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## Insights into the molecular mechanisms of pesticide tolerance in the *Aporrectodea caliginosa* earthworm

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1           **Insights into the molecular mechanisms of pesticide tolerance in the *Aporrectodea***  
2                                   ***caliginosa* earthworm**

3  
4   **Running title:** Earthworms' pesticide tolerance

5  
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23 **Abstract**

24 Diffuse pollution of the environment by pesticides has become a major soil threat to non-target  
25 organisms, such as earthworms for which declines have been reported. However some endogeic species  
26 are still abundant and persist in intensively cultivated fields, suggesting they become tolerant to long-  
27 term anthropogenic pressure. We thus considered the working hypothesis that populations of  
28 *Aporrectodea caliginosa* earthworms from conventionally managed fields developed a tolerance to  
29 pesticides compared with those from organically managed fields. To investigate this hypothesis, we  
30 studied earthworm populations of the same genetic lineage from soils that were either lowly or highly  
31 contaminated by pesticides to detect any constitutive expression of differentially expressed molecular  
32 pathways between these populations. Earthworm populations were then experimentally exposed to a  
33 fungicide—epoxiconazole—in the laboratory to identify different molecular responses when newly  
34 exposed to a pesticide. State-of-the-art omics technology (RNA sequencing) and bioinformatics were  
35 used to characterize molecular mechanisms of tolerance in a non-targeted way. Additional physiological  
36 traits (respirometry, growth, bioaccumulation) were monitored to assess tolerance at higher levels of  
37 biological organization. In the present study, we generated the *de novo* assembly transcriptome of *A.*  
38 *caliginosa* consisting of 64,556 contigs with N50 = 2862 pb. In total, 43,569 Gene Ontology terms were  
39 identified for 21,593 annotated sequences under the three main ontologies (biological processes, cellular  
40 components and molecular functions). Overall, we revealed that two same lineage populations of *A.*  
41 *caliginosa* earthworms, inhabiting similar pedo-climatic environment, have distinct gene expression  
42 pathways after they long-lived in differently managed agricultural soils with a contrasted pesticide  
43 exposure history for more than 22 years. The main difference was observed regarding metabolism, with  
44 upregulated pathways linked to proteolytic activities and the mitochondrial respiratory chain in the  
45 highly exposed population. This study improves our understanding of the long-term impact of chronic  
46 exposure of soil engineers to pesticide residues.

47  
48 **Key-words:** Aporrectodea, transcriptomics, pesticides residues, mitochondrial respiratory chain,  
49 agrosystem, soil biodiversity

50 **Highlights**

- 51 • Earthworms chronically exposed to high pesticide concentrations in agriculture soils
- 52 • Tolerance to pesticide investigated in the endogeic earthworm *A.caliginosa*
- 53 • *de novo* assembly transcriptome of *A. caliginosa* was generated
- 54 • Specific gene expression revealed in populations differently exposed *in natura*
- 55 • Proteolytic activities and the respiratory chain pathways differently expressed

Pesticides are specifically designed to regulate the population dynamics of unwanted organisms such as weeds, pathogens or pests. Post-war they became a major foundation of worldwide agricultural performance (Tang et al., 2021). As they are applied directly to soil as sprays, drenches, and granules or, increasingly, as seed coatings (Gunstone et al., 2021), most topsoils in agrosystems (up to 83% in Europe (V. Silva et al., 2019)) contain one or more pesticide residues, causing adverse effects to ecosystem and human health (Alpizar et al., 2019; Bernhardt et al., 2017). Soils are arguably the most complex and biodiverse ecosystems on Earth, containing nearly a quarter of the planet's biodiversity, which play crucial roles in many ecosystem functions and services (Bardgett & van der Putten, 2014). In agricultural landscapes, soil sustainability and fertility depend on its biological component (Swift et al., 2004). Earthworms, the first animal biomass on Earth, support numerous ecological functions: by foraging, they fragment and decompose organic litter (Phillips et al., 2021); by creating biopores, they affect soil structure, water permeability and aeration (Bastardie et al., 2005; Pérès et al., 2010), by ingesting soil, they stimulate microbial communities (Kersanté et al., 2006) and regulate pesticide biodegradation (Monard et al., 2008, 2011). The survival of earthworm populations and the maintenance of a high biomass are crucial in view of these ecosystem services, which are part of their engineering function. Agricultural intensification (including habitat loss, soil tillage and agrichemical use) has, however, been identified as a major driving factor behind the observed decline of terrestrial invertebrates (Forister et al., 2019; Hallmann et al., 2017; Sánchez-Bayo & Wyckhuys, 2019; Seibold et al., 2019; Tsiafouli et al., 2015). Earthworms have suffered a drastic drop in both abundance and diversity: sustainably cropped soils (maize/wheat rotation) support ten times less biomass than grassland soils, and are less functionally diverse due to the scarcity of epigeic species and a higher proportion of immature individuals (Curry et al., 2002; Riley et al., 2008). These changes in soil macrofauna have long been attributed to tillage and soil compaction by agricultural machinery and to larger plots, but recent ecotoxicological studies strongly suggest harmful sublethal effects of pesticide mixtures circulating in soils at the (infra)individual, the populational and the community level (Pelosi et al., 2014, 2021). Although earthworm biodiversity in agroecosystems is declining (Smith et al., 2008), some species persist despite the harsh conditions caused by soil disturbance and pesticide applications. This is particularly true of endogeic species such as genera *Aporrectodea* and *Allolobophora* sp (Givaudan, Binet, et al., 2014; Givaudan, Wiegand, et al., 2014; Pelosi, Toutous, et al., 2013). Certain species may thus have developed tolerance mechanisms to pesticide exposure. Excessive concentrations of pollutants can be tolerated via two mechanisms: either by plastic physiological acclimation or by genetic adaptation over several generations (Morgan et al., 2007). The dependence of agriculture on the extensive use of pesticides may now cover a sufficiently long period (~70 years) that adaptations may have occurred in different species, the mechanisms of which are not yet fully understood. This is particularly so for earthworms, in contrast to insects (Bass et al., 2015; Oakeshott et al., 2005). The evolutionary impacts

92 of contamination by pesticides on non-target species are rarely documented and therefore poorly  
93 understood, yet they may have important consequences on ecosystem functions. Agricultural soils can  
94 be managed in different ways, the major differences including tillage and the application of pesticides.  
95 Some crops are grown according to the principles of organic farming and others according to  
96 conventional agriculture, pesticide application and concentration varying greatly between and within  
97 each production method. Depending on their location, earthworm populations therefore have a history  
98 of contrasting exposures to chemicals. Because earthworm populations from plots farmed  
99 conventionally evolve in a contaminated environment, they could respond to residual contamination by  
100 pesticides through acclimation and/or adaptation mechanisms. Earthworm acclimation/adaptation  
101 processes have been evidenced so far for soil contamination by metals (Fisker et al., 2011; Posthuma &  
102 van Straalen, 1993). The main question is whether the soil fauna (earthworms) can cope and develop  
103 tolerance mechanisms to chronic multi-residual contamination by pesticides. To date and to our  
104 knowledge, the process of acclimation *in natura* to long-term pesticide exposure of earthworms has only  
105 been observed in the species *A. caliginosa* (Givaudan, Binet, et al., 2014; Givaudan, Wiegand, et al.,  
106 2014). It has been shown that earthworms from soil managed in a conventional farming framework have  
107 improved their biotransformation and antioxidant response capabilities. Therefore, there is an urgent  
108 need to understand and predict *in natura* the sublethal effects and evolutionary consequences of  
109 agricultural pesticides on soil biodiversity. We continued to study the endogeic species *Aporrectodea*  
110 *caliginosa* as a model earthworm because it is dominant in most European temperate agrosystems (Boag  
111 et al., 1997; Curry et al., 2008; Jordan et al., 2004; Lamandé et al., 2003; Nuutinen, 1992; Pérez-Losada  
112 et al., 2009). *A. caliginosa* is thus representative of cultivated fields in temperate regions and has recently  
113 been identified as a relevant model species for research in soil ecotoxicology (Bart et al., 2018; Pelosi,  
114 Joimel, et al., 2013). To document any evolutionary effects of pesticides, the current study examined  
115 two working hypotheses: H1) Populations of *A. caliginosa* earthworms from conventionally managed  
116 fields have developed a tolerance to pesticides compared with those from organically managed fields;  
117 H2) This tolerance to pesticides is expressed by a faster and/or more intense response of the molecular  
118 pathways involved in biotransformation/detoxification, oxidation and general response to stress. In order  
119 to investigate these hypotheses, we sampled *A. caliginosa* earthworms from sites with either low-level  
120 contamination (organic) or high-level contamination (conventional) to detect any constitutive  
121 expression of differentially expressed molecular pathways among these populations. These populations  
122 (F0) were then experimentally exposed in the laboratory to pesticide stress to identify differences in  
123 molecular responses when again exposed to pesticide (in this case epoxiconazole, a persistent fungicide  
124 frequently applied in European agriculture to protect cereals and sugar beet against *Septoria* sp. and  
125 rust). State-of-the art omics technology (RNA sequencing) and bioinformatics were used to characterize  
126 the molecular mechanisms of tolerance in a non-targeted way. Overall, our study sheds new light on the  
127 molecular mechanisms of tolerance to chemical pollution in soil of terrestrial invertebrates and helps to  
128 clarify how species are able to cope with pesticide contamination in agrosystems.

## 129 2. Materials and Methods

### 130 2.1. Study area, agricultural context and earthworm populations

131 This study took place in agricultural landscapes that are representative of the Atlantic biogeographical  
132 region of Europe with a temperate climate (mean rainfall of 696 mm year<sup>-1</sup>, mild annual mean  
133 temperature of 12.1°C). Agricultural land management is focused on intensive cropping dedicated to  
134 dairy and livestock productions and is mainly conventional, based on systematic use of chemicals  
135 (mineral fertilizers, pesticides) but with an increasing trend toward organic management.

