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

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Abstract :

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

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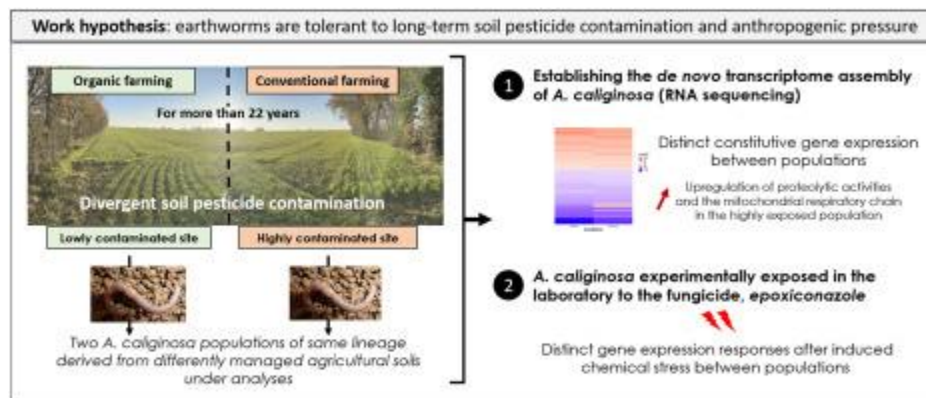
Graphical abstract



Highlights

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

Keywords : Aporrectodea, Transcriptomics, Pesticides residues, Mitochondrial respiratory chain, Agrosystem, Soil biodiversity

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1. Introduction

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 genral 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^{-1}, 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_w 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_w 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^2. AITC
168 stock solution 5 g L^{-1} in isopropanol (propan-2-ol) was further water diluted to a concentration of 0.1 g
169 L^{-1} (Zaborski, 2003). After collecting emerging individuals, a block of soil 31.7x31.7 cm² 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^{-1}), 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^{-1}, considering a field application
217 rate of 125 g ha^{-1} 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°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 H_2O. 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°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.
248

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. 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.
257

