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Research Article

How to resist soil desiccation: Transcriptional changes in a Mediterranean earthworm during aestivation

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ABSTRACT

Earthworms have a central role in ministering the terrestrial ecosystems and are proving to have an important role in modulating the effects climate change has on soil. Aestivation is a form of dormancy employed by the organisms living in deserts and arid environments, when confronted with prolonged periods of drought. Understanding global metabolic adjustments required for withstanding the harsh conditions of the ever more severe Iberian drought, we performed a global transcriptomic exploration of the endogeic earthworm *Carpetania matritensis* during aestivation. There were a total of 6352 differentially expressed transcripts in the aestivating group, with 65% being downregulated. Based on GO and KEGG enrichment analyses, downregulated genes seem to be indicative of an overall metabolic depression during aestivation. Indeed we noted a reduction of protein turnover and macromolecule metabolism coupled with suppression of genes involved in digestion. Upregulated genes, namely antioxidant genes and DNA repair genes showed clear signs of abiotic stress caused by ROS generation. Abiotic stress led to transcriptomic changes of genes involved in immune response, mostly affecting the NF-kb signaling pathway as well as changes in apoptotic genes indicating the necessity of investigating these processes in a tissue specific manner. Lastly we uncovered a possible mechanism for water retention by nitrogenous waste accumulation. This study provides the first ever transcriptomic investigation done on aestivating earthworms and as such serves as a general framework for investigation on other earthworm species and other soil invertebrates, which is becoming increasingly important with the current scenario of climate change.

1. Introduction

Earthworms are some of the most important soil dwelling invertebrates and make up approximately 40–90% of the macro-faunal biomass in many terrestrial ecosystems as well as being the most abundant soil animal group in agricultural soils (Fragoso et al., 1999; Plaas et al., 2019; Van Groenigen et al., 2014). They have multiple roles in soil, such as improving soil structure by burrowing, improving nutrient composition by mixing soil and decomposing organic residues and act as biological regulators of pathogens (Plaas et al., 2019; Toyota and Kimura, 1994). Since their activity affects both biotic and abiotic soil properties they are known to increase plant growth and plant production (Van Groenigen et al., 2014).

When analyzing the importance of earthworms through the 'climate change' lens two important things stand out. Firstly, earthworms' activity in soil contributes to C stabilization, which translates to a positive effect on C sequestration in soil (Angst et al., 2019; Thomas et al., 2020; Zhang et al., 2013). Secondly, earthworms help maintain the soil biota, especially in periods of desiccation by buffering the effects of elevated temperatures. Earthworms' structures can shelter other soil species in periods of soil desiccation (Siebert et al., 2019). Therefore, investigations aimed at gaining a greater level of understanding of the survival mechanisms of earthworms to climate change scenarios are of special importance. This is especially true for the Iberian Peninsula, which is becoming increasingly drier (Páscoa et al., 2017) and this might cause big changes in the biodiversity of the terrestrial ecosystems (Feehan et al., 2009). Unfavourable conditions such as droughts brought about by climate change could have severe consequences on earthworm composition and functioning of communities in the soil. (Singh et al., 2021; Singh et al., 2019).

Aestivation is a state of animal dormancy most commonly witnessed in deserts or semi-arid environments, such as the environment found in the central part of the Iberian Peninsula. Aestivating animals undergo important physiological and biochemical adjustments, such as changes in energy consumption, metabolic activity, and immune response (Storey and Storey, 2010). The duration of aestivation can vary among

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species from just a few weeks to a longer period. The decrease in metabolic rate in aestivating animals, which can reach 70–80% of the resting value, and nearly 100% in some species, conserves energy to extend survival time (Guppy and Withers, 1999; Pinder et al., 1992; Storey and Storey, 2010; Wang et al., 2015). Aestivation has been studied in both vertebrates and invertebrates such as land snails, sea cucumbers, sea urchin, earthworms, crocodiles, fish and frogs (Bayley et al., 2010; Díaz Cosín et al., 2006; Giokas et al., 2007; Kennett and Christian, 1993; Loong et al., 2012; Pérez-Portela et al., 2020; Yoshida and Kaito, 2019). Earthworms are only active when free water is available in the soil (Lee, 1985), which makes them suitable for aestivation studies. Soil moisture has significant effects on growth and burrowing rate in earthworms, in particular low soil moisture rates found in conditions of drought can cause reduction in aerobic metabolism, negatively affect growth and decrease cocoon numbers (Diehl and Williams, 1992; Holmstrup, 2001; Saroja, 1964). Many earthworm species go into diapause or become dormant and often migrate to deeper soil layers when higher temperatures cause water limitation in the soil (J. Jiménez et al., 2000; Kretzschmar and Bruchou, 1991; McDaniel et al., 2013). Other species, such as epigeic (living at the soil surface) species have a limited ability to migrate into the soil (Eggleton et al., 2009) and produce drought-resistant cocoons close to the soil surface as a main strategy to survive during summer drought (Holmstrup and Loeschcke, 2003; Petersen et al., 2008). Endogeic species (living in deep layers of soil) form non-permanent horizontal burrows to survive shorter periods of drought and when needed also form aestivation chambers covered with mucus and gut content, which prevents water loss (Bayley et al., 2010). Increase in free amino acid concentrations is another survival strategy for desiccation for at least 3 endogeic and anecic species from the genus *Aporrectodea* (Bayley et al., 2010; Holmstrup et al., 2016). The increase in free amino acid concentrations, in particular alanine, reduced the rate of water loss in the body thus providing protection against deleterious effects of desiccation (Holmstrup et al., 2016). There are not a lot of available studies on the accumulation of this molecule or other molecules, which may provide protection in unfavourable abiotic conditions in other earthworm species. Furthermore very little is known about the molecular mechanisms controlling all of the above mentioned physiological responses in earthworms. From what we know from studying other aestivating species we can infer that aestivation is regulated by a complex web of signaling cascades, which participate in the global suppression of gene transcription, histone modification and inhibition of translation (Storey and Storey, 2012). This allows the aestivating animal to have a minimum suite of vital functions for survival (Hochachka and Guppy, 2013).