136 Earthworm *A. caliginosa*, an endogeic species distributed worldwide in temperate areas, was chosen as  
137 an ecologically-relevant biological model (Klobučar et al., 2011). We studied two populations of *A.*  
138 *caliginosa*, one from a conventionally cropped site (henceforth referred to as “population from the  
139 highly contaminated sampling site” or “the HC population”) and one from an organically cropped site  
140 (henceforth referred to as “population from the lowly contaminated sampling site” or “the LC  
141 population”) (GPS coordinates: HC sampling site: 48.111180, -1.775199; LC sampling site: 48.127222,  
142 -1.725714; approximate coordinates to maintain the farmer’s privacy). The cropped sampling sites  
143 studied all lie within the same geological basin (silty Basin of Rennes, Brittany, France). Soils are loamy  
144 (conventional and organic sites, respectively: Clay 14.0% and 17.9%; Silt 71.2% and 63.2%; Sand  
145 14.8% and 18.9%; organic matter 1.86% and 2.89%; pH<sub>w</sub> 6.5 and 7.2). The conventionally managed site  
146 had been cropped under rotations of corn/cereals (wheat, triticale)/protein crops (peas and fava beans)  
147 for at least 22 years, and treated annually with pesticides. The organic site had been under a rotation of  
148 grassland (3 years), corn, wheat, rapeseed, and barley without any pesticides for 29 years. Similar tillage  
149 and farming practices were applied to both the LC and HC sites. In order to study earthworm tolerance  
150 to pesticide pressure in their environment, adults from both the HC and LC populations were reared in  
151 another common soil (henceforth termed “rearing soil”) whose properties were similar to the LC and  
152 HC sites (15.4% clay, 72.2% silt, 12.4% sand, 2.63% organic matter, pH<sub>w</sub> 6.6) and was chemical-free  
153 [i.e, taken from a site that had been managed organically since 1991 under a rotation of grassland (3  
154 years), corn, wheat, rapeseed, and barley (GPS coordinates: 48.132686, -1.718434)].

155

### 156 2.2. Characterization of soil and *A. caliginosa* populations in the LC and HC sites

#### 157 2.2.1. Soil and earthworm sampling

158 In 2018, soil and *A. caliginosa* individuals were collected from the LC and HC sampling sites for general  
159 characterization and for the experimental epoxiconazole exposure. For pesticide residue analysis, soil  
160 and *A. caliginosa* individuals were concomitantly sampled at three points 50 m apart. A total of six soil  
161 cores per sampling point were taken with an auger at a depth of 20 cm and pooled as a composite soil  
162 sample. At least three adult specimens of *A. caliginosa* were manually collected per sampling point and  
163 pooled together for pesticide analysis (once gut-voided). For genetic characterization of *A. caliginosa*  
164 populations, 15 individuals were manually collected from each site.

165 In order to compare HC and LC population abundance, earthworms were sampled using chemical  
166 extraction followed by hand-sorting (Bouché, 1972; Duriez et al., 2006). A diluted expellant solution of  
167 allyl isothiocyanate (AITC) was applied on the soil within a 100x100 cm metal frame i.e., 1 m<sup>2</sup>. AITC  
168 stock solution 5 g L<sup>-1</sup> in isopropanol (propan-2-ol) was further water diluted to a concentration of 0.1 g  
169 L<sup>-1</sup> (Zaborski, 2003). After collecting emerging individuals, a block of soil 31.7x31.7 cm<sup>2</sup> and 25 cm  
170 deep was excavated from where the expellant solution was applied, and the remaining earthworms were  
171 hand-sorted. Four sampling replicates, 50 m apart, were performed per site.

172

### 173 **2.2.2. Analyses of pesticide residues in HC/LC soil and *A. caliginosa***

174 A total of 73 molecules covering herbicides, fungicides, and insecticides were targeted (Table S1)  
175 following a survey of farmers on their crop protection practices (i.e., frequency and application doses).  
176 In order to quantify the residues of selected pesticides, a QuEChERS extraction method was applied to  
177 soil and earthworm samples, followed by liquid chromatography analysis coupled with tandem mass  
178 spectrometry (LC-MS/MS) as described in Daniele et al., 2018.

179

### 180 **2.2.3. Genetic characterization of *Aporrectodea caliginosa* populations**

181 The *A. caliginosa* species complex contains at least three cryptic lineages/species (L1, L2 and L3) that  
182 can be distinguished using a DNA barcoding approach (Shekhovtsov et al., 2016). The cytochrome c  
183 oxidase subunit I mitochondrial gene (COI) fragment was used to determine to which lineage belonged  
184 the two studied populations. The COI is proposed as a standard DNA barcode for animals (Hebert et al.,  
185 2003). The fragment was amplified according to Folmer et al., 1994 and details are given in supporting  
186 information S1.

187

### 188 **2.2.4. Onsite population densities of *A. caliginosa***

189 After being collected at the sampling sites, *A. caliginosa* individuals were weighed back at the laboratory  
190 without emptying their gut content (fresh weight basis), counted and taxonomically identified based on  
191 morphological criteria (Bouché, 1972). The abundance and biomass of *A. caliginosa* populations were  
192 calculated for both the HC and LC sites.

193

## 194 **2.3. Experimental epoxiconazole exposure**

195 After being collected from the HC and LC sites, *A. caliginosa* earthworms were acclimatized for 14  
196 days in the common rearing soil in a climate chamber (air temperature: 15°C; light day/night cycle: 16/8  
197 h; air moisture: 80±5%). Upon retrieval, the 25 cm top soil dedicated to earthworm rearing was air-  
198 dried until it reached 14% of WHC then sieved (2 mm sieve) and kept in sealed containers (100 l) prior  
199 to use. The rearing soil was then subjected to chemical analyses for pesticides (300 molecules, GIRPA  
200 lab Beaucouzé, France) and for polycyclic aromatic hydrocarbons (PAH) and metal trace elements by



201 the INRAE soil analysis laboratory (Arras, France). These facilities are certified by the French Ministry  
202 of the Environment and have a COFRAC accreditation. The condition that the soil had to meet in order  
203 to be used as a rearing soil for our experiment was the absence or minimal contamination by these  
204 pollutants (see Table S2).

205 Prior to the start of the exposure, each *A. caliginosa* individual was rinsed with distilled water, gently  
206 dried on filter paper and weighed. Ten adults were then transferred into each mesocosm, insuring a  
207 similar mean earthworm weight at each treatment. The sampling design is detailed in Table S3. Soil  
208 mesocosms consisted of polypropylene boxes (175 mm x 159 mm x 117 mm) whose lid was pierced  
209 with tiny holes to ensure sufficient aeration. The mesocosms were filled with 1 kg of either  
210 epoxiconazole spiked or control soil, and 10 g of dry horse manure was added onto the soil surface for  
211 earthworm feeding. Epoxiconazole is a persistent fungicide that was chosen due to 1) its high frequency  
212 of application in European agriculture (V. Silva et al., 2019) and 2) its detection in the highly  
213 contaminated site under study (HC). *A. caliginosa* earthworms were exposed to epoxiconazole as  
214 BASF's "OPUS®" commercial formulation (125 g active ingredient l<sup>-1</sup>), a broad-spectrum contact and  
215 systemic fungicide to protect cereals and sugar beet against *Septoria sp.* and rust. OPUS® was diluted  
216 in distilled water at the recommended dose for farmers of 0.17 mg kg<sup>-1</sup>, considering a field application  
217 rate of 125 g ha<sup>-1</sup> and assuming a single application with a homogenous distribution and no crop  
218 interception in the soil's top 5 cm (Dittbrenner et al., 2010). Soil was spiked by manually adding the  
219 diluted pesticide solution or distilled water (for the controls) on the soil with a 14% water content until  
220 reaching a final soil water content of 25%, and by renewing it after 14 days to sustain the chemical  
221 pressure. Exposure lasted 2 days for the transcriptomic responses and 28 days in total for all other  
222 endpoints. In the following text, the term "basal" refers to control individuals or conditions, and the term  
223 "stress" refers to epoxiconazole-exposed individuals or conditions.

224

### 225 **2.3.1. Epoxiconazole analysis in experimental soil and *A. caliginosa***

226 Soil samples and gut-voided earthworms were separately freeze-dried, ground, sieved to 250 µm and  
227 then stored at -18°C until epoxiconazole analysis. Epoxiconazole was extracted using a pressurized  
228 liquid ASE (Accelerated Solvent Extractor) 350 (Dionex Corporation, Sunnyvale, USA) followed by  
229 GC/MS/MS analysis according to (Mercier et al., 2014) with specific adjustments for both soil and  
230 earthworm tissues, details are given in Supporting information S2. To assess the earthworms'  
231 epoxiconazole accumulation, bioaccumulation factors (BAFs) defined as the ratio of total earthworm  
232 epoxiconazole concentration to total soil epoxiconazole concentration at T0 were calculated. Both  
233 concentrations were based on dry weight.

234

235

236

237 **2.3.2. *A. caliginosa* transcriptomic responses**

238 At T0 and after two days of exposure to epoxiconazole, *A. caliginosa* earthworms were individually  
239 frozen and ground (whole body, gut voided) with a CryoMill (Retsch) under liquid nitrogen conditions.  
240 Frozen earthworm powders were stored at  $-80^{\circ}\text{C}$  until use. Aliquots containing 30 mg of individual  
241 earthworm powder were used for total RNA extraction using a NucleoSpin RNA kit (Macherey-Nagel)  
242 according to the manufacturer's protocol. Genomic DNA was removed by DNase digestion using a  
243 column then total RNA was eluted in RNase-free  $\text{H}_2\text{O}$ . RNA purity and quantity were assessed using a  
244 Nanodrop (ND-1000, ThermoFisher), and RNA integrity was checked using a Bioanalyzer 2100  
245 (Agilent, CA, USA). Total RNA samples were stored at  $-80^{\circ}\text{C}$ . Samples were sent to GeT-PlaGe core  
246 facility (INRA Toulouse) in dry ice. RNA sequencing, *de novo* assembly, annotation, quantification,  
247 differential expression analysis, and enrichment analyses are described in supporting information S3.

249 **2.3.3. Analysis of *A. caliginosa* metabolism**

250 The weight of 30 gut-voided earthworms from each of the control and epoxiconazole exposure groups  
251 was individually monitored during the experiment at T0 and after 7 and 28 days of exposure.  
252 Respirometry measurements were taken on T0 and after 7 and 28 days of exposure. Ten earthworms  
253 from each treatment group were removed from the soil microcosm, rinsed, gently blotted dry on filter  
254 paper, and placed in a 250 ml glass jar hermetically closed for two hours.  $\text{CO}_2$  was measured by a micro-  
255 gas chromatograph (3000A, SRA Instruments) equipped with a single PoraPLOT U capillary column  
256 coupled with a thermal conductivity detector.

