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## Acclimation of earthworms to chemicals in anthropogenic landscapes, physiological mechanisms and soil ecological implications

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1 **Acclimation of earthworms to chemicals in anthropogenic landscapes, physiological**  
2 **mechanisms and soil ecological implications**

3  
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34

35 **Summary**

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

61

62

63 **Keywords**

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

66

67

68

## 70 **1. Introduction**

71 Often representing the largest animal biomass, earthworms are present in most terrestrial  
72 ecosystems and are considered as efficient ecosystem engineers as they actively modify the  
73 physical, chemical and biological properties of the soil (Binet et al., 1998; Bottinelli et al.,  
74 2010; Jones et al., 1997; Monard et al., 2008). They sustain several key ecosystem services by  
75 enhancing soil structure and nutrient cycling, and play a role in ecosystemic services such as  
76 water regulation, pollution remediation and primary production (Blouin et al., 2013). In  
77 anthropogenic landscapes, soil biodiversity and mainly earthworm communities have to face  
78 disturbances by intensive land-use due to agricultural practices such as ploughing and tillage,  
79 application of fertilizers and chemical pesticides (Paoletti, 1999; Thompson, 1971). The soil  
80 compartment is the primary sink for agricultural pesticides, as they are frequently applied  
81 several times a year. Concerning fungicides, a large part either do not reach its plant target, or is  
82 washed off from treated foliage, leading to major losses to the soil. Bromilow *et*  
83 *al.* (1999a) reported in a field study that only 30% of the fungicide sprays were intercepted by  
84 the barley crop. The frequent application of agricultural pesticides and the persistence of some  
85 of them eventually leads to increasing amounts of residual compounds in the soil, either as  
86 free or bound residues (Gevao et al., 2000; Mordaunt et al., 2005), which can be a threat to  
87 lumbricid species. It has been shown that abundance and diversity of earthworm  
88 communities are dramatically reduced by chemical and mechanical stress (Decaëns and  
89 Jiménez, 2002; Smith et al., 2008). Despite these impacts however, worm populations persist  
90 in conventionally (with pesticides usage) cropped fields, albeit in reduced numbers. Assuming  
91 that immigration rate is low in earthworm populations (Lavelle and Spain, 2001), this suggests  
92 that earthworm populations living in pesticide-polluted soils can cope with chronic chemical  
93 stress, either via avoidance behaviour or physiological resistance (Posthuma and Van  
94 Straalen, 1993).

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

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

105 Adaptation, either via physiological or genetically-mediated mechanisms, is an expression of  
106 the speciesecological plasticity allowing protection against stresses. However, it is known to  
107 be costly in terms of metabolism and energy usage, especially when it involves over-  
108 production of compounds such as protection enzymes (Calow, 1991). Increased metabolic  
109 rate or increased energy allocation to detoxification mechanisms can be at the detriment of  
110 energy storage, and thus impair other functions such as reproduction and growth (Jansen et al.,  
111 2011; Yasmin and D'Souza, 2010). This is particularly important when animals are exposed to  
112 low but chronic exposures, such as earthworms in agricultural fields, due to the persistence of  
113 residues of pesticides in the soils for several years after application (Gevao et al.,  
114 2000). Available energy resources, as measured by main storage compounds, can then reflect  
115 energy demands associated with different adaptation strategies. In the earthworm  
116 *Dendrobaena octaedra*, Holmstrup et al. (2011) reported high energetic costs reflected by  
117 glycogen depletion of the internal regulation of Al and Ni metals. On a lower level of  
118 organization, and possibly on a shorter timescale, metabolomics have recently been used in  
119 ecotoxicology studies to investigate the responses of the metabolism to contaminants (Brown  
120 et al., 2010; Bundy et al., 2008; Simpson and McKelvie, 2009), and we think they can be a  
121 valuable tool to study adaptation to contaminants.

122 One of the most common cultures in Brittany (France) is winter wheat, with a mean number  
123 of 6 pesticides applications per year, the majority of them being fungicides (Agreste, 2006).  
124 In particular, members of the triazole family, which act by inhibiting the biosynthesis of  
125 ergosterol, have attracted interest because of their high persistency in soils (Bromilow et al.,  
126 1999a, 1999b; Passeport et al., 2011), although data available on their toxicity is still scarce.

