of them, except for the paper of Dittbrenner et al (2010), have shown a decrease in activity or an impact on the characteristics of the burrow systems, e.g length, depth, and branching rate (Pelosi et al., 2013). Interestingly, in the present study, the increase in cast production was only observed in the pre-exposed earthworms. Therefore it could suggest that tolerance to this environmentally realistic level of fungicide is associated with a compensatory increased activity. This increase in burrowing behaviour could be induced by the metabolic changes observed in energetic depletion and metabolites rearrangements, similarly to the phenomenon of hormesis (Zhang et al., 2009). An alternative hypothesis would be that it is related to avoidance behaviour, but unsuccessful, as the earthworm is confined to the microcosm, resulting in an increased amount of casts. It is also known that geophagous earthworms are able to alter their burrowing behaviour and display different behaviours (in terms of soil ingestion) when they are feeding on organic matter in the soil or moving through the soil (possibly as part of an avoidance response)(Capowiez and Bérard, 2006; Hugnes et al., 1996). This may lead to reduced cast production in contaminated

soils rather than increased cast production but it is dependent on the level of contamination and the contaminant (Dittbrenner et al., 2010).

The fate of pesticides in soil can be affected by earthworms bioturbation via several mechanisms. It increases pesticides sorption on soil particles on the long-term, leading to the formations of non-extractable residues. Therefore it can increase the pesticide persistence, as it was previously shown for atrazine (Binet et al., 2006; Farenhorst et al., 2000). On the other hand, earthworms' activity was also reported to stimulate microorganisms activity, and enhance the activity of atrazine- or MCPA-specific bacterial degraders, accelerating its mineralisation (Liu et al., 2011; 2011, 2008). *A. caliginosa*alsoparticipated in the breakdown of four fungicides (folpet, fosetyl-Al, metalaxyl, myclobutanil) and two insecticides (Chlorpyrifos-Ethyl and λ-Cyhalothrin)(Schreck et al., 2008). In our study, pesticide concentration is lower in the microcosms containing earthworms from the conventional field (Table 2). The increase in bioturbation observed in these earthworm'smicrocosms suggest that they play a part in the pesticide's disappearance either by enhancing sorption or by enhancing microbial mineralization of epoxiconazole.

5. Conclusion

This study shows that an environmentally realistic concentration of epoxiconazole applied as OPUS® induced distinct physiological changes in two populations of the earthworm *A. caliginosa.*Biological responses in energy storage and metabolic profiles differed between earthworms derived from conventional farming and those from organic farming, indicating that an acclimation mechanism to the agricultural pesticide occurs in the long-term pre-exposed animals.Theacclimationin pre-exposed animals was also evidenced by their higher reaction to the chemical, withincreasedmetabolicrate and burrowingactivity compared to the naïve animals, which ecological consequence is alower pesticide concentrationin the soil. This original finding is of major interest in the frame of ecosystem resilience to global changes. Whether this physiological adaptation is a general pattern of response against fungicides or other pesticides would need to be confirmed with other molecules and agricultural contexts.

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Fig1

Metabolic rate (µg CO_2 g^{-1} worm (fresh weight) $hour^{-1}$) of pre-exposed (*square*) and naïve (*triangle*) *A. caliginosa* exposedto Epoxiconazole (*solid symbols, full lines)* or not exposed (o*pen symbols, dashed lines*) at day 0, 7 and 28. Mean values (N=5 worms per group) are presented with standard deviations as error bars. Significant differences are indicated with * between exposed and control worms and # between populations at each sampling time. Different letters (a or b) denote statistical differences between sampling times within the same group.