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.
267

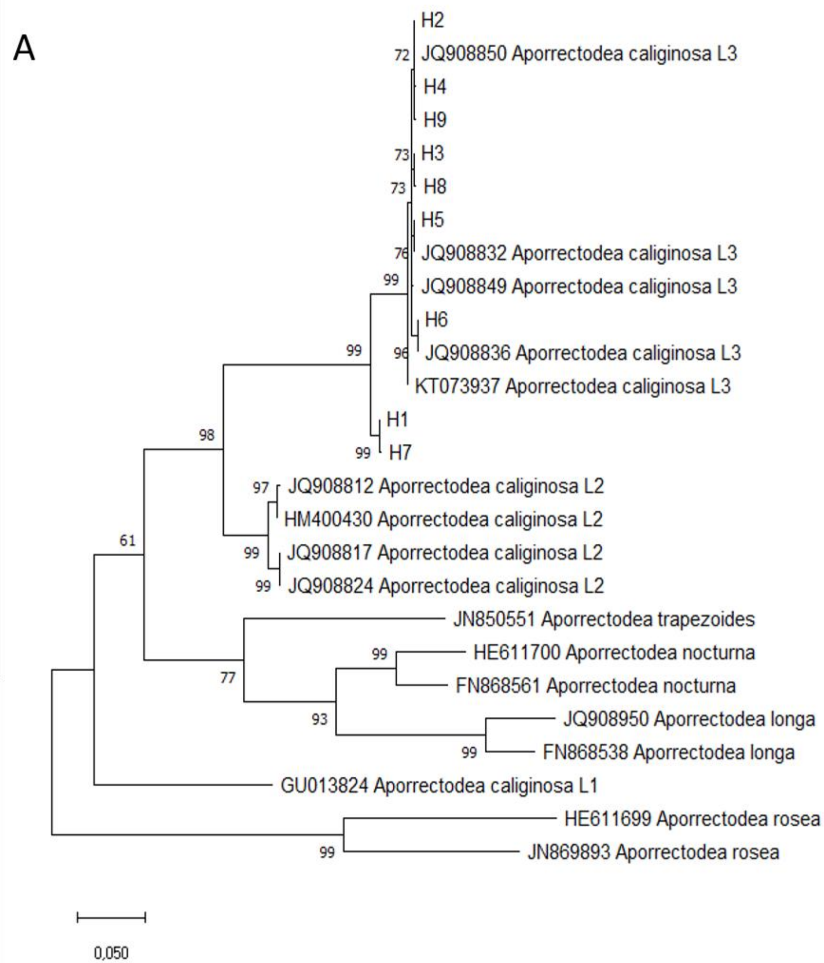
268 ## 3. Results

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	π
HC	15	5	26	0.705	0.0186
LC	15	6	8	0.829	0.0034
Total	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 (Nh), number of polymorphic sites (S), haplotype
286 diversity (Hd) and nucleotide diversity (π).
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 ± 28.1 and 15.3 ± 7.0 individuals.m^{-2} in LC and HC, respectively). The corresponding mean *A.*
292 *caliginosa* biomasses were 18.5 ± 9.8 and 3.0 ± 0.9 g.m^{-2} in the LC and HC sites, respectively.

293

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

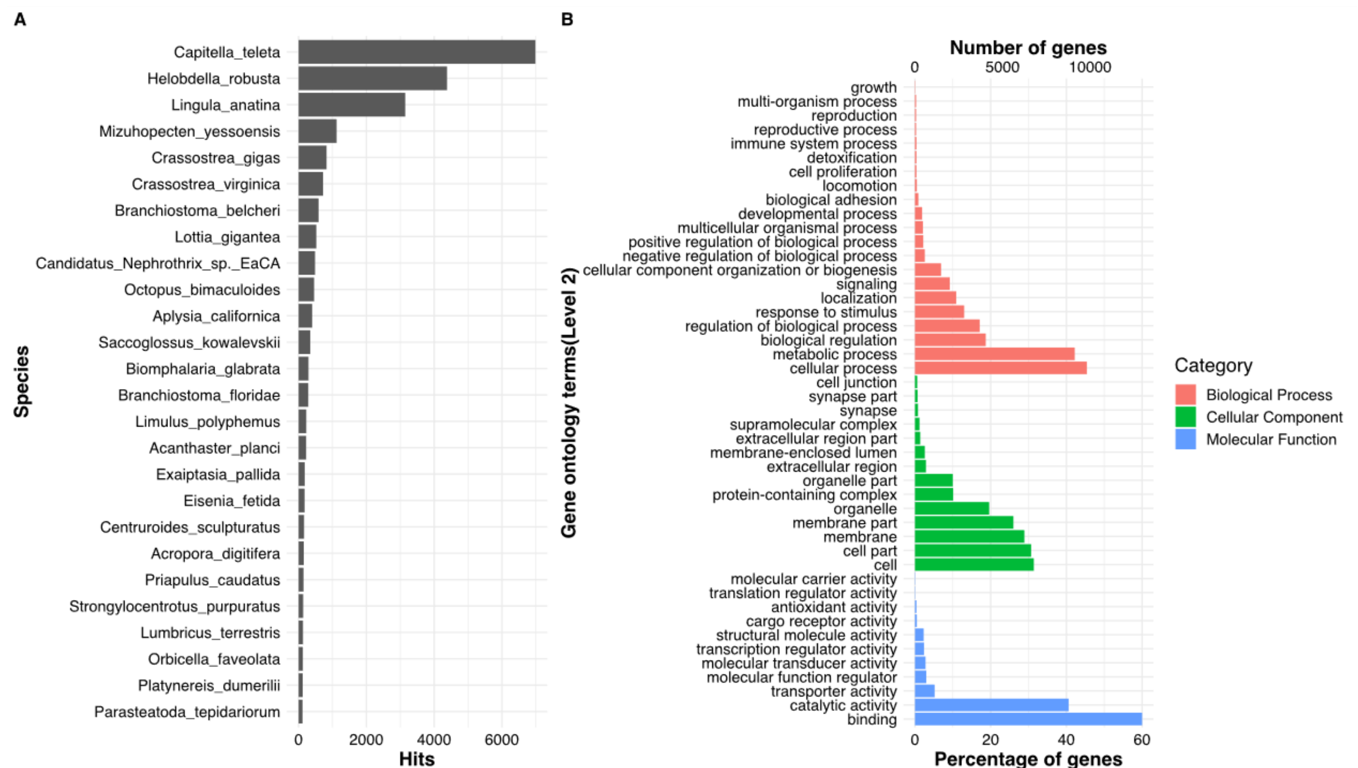
295 The multi-residual analysis showed that all samples contained at least one and up to 20 and 11 pesticides
296 for soil and earthworms, respectively. Out of the 73 targeted pesticides, 28 and 15 had been detected at
297 least once in the soil and *A. caliginosa*, respectively. Soil and *A. caliginosa* 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 aclonifen, 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 (± 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 ± 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