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

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

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

143

## 144 **2. Materials and Methods**

145

### 146 2.1. Earthworm populations, soil and agricultural context

147

148 Earthworms used in this study originated from two agricultural fields, one conventionally  
149 cropped and one cropped according to organic agriculture requirements. Both of these fields  
150 have been in these agricultural management strategies for more than 20 years and are located  
151 in the same agricultural basin (Vézin-le-Coquet, Brittany, France). Soils are slightly acid  
152 silt-clay loams (conventional and organic field, respectively: Clay 14.8 % and 16.6 %; Silt  
153 71.6 % and 71%; Sand 13.6 % and 12.4 %; organic matter 1.67 % and 2.55 %; pH (water  
154 suspension) 6.4 and 6.9). The conventional field had been cropped under rotations of  
155 wheat/maize/leguminous for 20 years, and annually treated with pesticides. The fungicide  
156 epoxiconazole was used each year a cereal was planted. Epoxiconazole is a triazole fungicide  
157 present in two pesticides formulations (OPUS® and OGAM®) and mainly used on wheat. The  
158 organic field has been under rotation with a cereal (2 years) / maize (1 year) / lucerne (3 years)  
159 without any pesticides for 20 years, and was not tilled during the lucerne periods.

160 Soil used for the exposure experiment was collected from the first 30 cm of a permanent (since  
161 1960) organic pasture (17.6% clay, 69.3 % silt, 13.1% sand, 4.0 % organic matter, pH (water  
162 suspension) 6.0) located in the same area where no initial epoxiconazole residuals were  
163 detected (see method in 2.3). Upon retrieval, it was air-dried until it reached 14% of humidity,  
164 then sieved to remove all soil particles larger than 2 mm and kept in sealed containers (100  
165 l) until used for the experiment.

166 The endogeic species *Aporretodea caliginosa* was chosen for this experiment. It is an  
167 environmentally relevant species for toxicological tests, since it is commonly found in  
168 agricultural fields and reported as a dominant species (Jordan et al., 2004; Lamandé et al.,  
169 2003; Nuutinen, 1992; Söchtig and Larink, 1992). Most standard ecotoxicity tests are  
170 conducted on epigeic species mainly with *Eisenia fetida* or *Eisenia andrei*, but these species

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

179

## 180 2.2. Experimental setup

181 Soil contamination: Epoxiconazole was applied as commercial formulation OPUS® (125 g  
182 active ingredient l<sup>-1</sup>, obtained from Bayer Crop Science) diluted in distilled water at 0.1 µg g<sup>-1</sup>  
183 soil, which is equivalent to a predicted field concentration calculated for a field application  
184 rate of 125 g.ha<sup>-1</sup> assuming a single application with a homogenous distribution and no crop  
185 interception in the top 5 cm of the soil (Dittbrenner et al., 2010). Soil spiking was conducted  
186 by manually adding 175 ml of the diluted pesticide solution or distilled water (for the  
187 controls) on each 2 kg of soil at 14% water content (1.75 kg dry weight) reaching a final soil  
188 water content of 24%. To insure homogeneity of pesticide distribution in the soil, the solution  
189 was added in two parts, the soil being thoroughly mixed, sieved using a 2 mm  
190 aperture and redispersed as a fine layer. Soil microcosms consisted of polycarbonate boxes  
191 (80mm x 50mm, Caubère, Yebles, France) with a lid pierced with tiny holes to ensure  
192 sufficient aeration. The microcosms were filled with 100 g of contaminated or control soil,  
193 then 0.2 g of dry grass meal was added to the surface of the soil. Then the microcosms were  
194 left two days in a cool dark room to ensure aeration of the soil after re-humidification. Water  
195 content was checked again in 3 additional control boxes and adjusted to 25% prior to  
196 introduction of animals, then checked again each week.

197 Experimental design: The experimental design is described in table 1. It comprised 11  
198 specimens from each population (pre-exposed and naïve) for each treatment (epoxiconazole or  
199 control) and sampling time (7 and 28 days) plus an initial control group at day 0 (unexposed).  
200 Prior start of exposure, each earthworm was rinsed in tap water, gently dried on filter paper,  
201 weighed and placed in individual Petri dishes for 48 hours for gut voiding. Then animals were  
202 transferred individually to the exposure microcosms (day 0) according to a size-class  
203 procedure, insuring a similar mean earthworm weight in each treatment. Soil was spiked with  
204 epoxiconazole at 0.15 µg.g<sup>-1</sup> soil or control soil. Exposure lasted for 7 and 28 days, with an

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

213 In addition, eight uncontaminated soil microcosms without worms were used as controls for  
 214 the cast production test to assess the potential formation of non-biogenic aggregates at 7 and  
 215 28 days. Another 3 soil microcosms were filled with contaminated soil to track the fate of  
 216 pesticide without the presence of worms. Humidity control was conducted in 3 additional,  
 217 non-contaminated soil microcosms, in order to adjust humidity when necessary.