258 **2.4. Statistical analyses**

259 Statistical analyses were carried out using the statistical software R (RStudio, R 4.0.3). Details on  
260 transcriptomics analysis are given in supporting information S3. For epoxiconazole analysis in soil and  
261 earthworms, weight monitoring, and respirometry measurements, all data were checked for normality  
262 distribution (Shapiro-Wilk test) and homogeneity of variances (Bartlett's test), with visual examination  
263 of QQ-plots. When assumptions were met, a one-way ANOVA was run with Tukey's *post hoc* tests.  
264 The non-parametric Kruskal-Wallis test was used if assumptions were not met; treatment groups were  
265 compared using a Dunn's pairwise comparison with Bonferroni correction. All tests were performed  
266 with a 0.05 significance level.

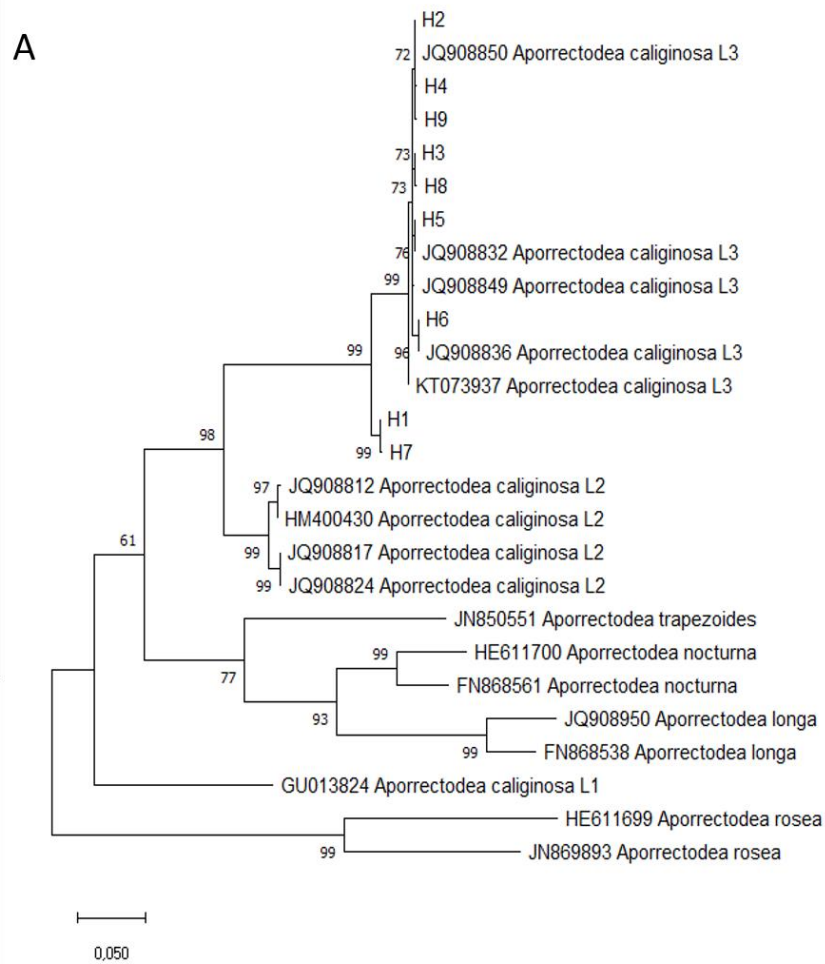
268 **3. Results**

269 **3.1. Characterization of *A. caliginosa* populations from the sampling sites**

270 **3.1.1. *Aporrectodea caliginosa* COI genetic diversity**

271 A total of nine haplotypes (H1-H9) were obtained for the 30 individuals sampled. All sequences were  
272 assigned to *Aporrectodea caliginosa* (match score  $> 99\%$ ) and several haplotypes (H2, H4, H8 and H9)

273 matched *A. caliginosa* L3 in The Barcode of Life Data System (BOLD). The maximum likelihood tree  
 274 confirmed the BOLD assignment and further revealed that all study haplotypes belonged to the same  
 275 well-supported clade formed by the *A. caliginosa* L3 lineage (Figure 1). A higher haplotype diversity  
 276 was observed in the LC population whereas a higher nucleotide diversity was observed in the HC  
 277 population (Figure 1). This higher nucleotide diversity is due to the presence in the HC population of  
 278 two divergent haplotypes (H1 and H7, Figure 1) that are absent in the LC population.



**B**

Population	N	$N_h$	S	$H_d$	$\pi$
HC	15	5	26	0.705	0.0186
LC	15	6	8	0.829	0.0034
<b>Total</b>	30	9	31	0.798	0.0135

279  
 280 **Figure 1.** (A) Maximum likelihood tree estimated from the COI sequences of the study *Aporrectodea*  
 281 *caliginosa* specimens (H1 – H9) and reference sequences of *A. caliginosa* spp. from Genbank. The  
 282 TN93+G+I model was used as the model for nucleotide substitution. Branch lengths are proportional to  
 283 the estimated number of nucleotide substitutions. The numbers above the branches are bootstrap values.  
 284 (B) Genetic diversity in *A. caliginosa* populations. The sample size (N) and the following measures of  
 285 COI genetic diversity are given: number of haplotypes ( $N_h$ ), number of polymorphic sites (S), haplotype  
 286 diversity ( $H_d$ ) and nucleotide diversity ( $\pi$ ).  
 287

288

289 **3.1.2. Onsite abundance and biomass of *A. caliginosa* populations**

290 The mean abundance of *A. caliginosa* was more than three times higher in the LC site than the HC one  
291 ( $56.5 \pm 28.1$  and  $15.3 \pm 7.0$  individuals.m<sup>-2</sup> in LC and HC, respectively). The corresponding mean *A.*  
292 *caliginosa* biomasses were  $18.5 \pm 9.8$  and  $3.0 \pm 0.9$  g.m<sup>-2</sup> in the LC and HC sites, respectively.

293

294 **3.2. Onsite soil and earthworm pesticide contamination**

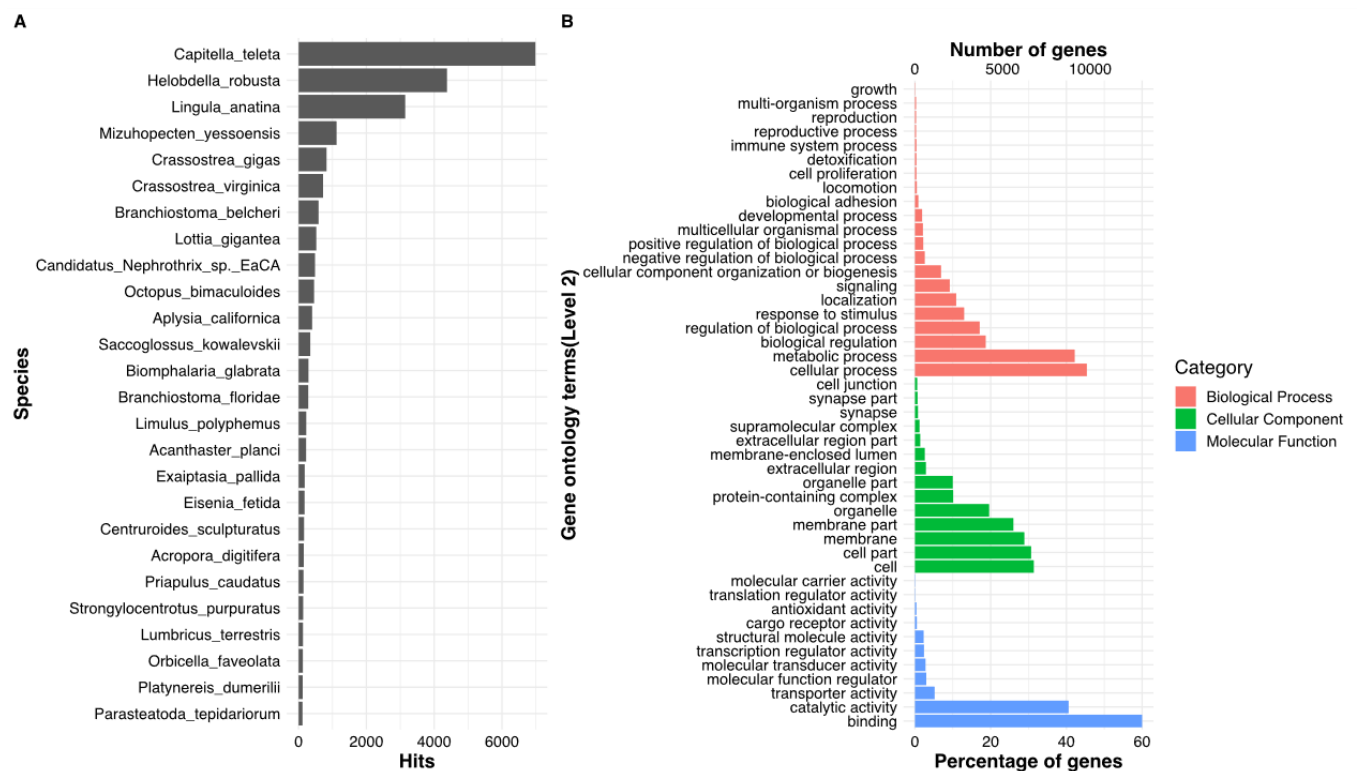
295 The multi-residual analysis showed that all samples contained at least one and up to 20 and 11 pesticides  
296 for soil and earthworms, respectively. Out of the 73 targeted pesticides, 28 and 15 had been detected at  
297 least once in the soil and *A. caliginosa*, respectively. Soil and *A. caliginosa* from the HC site contained  
298 a higher number and higher concentrations of pesticides than the LC site. The highest concentrations in  
299 soil from the HC site were for the herbicides acetonifin, pendimethalin and clomazone, with 417.0, 15.6,  
300 and 113.0 ng/g dw. The highest concentrations of pesticides in earthworms from the same site were for  
301 metolachlor ESA (herbicide) and imidacloprid (insecticide) with 747.0 and 99.2 ng/g ww, respectively).  
302 In both soil and *A. caliginosa* earthworms from the LC site, only five (atrazine, carbendazim, flusilazole,  
303 metconazole, fluxapyroxad) and three molecules (imidacloprid, tebuconazole and metolachlor ESA),  
304 respectively, were detected and all were at concentrations below 5.0 ng/g ww (Table S4).