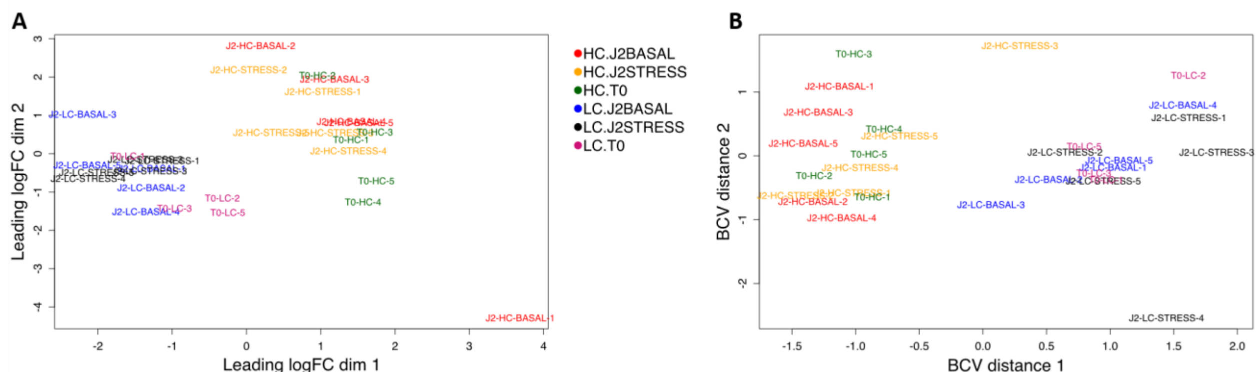
330 ### 3.4. Functional annotations of the *de novo* transcriptome assembly