In this paper we focus on identifying the key transcriptional changes during the aestivation process in earthworms by interrogating data from an endogeic Mediterranean species *Carpetania matritensis.* This species is adapted to sandy soils that are poor in organic matter in which most earthworms cannot survive (Hernández et al., 2007). When the conditions are unfavourable and soil humidity is low this earthworm species undergo a paradiapause and form aestivation chambers (Díaz Cosín et al., 2006). This hypometabolic state is not strict, meaning it is not controlled by an 'internal clock', but rather by a decrease in soil moisture. Soil moisture is the most important factor, however, temperature and season exert some influence on the aestivation (Díaz Cosín et al., 2006). Díaz Cosín et al. (2009) reported a significant decrease in cocoon production between 15% soil moisture and 20% soil moisture, but there is no information on lower percentages of soil moisture. We compare the global responses of control and aestivating earthworms and identify the main affected pathways and the most important candidate genes involved. The findings can provide a general framework for drought stress mitigation of aestivating earthworm species. Moreover as previously emphasized it is important to understand the molecular basis of a process that is becoming increasingly important with the current scenario of climate change.

2. Material and methods

2.1. Earthworms and experimental design

Soil and earthworms of the species *Carpetania matritensis* (formerly *Hormogaster elisae,* see Marchán et al. (2020)) were manually collected in May 2020 in El Molar, Spain (40°4422.9N, 3°3353.1W). Details of soil, climatic, and vegetation characteristics of El Molar can be found in (Garvín et al., 2000). Soil was air-dried and earthworms were acclimatized to laboratory conditions for 15 days by keeping them in plastic containers in soil sieved to 4 mm from the collection site and 20% moisture of dry weight (320 g dry soil plus 80 ml distilled water). The containers were kept at 16 °C and in darkness. Since the aim of the experiment was to compare the transcriptomic response during aestivation compared to normal conditions, we followed Díaz Cosín et al. (2006) to induce aestivation with high probability. The experiment included two treatments: control and aestivation (Fig. 1A and B). Both types of experimental units were prepared in 0.5 L plastic containers with 320 g of 4 mm sieved dry soil from the collection site. Díaz Cosín et al. (2006) suggested that the main factor inducing aestivation is soil moisture while temperature has a lesser influence. Therefore, we kept temperature constant between treatments and varied moisture. Control mesocosms were prepared at 20% moisture and aestivation mesocosms at 5% moisture by adding distilled water to dry soil, sprayed in different layers. The amount of dry soil used was sufficient to prevent food from becoming a limiting factor for activity (Díaz Cosín et al., 1996). A single earthworm, previously washed in distilled water, dried in filter paper and weighed, was placed on top of the soil of each container 24 h after preparation to avoid a temperature rise in the medium with the possible lethal effects for earthworms, due to a possible bacterial overgrowth after the addition of the water (Díaz Cosín et al., 1996). Twelve replicates per treatment were performed. The average initial weight of individual mature earthworms was 4.22 g ± 0.6 and did not differ between treatments. Containers were maintained in growth chambers during four weeks (28 days) at 16 °C and darkness. Each week we checked for moisture loss (by measuring the total weight of the containers) and added water as needed, but generally it was not the case. After 28 days, earthworms were removed, washed with distilled water and weighed and the presence of aestivation chambers was checked. Earthworms were then snap frozen in dry ice, crushed to powder with a mortar and pestle keeping the temperature low with dry ice, and stored at −80 until RNA extraction.

2.2. RNA extraction and sequencing

RNA was extracted from five randomly selected earthworms per treatment from 50 mg aliquots of powdered tissue. Three RNA extractions were performed for each earthworm to capture total tissue variability. Powdered tissue was homogenized in 1.5 ml Trizol (Invitrogen) and RNA was extracted according to the manufacturer's protocol. Samples were then treated with RNase-free DNase (Roche) for 90 min and organic extraction was performed using phenol-chloroform-isoamyl alcohol and Phase Lock Gel Light tubes (5Prime) to maximize aqueous phase recovery. Concentration and quality of RNA were checked using Nanodrop and RNA integrity was verified using Agilent 2100 Bioanalyzer. Equal amounts of the three RNA samples per individual were combined in a single tube and ten cDNA libraries (five individuals per treatment) were prepared by Novogene using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) and following manufacturer's recommendations. Libraries were sequenced with IlluminaNovaseq6000 using a 150 cycle paired-end protocol. Raw reads were trimmed by Novogene through in-house scripts, removing reads with adapters, poly-N sequences ($N > 10\%$) and reads with low quality. Downstream analyses were based on clean data, 93.4% of the reads presented a Phred score of Q30 or higher and 97.6% a Phred score of 20 or higher.

