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

Running title: Earthworms' pesticide tolerance

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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.

Key-words: Aporrectodea, transcriptomics, pesticides residues, mitochondrial respiratory chain, agrosystem, soil biodiversity

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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

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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

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

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2. Materials and Methods

2.1. Study area, agricultural context and earthworm populations

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

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

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

2.2.1. Soil and earthworm sampling

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

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

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

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

2.2.3. Genetic characterization of *Aporrectodea caliginosa* populations

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

2.2.4. Onsite population densities of *A. caliginosa*

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

2.3. Experimental epoxiconazole exposure

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

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

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

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

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

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2.3.2. *A. caliginosa* transcriptomic responses

At T0 and after two days of exposure to epoxiconazole, *A. caliginosa* earthworms were individually frozen and ground (whole body, gut voided) with a CryoMill (Retsch) under liquid nitrogen conditions. Frozen earthworm powders were stored at −80°C until use. Aliquots containing 30 mg of individual earthworm powder were used for total RNA extraction using a NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's protocol. Genomic DNA was removed by DNase digestion using a column then total RNA was eluted in RNase-free H_2O. RNA purity and quantity were assessed using a Nanodrop (ND-1000, ThermoFisher), and RNA integrity was checked using a Bioanalyzer 2100 (Agilent, CA, USA). Total RNA samples were stored at −80°C. Samples were sent to GeT-PlaGe core facility (INRA Toulouse) in dry ice. RNA sequencing, *de novo* assembly, annotation, quantification, differential expression analysis, and enrichment analyses are described in supporting information S3.

2.3.3. Analysis of *A. caliginosa* metabolism

The weight of 30 gut-voided earthworms from each of the control and epoxiconazole exposure groups was individually monitored during the experiment at T0 and after 7 and 28 days of exposure. Respirometry measurements were taken on T0 and after 7 and 28 days of exposure. Ten earthworms from each treatment group were removed from the soil microcosm, rinsed, gently blotted dry on filter paper, and placed in a 250 ml glass jar hermetically closed for two hours. CO_2 was measured by a micro-gas chromatograph (3000A, SRA Instruments) equipped with a single PoraPLOT U capillary column coupled with a thermal conductivity detector.

2.4. Statistical analyses

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

3. Results

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

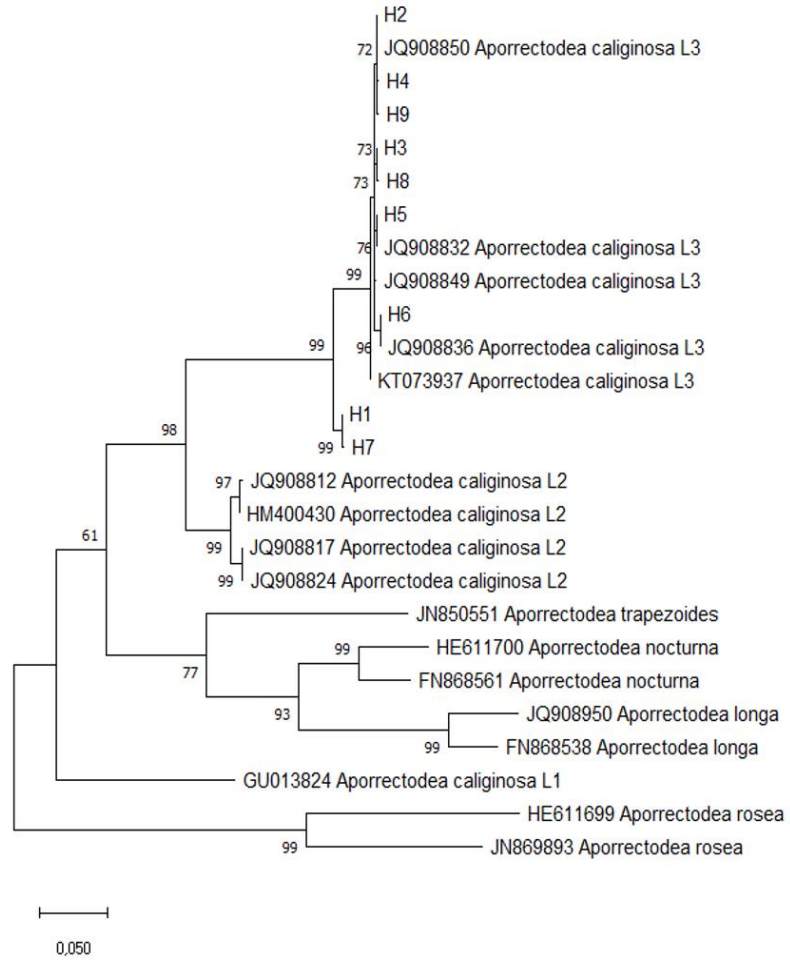
3.1.1. *Aporrectodea caliginosa* COI genetic diversity

