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The use of muscle biomarkers for assessing physiological effects of heavy metal pollution in the greater white-toothed shrew (*Crocidura russula*)

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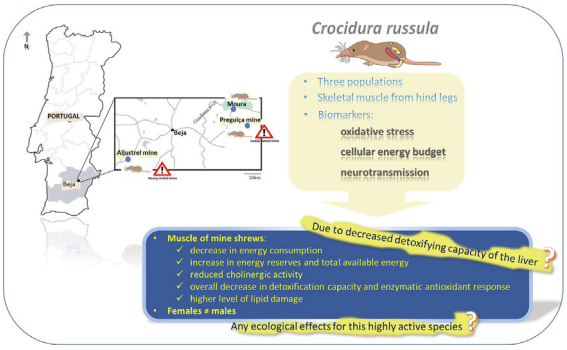
HIGHLIGHTS

- Muscle biomarkers were analyzed in the shrew *Crocidura russula* from mining areas.
- Increased lipid peroxidation in muscle shrews from mining areas shows oxidative stress.
- Shrews also showed decreased energy consumption and increased energy reserves.
- Decreased detoxification and antioxidant capacities were observed in mine shrews.
- Neuromuscular activity is lower in muscle of shrews inhabiting mining areas.

GRAPHICAL ABSTRACT



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ABSTRACT

The greater white-toothed shrew *Crocidura russula* has been used as a sentinel species for estimating environmental risks to human populations. Previous studies in mining areas have focused on the liver of shrews as the primary target of physiological and metabolic changes due to heavy metal pollution. However, populations persist even when detoxification by the liver seems to be compromised and damage is observed. These pollutant-adapted individuals inhabiting contaminated sites may exhibit altered biochemical parameters that confer increased tolerance in various tissues other than the liver. The skeletal muscle tissue of *C. russula* might be an alternative tissue that allows the survival of organisms inhabiting historically polluted sites due to the detoxification of redistributed metals. Organisms from two heavy metal mine populations and one population derived from an unpolluted site were used to determine the detoxification activities, antioxidant capacity, and oxidative damage, as well as cellular energy allocation parameters and acetylcholinesterase activity (a biomarker of neurotoxicity). Muscle biomarkers differ between shrews from polluted sites and shrews from the unpolluted location, with the mine animals showing: (1) a decreased energy consumption concomitant with increased energy reserves and total available energy; (2) reduced cholinergic activity, suggesting an impairment of neurotransmission at the neuromuscular junction; (3) an overall decrease in detoxification capacity and enzymatic antioxidant response and a higher level of lipid damage. Also, some of these markers differed between females and males. These changes may have resulted from a decreased detoxifying capacity of the liver and could potentially bring about significant ecological effects for this highly active species. Heavy metal pollution induced physiological changes in *Crocidura russula* showing that skeletal muscle may serve as a backup sink organ allowing rapid species adaptation and evolution.

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1. Introduction

The health effects of metal pollution may not only depend on the metal, dose, and duration of exposure, but also on intrinsic (age, sex, physiological, biochemical, and genetic characteristics of individuals), and environmental factors (e.g., season, food, and water availability and quality) (Wren, 1986). Metal absorption can occur through the gastrointestinal tract, skin, or lungs, and distributed through the bloodstream to various organs and tissues, where, depending on the metal, its chemical form, and the tissue itself, they tend to bioaccumulate (e.g., Komarnicki, 2000; Núñez-Nogueira et al., 2019).

Small mammals chronically exposed to environmental metals are good sentinel species for estimating risks to human populations, allowing the identification of adverse effects. Previous studies on greater white-toothed shrews *Crocidura russula* (Hermann, 1780) living in mining areas suggested that the liver may be a primary target for physiological and metabolic changes, as differences in liver metal levels, glutathione-S-transferase activity, mass, and histology were found (Marques et al., 2007; Sánchez-Chardi et al., 2007, 2008). This may be related to the important function of the liver in detoxifying deleterious compounds produced by various metals and other environmental pollutants, thereby generating reactive oxidative species (ROS) that can ultimately result in liver injury. Moreover, the liver has been shown to store several metals that can directly interact with and damage macromolecules such as lipids, proteins, and DNA (Gu and Manautou, 2012). Therefore, the detoxification and removal of drugs and toxic chemicals by the liver might be overwhelmed and compromised. According to previous works, the redistribution of drugs and toxic chemicals would be a consequence of liver detoxification failure as well as their accumulation in other organs and tissues (e.g., Almeida et al., 2012; Nunes et al., 2001; Rodrigues et al., 2022; Turna Demir and Yavuz, 2020). Nevertheless, these secondary targets may also participate in part in the detoxification processes, acting as a backup, since detoxification rates are slower in these tissues, and allowing the organisms to avoid excessive liver damage. Adaptation to chronic exposure to pollutants often involves systemic changes in pathways linked to metabolism and stress response (Pedrosa et al., 2017).