218

		worms at start	Sampling days and number of worms sampled			treatment
			0	7	28	
Microcosms 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

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

222

### 223 2.3. Pesticide concentration in soil

224

225 Sub-samples of 2g of soil were retrieved the day of the pollution (day 0), and after 7 and 28  
 226 days, from three randomly chosen microcosms out of the 11 replicates. They were dried at  
 227 30°C overnight, then kept frozen until pesticide analysis. Epoxiconazole in soil sub-samples  
 228 was measured by liquid chromatography coupled with mass spectrometer (LC-MS) (Waters  
 229 alliance 2690, Waters, Saint Quentin en Yvelines, France). 2g of soil sample were extracted in  
 230 500 ml of mineral water. After 15 min of ultrasound treatment, the sample was mixed by



231 rotary shaking for one hour, and subjected to another 15 min of ultrasound treatment. 0.25 µg  
232 of triadimenol was added as internal standard and 1 ml of analytical-grade nitric acid (HNO<sub>3</sub>)  
233 to acidify the mixture. Then pesticides were extracted from the aqueous mixture in two steps  
234 with 25 ml of HPLC-grade dichloromethane followed by 15 min of agitation. The  
235 dichloromethane extracts were combined and evaporated to a drop (ca. 10 µl), then 500 µl  
236 acetonitrile were added and 0.25 µg of pentabromophenol were added as second internal  
237 standard. The extract was evaporated again to a drop and adjusted to 500 µl with 10%  
238 acetonitrile acidified (0.1 % formic acid).

239 This sample was analysed by LC-MS using a high-performance liquid chromatography  
240 (Alliance 2695, Waters, Saint Quentin en Yvelines, France) coupled to a quadrupole mass  
241 spectrometer model ZQ (Waters-Micromass, Saint Quentin en Yvelines, France) equipped  
242 with an electrospray source. Epoxiconazole was separated on a X Terra MS C18 column (150  
243 x 2.1 mm, 3.5 mm particle size, Waters, Saint Quentin en Yvelines, France) at 35 °C. A  
244 binary mobile phase gradient (A: ultrapure water with 0.1% formic acid; B: acetonitrile with  
245 0.1% formic acid) was used for pesticide separation. The chromatographic method held the  
246 initial mobile phase composition (82% A, 18% B) constant for 10 min, followed by 70% A /  
247 30% B (10min), 50% A / 50% B (15 min), 20% A-80% B (5 min), then again 82% A / 18% B  
248 for 10 min. Quantification limit was 2.5 ng.g<sup>-1</sup> dry soil and extraction yield was 75 ± 7%. A  
249 standard curve of epoxiconazole was made with several aliquots of 2 g of dry soil (the same  
250 uncontaminated pasture soil used for the microcosms) spiked manually with the purified  
251 compound, air-dried for two hours, and extracted the same way as the real samples for  
252 quantification, relating to the two internal standards. Pesticides analytical standards were  
253 purchased from Dr Ehrenstorfer (Ausburg, Germany).

254 Recovery of epoxiconazole in spiked soil was initially 80% of the desired concentration (0.1  
255 µg.g<sup>-1</sup>), with a coefficient of variation of 10%, which was considered highly satisfactory, as  
256 we used the commercial formulation of the pesticide. Concentrations of epoxiconazole in soil  
257 (Table 2) were still two thirds of the initial concentration after 28 days, which is consistent  
258 with the long persistencies (half-life > two years) reported in the literature (Bromilow et al.,  
259 1999a, 1999b; Liang et al., 2012).

260

#### 261 2.4. Respirometry measurements

262

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

265 filter paper for gut voiding. Thereafter, the glass jar was hermetically closed for two hours,  
266 and CO<sub>2</sub> was measured by a Micro-Gas Chromatograph (3000A, SRA Instruments) equipped  
267 with a single capillary column Poraplot U and coupled with a thermal conductivity detector.  
268 Then the worm was frozen in liquid nitrogen for further measurements of energy resources  
269 (2.6) and metabolomics (2.7).

270

## 271 2.5. Cast production

272

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

277

## 278 2.6. Energy resources measurement

279

280 Frozen worms were freeze-dried, and ground to a fine powder by multiple 30 sec agitations  
281 with inox beads in 2 ml test tubes in a bead-beater (Retsch MM400, Retsch GbmH, Haan,  
282 Germany). Each ground sample was separated in several aliquots, 2 mg for total lipids, 5mg  
283 for soluble proteins, and 2 x 10 mg for glycogen and metabolites measurements. Lipid  
284 aliquots were extracted according to Folch(1957), lipids in the chloroform extract were  
285 assayed by the sulfo-vanillin method with a calibration curve of commercial vegetable oil at  
286 525 nm. Proteins aliquots were homogenized in 0.1 M phosphate buffer (pH 6.5), centrifuged  
287 and measured according to Bradford (1976) using a calibration curve of bovine serum  
288 albumin. Glycogen was measured according to the method of Nicolai *et al*(2012). Briefly,  
289 aliquots were homogenized in 600 µl trichloroacetic acid (4%) and centrifuged at 5000 G  
290 (rotor N° 12145, SIGMA, 8000 rpm) for 10 minutes. Then 500 µl of supernatant was  
291 recovered and glycogen was precipitated by adding 1.5 ml of ethanol and stirring for 10  
292 minutes. The extract was centrifuged (5000 G) and the ethanol eliminated with a glass pipette.  
293 The pellet was washed with 2ml ethanol, then the remaining ethanol was totally evaporated at  
294 70°C. The pellet was redissolved by stirring it overnight in ultrapure water, and centrifuged  
295 again. Absorbance was measured at 425 nm with a microplate reader (Fischer Scientific  
296 Multiskan FC) after addition of Lugol using a calibration curve of purified glycogen (Oyster  
297 type II, Sigma).