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

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

A



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

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

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

The mean abundance of *A. caliginosa* was more than three times higher in the LC site than the HC one (56.5 ± 28.1 and 15.3 ± 7.0 individuals.m^{-2} in LC and HC, respectively). The corresponding mean *A. caliginosa* biomasses were 18.5 ± 9.8 and 3.0 ± 0.9 g.m^{-2} in the LC and HC sites, respectively.

3.2. Onsite soil and earthworm pesticide contamination

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

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

A total of 2,068,522,212 raw reads were generated, with an average of 23,729,267 (± 1,892,470) reads for each sample. Of these, a total of 139,950,716 reads were used for the transcriptome assemblies. The clean reads were assembled into 64,556 contigs with an average length of 2,010 pb ranging from 203 to 28,628 pb and N50 of 2,862 pb. The assembled transcriptome size was 129,787,458 bp. The assembly quality was then assessed by calculating the read on contig re-alignment rate for every sample, and by processing the contigs with BUSCO (version 2) using the metazoa_odb9 database (Simao et al. 2015). This analysis was to check the presence and completeness of a set of expected single-copy protein-coding genes for a given branch of the evolution tree. Among the 978 metazoa_odb9 proteins searched by BUSCO (3.0.2), 964 were found to be complete in single or multiple copies. This represents 98.6% of the proteins expected in the genome. The remaining ones were missing (1.1%) or fragmented (0.3%). With respect to the assembly quality, the mean per sample read mapping rate was 97.02 ± 0.60% across libraries. RNA-seq raw sequences and the de novo assembled transcriptome assemblies have been deposited in NCBI under BioProject PRJNA883218. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GKBY00000000. The version described in this paper is the first version, GKBY01000000.