305

306 **3.3. Establishing the *de novo* transcriptome assembly of earthworm species *A. caliginosa***

307 A total of 2,068,522,212 raw reads were generated, with an average of 23,729,267 ( $\pm 1,892,470$ ) reads  
308 for each sample. Of these, a total of 139,950,716 reads were used for the transcriptome assemblies. The  
309 clean reads were assembled into 64,556 contigs with an average length of 2,010 pb ranging from 203 to  
310 28,628 pb and N50 of 2,862 pb. The assembled transcriptome size was 129,787,458 bp. The assembly  
311 quality was then assessed by calculating the read on contig re-alignment rate for every sample, and by  
312 processing the contigs with BUSCO (version 2) using the metazoa\_odb9 database (Simao et al. 2015).  
313 This analysis was to check the presence and completeness of a set of expected single-copy protein-  
314 coding genes for a given branch of the evolution tree. Among the 978 metazoa\_odb9 proteins searched  
315 by BUSCO (3.0.2), 964 were found to be complete in single or multiple copies. This represents 98.6%  
316 of the proteins expected in the genome. The remaining ones were missing (1.1%) or fragmented (0.3%).  
317 With respect to the assembly quality, the mean per sample read mapping rate was  $97.02 \pm 0.60\%$  across  
318 libraries. RNA-seq raw sequences and the *de novo* assembled transcriptome assemblies have been  
319 deposited in NCBI under BioProject PRJNA883218. This Transcriptome Shotgun Assembly project has  
320 been deposited at DDBJ/EMBL/GenBank under the accession GKBY00000000. The version described  
321 in this paper is the first version, GKBY01000000.

322



323

324 **Figure 2.** (A) Top hit species distribution on the basis of best sequence alignments and lowest E  
 325 values and (B) Distribution of Gene Ontology (GO) assignments of assembled *A. caliginosa* contigs.  
 326 GO categories are shown on the x-axis grouped into three main categories: biological processes,  
 327 cellular components and molecular functions. The y-axis indicates the percentage and total number of  
 328 genes in each category.  
 329

329

### 330 3.4. Functional annotations of the *de novo* transcriptome assembly

331 A total of 25,725 contigs could be annotated, with reference to the sequences recorded in the NR protein  
 332 database. The marine polychaete *Capitella teleta* (Phylum: Annelida) was the species found most often  
 333 in the NR protein annotations, corresponding to 27.16% of the contigs (6,988 contigs). The other species  
 334 found as best hit annotations were the leech *Helobdella robusta* (4,371 contigs, 16.99%) and the  
 335 brachiopod *Lingula anatina* (3,146 contigs, 12.23%) (Figure 2A).

336 In total, 43,569 GO terms were identified for 21,593 annotated sequences under the three main  
 337 ontologies. From these sequences, 18,160 (41.7%) were assigned to molecular functions (GO:0003674),  
 338 13,782 (31.6%) to biological processes (GO:0008150), and 11,626 (26.7%) to cellular components  
 339 (GO:0005575). Within the molecular function category, binding (GO:0005488) and catalytic activity  
 340 (GO:0003824) were the most represented GO terms with 12,956 sequences and 8,763 sequences,  
 341 respectively. The top three GO terms for biological processes were cellular process (GO:0009987, 9802  
 342 contigs) including metabolic process (GO:008152, 9120 contigs), biological regulation (GO:0065007,  
 343 4040 contigs) and response to stimulus (GO:0050896, 2810 contigs). Furthermore, in the cellular  
 344 component category, the predominant GO terms were grouped into cell (GO:0005623, 6785 contigs),

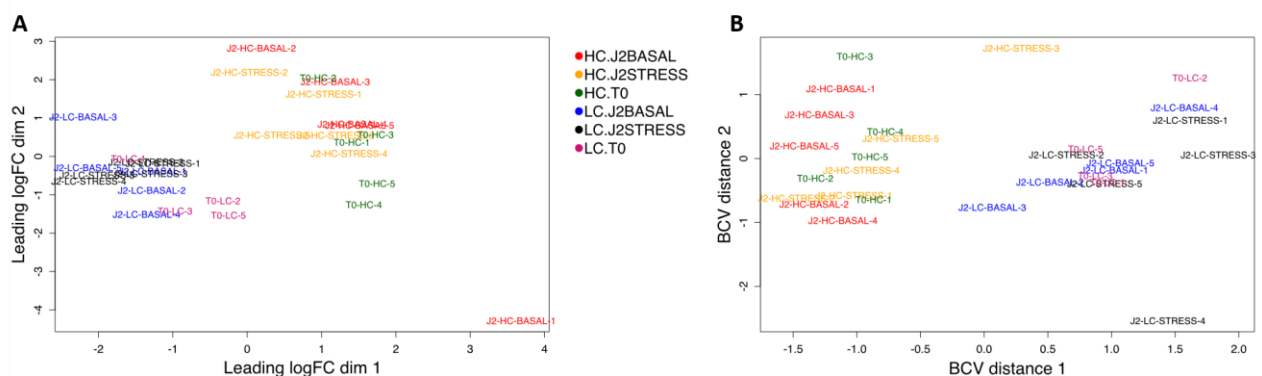
345 membrane (GO:0016020, 6245 contigs), organelle (GO:0043226, 4242 contigs) and protein-containing  
346 complex (GO:0032991, 2186 contigs) (Figure 2B).

347 In order to identify active functional pathways in *A. caliginosa*, contigs were mapped to the reference  
348 pathways in the KEGG database. In total, 16,933 contigs were mapped to 404 KEGG pathways and  
349 5,847 KEGG orthologs. The KEGG annotations were helpful for identifying contigs related to  
350 xenobiotics biodegradation and metabolism-related pathways (Table S5). The pathways with the highest  
351 number of sequence hits to enzyme genes were Metabolism of xenobiotics by cytochrome P450  
352 (map00980), Drug metabolism – cytochrome P450 (map00982) and Drug metabolism – other enzymes  
353 (map00983).

354

### 355 3.5. Molecular responses of LC and HC populations under basal and stress 356 conditions

357 Analyses were performed to check the overall reproducibility and variation between earthworm samples  
358 belonging to the same population (HC or LC) and treatment (exposed (stress) or not (basal)). A heatmap  
359 of Pearson correlations based on log10 expression counts and an MDS plot were generated to evaluate  
360 the sample relatedness and identify outliers (Figure 3). The heatmap and MDS plot showed that  
361 individuals belonging to the same *A. caliginosa* population were highly correlated. However, one sample  
362 from the LC site was identified as an outlier (T0-LC-4) and was therefore removed from the downstream  
363 analysis.



364

365 **Figure 3.** Multidimensional scaling plots (MDS) generated using the *limma plotMDS* function where  
366 A) distances correspond to leading log-fold change between samples and B) distances between samples  
367 correspond to the leading biological coefficient of variation (BCV). In both plots, dimension 1 separates  
368 LC and HC populations, indicating the paired nature of the samples in each population. (T0: beginning  
369 of the experiment; J2: two days after exposure, Basal: control individuals, Stress: exposed individuals)

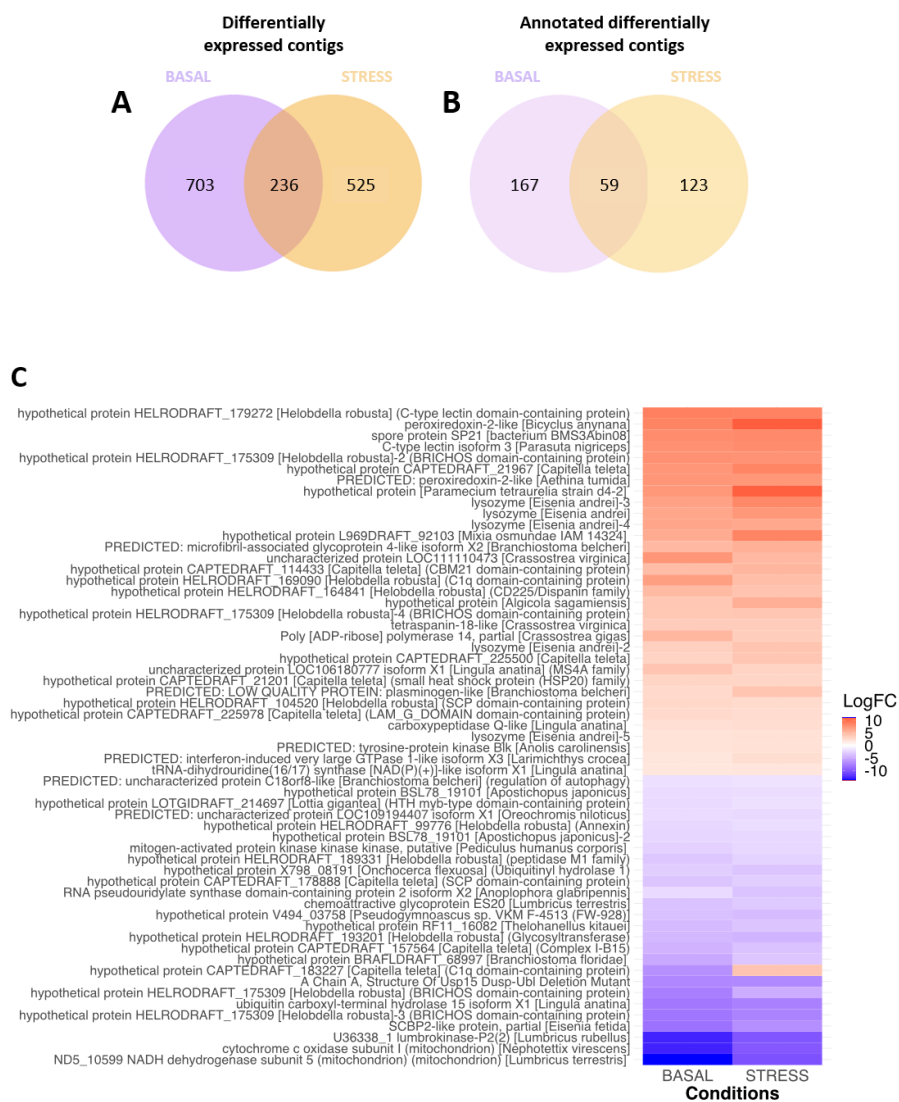
370

#### 371 3.5.1. Investigation of differential molecular pathways between LC and HC populations under 372 basal and chemical stress conditions (LC vs. HC populations)