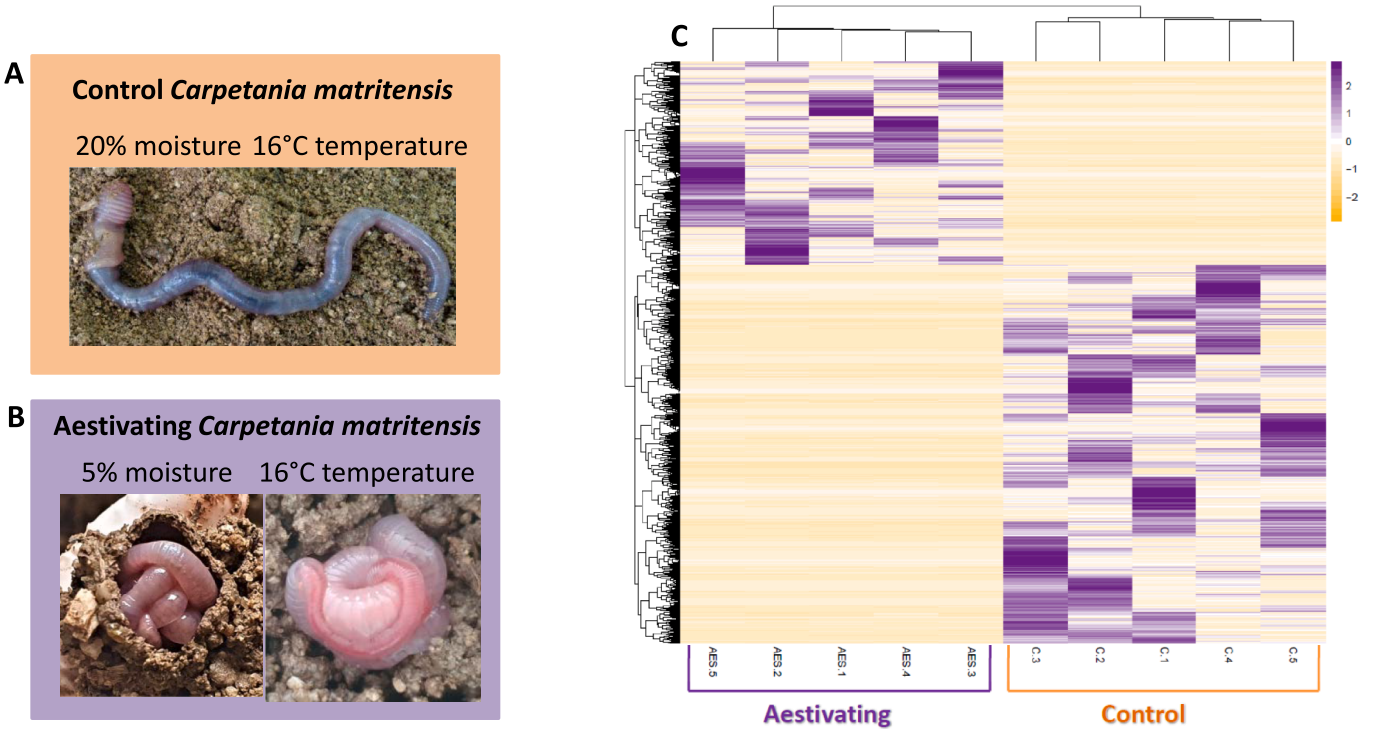

Fig. 1. Visual representation of control (A) *C.matritensis* and aestivating (B) *C.matritensis.* (C) represents the hierarchical heatmap of all DEGs based on the centered and normalized log10 (FKPM+1). Purple colour indicates high expression value, while yellow colour indicates low expression value. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Assembly and annotation of a reference transcriptome

A reference transcriptome representing the integration of data from both control and aestivating earthworms was assembled using Trinity 2.6.6 (Grabherr et al., 2011). Subsequently, CORSET was used to remove redundancy through hierarchical clustering (Davidson and Oshlack, 2014). BUSCO (Seppey et al., 2019) was used to perform a quantitative assessment of completeness in terms of expected gene content of the transcriptome using metazoa_odb10. The resulting contigs were functionally annotated against seven databases: Diamond 0.8.22 (Buchfink et al., 2015) was used for annotation against NR (NCBI non-redundant protein sequences), Swissprot (reviewed) and KOG (euKaryotic Orthologous Groups) with a threshold of 1e-5; NCBI BLAST (Altschul et al., 1997) was used for annotation with NT (NCBI nucleotide sequences) and a threshold of 1e-5; hmm scan in the HMMER package (http://hmmer.org) was used for protein structural domain prediction with PFAM (Finn et al., 2010) and a threshold of 0.01; Blast2GO (Götz et al., 2008) was used for annotation with GO (Gene Ontology) based on the protein annotations of NR and PFAM with a threshold of 1e-6; KAAS (KEGG Automatic Annotation Server:http://www.genome.jp/kegg/kaas/, Moriya et al., 2007) was used for annotation with KEGG (Kyoto Encyclopedia of Genes and Genome) and a threshold of 1e-5.

2.4. Differential expression and pathway analyses

Clean reads were mapped back to the assembled transcriptome with RSEM for estimating gene expression levels for each sample. Read counts for each gene were obtained and differential expression analysis between aestivating and control earthworms (five biological replicates per condition) was carried out with the DESeq2 R package. Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR) was used to adjust the resulting *P* values. Genes with an adjusted P value < 0.05 were considered as differentially expressed.

Enrichment analyses were performed in order to search shared functions among differential expressed genes and incorporate biological knowledge. Statistical GO enrichment of differentially expressed genes was performed with the R package clusterProfiler. GO terms with adjusted P value < 0.05 were considered significantly enriched. Furthermore we used the REVIGO web server (Supek et al., 2011) to facilitate the visualization and interpretation of the GO terms obtained from the GO enrichment analysis. Statistical enrichment of differentially expressed genes in KEGG pathways was also performed using KOBAS software. These enrichment analyses were performed including all the differentially expressed genes, only upregulated genes and only downregulated genes.

3. Results and discussion

3.1. Aestivating earthworms and sequencing output

At the end of the experiment (28 days), all earthworms at 5% moisture were aestivating (curled up into a tight ball in a chamber lined with mucus) and all earthworms at 20% moisture were active. All earthworms lost some weight during the experiment, but aestivating ones showed a statistically significant higher weight loss ($t(11) = -5.54$, $p = 0.000014$). They lost 2.07 ± 0.69 g while control worms lost 0.6025 ± 0.603 g.

Reads were submitted to European Nucleotide Archive (ENA) under study number PRJEB45058 (Samples ERS6482298-ERS6482307). For each sample, 30–40 million clean pair-ended reads were used for analyses (total 350 million reads for the reference transcriptome). The average GC content was 44.2%. The number of reads before and after cleaning for each sample is provided in Supplementary File 2.

3.2. Reference transcriptome

The Trinity assembly yielded 435,160 transcripts with an N50 of 1425 and an N90 of 487. The maximum length of transcript was 28,200 bp and 35.3% of the transcripts were 1000 bp or longer. Graphical representations of the length distribution of the transcripts can be seen

in Supplementary File 1. BUSCO showed 88.1% completeness and high duplication (24.4% complete and single, 63.7% complete and duplicated). Fragmentation was found in 9.5% of genes and 2.4% were missing.

Annotation details are provided in Supplementary File 1. The percentage of contigs that were annotated in at least one database was 32.43%. The databases with the highest annotation of contigs were NR and Swissprot with 31.03% and 21.72% contigs, respectively. Within the NR annotation, two annelids provided the highest hits with 32% of the annotated contigs: the polychaete *Capitella teleta* and the leech *Helobdella robusta* (19.6% and 12.4% of the highest hits, respectively). Estimates of the GO, KEGG and KOG classifications are shown in the figures in Supplementary File 1.

3.3. Characterization of differentially expressed transcripts in response to aestivation

Around 73.2–75.2% of the reads from each sample mapped against the reference transcriptome, and were therefore informative for subsequent quantitative analyses.

Hierarchical clustering analysis of DE transcripts showed a clear distinction in gene expression between the control and the aestivating earthworms (Fig. 1C). Paired comparisons of genes with an adjusted P value < 0.05 between aestivating earthworms and control earthworms yielded a total of 6352 differentially expressed genes (DEGs), with 4131 downregulated (65% of the total DEGs) and 2221 upregulated (35% of the total DEGs, Supplementary File 1). A complete list of the differentially expressed genes and significant BLAST hits is available in the Supplementary File 2.