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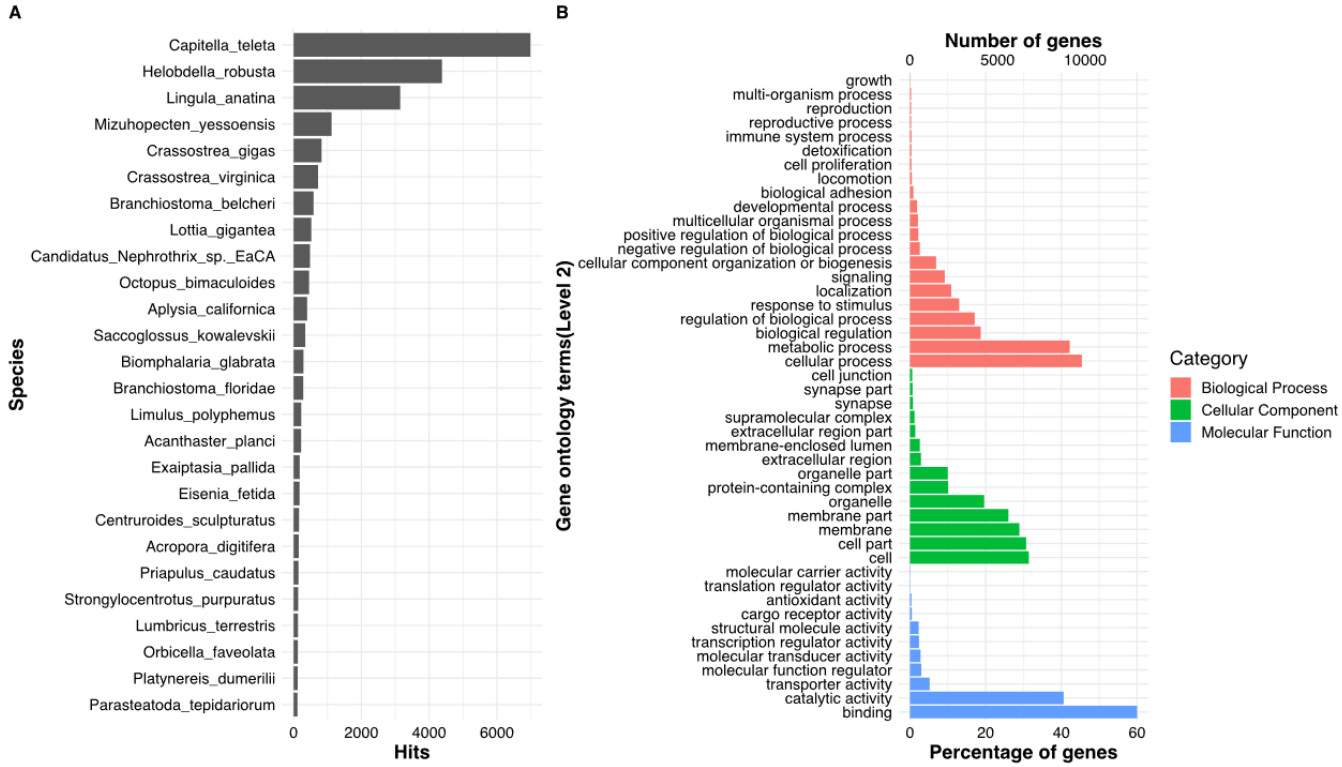


Figure 2. (A) Top hit species distribution on the basis of best sequence alignments and lowest E values and (B) Distribution of Gene Ontology (GO) assignments of assembled *A. caliginosa* contigs. GO categories are shown on the x-axis grouped into three main categories: biological processes, cellular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