298

## 299 2.7. Metabolomics

### 300 2.7.1. Sample extraction and derivatization

301

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

315

### 316 2.7.2. GC-MS analyses

317

318 Gas chromatography coupled with mass spectrometry (GC–MS) was used to measure up to 58  
319 small metabolites belonging to different classes of molecules: amino-acids, polyols, sugars,  
320 intermediates of the citric acid cycle and other unclassified biological molecules. The GC–MS  
321 system was comprised of a Trace GC Ultra chromatograph, and a Trace DSQII quadrupole  
322 mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA, USA). The injector  
323 temperature was set at  $250\text{ }^\circ\text{C}$ . The oven temperature was increased from  $70\text{ }^\circ\text{C}$  to  $170\text{ }^\circ\text{C}$  at  
324  $5\text{ }^\circ\text{C}\text{ min}^{-1}$ , from 170 to  $280\text{ }^\circ\text{C}$  at  $7\text{ }^\circ\text{C}\text{ min}^{-1}$ , from 280 to  $320\text{ }^\circ\text{C}$  at  $15\text{ }^\circ\text{C}\text{ min}^{-1}$ , then the oven  
325 remained for 4 min at  $320\text{ }^\circ\text{C}$ . A 30 m fused silica column (TR5 MS, I.D. 2.5 mm, 95%  
326 dimethyl siloxane, 5% Phenyl Polysilphenylene–siloxane) was used, with helium as the  
327 carrier gas at a rate of  $1\text{ mL}\cdot\text{min}^{-1}$ . One microliter of each sample was injected using the split  
328 mode (split ratio: 25:1). We completely randomized the injection of the samples. The  
329 temperature of the ion source was set at  $250\text{ }^\circ\text{C}$  and the MS transfer line at  $300\text{ }^\circ\text{C}$ . Detection  
330 was achieved using MS detection in electronic impact (EI). We used the selective ion-  
331 monitoring mode (SIM) (electron energy:  $-70\text{ eV}$ ), allowing a precise annotation of the  
332 detected peaks. The peaks were identified according to both their mass spectra (two ions) and

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

337

## 338 2.8. Statistical analyses

339

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

357

## 358 3. Results

359

### 360 3.1. Energy dissipation

361

362 Fungicide treatment increased CO<sub>2</sub> production in both pre-exposed and naïve populations  
363 after 7 and 28 days compared to their non-treated controls (Fig 1). Respiration rate in the  
364 fungicide-treated group was higher after 7 and 28 days compared to day 0 in the pre-exposed  
365 population. Differences became significant between populations at 28 days, where

366 metabolic rates of the pre-exposed population (both exposed and control groups) were still  
367 higher than at the outset, while it remained constant in the naïve population from day 7 to 28.

368

### 369 3.2. Cast production (CP)

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

379

### 380 3.3. Energy resources

381

382 Mean glycogen content was decreased by nearly 20 mg in fungicide-treated compared to  
383 control groups after 7 days in the naïve earthworms, and after 28 days in the pre-exposed  
384 earthworms as shown in (Figure 3-A). At the end of the exposure, the worms originating from  
385 the conventional-treated field had a slightly lower glycogen tissue level than the naïve ones.

386 The lipid tissue levels (Figure 3-B) did not show any significant differences between un-  
387 exposed and fungicide exposed earthworms or between earthworms originating from the  
388 conventional or the organic-treated field. Lipid levels were lower in all treated groups  
389 compared to their respective controls, however it did not achieve significance. Protein  
390 contents (Figure 3-C) decreased similarly in the 4 groups of worms during the first 7  
391 days. They were then significantly increased by fungicide treatment in both exposed and  
392 naïve populations compared to their respective controls after 28 days, with pre-exposed  
393 worms having a final protein amount almost twice as high as naïve ones.