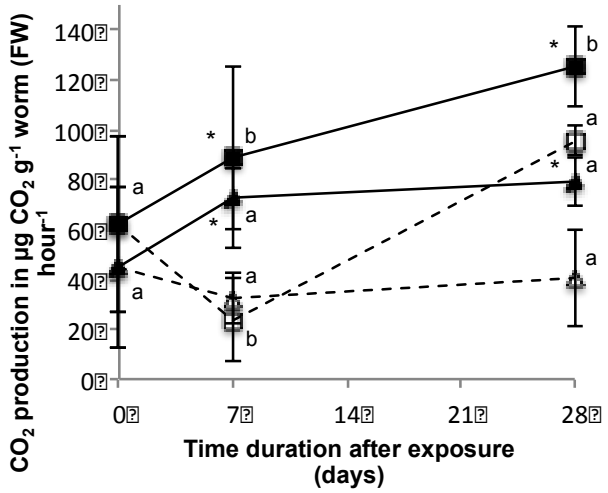

Fig2

784 Mean cast production (in g dry cast weight g^{-1} earthworm body mass day^{-1}) of pre-exposed and naïve
785 *Aporrectodea caliginosa* after exposure to epoxiconazole for 7 and 28 days (n=8). Error bars are standard
786 deviations (SD). * indicates significant differences between exposed and control groups (student-t-test, ° $p<0.1$,
787 * $p<0.05$, ** $p<0.01$) and # between earthworm populations.

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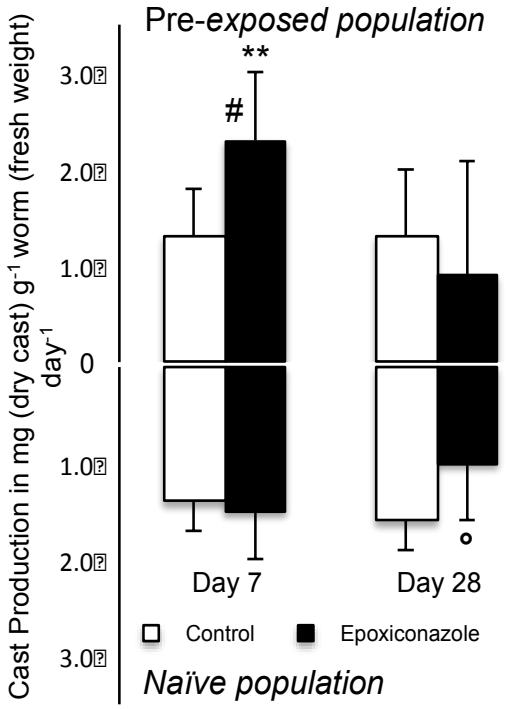

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802 **Fig3**

Glycogen (A), Lipids (B), and Proteins (C) contents (percentage of mean of day 0 value) of pre-exposed and naïve *A. caliginosa* exposedto Epoxiconazole at day 7 and 28. Mean values (N=5 worm per group) are presented with standard deviations as *error bars*. Significant differences are indicated with * between exposed and control worms (Student-t-tests, p<0.1).