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

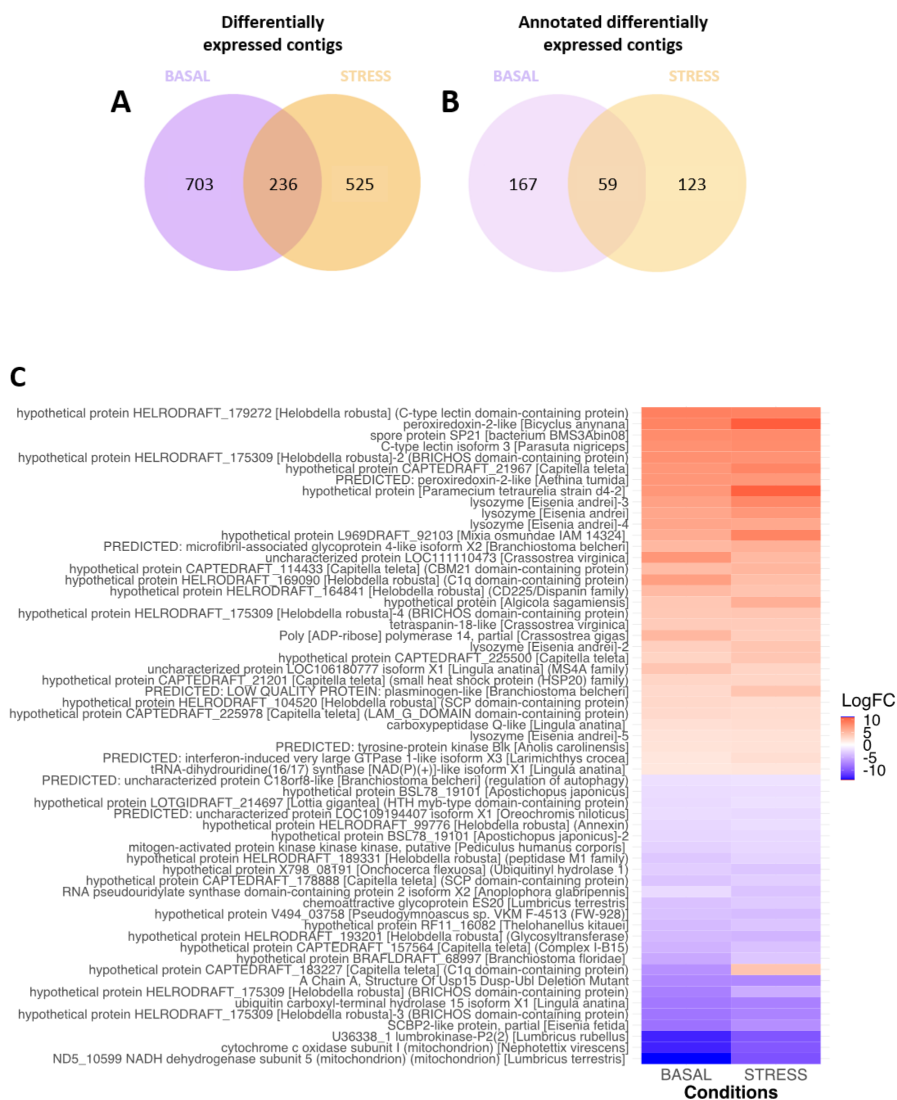
357 Analyses were performed to check the overall reproducibility and variation between earthworm samples
358 belonging to the same population (HC or LC) and treatment (exposed (stress) or not (basal)). A heatmap
359 of Pearson correlations based on log10 expression counts and an MDS plot were generated 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
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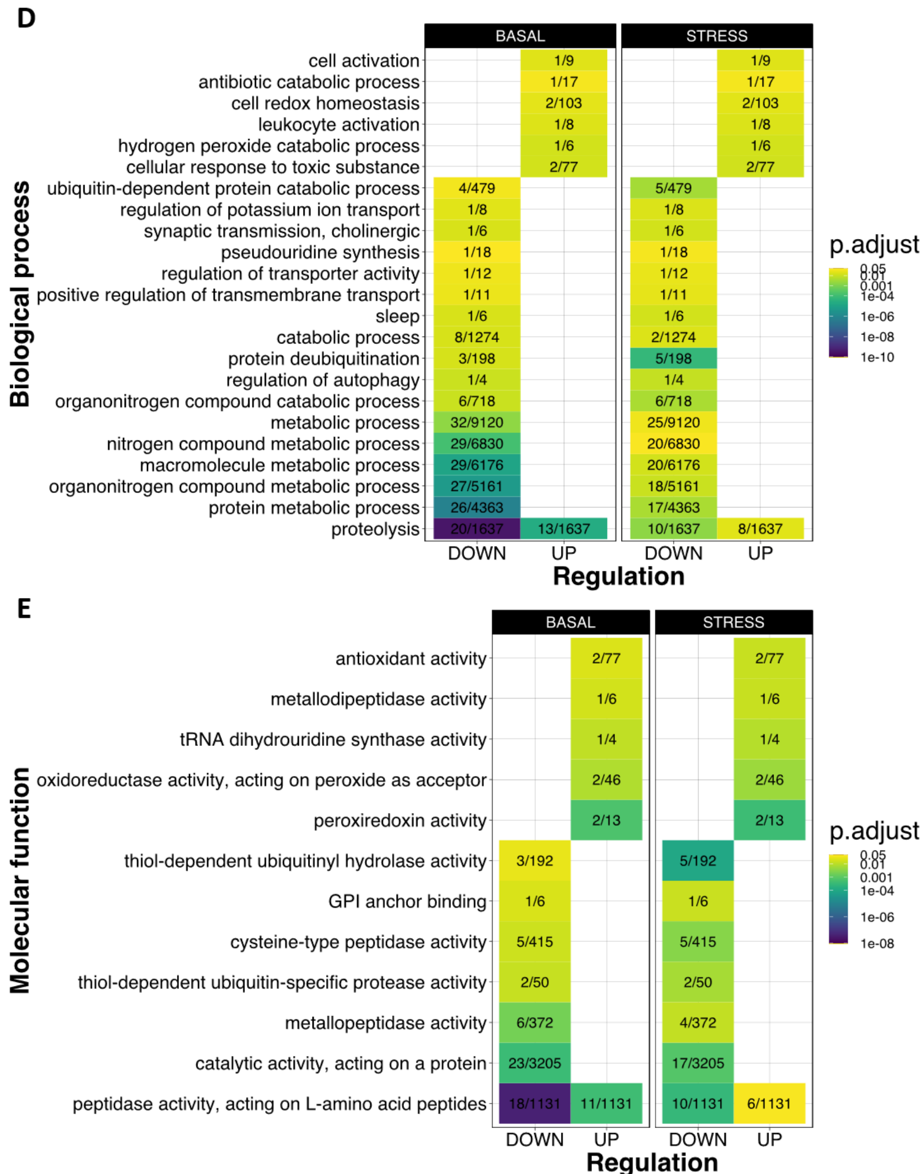