394

### 395 3.4. Metabolomics

396

#### 396 3.4.1. Metabolic profiles of fungicide-exposed and control populations

397

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

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

405

406 3.4.2. Metabolic changes in the worms populations

407

408 In the loadings plots of the PCAs (figure 4C and 4D), several coordinated responses were  
409 identifiable. In the conventional population (Figure 4C), the 7 variables that have loadings  
410 lower than -0.5 on axis 2 form a first cluster containing mostly lipophilic amino-acids. Another  
411 cluster is in the lower half of the plot corresponding to variables having low loadings (roughly,  
412 <-0.2) on axis 3, composed of the three sugars, succinate, ornithine, the amino-acids glycine  
413 and proline and putrescine. On the other hand in the organic population (Figure 4D), we see  
414 two clear clusters that could correspond to the incubation time: the top right corner for seven  
415 days and the top left corner for 28 days. Day zero would correspond to the sugars Glu and Rib  
416 (lower half). The induced changes in metabolite concentrations after exposure was then  
417 measured as normalized concentrations in percentage of the control value and compared  
418 according to functional groups (Figure 5). In the conventional worms, several metabolites  
419 increased after 28 days of epoxiconazole exposure in all four biochemical groups (amino-  
420 acids, sugars, organic acids and ornithine-putrescine), up to 7-fold for aspartate, 4-fold for  
421 aminobutyrate, and 2-fold for ornithine and putrescine. This general increase in metabolite  
422 levels was not observed in the naïve population, which displayed different trends. Indeed, in  
423 Figure 5E, certain amino-acids (Asn, Asp, Phe and Val) increased slightly, whereas  
424 others dropped below 100% or stayed stable at 28 days. Not much variation was observed in  
425 organic acids of the organic earthworms at 28 days, except for aminobutyrate which dropped to  
426 50% of the control value, contrasting to its 4-fold increase in the pre-exposed animals.  
427 Putrescine level increased by 2-fold after 28 days in both populations whereas ornithine  
428 decreased to below 50% of the control value in the organic population only.

429

## 430 4. Discussion

431

432 4.1. Acclimation in energetic processes and metabolism

433

434 Animals can tolerate pollutants via biotransformation, excretion or scavenging of free radicals  
435 (when pollutants induce oxidative stress). For example, detoxification of several pollutants,  
436 e.g. atrazine or paraquat, is mediated via the enzyme Glutathione-S-Transferase. This enzyme,  
437 belonging to phase II detoxification mechanisms, acts through binding xenobiotics to  
438 glutathione and facilitating its excretion (Anderson and Gronwald, 1991; 2007, 2000).  
439 Another common detoxification pathway to xenobiotics is the group of cytochrome p450  
440 oxidases. This family of enzymes transform the structure of organic chemicals, hence greatly  
441 altering their toxicity (Ribera et al., 2001; Rodríguez-Castellanos and Sanchez-Hernandez,  
442 2007). However, no data on epoxiconazole tolerance pathways e.g. detoxification  
443 mechanisms was available in the literature.

444 On the other hand, the literature suggests that there are energetic costs in organisms for  
445 coping with pollutants (Fisker et al., 2011; Holmstrup et al., 2011; Wiegand et al., 2007). The  
446 way organisms handle energetic processes can therefore inform us on adaptation mechanisms.  
447 As an example by measuring the main energy resources, Pook et al (2009) showed that a  
448 metal-resistant population of marine harbour ragworm (*N. diversicolor*) had a lower scope for  
449 growth than a reference (non-resistant) population and demonstrated a metabolic cost, or  
450 tradeoff, of resistance. Other tradeoffs can be the co-selection of traits along with tolerance to  
451 xenobiotics, such as life history traits. Interestingly, in the conventionally cropped field, the  
452 worms sampled had a lower initial mean weight but with constant tissue composition  
453 compared to the organic worms (Table 1). This could be a result of the selection of smaller  
454 individuals, by the global agricultural management of the field, as a possible combined effect  
455 of fertilizers, pesticides and tillage (tillage was less frequent in the organic field due to the  
456 lucernecropping). This assumption is supported by the fact that the bigger anecic species such  
457 as *L. terrestris* or *A. giardiare* usually the most impacted in cropped soil (Edwards and Bohlen,  
458 1996).

459 To investigate such energetic costs, we measured the worm's metabolic rate ( $\mu\text{g CO}_2 \text{ g}^{-1}$   
460 worm (fresh weight)  $\text{hour}^{-1}$ ) as a proxy of energy dissipation, and the main energy storage  
461 compounds which are glycogen (main sugar resource), total proteins and lipids. Metabolic  
462 rate was increased in both populations after 7 days of exposure, showing that the fungicide  
463 increased metabolic rate, but to a greater extent in the pre-exposed earthworms. Energy  
464 dissipation in both control and exposed groups were indeed higher than the naïve earthworms  
465 after 28 days. The increase in putrescine and alanine observed in the two populations when  
466 exposed suggests a stress response in both groups of earthworms, these two metabolites have  
467 previously been suggested as universal biomarkers in metabolomics studies (Groppa and