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

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

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

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

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

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

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

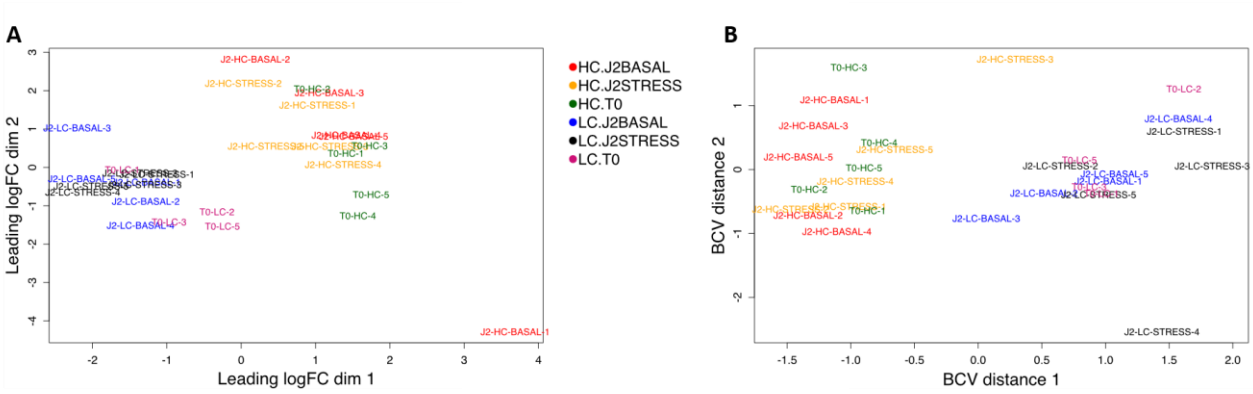


Figure 3. Multidimensional scaling plots (MDS) generated using the *limma plotMDS* function where A) distances correspond to leading log-fold change between samples and B) distances between samples correspond to the leading biological coefficient of variation (BCV). In both plots, dimension 1 separates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

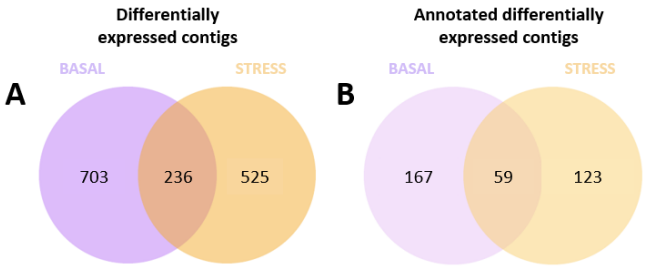
3.5.1. Investigation of differential molecular pathways between LC and HC populations under basal and chemical stress conditions (LC vs. HC populations)

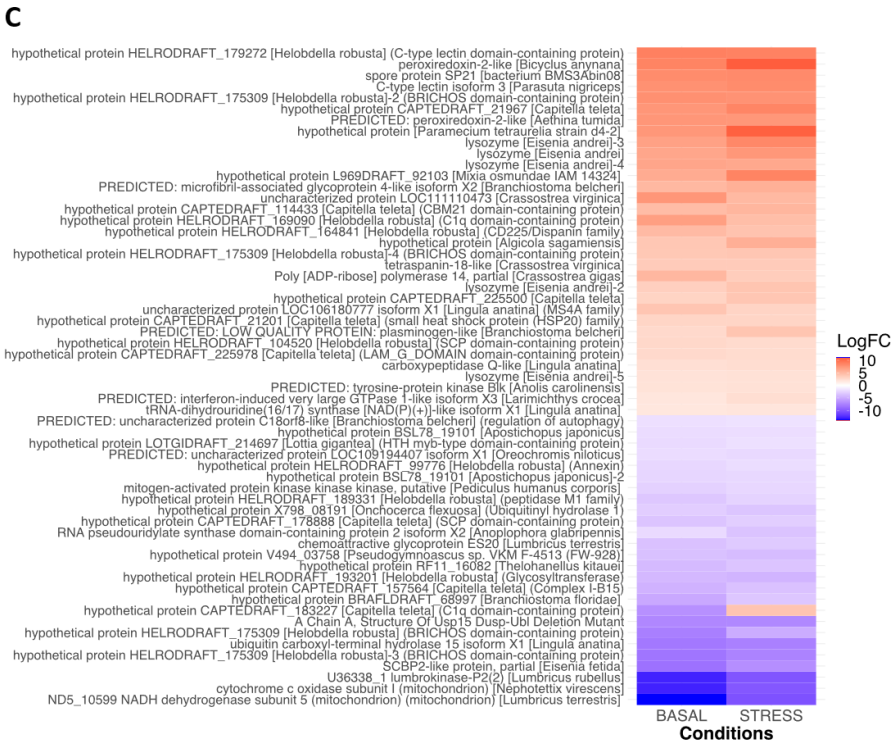
Under basal condition (LC.basal versus HC.basal), analysis showed that 939 contigs were differentially expressed with 569 contigs upregulated and 370 downregulated when comparing LC and HC populations that had a contrasting site history with respect to agricultural practices and pesticide use

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(Figure 4A). Annotations enabled us to identify 226 proteins (24% of all the differentially expressed contigs) (Figure 4B, Table S6). Results of the gene ontology enrichment analysis for biological process and molecular function are presented in Figures 4D and 4E. KEGG enrichment analysis revealed that one pathway was upregulated under the basal condition: protein digestion and absorption (map04974) and three pathways were downregulated: ECM-receptor interaction (map04512), pancreatic secretion (map04972) and protein digestion and absorption (map04974) (Table S8).