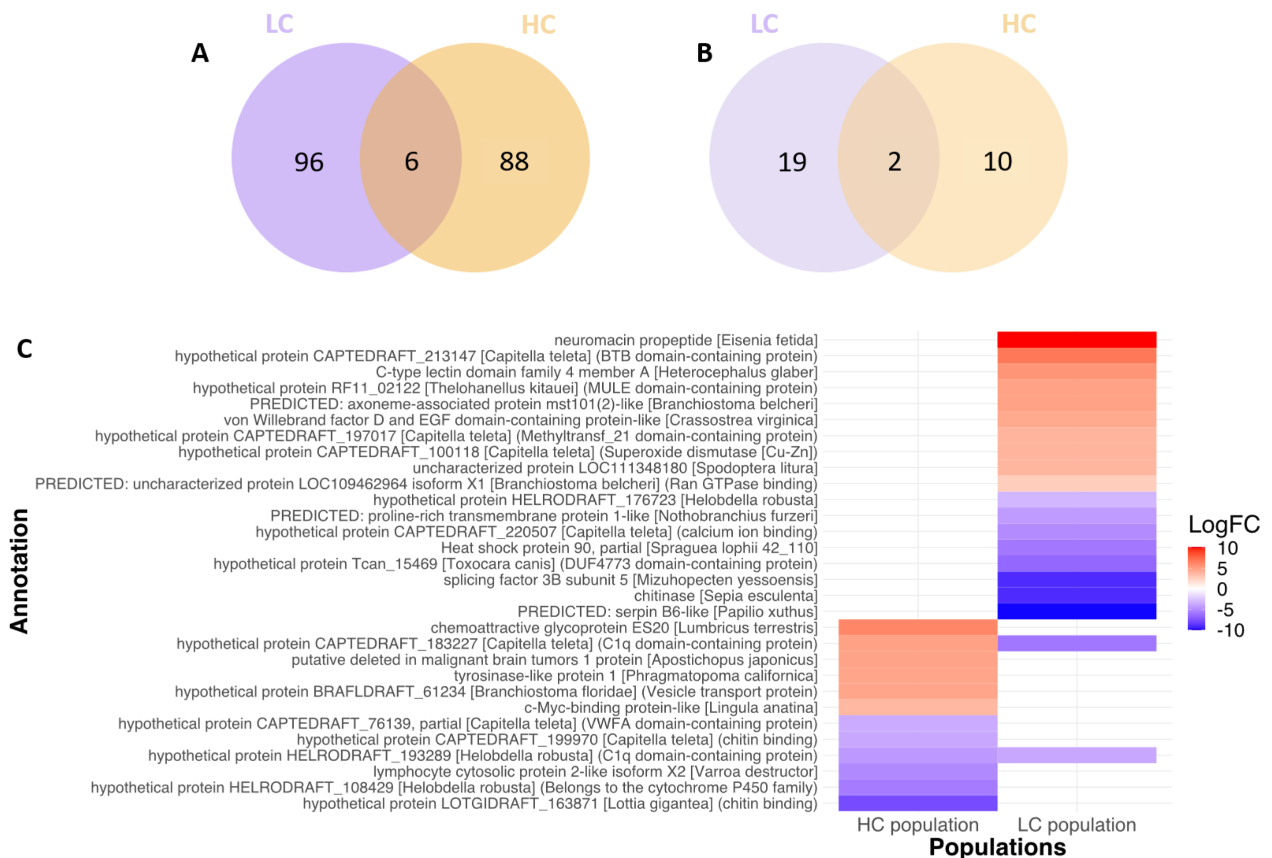
Figure 4. Venn diagram depicting the total number of differentially expressed contigs (A) and Venn diagram depicting the total number of annotated differentially expressed contigs (B) when comparing LC and HC populations under stress and basal conditions, as well as the number of common contigs in both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