373

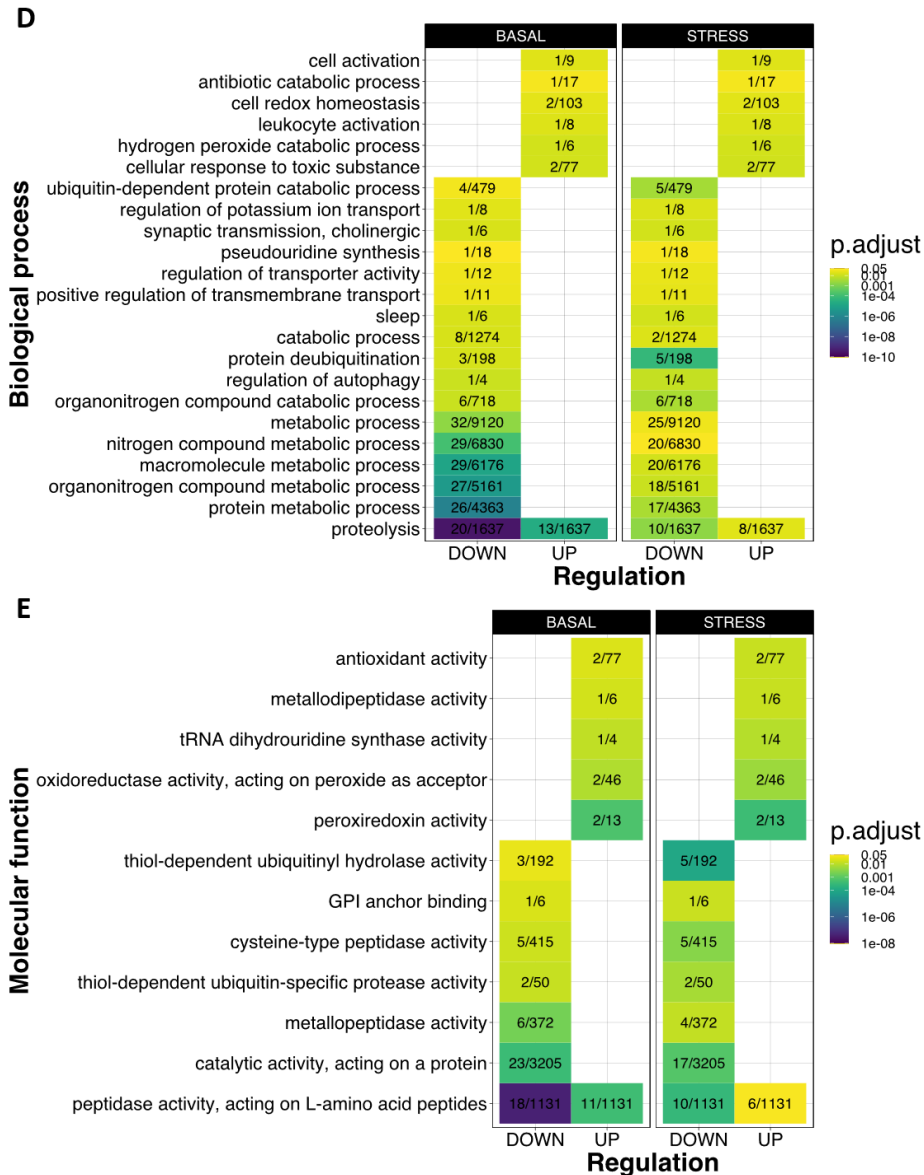
374 Under basal condition (LC.basal versus HC.basal), analysis showed that 939 contigs were differentially  
375 expressed with 569 contigs upregulated and 370 downregulated when comparing LC and HC  
376 populations that had a contrasting site history with respect to agricultural practices and pesticide use

377 (Figure 4A). Annotations enabled us to identify 226 proteins (24% of all the differentially expressed  
 378 contigs) (Figure 4B, Table S6). Results of the gene ontology enrichment analysis for biological process  
 379 and molecular function are presented in Figures 4D and 4E. KEGG enrichment analysis revealed that  
 380 one pathway was upregulated under the basal condition: protein digestion and absorption (map04974)  
 381 and three pathways were downregulated: ECM-receptor interaction (map04512), pancreatic secretion  
 382 (map04972) and protein digestion and absorption (map04974) (Table S8).  
 383 Under stress condition (LC.stress versus HC.stress), 761 contigs were differentially expressed by the  
 384 two populations (321 downregulated and 440 upregulated) (Figure 4A) and 182 contigs were annotated  
 385 (24%) (Figure 4B, Table S7). Results of the gene ontology enrichment analysis for biological process  
 386 and molecular function are presented in Figures 4D and 4E. KEGG analysis showed that three pathways  
 387 were specifically downregulated under the stress condition: autophagy (map04136, map04138) and  
 388 mitophagy (map04139) (Table S8).  
 389  
 390



391

392



393  
 394 **Figure 4.** Venn diagram depicting the total number of differentially expressed contigs (A) and Venn  
 395 diagram depicting the total number of annotated differentially expressed contigs (B) when comparing  
 396 LC and HC populations under stress and basal conditions, as well as the number of common contigs in  
 397 both conditions. Heatmap of the 59 differentially expressed contigs shared (FDR<0.05) by both LC and  
 398 HC populations (C). Comparison of enriched biological processes (D) and enriched molecular functions  
 399 (E) between LC and HC populations under basal and stress conditions (numbers indicate the number of  
 400 significant contigs corresponding to a given function, followed by the number of annotated contigs).  
 401

402 **3.5.2. Testing for differential molecular responses to an induced chemical stress**  
 403 **(fungicide/epoxiconazole) in the LC and HC populations**

404  
 405 After 2 days of the earthworms' exposure to epoxiconazole, 52 contigs were upregulated and 50 contigs  
 406 downregulated in the LC population compared with 42 upregulated contigs and 52 downregulated  
 407 contigs in the HC population.

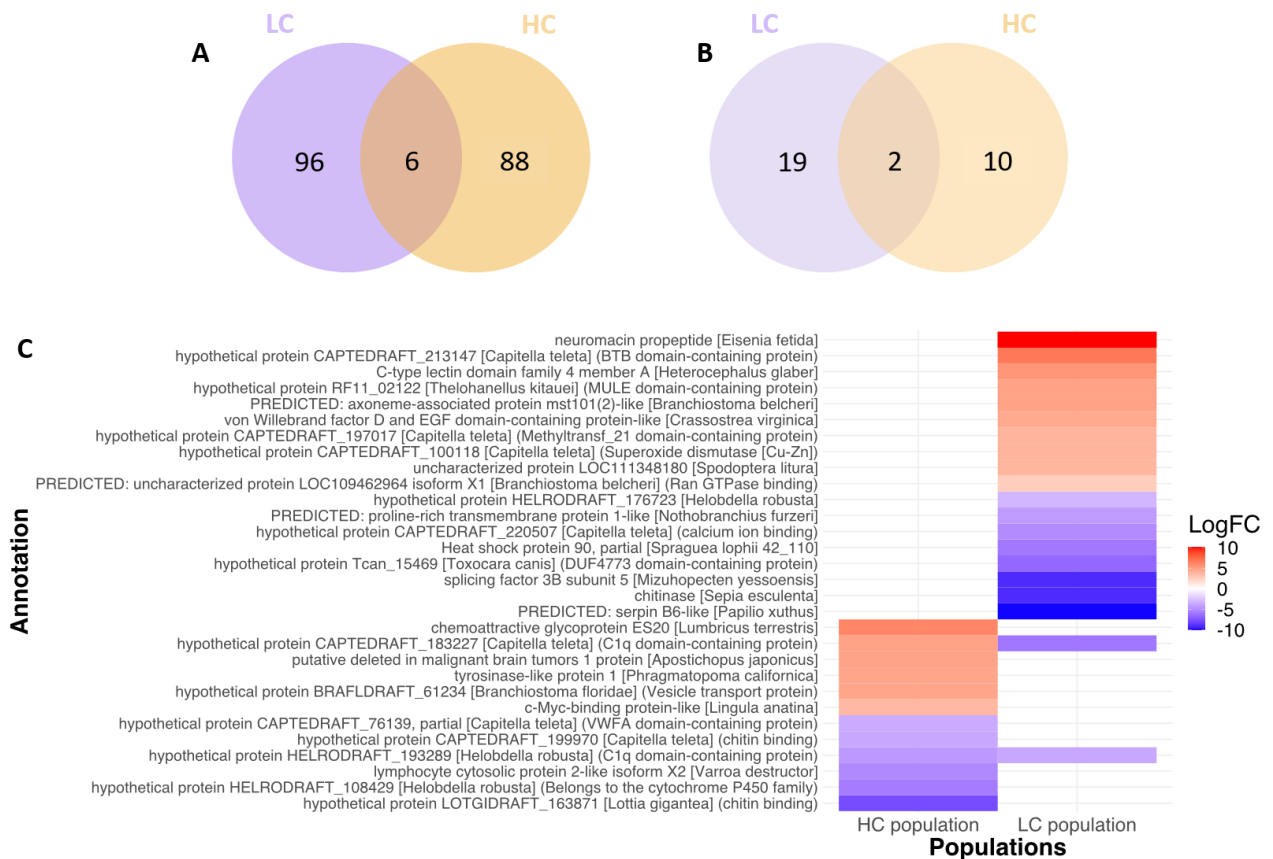
408 In the LC population (LC.basal vs. LC.stress), 21% of differentially expressed contigs were annotated.  
 409 Epoxiconazole exposure induced both upregulation and downregulation of different immune-related



410 proteins (neuromacin, Serpin B6, C-type lectin domain family 4 member A, C1q domain-containing  
411 protein), and upregulation of the RNA splicing protein (splicing factor 3B subunit 5), stress response  
412 protein (Heat shock protein 90), and hydrolytic enzymes such as chitinase. Epoxiconazole exposure also  
413 induced downregulation of stress oxidative responses such as superoxide dismutase, and the sperm  
414 axoneme assembly protein (axoneme-associated protein mst101(2)-like) in the exposed group (Figure  
415 5C). In the HC population (HC.basal vs. HC.stress), 13% of differentially expressed contigs were  
416 annotated. Epoxiconazole exposure induced upregulation of the chitin binding-related protein,  
417 cytochrome P450 family protein (CYP2 family), immune-related proteins (C1q domain-containing  
418 protein, lymphocyte cytosolic protein 2-like isoform X2), and the blood glycoprotein (VWFA domain-  
419 containing protein). Exposure also induced downregulation of a protein involved in reduction-oxidation  
420 mechanisms (tyrosinase-like protein 1), chemoattractive glycoprotein ES20, an RNA biosynthetic  
421 process-related protein (c-Myc-binding protein like), the vesicle transport protein, and immune response  
422 protein (C1q domain-containing protein, putative deleted in malignant brain tumors 1 protein) (Figure  
423 5C). Overall, both populations shared only six contigs (Figure 5A) in response to epoxiconazole  
424 exposure, just two of these being annotated (Figure 5B) (C1q domain-containing protein), and one BPs  
425 (chitin metabolic process) was enriched in both populations.