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

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

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

498

499

500 4.2. The link with earthworm burrowing and the pesticide fate in soil



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

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

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

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

550

## 551 **5. Conclusion**

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

564

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566

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578

579

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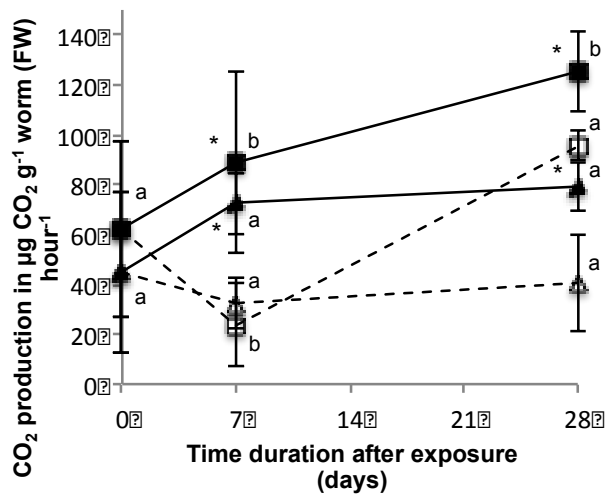
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 762

763 **Fig1**

764 Metabolic rate ( $\mu\text{g CO}_2 \text{ g}^{-1} \text{ worm (fresh weight) hour}^{-1}$ ) of pre-exposed (*square*) and naïve (*triangle*) *A.*  
 765 *caliginosa* exposed to Epoxiconazole (*solid symbols, full lines*) or not exposed (*open symbols, dashed lines*) at  
 766 day 0, 7 and 28. Mean values (N=5 worms per group) are presented with standard deviations as error bars.  
 767 Significant differences are indicated with \* between exposed and control worms and # between populations at  
 768 each sampling time. Different letters (a or b) denote statistical differences between sampling times within the  
 769 same group.



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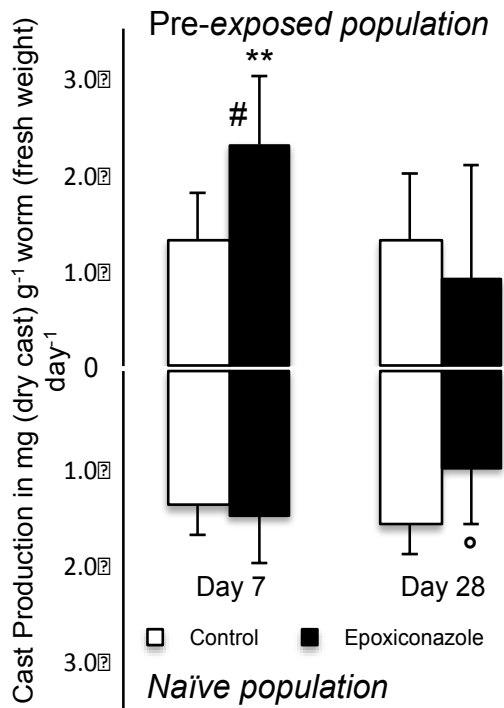
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783 **Fig2**

784 Mean cast production (in g dry cast weight g<sup>-1</sup> earthworm body mass day<sup>-1</sup>) 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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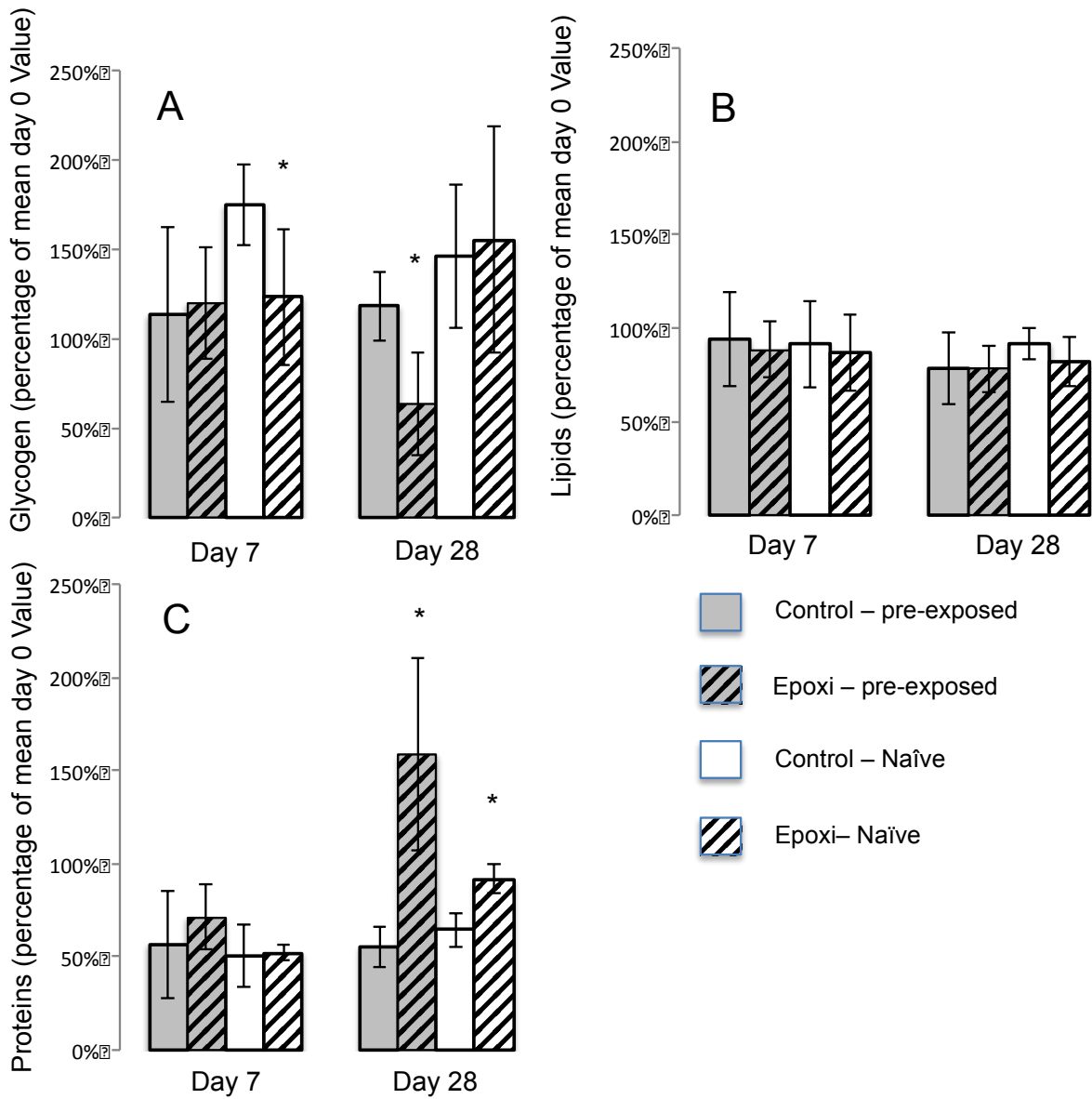
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802 **Fig3**