Under stress condition (LC.stress versus HC.stress), 761 contigs were differentially expressed by the two populations (321 downregulated and 440 upregulated) (Figure 4A) and 182 contigs were annotated (24%) (Figure 4B, Table S7). Results of the gene ontology enrichment analysis for biological process and molecular function are presented in Figures 4D and 4E. KEGG analysis showed that three pathways were specifically downregulated under the stress condition: autophagy (map04136, map04138) and mitophagy (map04139) (Table S8).





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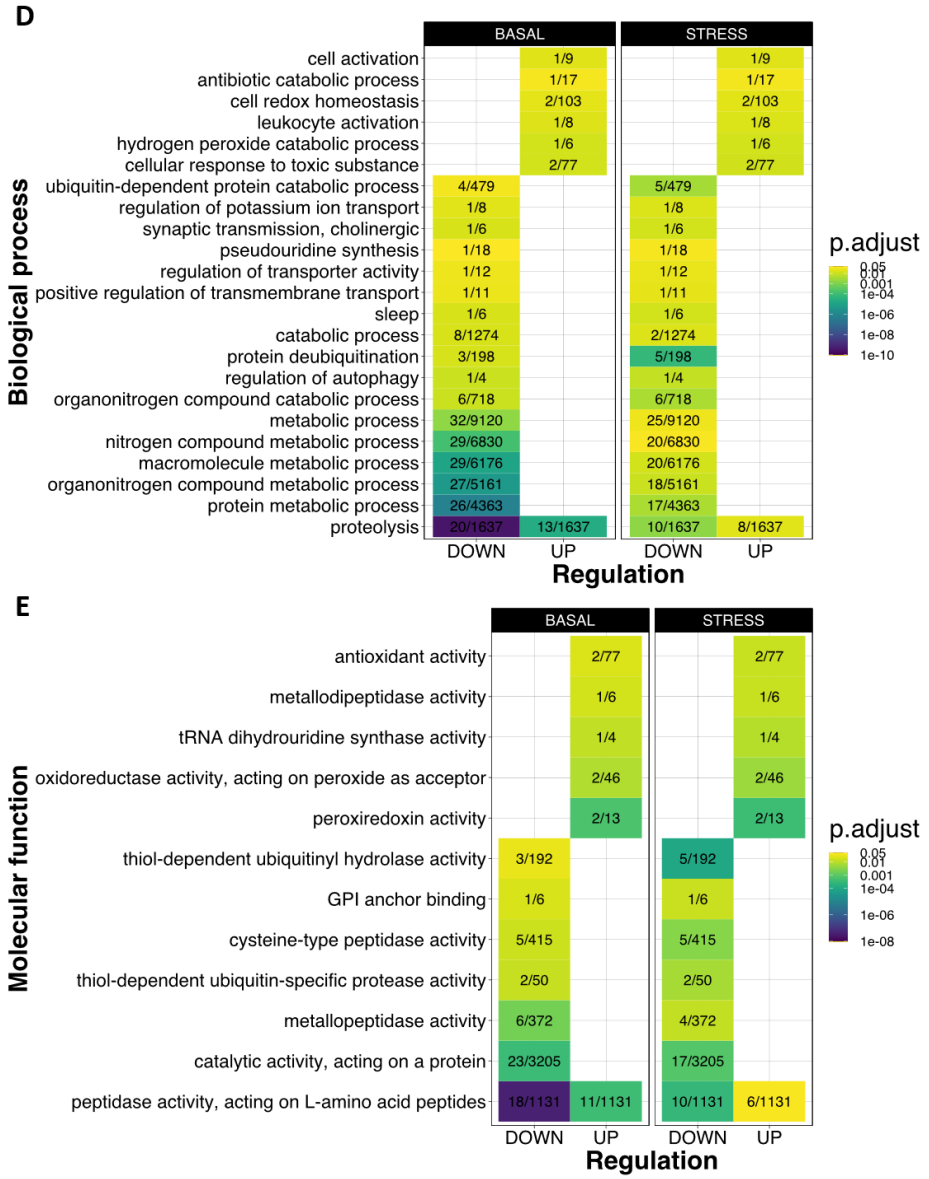