Thus, it is hypothesized that organisms inhabiting historically polluted sites can rapidly turn pollutant-adapted due to the backup provided by slow detoxification processes of secondary organs that might confer greater tolerance to pollutants when compared to animals from pristine sites. To test this possibility, the skeletal muscle tissue of the legs of the specimens of *Crocidura russula* from two populations of heavy metal mining sites and one population derived from an unpolluted site were studied to achieve its role as a preferred systemic secondary target. Skeletal muscle tissue comprises a large proportion of total body mass and may suffer from oxidative imbalance due to its important role in systemic energy homeostasis. Muscle tissue is also characterized by an abundance of mitochondria and, consequently, high ROS generation. Due to its increased nutrient demand, skeletal muscle profoundly impacts body energy consumption and systemic glucose and lipid homeostasis. Animals inhabiting polluted areas may have a high energy demand for detoxifying and oxidative responses from the liver, leading to an unbalanced trade-off and metabolic impairment in other organs, such as skeletal muscle.

In particular, for the above-referred *C. russula* specimens inhabiting heavy metal polluted-sites, it was previously found an increase in the frequency of micronuclei which could indicate a redistribution of metals in non-target organs (Sánchez-Chardi et al., 2008). Thus, this research work aims to determine the role of muscle from the same specimens, from two heavy metal-polluted sites and one non-polluted site, concerning the detoxification activities, antioxidant capacity, and oxidative damage, as well as cellular energy allocation parameters. The activity of acetylcholinesterase, an important enzyme that recycles acetylcholine at mammalian neuromuscular junctions, was also determined as a biomarker of neurotoxicity. This different approach using biomarkers of damage and defense in the skeletal muscle of shrews will show the importance of this large secondary tissue for the survival of populations inhabiting historically metal-polluted sites, and also the metabolism changes associated with adaptation processes and effects at higher levels of biological organization. This is extremely important for biomonitoring purposes, because usually non-target organs are not considered, but its use might represent an advantage since the liver of organisms is also the primary target for damage induced by pollutants.

2. Material and methods

2.1. Studied organisms and sites

The specimens of *Crocidura russula* used in this study had been previously analyzed (Marques et al., 2007; Quina et al., 2021; Sánchez-Chardi et al., 2007, 2008). Briefly, the polluted sites correspond to the Aljustrel mine (37°53′08″N; 08°08′32″W), located in the Iberian Pyrite Belt and containing large polymetallic sulphide deposits that were exploited by Phoenicians and Romans and formally operational from 1867 to 1996; and the Preguiça mine (38°02′15″N; 07°17′01″W), located in the Magnetite-Zinc Belt of the Ossa-Morena zone in the Iberian Peninsula, which operated from 1911 to 1964, with main extractions of zinc and lead ores. For comparative purposes, a site located ~72 km and 20 km, respectively, from the Aljustrel and Preguiça mining areas was chosen, without known exogenous sources of heavy metals and with a climate and vegetation comparable to those of the two mining areas (Moura; hereafter referred as the Reference site; 38°11′13″N; 07°24′34″W). Sampling took place in the spring and autumn of 2002 and 2003, and collected specimens from the three sites and sexes were equally distributed among the seasons. All captures were taken along riparian areas within the vicinity of the three sampling sites. In 2002–2003, the Aljustrel and Preguiça mines were inactive but still polluted, with the presence of manganese, iron, zinc and lead in soil and plants, and also of arsenic in Aljustrel. Metal concentrations measured in soil samples, vegetation samples and a vegetation pool can be seen in Table S1 of Quina et al. (2019).