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

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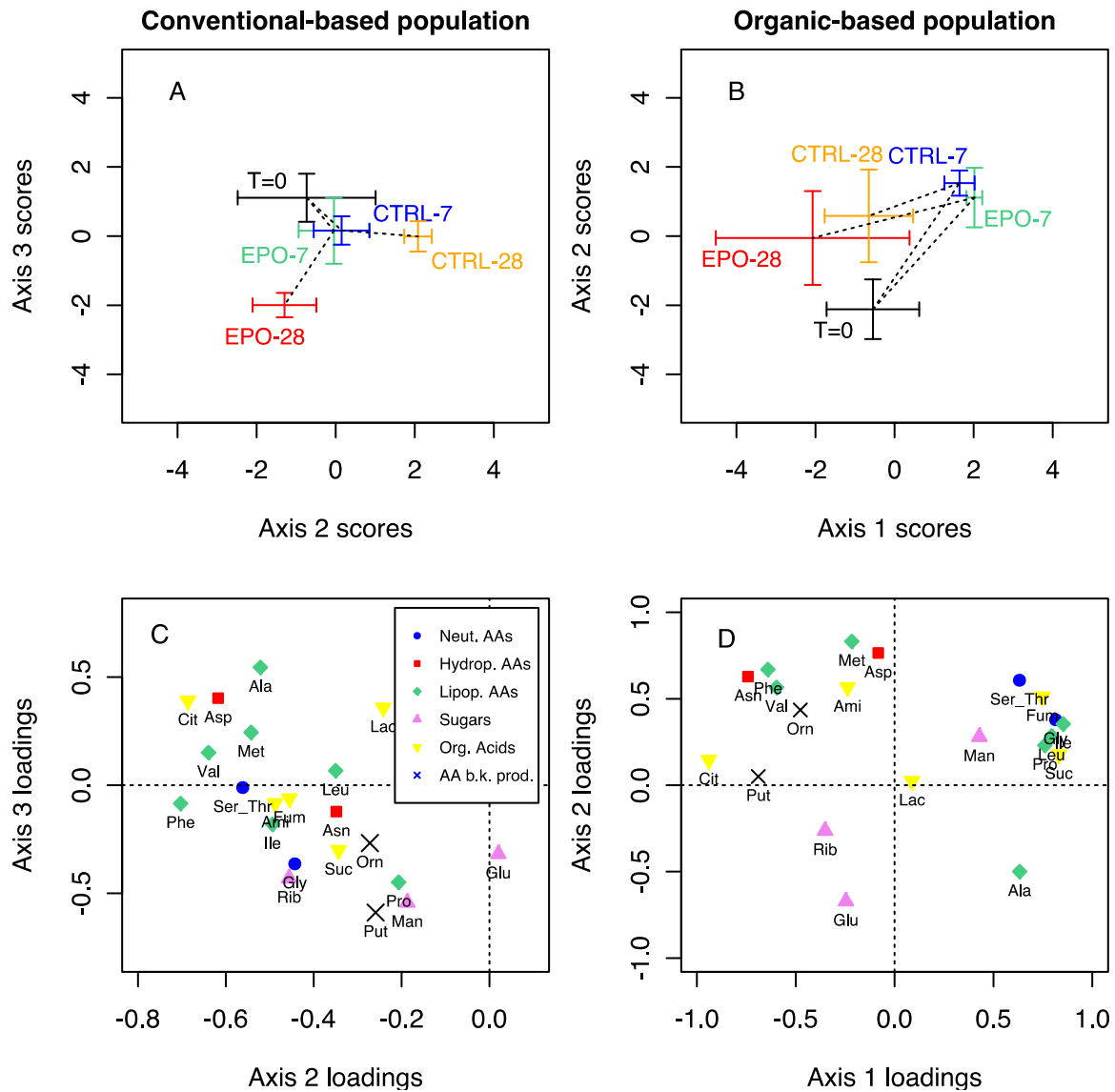
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815 **Fig4**