426 In order to understand the function of the differentially expressed contigs in the control and exposed  
427 groups in both the LC and HC populations, GO enrichment analysis was performed. In the LC  
428 population, epoxiconazole caused upregulation of 16 BPs linked to four main functions: cellular  
429 response to heat (GO:0034605), protein stabilization (GO:0050821), RNA splicing (GO:0000398), and  
430 the chitin metabolic process (GO:0006030). Moreover, four MF terms were upregulated in the exposed  
431 group linked to protein binding and chitin binding (Table S9).

432 In the HC population, exposure to epoxiconazole downregulated 10 BP GO terms linked to positive  
433 regulation of RNA biosynthetic process (GO:1902680) and endocytosis (GO:0006897) while 11 BP  
434 terms were upregulated, most being linked to the chitin metabolic process (GO:0006030), and response  
435 to xenobiotic stimulus (GO:0009410). Four MF GO terms downregulated in the exposed group were  
436 linked to scavenger receptor activity (GO:0005044) and transcription coactivator activity  
437 (GO:0003713), while 3 MF GO terms were upregulated; these were linked to extracellular matrix  
438 structural constituent (GO:0005201), chitin binding (GO:0008061) and oxidoreductase activity  
439 (GO:0016712) (Table S9).



440

441 **Figure 5.** Venn diagram depicting the total number of differentially expressed contigs (A) Venn diagram  
 442 depicting the total number of annotated differentially expressed contigs (B) and Heatmap of  
 443 differentially expressed contigs (C) in the LC and HC populations after epoxiconazole exposure (stress  
 444 condition).  
 445

### 446 3.6. Bioaccumulation and physiological responses after chemical stress

#### 447 3.6.1. Epoxiconazole analysis in soil and *A. caliginosa* earthworms

448 Our analysis revealed that epoxiconazole concentrations in soil matched nominal concentrations on T0,  
 449 except for a slightly lower concentration in soil containing earthworms from the HC site, probably due  
 450 to heterogeneity in pesticide application. The fate of epoxiconazole during the experiment did not differ  
 451 significantly between the two populations, with a similar decrease in concentration of 2% vs. 8% (day  
 452 7) and 11% vs. 15% (day 28) in the HC and LC mesocosms, respectively. Analyses of epoxiconazole in  
 453 earthworm tissues showed a similar bioaccumulation factor (BAF around 2) on day 7 in the two  
 454 populations. Lower bioaccumulation was observed on day 28 in the HC population, although not  
 455 significant (BAF of 2 and 1.4 for LC and HC respectively) (Figure 6A).  
 456

#### 457 3.6.2. *A. caliginosa* weight monitoring throughout the experiment

458 The initial mean weight of adult *A. caliginosa* earthworms (n=30) collected from the HC and LC sites  
 459 was  $0.562 \pm 0.116$  g and  $0.556 \pm 0.099$  g, respectively. Survival was 100% in all the mesocosms.

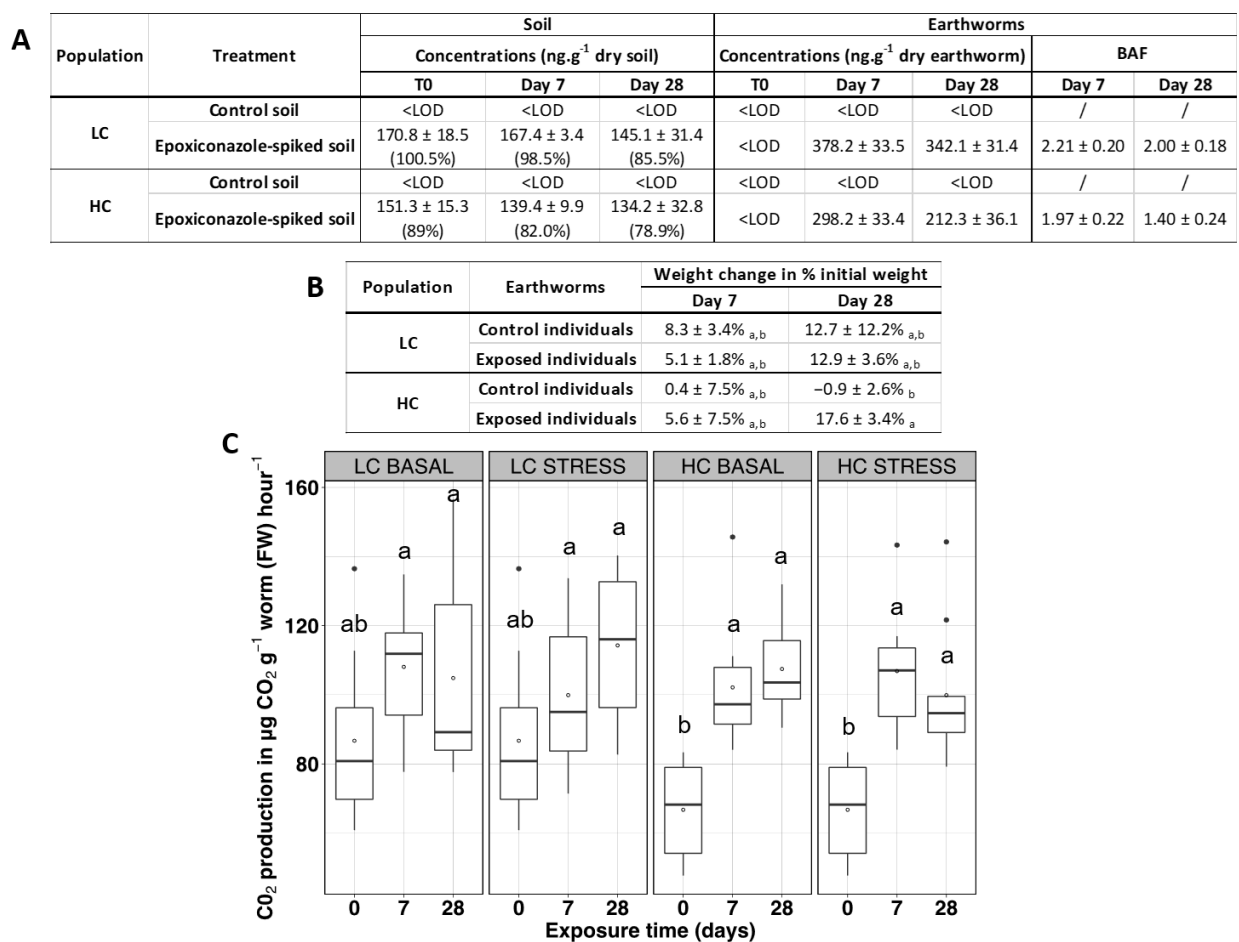
460 Throughout the experiment, all earthworms gained weight similarly except those of the HC control that  
 461 slightly decrease their initial weight. On day 28, a significant weight increase was observed in the HC  
 462 exposed group ( $p=0.0355$ ) compared to HC control group's weight (Figure 6B).

463

### 464 3.6.3. *A. caliginosa* metabolism monitoring throughout the experiment

465 At the beginning of the experiment (T0), earthworm respiration measured as CO<sub>2</sub> production was higher  
 466 in the LC population than in the HC one, although not significantly due to the high variability between  
 467 individual earthworms. The metabolic rate of the HC populations increased significantly over time,  
 468 whether exposed or not to epoxiconazole. Conversely, an insignificant variation in energy dissipation  
 469 was observed in the LC earthworms throughout the experiment ( $p>0.05$ ) (Figure 6C).

470



471

472 **Figure 6.** Epoxiconazole concentrations in soil ( $n=3$ ) and earthworms ( $n=5$ ) as well as earthworm  
 473 bioaccumulation factor (BAF) on days 0, 7, and 28. Results are expressed as a mean ± standard deviation  
 474 (A). Weight change of earthworms from the HC and LC sites on days 7 and 28. Values are the mean out  
 475 of 30 replicates ( $N=30$ ) ± standard deviation (SD) (B). Metabolic rate ( $\mu\text{g CO}_2 \text{ g}^{-1} \text{ worm (fresh weight)}$   
 476  $\text{h}^{-1}$ ) of earthworms from the HC and LC sites exposed to epoxiconazole (STRESS) or not (BASAL) on  
 477 days 0, 7, and 28. A dot on the boxplot indicates a mean value ( $N= 10$  worms per group) (C). Different  
 478 letters (a or b) denote statistical differences ( $p<0.05$ ).

479

480 **4. Discussion**

481 **4.1.A. *caliginosa* facing pesticide soil contamination**

482 Conventional farming leads to a residual stock of a cocktail of pesticides in soil; up to 20 different  
483 chemicals were detected in the soil of the HC site. Consequently, earthworms bioaccumulated a high  
484 level of pesticides in their tissue (up to 987.9 ng g<sup>-1</sup> wet weight). Most were insecticides (neonicotinoids:  
485 imidacloprid, thiacloprid) or herbicides (mesotrione, metolachlor ESA). Very few studies have  
486 investigated the bioaccumulation of pesticides in the tissue of earthworms from arable land, though  
487 many have focused on metal bioaccumulation (Beaumelle et al., 2017). Recently, Pelosi et al., 2021 also  
488 found high concentrations of currently used pesticides in earthworms, within a range similar to our own  
489 observations. The population-level consequences of such chemical pressures can include different  
490 responses, such as a decline leading to future extinction with the loss of the associated ecological  
491 services, or survival mediated by either physiological or behavioral plasticity, or by adaptation through  
492 natural selection (Chevin & Lande, 2010).