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

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

In the LC population (LC.basal vs. LC.stress), 21% of differentially expressed contigs were annotated. 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. Expoxiconazole 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

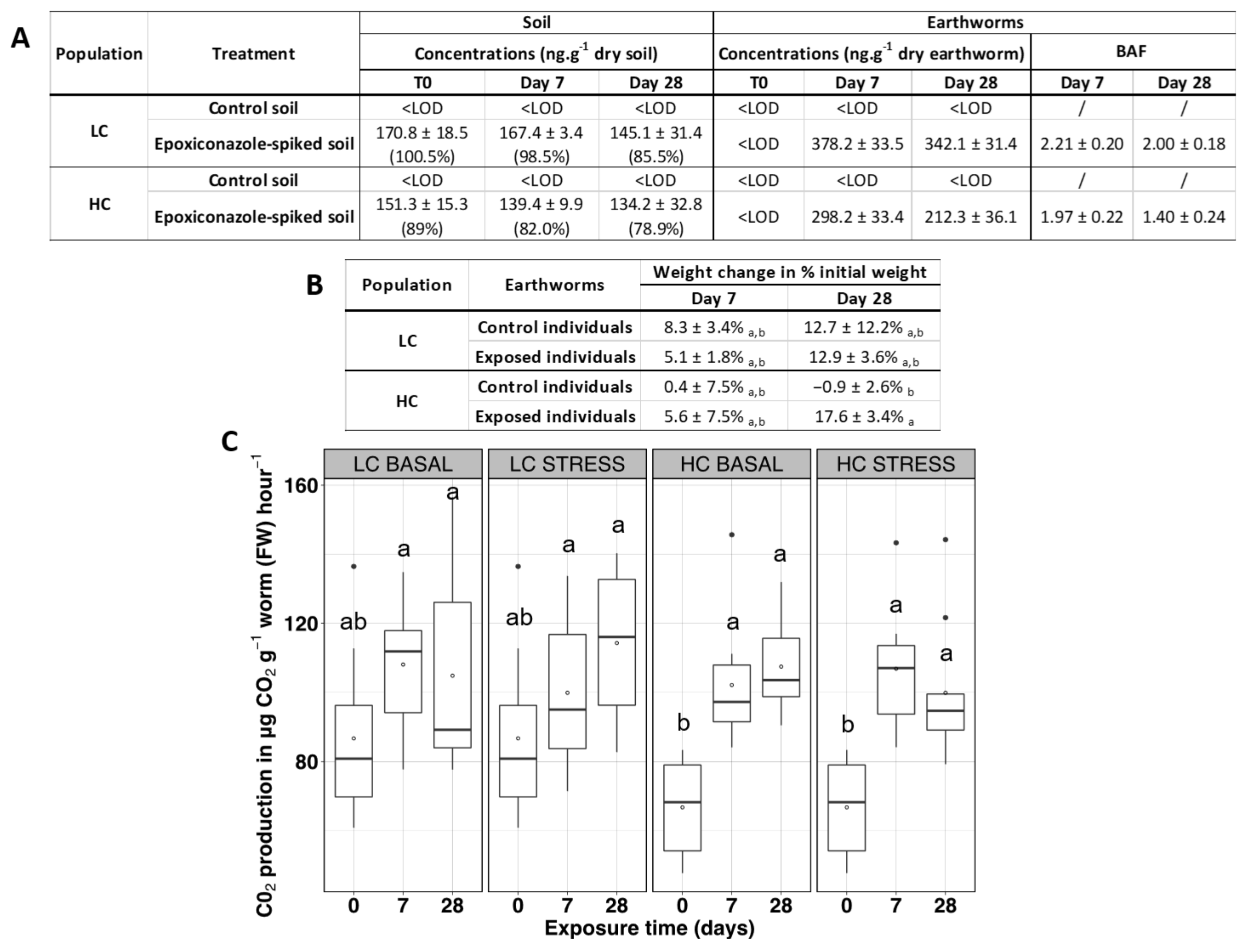
457 ### 3.6.2. *A. caliginosa* weight monitoring throughout the experiment