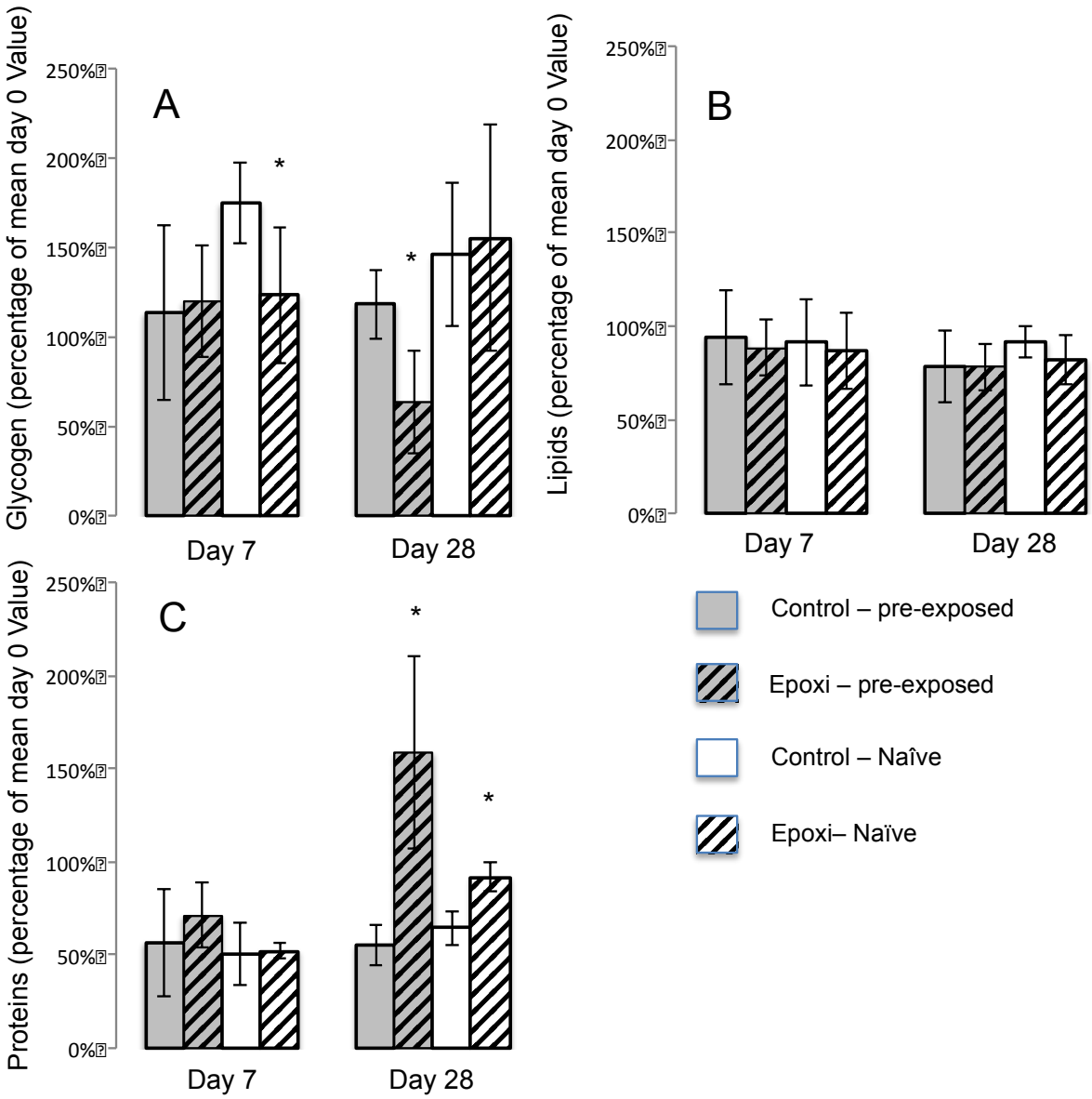

Fig4

Principal Component Analysis of metabolites data (22 variables) showing relationship between metabolite profiles and epoxiconazole exposure along time. A, B: Scores plots for conventional population (Axes 2 and 3) and organic population (Axes 1 and 2). Data are shown as crosses for both exposed and control groups means ± standard error of the mean (SEM). Exposed and Control groups are joined by time order with dashed lines. C, D: Loadings plots for individual metabolites in the conventional population (axes 2 and 3) and in the organic population (axes 1 and 2). Metabolites are identified by their abbreviations and colored by functional groups listed in supplementary material (Table S4).