The shrews were live-trapped, transported to the laboratory, lightly anaesthetized, and killed by cervical dislocation, in strict accordance with the directive 86/609/EEC on the protection of laboratory animals. The liver, kidneys, spleen were removed, blood samples were taken, and sex was determined. Thereafter, the animals were kept frozen (−80 °C) until sample processing for the present study in 2018 (see next section). A total of 63 shrews were studied (Females; Males): 25 from Aljustrel (14; 10; 1 undetermined), 16 from Preguiça (9; 7), and 22 from the Reference site (12; 10)

2.2. Oxidative stress, cellular energy allocation, and AChE analyses

Muscle tissue from hind legs was removed from frozen specimens, weighted, defrosted on ice, and homogenized in 1600 μL of ultrapure water using an ultrasonic homogenizer (Ystral GmbH D-7801 Dottingen). Each homogenate was split in three 300 μL aliquots for measurements of lipids content, carbohydrates + proteins content, and the activity of the electron transport system (ETS); one aliquot of 200 μL, containing 4 μL of 4 % butylated hydroxytoluene (BHT) prepared in methanol, was used for lipid peroxidation (LPO) determination; to the remaining 500 μL was added the same volume of 0.2 M K-phosphate buffer (pH 7.4) followed by centrifugation for 20 min at 9000*g* (4 °C). Aliquots of the supernatant (post-mitochondrial supernatant: PMS) were obtained for analysis of the protein content (100 μL), catalase (CAT) activity (100 μL), glutathione-S-transferase (GST) activity (250 μL), total glutathione (TG) levels (250 μL), and acetylcholinesterase (AChE) activity (250 μL). Protein concentrations at the PMS were determined at 600 nm, using the Bradford method (Bradford, 1976) adapted to microplate by Guilhermino et al. (1996) and with bovine γ-globulin as standard. Activity of AChE from PMS was determined at 414 nm using the $\varepsilon = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$ following a the Ellman's method (Ellman et al., 1961) adapted to microplate. Catalase activity was determined in PMS by measuring decomposition of the substrate H_2O_2 at 240 nm (Clairborne, 1985) adapted to microplate. Glutathione-S-transferase activity was determined following the conjugation of GSH

with 1-chloro-2,4- dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974) adapted to microplate (Rodrigues et al., 2017). Enzyme activities were expressed in nmol/min/mg protein (AChE and GST) or μmol/min/mg protein (CAT). Total glutathione content was determined at 412 nm using a recycling reaction of reduced glutathione (GSH) with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of glutathione reductase (GR) excess (Tietze, 1969; Baker et al., 1990) adapted to microplate (Rodrigues et al., 2017). TG content was calculated as the rate of TNB^{2-} formation with an extinction coefficient of DTNB chromophore formed, $\varepsilon = 14.1 \times 10^3\ M^{-1}\ cm^{-1}$ (Baker et al., 1990; Rodrigues et al., 2017), and expressed as nmol TNB^{2-}/min/mg protein. Endogenous lipid peroxidation was established by measuring thiobarbituric acid-reactive substances (TBARS) at 535 nm (Bird and Draper, 1984), and expressed as nmol TBARS/g wet tissue weight using the $\varepsilon = 1.56 \times 105\ M^{-1}\ cm^{-1}$. The sum of lipids, carbohydrates and proteins content was used to estimate the Energy Available (Ea), transforming them into energetic equivalents by enthalpy of combustion (39.5 KJ/g lipids, 17.5 KJ/g carbohydrates and 24 KJ/g proteins), according to De Coen and Janssen (1997). Total lipid contents were determined with concentrated H_2SO_4 at 375 nm, using tripalmitin as standard. Total carbohydrate contents were determined with 5 % phenol and concentrated H_2SO_4; after 30 min incubation at 25 °C, the absorbance was measured at 492 nm, using glucose as standard. Protein contents were determined at 595 nm using Bradford reagent and bovine serum albumin as standard. The Ea was expressed in mJ mg^{-1} wet tissue weight. The ETS activity (or energy consumption; Ec) was measured as the rate of INT (Iodonitrotetrazolium) reduction in the presence of the non-ionic detergent Triton X-100, with the absorbance read kinetically at 490 nm (De Coen and Janssen, 1997) adapted to microplate (Rodrigues et al., 2017). The cellular oxygen consumption rate was calculated based on the stoichiometric relationship of 1 μmol of oxygen consumed per 2 μmol of formazan formed. Energy consumption was estimated by the conversion to energetic values using the specific oxyenthalpic equivalent for an average lipid, protein and carbohydrate mixture of 480 KJ mol^{-1} O_2 (Gnaiger, 1983). The activity was expressed in mJ h^{-1} mg^{-1} wet tissue weight. Cellular Energy Allocation (CEA) was calculated according to Verslycke et al. (2003) using the formula CEA = Ea/Ec. For each sample, all biochemical analyses were performed in quadruplicate, in 96 well flat-bottom plates, at 25 °C using Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, USA).