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

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

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

At the beginning of the experiment (T0), earthworm respiration measured as CO_2 production was higher in the LC population than in the HC one, although not significantly due to the high variability between individual earthworms. The metabolic rate of the HC populations increased significantly over time, whether exposed or not to expoxiconazole. Conversely, an unsignificant variation in energy dissipation was observed in the LC earthworms throughout the experiment ($p>0.05$) (Figure 6C).



A

Population	Treatment	Soil: Concentrations (ng.g^{-1} dry soil) T0	Day 7	Day 28	Earthworms: Concentrations (ng.g^{-1} dry earthworm) T0	Day 7	Day 28	BAF Day 7	BAF Day 28
LC	Control soil	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	/	/
	Epoxiconazole-spiked soil	170.8 ± 18.5 (100.5%)	167.4 ± 3.4 (98.5%)	145.1 ± 31.4 (85.5%)	<LOD	378.2 ± 33.5	342.1 ± 31.4	2.21 ± 0.20	2.00 ± 0.18
HC	Control soil	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	/	/
	Epoxiconazole-spiked soil	151.3 ± 15.3 (89%)	139.4 ± 9.9 (82.0%)	134.2 ± 32.8 (78.9%)	<LOD	298.2 ± 33.4	212.3 ± 36.1	1.97 ± 0.22	1.40 ± 0.24

B

Population	Earthworms	Weight change in % initial weight: Day 7	Day 28
LC	Control individuals	8.3 ± 3.4% $_{a,b}$	12.7 ± 12.2% $_{a,b}$
	Exposed individuals	5.1 ± 1.8% $_{a,b}$	12.9 ± 3.6% $_{a,b}$
HC	Control individuals	0.4 ± 7.5% $_{a,b}$	−0.9 ± 2.6% $_{b}$
	Exposed individuals	5.6 ± 7.5% $_{a,b}$	17.6 ± 3.4% $_{a}$

Figure 6. Epoxiconazole concentrations in soil (n=3) and earthworms (n=5) as well as earthworm bioaccumulation factor (BAF) on days 0, 7, and 28. Results are expressed as a mean ± standard deviation (A). Weight change of earthworms from the HC and LC sites on days 7 and 28. Values are the mean out of 30 replicates (N=30) ± standard deviation (SD) (B). Metabolic rate (µg CO2 g^{-1} worm (fresh weight) h^{-1}) of earthworms from the HC and LC sites exposed to epoxiconazole (STRESS) or not (BASAL) on days 0, 7, and 28. A dot on the boxplot indicates a mean value (N= 10 worms per group) (C). Different letters (a or b) denote statistical differences ($p<0.05$).

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^{-1} 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_2 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, letting 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

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

Conclusion

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

Credit author Statement

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