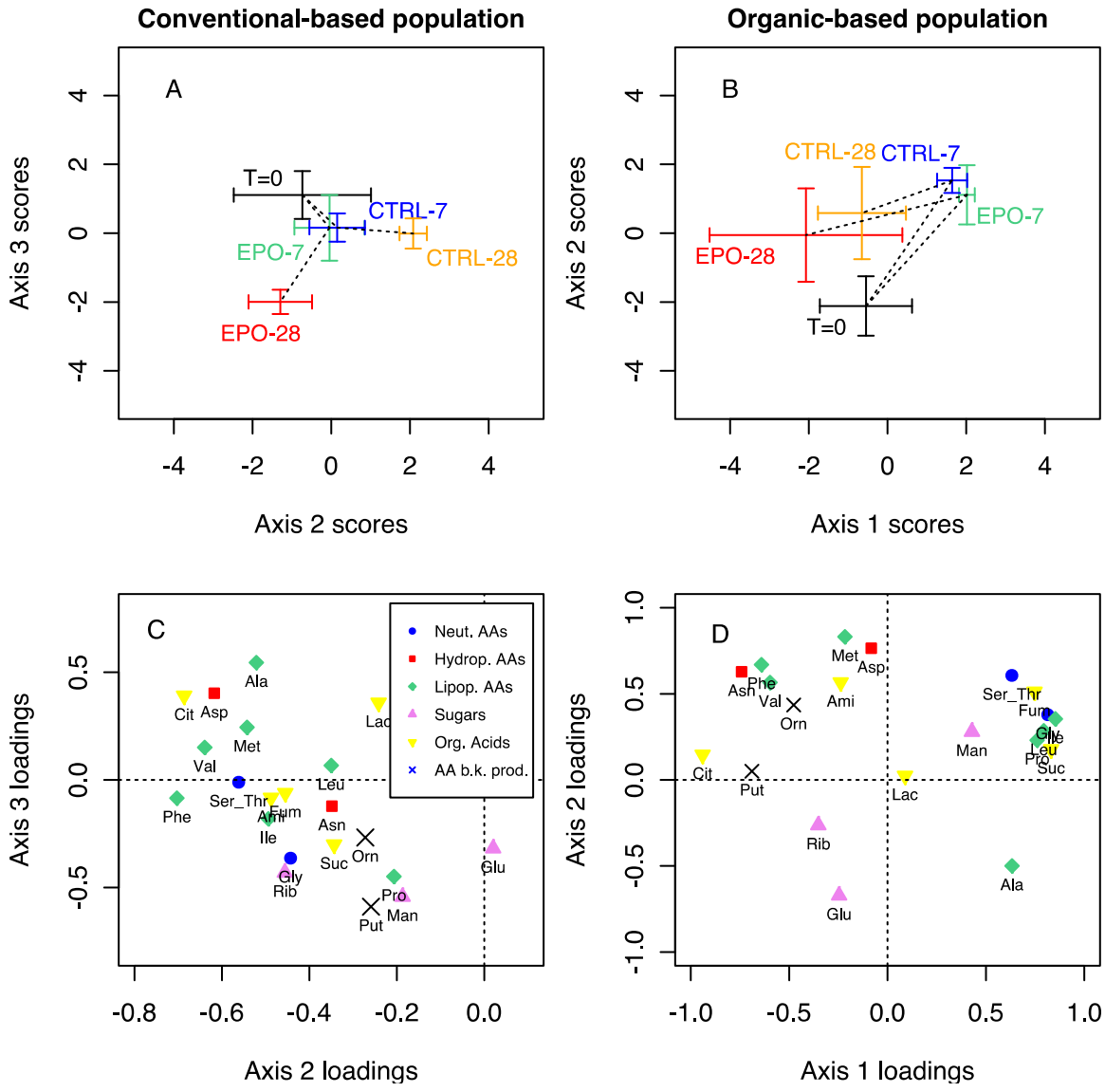

Fig5

Metabolite functional group responses to epoxiconazole, concentrations expressed as percentage of mean control value. A and E: amino-acids (blue=neutral, black=lipophilic, red=hydrophilic) B and F: sugars (blue=glucose, black=mannose, red=ribose) C and G: Krebs' cycle intermediates (blue = aminobutyrate, black = citrate, red = fumarate, green= lactate, violet= succinate) D and H: amino acids degradation products (black=ornithine, blue=putrescine). * indicates significant differences between exposed and control groups (Student t-tests, $p<0.1$).