Figure 4. Venn diagram depicting the total number of differentially expressed contigs (A) and Venn diagram depicting the total number of annotated differentially expressed contigs (B) when comparing LC and HC populations under stress and basal conditions, as well as the number of common contigs in both 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

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

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

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

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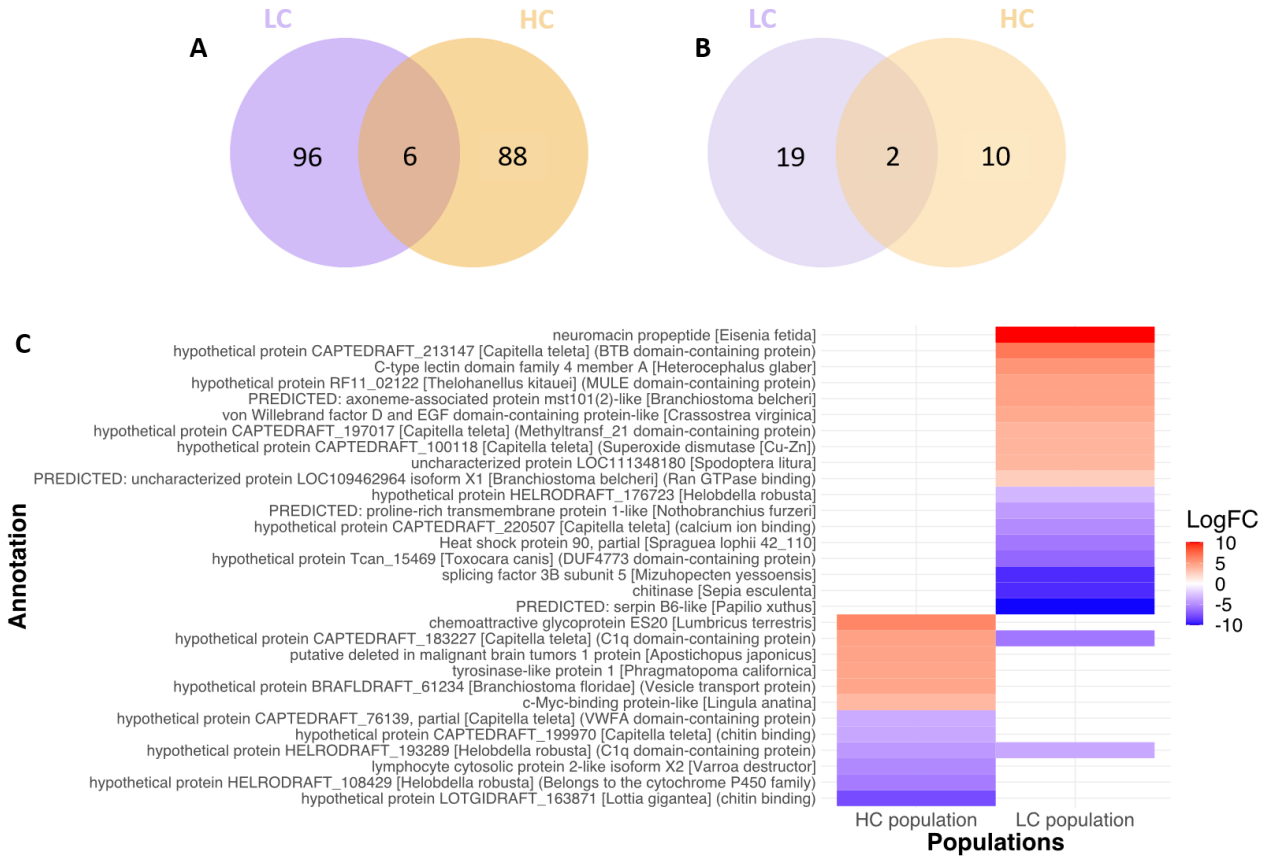