3.4. GO and KEGG enrichment analysis

To better understand the biological function presented by the DE genes we performed GO and KEGG enrichment analysis. Complete tables of the enrichment analysis are provided in Supplementary File 2 and bar charts are provided in the Supplementary File 1. In order to get more insight into possible connections between the GO terms obtained with the differentially expressed genes (DEGs) we visualized them with the REVIGO online tool (Supplementary File 1), however we found the plotting with the R package ggplots2 to be more suitable for

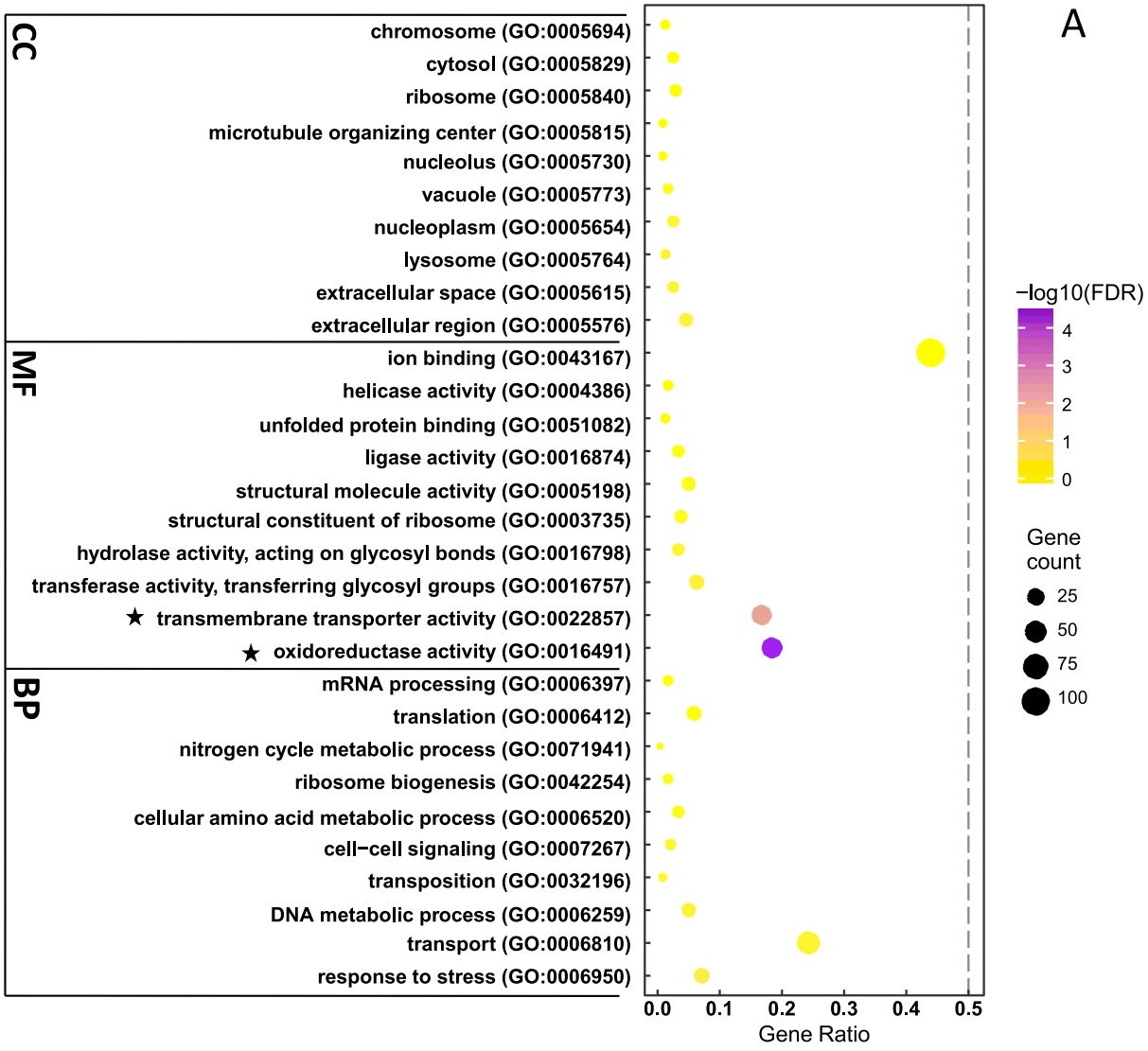

Fig. 2. Dot plot of GO enrichment analysis obtained with differentially expressed genes (DEGs). A and B represent the 10 most enriched GO terms obtained from upregulated and downregulated DEGs, respectively, in the aestivating samples. Asterisks represent significantly enriched (adjusted P value < 0.05) GO terms.