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

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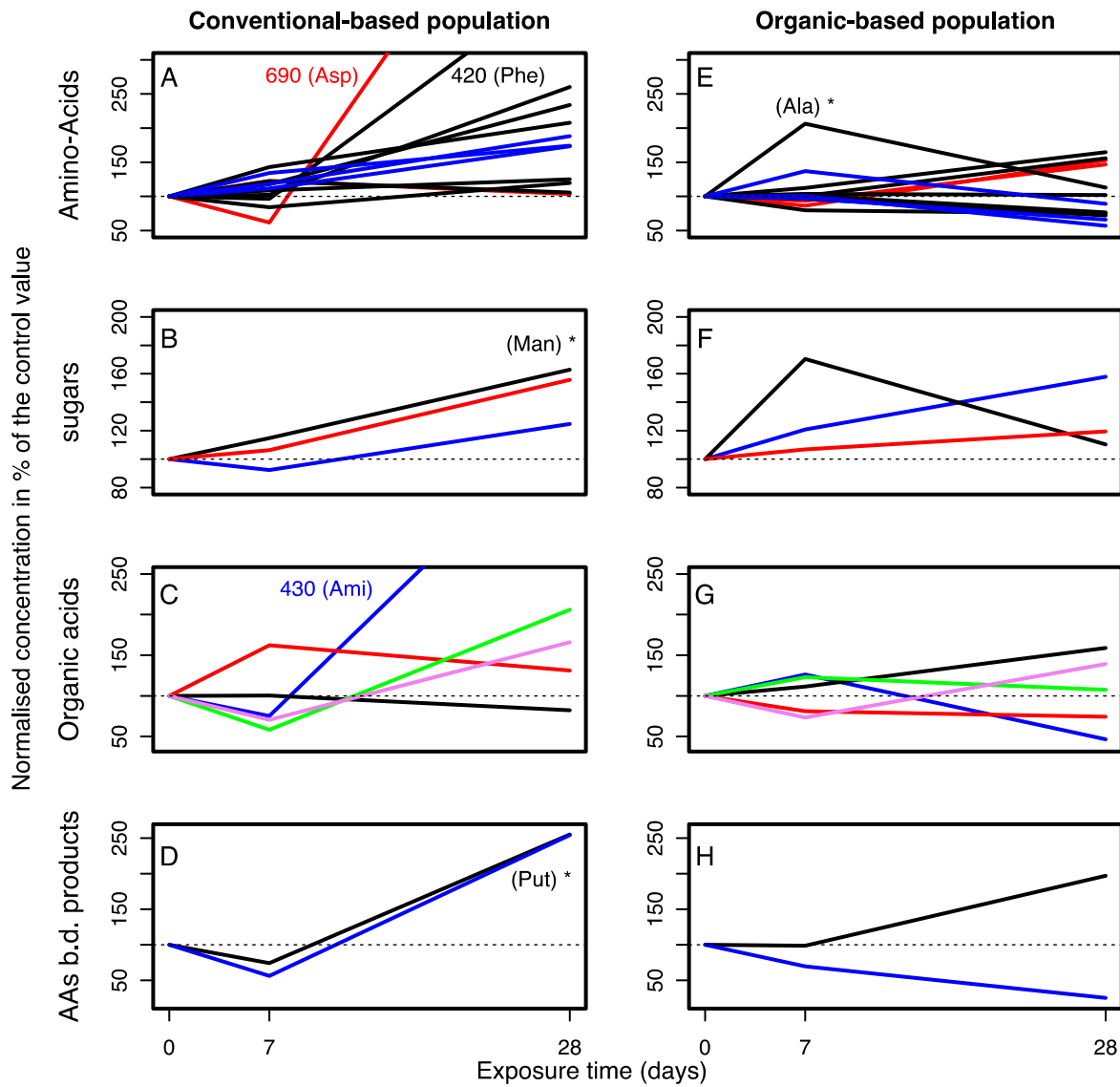
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830 Fig5

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

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