Figure 5. Venn diagram depicting the total number of differentially expressed contigs (A) Venn diagram depicting the total number of annotated differentially expressed contigs (B) and Heatmap of differentially expressed contigs (C) in the LC and HC populations after epoxiconazole exposure (stress condition).

3.6. Bioaccumulation and physiological responses after chemical stress

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

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

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

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

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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			Earthworms				
		Concentrations (ng.g^{-1} dry soil)			Concentrations (ng.g^{-1} dry earthworm)			BAF	
		T0	Day 7	Day 28	T0	Day 7	Day 28	Day 7	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% $_{a}$

C

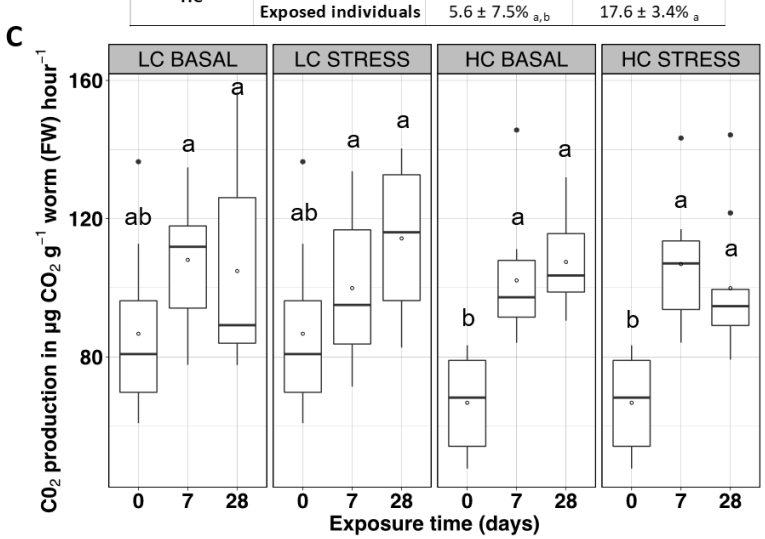


Figure 6. Epoxiconazole concentrations in soil (n=3) and earthworms (n=5) as well as earthworm bioaccumulation factor (BAF) on days 0, 7, and 28. Results are expressed as a mean ± standard deviation (A). Weight change of earthworms from the HC and LC sites on days 7 and 28. Values are the mean out of 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).

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4. Discussion

4.1. *A. caliginosa* facing pesticide soil contamination

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

4.2. Genetic diversity among the studied populations

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

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

4.3. Interpopulation differences: differentially expressed contigs under basal and stress conditions

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

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

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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),

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

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

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

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this response, but it is well known that ion channels remain the primary target of most of the small molecule insecticides, and are involved in pesticide resistance (French-Constant, 1994).

In the HC population, upregulation of CytochromeP450 CYP2J2 was observed under the stress condition. In humans, this cytochrome is involved in phase I xenobiotics metabolism (Xu et al., 2013). This response was only observed in the HC population and could be a specific mechanism in response to pesticide exposure developed in this population.

4.4. Both populations expressed different responses to additional stress

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

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

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

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

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

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

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

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

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

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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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Data availability statement

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

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