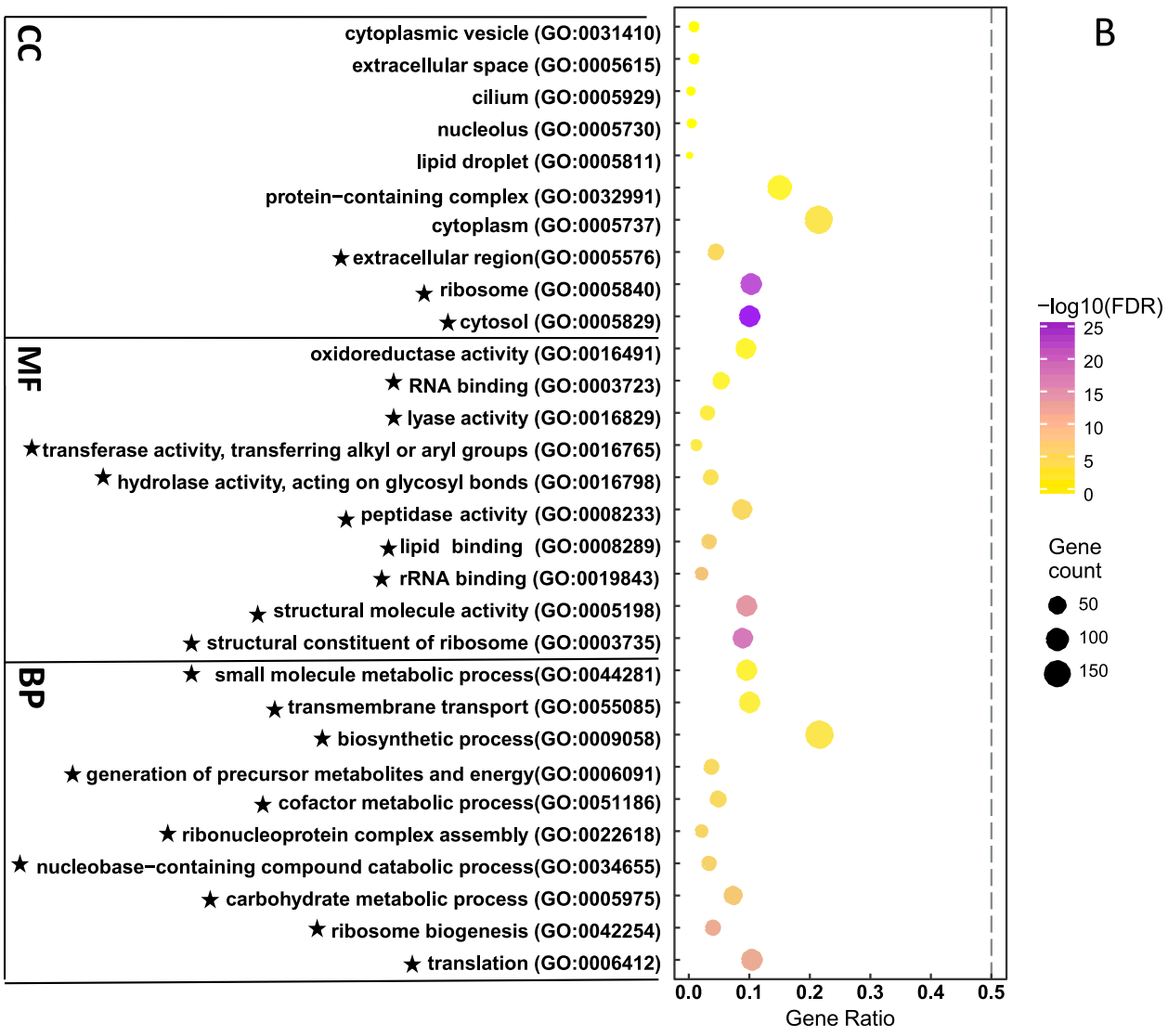

Fig. 2. (*continued*).

interpretation of the enrichment analysis. GO enrichment analysis of the downregulated DEGs yielded a bigger number of statistically enriched GO terms in all of the three categories (Fig. 2B). When performing enrichment analysis with the downregulated DEGs we got 25 statistically enriched GO terms whereas enrichment analysis with the upregulated DEGs only resulted in statistical enrichment of 2 GO terms from the molecular function category (Fig. 2A). A similar pattern was noted in the KEGG enrichment analysis (Supplementary Files 1 and 2).

Greater statistical significance with downregulated DEGs is expected considering the fact that aestivation is defined as the animals' ability to arrest activity during unfavourable arid conditions (Storey and Storey, 2012). As such, it is a survival strategy for many invertebrates and has been widely reported in land snails, sea cucumbers and earthworms (Bayley et al., 2010; Díaz Cosín et al., 2006; Giokas et al., 2007; Yang et al., 2021). Endogeic earthworm species *Carpetania matritensis* enter aestivation when confronted with prolonged periods of drought in the Iberian Peninsula. In this study we aimed to understand the transcriptomic changes that drive this hypometabolic state.

The overall downregulation of gene expression in the aestivating group demonstrates a marked suppression of energy requiring biosynthetic processes most notably translation, coupled with an overall reduction of protein turnover, anabolic metabolism suppression and some signs of cellular stress.

3.4.1. Indications of abiotic stress and possible cross- tolerance

From our enrichment results plotted in Fig. 2A, a clear trend for response to stress (GO: 0006950) is visible. Upregulated genes clustered in this GO term are summarized in Table 1. In general we saw genes for proteins involved in DNA repair such as E3 ubiquitin-protein ligase RAD18, UV excision repair protein RAD23 homolog B, N-glycosylase/DNA lyase and Apurinic-apyrimidinic endonuclease as well as genes for oxidative stress enzymes such as Protein BTG1, Catalase and Calcium-binding mitochondrial carrier protein SCaMC-1-B, which suggest that during aestivation some concentration of ROS is generated resulting in possible DNA damage. Another potential gene acting in the presence of elevated ROS concentrations is the gene for Cathepsin B (Table 1), a proteolytic enzyme with multiple functions. Shi et al. (1994) reported a correlation between increased concentrations of Cathepsin B

Table 1
Representative differentially expressed genes (DEG, p adjusted <0.05) and the proteins they encode for the different physiological responses of aestivating *C. matritensis* discussed in this study. Positive log2 fold change values represent upregulated DEGs (in dark gray), while negative log2 fold change values represent downregulated DEGs (in light gray). DEGs that were not clustered in the enriched GO terms are represented with (−).