493

494 **4.2. Genetic diversity among the studied populations**

495 The differential tolerance observed in some population samples from reference and contaminated sites  
496 may represent a loss of species diversity through local contaminant-caused extinction of one or more  
497 members of a cryptic species complex rather than a within-species loss of putatively less-tolerant  
498 genotypes or haplotypes (Rocha-Olivares et al., 2004). Recent genotyping studies have begun to  
499 challenge current understanding of diversity through the identification of genetically distinct cryptic  
500 lineages within previously established earthworm morphospecies. There is some evidence that different  
501 earthworm lineages have different ecological properties, as observed in numerous other invertebrate  
502 species (Spurgeon et al., 2016). Kille et al., 2013 demonstrated that two cryptic lineages of earthworm  
503 *L. rubellus* react differently to soil contamination by arsenic, the specimens from lineage A employing  
504 genetic adaptation while the lineage B specimens use an epigenetic strategy. Although rarely performed  
505 in ecotoxicological study, checking for cryptic species in earthworms should thus be mandatory. For  
506 instance, *A. caliginosa*—considered a relevant model species for ecotoxicology research—is a complex  
507 of three highly divergent genetic lineages (Fernández et al., 2012; Pérez-Losada et al., 2009; Porco et  
508 al., 2013). The nature of these lineages is not completely understood: The number of nucleotide  
509 substitutions between them in the mitochondrial COI gene is much higher than within the lineages  
510 (Porco et al., 2013), and based on these criteria, the lineages may represent cryptic species. In this study,  
511 we showed that all the specimens of both populations belonged to the same L3 lineage, which is known  
512 to be present both in Western Europe (native populations) and North America (introduced populations).  
513 Thus, we confirmed using COI DNA barcoding that our ecotoxicological experiment was performed on  
514 a single species. As tolerance to environmental pollution in a population with a complex genetic  
515 structure can be achieved by various means, the COI data was also used to analyze the genetic variation

516 of the populations under study, keeping in mind that information obtained using COI mtDNA haplotypes  
517 is limited because only one locus is screened (Lazrek et al., 2011). We showed that the HC population  
518 had a higher nucleotide diversity than the LC population, a result mainly explained by the presence in  
519 this population of two divergent haplotypes that were absent in the LC population. Genetic diversity in  
520 earthworms can be affected by many factors, such as ecological preferences or life history traits, but  
521 also pollution, geographic distance, and geographic barriers for example (Dupont et al., 2019). To date,  
522 it is still difficult to draw general conclusions about spatial genetic variation in earthworms, especially  
523 at the fine scale (Dupont et al., 2015) as in our study. One of the possible environmental pressures that  
524 might modify genetic diversity between our populations could be the soil's contamination by pesticides,  
525 all other things being equal (i.e., pedo-climate context, soil properties and nutrients, metallic and HAP  
526 pollution). In contaminated environments, significant changes in genotype and allele frequencies may  
527 occur as a result of different processes: (i) an increase in mutation rates, (ii) selection of resistant  
528 genotypes, (iii) bottlenecks where population sizes are drastically reduced, and (iv) reduction of  
529 migration rates, preventing gene exchange among populations (Dupont et al., 2019; van Straalen &  
530 Timmermans, 2002).

531

#### 532 **4.3. Interpopulation differences: differentially expressed contigs under basal and stress** 533 **conditions**

534 Gene ontology analysis revealed that terms with a high number of differentially expressed contigs and  
535 a low p-value were linked to proteolysis (BP) and peptidase activity (MF) (Figure 4). These terms were  
536 mainly upregulated in the HC population rather than the LC population under both basal and stress  
537 conditions. Proteases or peptidases play a key role in protein turnover, along with transcription and  
538 expression of the active genome (Wilkins, 2017). Elevated proteolytic activities are one of the responses  
539 to pesticide exposure or in pesticide-resistant strains of insect pests (see Wilkins, 2017 for a detailed  
540 review on the subject). It has even been proposed as a basis for resistance detection (Gong et al., 2005).  
541 In our study, we can note the upregulation of different proteins linked to the ubiquitin-proteasome  
542 system (UPS) (A Chain A, Structure Of Usp15 Dusp-Ubl Deletion Mutant; ubiquitin carboxyl-terminal  
543 hydrolase 15 isoform X1). The UPS is a selective proteolytic system in which the conjugation of  
544 ubiquitin to damaged or unneeded proteins induces their degradation by the proteasome. Through  
545 overexpression of the proteasome Subunit Beta Type 6 (PSMB6), mosquitoes had acquired stable  
546 resistance to the insecticide deltamethrin (L. Sun et al., 2013).

547 The HC population also showed high lumbrokinase P2 enzyme upregulation. Earthworm fibrinolytic  
548 enzyme (EFE) has similar characteristics to alkaline trypsin-, chymotrypsin- and elastinase-like serine  
549 proteases with a strong fibrinolytic activity in the digestive tract of earthworms (Zhao et al., 2005).  
550 Trypsin and chymotrypsin serine proteases were overexpressed in a deltamethrin-resistant strain of  
551 mosquito *Culex pipiens pallens* ensuring a greater chance of survival under pesticide pressure (Wu et  
552 al., 2004). We can note upregulation of cathepsin L1, mitogen-activated protein kinase (MAPK), and

553 matrix metalloproteinase-18-like in the HC population, which were also implicated in responses or  
554 resistance to pesticides (Wilkins, 2017). Different explanations have been proposed for increased  
555 proteolytic activity linked to pesticide resistance or exposure (A. Silva et al., 2012). Peptidases may be  
556 involved in protein degradation to interact with damaged protein or to fulfill higher energy demands,  
557 which is usually a response to stress (Pedra et al., 2004). Peptidases may also play a role during protein  
558 biosynthesis or in modification of enzyme conformation related, for example, to the metabolic  
559 machinery required to detoxify insecticides (Ahmed et al., 1998). In our study this elevated peptidase  
560 activity in the HC population could be due to: 1) an actual response to onsite pesticide exposure of HC  
561 earthworms (they were exposed to pesticides at the sampling site shortly before being collected,  
562 although there was a 14-day acclimation period in the lab in soil without pesticides, see materials and  
563 methods section), 2) or conversely an additional stress response due to the absence of pesticides in the  
564 rearing soil and/or 3) a response due to acclimation or adaptation mechanisms. Indeed, we can observe  
565 that under the stress condition there are fewer differentially expressed contigs linked to proteolysis in  
566 the HC population. This could be explained by the fact that earthworms from the HC population were  
567 already adapted to the induced stress due to their long history of exposure to chemicals. The metabolic  
568 differences in terms of respirometry (CO<sub>2</sub> production) and growth performance that we observed  
569 between the HC and LC populations support this explanation.

570 Although the results are noisy, a general trend in differentiated metabolic rate was observed between  
571 the LC and HC populations irrespective of experimental treatments. It is also interesting to note that  
572 earthworms from the HC population were significantly smaller (mean weight of 0.196 g) than  
573 individuals from the LC population (mean weight of 0.327g) in concordance with the high metabolic  
574 activity observed in gene expression results, as already observed in other studies suggesting energetic  
575 costs in organisms coping with soil pollution (Fisker et al., 2011; Holmstrup et al., 2011; Wiegand et  
576 al., 2007). These results are in accordance with (Givaudan, Wiegand, et al., 2014), who showed that the  
577 metabolic rate increased after fungicide exposure in earthworms naturally pre-exposed to pesticides.

578 Other proteins linked to proteolysis were upregulated in the HC population; serine/threonine kinase  
579 Unc-51-like kinase-1 (Ulk1) is thought to be essential for inducing autophagy, a protective intracellular  
580 bulk degradation mechanism (Li et al., 2017) that is activated by various high stresses in cellular  
581 response to reactive oxygen and nitrogen species as well as toxic proteins (Li et al., 2017). The earliest  
582 identified degradation route for proteins is within lysosomes; foreign proteins and cell materials can be  
583 transferred to the lysosome by a number of mechanisms which include autophagy (Wilkins, 2017).  
584 These results are in accordance with KEGG analysis showing upregulation of pathways linked to these  
585 mechanisms in the HC population under both basal and stress conditions (lysine degradation, protein  
586 digestion and absorption, pancreatic secretion, autophagy, mitophagy).

587 Another interesting result observed regarding response linked to metabolism is the higher upregulation  
588 of three mitochondrial proteins in the HC population (NADH dehydrogenase subunit 5 (or NADH-  
589 ubiquinone oxidoreductase chain 5), complex I-B15 (NADH-ubiquinone oxidoreductase B15 subunit),

590 and cytochrome c oxidase subunit I (COI). At the cell level, mitochondria perform a variety of  
591 biochemical processes, but their main function is to produce most of the cellular ATP via oxidative  
592 phosphorylation (Stoker et al., 2019). Mitochondria are known to be impacted by pesticide exposure,  
593 even by non-conventional mitochondria-targeting pesticides (Cowie et al., 2017; Leung & Meyer, 2019;  
594 Nicodemo et al., 2014; van Pottelberge et al., 2009). Inhibition of electron transport at the mitochondrial  
595 respiratory chain has been a successful mode of action of pesticides (Lümmen, 2007; van Leeuwen &  
596 Dermauw, 2016). A number of mitochondrial respiratory chain genes are associated with pesticide  
597 resistance and specifically insecticide resistance, including those mentioned above (van Leeuwen et al.,  
598 2008). NADH dehydrogenase subunit 5 (or NADH-ubiquinone oxidoreductase chain 5) and complex I-  
599 B15 (NADH-ubiquinone oxidoreductase B15 subunit) are core and accessory subunits of the respiratory  
600 chain Complex I, respectively. Recently, (Bajda et al., 2017) reported the discovery of a mutation  
601 (H92R) in the PSST homolog of complex I in METI-I (mitochondrial electron transport inhibitor) of  
602 resistant phytophagous *Tetranychus urticae* mites. COI is one of three mitochondrial DNA encoded  
603 subunits of respiratory complex IV. An elevated expression of mitochondria cytochrome c oxidase as  
604 observed in the HC population was involved in the development of resistance to pesticides in several  
605 organisms: Chinese hamster ovary cells (Alemany et al., 2000), *Blattella germanica* (Pridgeon & Liu,  
606 2003), *A. aegypti* (Pridgeon et al., 2009), *Schistosoma mansoni*, (Pereira et al., 1998), and in whiteflies  
607 (Yang et al., 2013). It is interesting to note that this COI gene was used to analyze the genetic diversity  
608 of the LC and HC populations and showed that two divergent haplotypes were present in the HC  
609 population.