	Gene	Protein	log2fold change	Enriched GO term
Abiotic stress response & cross - tolerance	*Lbp*	Lipopolysaccharide-binding protein	−4.986	GO:0008289
	LBP	Lipopolysaccharide-binding protein	−3.024	GO:0008289
	USP2	Ubiquitin carboxyl-terminal hydrolase 2	−4.102	GO:0008233
	BPI	Bactericidal permeability-increasing protein	−3.475	GO:0008289
	TRIM45	Tripartite motif-containing protein 45	−4.693	–
	lysoz1	Lysozyme 1	10.801	GO: 0006950
	RAD23B	UV excision repair protein RAD23 homolog B	7.192	GO: 0006950
	CAT	Catalase	9.690	GO: 0006950
	TRIM56	E3 ubiquitin-protein ligase TRIM56	4.424	–
	apn-1	Apurinic-apyrimidinic endonuclease	5.825	GO: 0006950
	OGG1	N-glycosylase/DNA lyase	8.148	GO: 0006950
	Btg1	Protein BTG1	9.008	GO: 0006950
	RAD18	E3 ubiquitin-protein ligase RAD18	4.317	GO: 0006950
	Fstl1	Follistatin-related protein 1	11.194	–
	slc25a24-b	Calcium-binding mitochondrial carrier protein SCaMC-1-B	7.059	GO: 0006950
	CYP2F3	Cytochrome P450 2F3	5.341	GO: 0016491
	CYP2U1	Cytochrome P450 2U1	4.326	GO: 0016491
	Cyp18a1	Cytochrome P450 18a1	4.692	GO: 0016491
	CTSB	Cathepsin B	9.859	GO:0008233
Apoptosis and cell cycle regulation	*Diap2*	Death-associated inhibitor of apoptosis 2	−5.533	–
	Faim	Fas apoptotic inhibitory molecule 1	−3.290	–
	Faim2	Protein lifeguard 2	−5.694	–
	Flna	Filamin-A	−10.401	–
	usp16	Ubiquitin carboxyl-terminal hydrolase 16	−4.491	GO:0008233
	His3	Histone H3	−5.190	–
	H3-3A	Histone H3.3	−7.821	–
	H2B-3	Histone H2B.3	−5.433	–
	HTAY	Histone H2A.Y	−3.871	–
	H2A.F/Z	Histone H2A.V	−6.633	–
	Birc3	Baculoviral IAP repeat-containing protein 3	4.316	–
	N/A	Apoptosis regulatory protein Siva	4.124	–
Protein turnover	*RpS15Aa*	40S ribosomal protein S15Aa	−4.984	GO:0006412 GO:0006412
	RPS18	40S ribosomal protein S18	−6.294	GO:0042254
	RpLP1	60S acidic ribosomal protein P1	−5.245	GO:0006412
	AMFR	E3 ubiquitin-protein ligase AMFR	−9.874	GO: 0006950
	RpL36	60S ribosomal protein L36	−6.756	GO:0006412 GO:0006412 GO:0042254
	rpl-12	60S ribosomal protein L12	−4.014	GO:0022618
	TEF1	Elongation factor 1-alpha 1	−6.470	GO:0006412
	EEF2	Elongation factor 2	−5.403	GO:0006412 GO:0006412 GO:0042254
	eif6	Eukaryotic translation initiation factor 6	−5.783	GO:0022618
	iff-2	Eukaryotic translation initiation factor 5A-2	−3.439	GO:0006412
	SES1	Serine–tRNA ligase	−3.793	GO:0006412
	asnS	Asparagine–tRNA ligase	−4.613	GO:0006412
	CalpB	Calpain-B	−4.999	GO:0008233
	CTSC	Dipeptidyl peptidase 1	−5.329	GO:0008233
	Fur1	Furin-like protease 1, isoforms 1/1-X/2	−7.046	GO:0008233
	Psmb7	Proteasome subunit beta type-7	−3.903	GO:0008233
	PEPD	Xaa-Pro dipeptidase	−3.944	GO:0008233
	Uchl5	Ubiquitin carboxyl-terminal hydrolase isozyme L5	−4.999	GO:0008233
	PRKG1	cGMP-dependent protein kinase	−5.223	–
	Prkag2	5'-AMP-activated protein kinase subunit gamma-1-like	5.209	–
Water loss prevention	*N/A*	Giant extracellular hemoglobin linker 2 chain	−13.322	–
Digestion	*N/A*	Chymotrypsin B	−4.577	GO:0008233
	TMPRSS15	Enteropeptidase	−4.933	GO:0008233

and ROS in ageing *D. melanogaster*. Upregulation of stress response mechanisms could serve as anticipatory preparation for the stress that follows prolonged hypometabolic states (Storey and Storey, 2010). Indeed upregulation of genes for proteins involved in DNA repair have been reported in aestivating African bullfrog (Yoshida and Kaito, 2019), while upregulation of genes for oxidative stress enzymes has been reported in land snail (Ramnanan et al., 2009), horseshoe bat (Xiao et al., 2015) and the springtail *Folsomia candida* (Timmermans et al., 2009). We found several upregulated genes for Cytochrome P450 (Table 1) enzymes within the significantly enriched GO term oxidoreductase activity (GO: 0016491) shown in Fig. 2A. The detoxifying activity of this enzyme family has been documented in the earthworm *Eisenia fetida* exposed to enrofloxacin stress (Li et al., 2018), and Doroszuk et al. (2012) reported that Cytochrome P450 had a role in the starvation resistance of *D.melanogaster* individuals. Contrastingly in mammals, namely the greater horseshoe bat, Cytochrome P540 was downregulated during torpor (Xiao et al., 2015).

Curiously downregulated genes for Bactericidal permeability-increasing proteins and Lipopolysaccharide-binding proteins (Table 1) clustered in the significantly enriched GO term lipid binding (GO:0008289, Fig. 2B) seem to contradict the upregulation trend of genes clustered in response to stress (GO:0006950) namely the antibacterial Lysozyme 1 (Table 1). Additionally genes regulating the immune response were present in varying degrees and are summarized Table 1. The downregulated gene for Ubiquitin carboxyl-terminal hydrolase 2 was clustered in significantly enriched GO term peptidase activity (GO:0008233). In *D. melanogaster* this enzyme negatively controls the Imd pathway (part of the NF-κB pathway). Namely in homeostasis this enzyme binds Imd protein to the proteasome and promotes its degradation (Engel et al., 2014). Consequently, our results seem to suggest that downregulation of this enzyme in aestivation might be involved in the regulation of the immune response. The tripartite motif (TRIM) family of proteins most of which exhibit E3 ubiquitin ligase activities regulate a variety of cellular functions that are of interest for aestivation research. Several genes for TRIM56, identified as having antiviral activity (Kawai and Akira, 2011), were upregulated in aestivating earthworms while TRIM45, a negative regulator for the NF-κB signaling pathway was downregulated (Hatakeyama, 2017). We also noted a significant upregulation of the gene for the Follistatin-related protein 1 shown in Table 1. This protein has a multi-specific binding nature, such as the CD14 protein and TLR4 receptor of the immune system. Its role as a proinflammatory factor has been studied in human cell lines as well as mouse cell lines (Murakami et al., 2012), hence we can assume that its function in the immune system response is conserved among different animal species. These findings seem to suggest some possible connection between response to abiotic stress and immune response; indeed, multiple stressors of different nature can induce the same protective mechanisms in organisms, and this phenomenon is defined as cross-tolerance (Sinclair et al., 2013).The activation of immune response has been observed in insects exposed to cold stress and summarized by Sinclair et al. (2013). Furthermore the author hypothesized the stress arising from diapause due to unfavourable conditions could create an opportunity for outgrowth of natural flora, pathogens and parasites, which may induce the hosts immune system. This interesting hypothesis might explain the transcriptional changes in genes involved in the immune system as well as antibacterial and antiviral genes in aestivating *C. matritensis*.

3.4.2. Transcriptional changes in apoptotic and cell cycle regulating genes

With the upregulation of the genes clustered in the response to stress (GO: 0006950) it is expected to see some changes in apoptotic and cell cycle regulating genes. The expression values of these genes are summarized in Table 1. For example we saw a downregulation of two genes for proteins involved in the Fas signaling pathway: Protein lifeguard and Fas apoptotic inhibitory molecule, which have a role in blocking the activation of caspase, a proteolytic enzyme in programmed cell death (Fernández et al., 2007; Schneider et al., 1999). Indeed elevated levels of negative regulators of apoptosis have been described in hibernating thirteen-lined ground squirrels as a cell protective mechanism in torpid state (Rouble et al., 2013). We saw both upregulated and downregulated genes for inhibitors of apoptosis proteins (IAPs), namely downregulated Death-associated inhibitor of apoptosis 2 and upregulated Baculoviral IAP repeat-containing protein 3. These proteins interfere with the activation of caspases and thus contribute to cell death resistance (Feoktistova et al., 2011; Hay et al., 1995). The gene for the apoptosis regulatory protein Siva, which functions as a positive regulator of apoptosis (Prasad et al., 1997) was upregulated in the aestivating group. Changes in apoptotic gene expression as a consequence of abiotic stress are oftentimes regulated by epigenetic changes. In aestivating *C. matritensis* the gene for the enzyme TAF5-like RNA polymerase II p300 (Table 1) was downregulated. This enzyme may maintain the integrity of the histone acetylase complex (HAT complex) in yeast, which enables acetylation of nucleosomal substrates (Struhl and Moqtaderi, 1998). Additionally in embryonic stem cells this protein has been shown to activate c-Myc, a well-studied transcription factor responsible for cell cycle and apoptosis (Seruggia et al., 2019), however no investigation so far has been reported on its apoptotic role in invertebrates. Aestivating worms also experienced downregulation of several genes for histone proteins (Table 1), which has been previously demonstrated in mammalian cells as a result of DNA damage. Su et al. (2004) suggest that upon DNA damage the G1 checkpoint downregulates histone gene expression through inhibition of cyclin E–Cdk2 activity. We saw the downregulation of the gene for Ubiquitin carboxyl-terminal hydrolase 16 (Table 1), clustered in the significantly enriched GO term peptidase activity (GO:0008233, Fig. 2B), which is involved in epigenetic transcriptional repression. When activated, this enzyme deubiquitinates 'Lys-120' of histone H2A and thus induces cell proliferation through mitosis (Joo et al., 2007). Suppression of said enzyme probably leads to suppression of cell proliferation in aestivating worms. Another possible mechanism that aestivating *C. matritensis* may employ in order to halt cell proliferation is the downregulation of the gene for Filamin A (Table 1). Filamin A is a versatile molecule interacting with over 30 proteins of great functional diversity (Feng and Walsh, 2004), one of which is assistance in the formation and activation of the cyclin B1-cdk1-cdc25C complex necessary for mitosis (Telles et al., 2011). Most of the research on this molecule is focused on human cells, and so far its binding pattern related to mitosis in invertebrates has not been yet investigated.

3.4.3. Downregulation of protein turnover

The clustering of downregulated genes for 60s and 40s ribosomal subunit genes, initiation factors genes and genes coding different tRNA ligases (Table 1) in the significantly enriched GO terms ribosome biogenesis (GO:0042254) and translation (GO:0006412, Fig. 2B) clearly indicate suppression of protein synthesis. This is not surprising since both of these GO terms contain processes that are ATP-expensive. Downregulation of protein synthesis has been reported in both vertebrates and invertebrates, namely in African lungfish (Loong et al., 2012), sea cucumber (Yang et al., 2021), greater horseshoe bat (Xiao et al., 2015) and land snail (Ramnanan et al., 2009) to name a few. Among downregulated genes clustered in the significantly enriched GO term peptidase activity (GO:0008233, Fig. 2B) were different cysteine and

serine proteases (Table 1) abundant in all organisms with variety of physiological functions ranging from digestive processes, immune responses, as elements in the apoptotic machinery, development, degradation of macromolecules, etc. (Grzonka et al., 2007; Krem and Di Cera, 2002). Protein degradation via the proteasome is responsible for the degradation of either damaged or normal proteins that are no longer needed (Papaevgeniou and Chondrogianni, 2014). Even though there is evidence of some abiotic stress, it seems that it's not severe enough to activate the proteasome being that the gene for the Proteasome subunit beta type-7 is downregulated (Table 1). Taking all of this into consideration we can assume that there is an overall reduction of protein turnover expressed by both the downregulation of translation genes and some proteolytic genes. Storey and Storey (2012) reported that reduced protein turnover corresponding to inhibition of the eukaryotic initiation factor and elongation factor allow for prolonged lifespan in the hypometabolic state.

Reversible phosphorylation, epigenetic changes and ubiquitination can regulate protein synthesis on different levels. Addition or removal of phosphate groups regulates many cell functions, which allow entry and maintenance of hypometabolic states, such as aestivation. In the case of protein synthesis, reversible phosphorylation controls the activity of ribosomal initiation and elongation factors (Storey and Storey, 2010), whose genes were noticeably downregulated in our samples. For example the gene for cGMP-dependent protein kinase was downregulated, while the gene for 5'-AMP-activated protein kinase subunit gamma was upregulated (Table 1). 5′-AMP-activated protein kinase a major role in regulating cellular energy metabolism, serving as a positive regulator of ATP production and it also acts as a negative regulator of protein synthesis by participating in the inhibition of elongation factors (Storey and Storey, 2012). Deubiquitinating enzymes have a broad function in the cell, many of which are involved in homeostasis maintenance during changing environmental conditions (He et al., 2016). In this respect, downregulation of the gene for E3 ubiquitin-protein ligase AMFR (Table 1) might cause suppression of the endoplasmic reticulum (ER)-associated protein degradation (ERAD). ERAD is a consequence of ER stress caused by defective ribosomal products or misfolded proteins (Shen et al., 2007). Since there seems to be a downregulation of protein synthesis and consequently low concentration of defective ribosomal products, it is reasonable to suppress ERAD. Additionally the downregulated gene for Ubiquitin carboxyl-terminal hydrolase isozyme L5 (Table 1) clustered in the significantly enriched GO term peptidase activity (GO:0008233, Fig. 2B) is responsible for the removal of ubiquitin from the distal subunit of the polyubiquitin chain, which leads to suppression of protein degradation (Ge et al., 2017). In mammalian cells the downregulation of this enzyme increases degradation of proteotoxic proteins (Matilainen et al., 2013). This indicates that even though the overall protein turnover is reduced there might be some upregulation of specific pathways intended for degradation targeting proteotoxic proteins, which might be present only in specific tissues.