610 In our study, the significant upregulation of some mitochondrial genes may be a compensatory  
611 mechanism for electron transport in the respiratory chain through a plastic response or a genetic  
612 adaptation. Higher mutation rates in animal mtDNA together with limited DNA repair mechanisms  
613 render these genes susceptible to rapid evolution through random drift or natural selection. Traits  
614 encoded by mtDNA have thus the potential to evolve, and reach fixation, very rapidly (van Leeuwen et  
615 al., 2008). It is also interesting to note that multiple lines of evidence have recently indicated that the  
616 cytosolic (Ubiquitin–proteasome system) UPS plays a crucial role in the quality control of mitochondrial  
617 proteins. The effects of the UPS go beyond the removal of damaged proteins and include the adjustment  
618 of mitochondrial proteome composition, the regulation of organelle dynamics, and the protection of  
619 cellular homeostasis against mitochondrial failure (Bragoszewski et al., 2017). However, the correlation  
620 between mitochondrial dysfunction and UPS dysfunction after pesticide exposure needs further  
621 investigation.

622 Interestingly, a shift regarding pathways linked to ion binding (metal ion binding, calcium ion binding)  
623 was observed; these pathways were upregulated in the LC population (downregulated in the HC  
624 population) under the basal condition and became upregulated in the HC population (downregulated in  
625 the LC population) under the stress condition. Further investigations should be performed to understand

626 this response, but it is well known that ion channels remain the primary target of most of the small  
627 molecule insecticides, and are involved in pesticide resistance (French-Constant, 1994).  
628 In the HC population, upregulation of CytochromeP450 CYP2J2 was observed under the stress  
629 condition. In humans, this cytochrome is involved in phase I xenobiotics metabolism (Xu et al., 2013).  
630 This response was only observed in the HC population and could be a specific mechanism in response  
631 to pesticide exposure developed in this population.

632

#### 633 **4.4. Both populations expressed different responses to additional stress**

634 It was also observed that the LC and HC populations displayed almost the same number of differentially  
635 expressed contigs in response to epoxiconazole exposure, but they only shared six contigs, indicating a  
636 specific response for each population. Unfortunately, the lack of annotation did not allow us to draw a  
637 particular pathway in response to exposure. This global result could be due to the fact that the response  
638 was analyzed only 2 days after induced epoxiconazole exposure, which is perhaps too short to observe  
639 a differentiated response from the two populations. A higher number of differential gene expressions in  
640 fungicide-exposed arthropods was observed after 4 days of exposure, compared with 2 and 7 days  
641 (Simões et al., 2019). Another possible explanation could be that the choice of epoxiconazole was maybe  
642 not the best pesticide to trigger a clear response in earthworms as evidenced in other studies:  
643 Epoxiconazole induced either no or moderate effects in earthworms at an environmentally relevant  
644 exposure (Bart et al., 2017; Givaudan, Binet, et al., 2014; Givaudan, Wiegand, et al., 2014; Nelieu et  
645 al., 2016; Pelosi et al., 2016).

646 In the LC population, processes involved in response to stress included upregulation of heat shock  
647 protein 90. HSPs are known to be rapidly synthesized in response to environmental stressors, including  
648 pesticide stress (Y. Sun et al., 2014). Different proteins were also up- or downregulated in response to  
649 epoxiconazole without a clear explanation of the exact nature of these changes, e.g., downregulation of  
650 the neuromacin propeptide, whose functions include bacterial killing, symbiostasis in the gut, immune  
651 defense, and regeneration of the damaged nerve cord (Bruno et al., 2019). The expression of genes  
652 related to these immune processes has been found to change following pesticide exposure (Costa et al.,  
653 2020). The BTB domain-containing protein was also downregulated, which is a versatile protein-protein  
654 interaction motif that participates in a wide range of cellular functions, including transcriptional  
655 regulation, cytoskeleton dynamics, ion channel assembly and gating, and targeting proteins for  
656 ubiquitination (Stogios et al., 2005). Contigs linked to the Ran GTPase binding function were  
657 downregulated. Ran (GTP-binding nuclear protein Ran) is a small GTPase involved in important cellular  
658 activities such as nucleocytoplasmic transport, mitotic spindle assembly, and nuclear envelope  
659 formation; it is known to be differentially expressed in response to stress (Bo et al., 2018; Nachury &  
660 Weis, 1999). Conversely, Serpin B6-like was upregulated in response to epoxiconazole exposure. Serpin  
661 B6 inhibits Cathepsin G, thereby inhibiting its functions to clear pathogens, regulate inflammation by



662 modifying the chemokines, cytokines, cell surface receptors, and C components, control blood pressure,  
663 and induce thrombogenesis (Mangan et al., 2008).

664 In the HC population, upregulation of the cytochrome P450 family protein belonging to the CYP2 family  
665 was evidenced again, as already observed in the interpopulation differences (upregulation of CYP2J2).  
666 This result confirmed the activation of a specific pathway involved in the phase I xenobiotic metabolism  
667 only in the HC population, requiring in-depth analysis of this mechanism.

668 As observed in the LC population, different proteins were modulated in response to epoxiconazole  
669 without an obvious explanation of their functions. We can note downregulation of tyrosinase-like  
670 protein 1; in humans, *Tyrp1* is a melanocyte-specific gene product involved in melanin synthesis. This  
671 response could be a direct effect of epoxiconazole. In *Mytilus galloprovincialis*, evidence of tissue  
672 damage was given by a massive deposit of melanin (melanosis) after exposure to an insecticide,  
673 highlighting the inflammation processes (Stara et al., 2020). Downregulation of the c-Myc-binding  
674 protein-like was also observed; this is a proto-oncogene that functions as a transcription factor, thought  
675 to regulate expression of more than 15% of cellular genes (Dang et al., 2006). Its expression promotes  
676 cell proliferation and genomic instability by accelerating cells through G1 and S phases of the cell cycle,  
677 abrogating cell cycle checkpoints, and increasing cell metabolism (Mladinic et al., 2012).

678 Different proteins linked to immune responses (lymphocyte cytosolic protein 2-like isoform X2, C1q  
679 domain-containing protein) were also modulated in response to epoxiconazole exposure. Pesticides are  
680 known to be immunotoxic and interfere with specific immunological functions of each type of immune  
681 cell (see (Lee & Choi, 2020) for a review).

682 A chemoattractive glycoprotein (ES20) was found to be downregulated in response to epoxiconazole  
683 exposure. Alarm pheromones have been detected in earthworms (Ressler et al., 1968), which can deter  
684 other members of the species but can act as a chemoattractant to other animals such as snakes (Jiang et  
685 al., 1990). In both populations, the chitin metabolic pathway was enriched due to the upregulation of  
686 chitinase. This response could be explained by the fact that epoxiconazole is known to induce  
687 stimulation of chitinase in wheat as a defense against fungi (Siefert et al., 1996).

688

#### 689 **4.5. Implications of long-term pesticide use for soil health**

690 The knowledge required to assess the implications of pesticide residue to soil biota and their  
691 repercussions on soil security is far from being complete, leaving a wide gap in the way regulations for  
692 pesticide approval and policies on environmental health are formulated. Within regulatory risk  
693 assessment of pesticides, there is particularly a need for more research on long-term impacts as  
694 highlighted by “EFSA (European Food Security Agency) Scientific Opinion addressing the state of the  
695 science on risk assessment of plant protection products for in-soil organisms” (EFSA, 2017). Our  
696 findings suggest on-going evolutionary responses of soil fauna to long-term anthropogenic pressure.  
697 Soil fauna adaptation to soil chemical pressure is a pivotal question. As soil engineer, earthworm  
698 tolerance to pesticides may hold important support for the soil sustainability and the agroecosystem

699 resilience. However, the costs to the population of such an acclimation/adaptation, especially in terms  
700 of reproduction, are still unknown. Our work advocates for further investigations that explicitly integrate  
701 the evolutionary effect of pesticides on soil biota and calls also for considering long-term effect of  
702 pesticides in deriving environmentally safe concentrations or registering new pesticide active  
703 ingredients.

704

## 705 **Conclusion**

706 Overall, we demonstrated for the soil engineer *A. caliginosa*, that two same lineage populations deriving  
707 from similar pedo-climatic environments, constitutively exhibit distinct gene expression pathways after  
708 they long-lived in differently managed agricultural soils. The main difference observed was regarding  
709 metabolism, with pathways linked to proteolytic activities and to the mitochondrial respiratory chain.  
710 Other specific responses were observed and should be further investigated, such as the cytochrome P450  
711 family CYP2 or responses linked to ion binding. It is important to keep in mind that these results need  
712 further in-depth investigations. Indeed, with the low annotation rates of the earthworm transcriptome  
713 (about 20%, which is standard for *de novo* assemblies), what we have discovered in the current study  
714 might be just the tip of the iceberg. The recent publication of the genome of *A. caliginosa* (Perry et al.,  
715 2022) and a future annotated *A. caliginosa* genome/transcriptome can empower the approach pursued  
716 in this study and will also aid future research. Finally, studies that integrate responses from the molecular  
717 scale to the individual and population scale should be pursued in order to understand the physiological  
718 processes involved and to predict their potential repercussions at higher levels of biological  
719 organization.

720

## 721 **Credit author Statement**

722 **Audrey Barranger:** Conceptualization, Methodology, Formal analysis, Investigation, Data Curation,  
723 Visualization, Writing – original draft, Project administration, Supervision. **Christophe Klopp:**  
724 Methodology, Formal analysis, Data Curation, Writing – review & editing. **Barbara Le Bot:**  
725 Methodology, Formal analysis, Resources, Writing – review & editing. **Gaëlle Saramito:** Methodology,  
726 Formal analysis, Data Curation. **Lise Dupont:** Methodology, Formal analysis, Data Curation, Writing  
727 – review & editing. **Stéphanie Llopis:** Investigation. **Claudia Wiegand:** Funding acquisition, Writing  
728 – review & editing. **Françoise Binet:** Conceptualization, Methodology, Investigation, Resources,  
729 Supervision, Writing – review & editing, Funding acquisition, Project administration.

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740

#### 741 **Data availability statement**

742 RNA-seq raw sequences and the *de novo* assembled transcriptome assemblies have been deposited in  
743 NCBI under BioProject PRJNA883218. This Transcriptome Shotgun Assembly project has been  
744 deposited at DDBJ/EMBL/GenBank under the accession GKBY00000000. The version described in  
745 this paper is the first version, GKBY01000000.

746

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