3.4.4. Downregulation of digestion

As previously mentioned the aestivating earthworms showed a significant weight loss of 2.07 ± 0.69 g. In accordance with our results, earlier investigations on aestivation mechanisms of *C. matritensis* also demonstrate a decrease of mean body mass (Díaz Cosín et al., 2006). While in the aestivation chamber the earthworms' metabolic activity reduces; all feeding stops and the worms empty their gut (Michon, 1954; Saussey, 1966). KEGG analysis demonstrated the suppression of the digestive system in the form of vitamin digestion and absorption (ko04977) and protein digestion and absorption (ko04974) shown in Supplementary File 1 and Supplementary File 2. Additionally we saw clustering of downregulated genes for digestive enzymes such as Chymotrypsin B, and Enteropeptidase (Table 1) in the significantly enriched GO term peptidase activity (GO:0008233, Fig. 2B). Reduced activity of some digestive enzymes including Chymotrypsin has been demonstrated in diapausing model organism *Nasonia vitripennis* (Wolschin and Gadau, 2009), suggesting that suppression of digestion is an important mechanism for any hypometabolic state in invertebrates. Furthermore Wang et al. (2015) reported that aestivating sea cucumbers go through apoptotic degradation of the digestive tract, which lead to reduction in energy consumption and storage

3.4.5. Downregulation of anabolic metabolism

Since any type of anabolic metabolism requires energy, the downregulation of genes clustered in the enriched GO terms biosynthetic process (GO:0009058), small molecule metabolic process (GO:0044281), and generation of precursor metabolites and energy (GO:0006091) shown in Fig. 2B, seems reasonable for a hypometabolic state such as aestivation. Downregulation of genes clustered in the significantly enriched GO term carbohydrate metabolic processes (GO:0005975, Fig. 2B) in aestivating *C. matritensis* seems to be compatible with the findings on aestivating (normoxia) African lungfish (Loong et al., 2012) and in diapausing *D. melanogaster* (Zhao et al., 2016). However Yang et al. (2021), reported aestivating sea cucumbers experienced elevated carbohydrate metabolism indicating that some aestivating species might increase their energy supply to maintain aestivation. The reduction of anabolic metabolism in aestivating *C. matritensis* seems to be supported even further with the sharp downregulation of the gene for the Giant extracellular hemoglobin linker 2 chain (Table 1). Hemoglobin is essential for oxygen transport, which drives metabolism and as such it is one of the most intensively studied proteins (Hsia, 1998; Jensen et al., 1998). In spite of all the investigation done on hemoglobin its function in animal dormancy is unclear. For example, decline of hemoglobin concentrations has been reported in juvenile female hibernating bats species *Myotis myotis* (Postawa and Nagy, 2016). In African lungfish there is an initial increase in production of hemoglobin as a result of oxidative stress and inflammation arisen through tissue reconstruction followed by a decrease during the maintenance phase of aestivation, while carbohydrate metabolism was upregulated only after arousal from aestivation (Hiong et al., 2015). These findings prompt the need to study the changes in gene expression in different aestivation phases of *C. matritensis*.

3.4.6. Possible mechanisms for water loss prevention

Reducing the rate of water loss is an important mechanism for drought tolerance in earthworms (Singh et al., 2019). KEGG enrichment analysis provided some insight on possible function of the arginine biosynthesis pathway (ko00220) (Supplementary File 1 and 2) in aestivating earthworms. Indeed increased concentration of free amino acids, in particular alanine, has been demonstrated in desiccated earthworms genus *Aporrectodea* (Holmstrup et al., 2016). So far the role of the amino acid arginine as a protector against water loss in earthworms has not been reported; however agrinine has recently been proposed as a novel osmoprotectant in springtails exposed to long-term desiccation (Holmstrup et al., 2015). Another possible option for water loss prevention in aestivating *C. matritensis* could be nitrogenous waste accumulation, since the arginine biosynthesis pathway (ko00220) encompases the urea cycle. Benoit et al. (2008) and Hadley (1994) reported that nitrogenous waste products are used by arthropods, ticks and spiders to maximize water conservation. Furthermore Storey (2005) described the link between increased urea concentrations and temperature resistance. Temperature increase goes hand in hand with soil moisture changes, so it would be interesting to investigate whether

nitrogenous waste has a double role in aestivating *C. matritensis*, which might provide this species an advantage in drought resistance.

4. Conclusion

In this study, we sequenced the transcriptomes of active and aestivating *C. matritensis* in order to gain insights into the physiological changes that control this hypometabolic state. This is the first ever in depth transcriptomic investigation done on earthworms in this condition of prolonged drought. As expected, aestivation led to a reduction of most of the major biosynthetic processes in particular anabolic processes of macromolecules, which was coupled with a decrease of protein turnover. The overall halt of the ATP wasteful primary metabolism was even further corroborated with the downregulation of genes involved in digestion. More importantly our investigation shed some light on the mechanisms for mitigation of the stress caused by aestivation. In summary, the stress experienced by aestivating *C. matritensis* is most probably of abiotic nature, categorized by ROS generation, which induced activation of expressed antioxidant proteins and DNA repair proteins. Of course stress is also expressed through changes in immune system responses and apoptosis. Lastly we stumbled upon a possible not yet investigated water retention mechanism in aestivating earthworms. Upregulation of genes, which lead to nitrogenous waste accumulation clearly have a role in mitigating aestivation stress. Future investigation should focus on transcriptional analysis of specific tissues and/or systems such as the digestive tissue, the central nervous system and the excretory system. Additionally the different stages of aestivation especially in prolonged periods should also be closely studied.

Data accessibility

Reads have been deposited to European Nucleotide Archive (ENA) and are available under study number PRJEB45058 (Samples ERS6482298-ERS6482307).

Author contributions

Marta Novo conceived the project and designed the experiment. Marta Novo also extracted the RNA from the samples and sent them for analysis. Natasha Tilikj interpreted the results and created the figures and tables. Natasha Tilikj wrote the first draft and Marta Novo contributed to its improvement. Both authors collaboratively wrote and revised the final version of the manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2021.111112.

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