2.3. Statistical analysis

Data was checked for normality with the Kolmogorov-Smirnov test, and for variance homoscedasticity with the Levene's test. To correct for normality, carbohydrates, ETS, LPO, CAT, CEA and Ea data were log10 transformed; lipids data was transformed with the reciprocal. For all parameters, statistically significant differences among sites or between sexes were estimated with one-way analysis of variance (ANOVA). Comparisons between pairs of sites were done with the Tukey HSD test. Statistical tests were performed using SPSS v.28 software (IBM Corp. Released 2021. IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY: IBM Corp), with the significance level set at 0.05.

3. Results

3.1. Population differences (all)

3.1.1. Muscle parameters of the oxidative response

Significant differences were found in muscle glutathione-S-transferase (GST) and catalase (CAT) activities ($F_{(2,60)} = 3872$, $p = 0.026$ and $F_{(2,60)} = 16.213$, $p < 0.001$, respectively). These differences originated mainly from the Aljustrel shrews, which had lower GST activity compared to the other two sites, and from the lower muscle CAT activities in both mine populations compared to the reference (Table 1; Fig. 1). In contrast, mean levels of total glutathione (TG) and lipid peroxidation (LPO) were higher in shrews from mining sites, although not statistically significant, compared to the values of the reference shrews.

3.1.2. Muscle parameters of energy metabolism

Mean muscle levels of energy content in carbohydrates ($F_{(2,60)} = 4437$, $p = 0.016$) and proteins ($F_{(2,60)} = 5162$, $p = 0.009$), and also in lipids (non-significant) were generally higher in the mine animals, particularly in Preguiça (Table 1; Fig. 1). Energy available (Ea) was thus higher in these animals compared to the reference (just about non-significant; $F_{(2,60)} = 2997$, $p = 0.057$). This energy was not consumed by the electron transport system (ETS), whose mean values tended to be lower in the mine shrews compared to reference shrews; conversely, cellular energy allocation (CEA) values were higher in the shrews from mining sites ($F_{(2,60)} = 3790$, $p = 0.028$), most notably in Preguiça (Table 1; Fig. 1).

3.1.3. Muscle parameters of neurotransmission

Mean muscle values in acetylcholinesterase (AChE) activity were lower in the shrews from polluted sites ($F_{(2,60)} = 5474$, $p = 0.007$), especially from Aljustrel (Table 1; Fig. 1).

3.2. Population differences by sex

3.2.1. Reference population

To identify sex-related effects in physiological parameters, we first analyzed the Reference population and statistically significant sex differences were found in muscle GST activity ($F_{(1,20)} = 8060$, $p = 0.01$), with females showing lower values than males (Table 2 and Fig. 2). This pattern was also

Table 1
Mean values and standard error of the mean (in parenthesis) of the measurements of oxidative stress, energy metabolism and neurotransmission parameters estimated from the muscle of shrews from the three sites: Ref – Reference site Alj – Aljustrel mine, Preg – Preguiça mine. Also depicted are the p values from the ANOVA analysis (comparison among sites) and the statistically significant pairwise comparisons obtained from the Tukey HSD test. The measured parameters were: total glutathione (TG; nmol TNB^{2-} min^{-1} mg^{-1} protein); glutathione S-transferase (GST) activity (nmol min^{-1} mg^{-1} protein); catalase (CAT) activity (nmol min^{-1} mg^{-1} protein); lipid peroxidation (LPO; nmol TBARS g^{-1} wt); lipid, carbohydrate and protein contents (expressed as energetic equivalents; mJ mg^{-1} wt); electron transport system (ETS; mJ h^{-1} mg^{-1} wt); cellular energy allocation (CEA; mJ mg^{-1} wt); available energy reserves (Ea; mJ mg^{-1} wt); acetylcholinesterase (AChE) activity (nmol min^{-1} mg^{-1} protein).

	Oxidative response				Energy metabolism						Neurotransmission
	TG	GST activity	CAT activity	LPO	Lipids	Carbohydrates	Proteins	ETS	CEA	Ea	AChE activity
Ref	25.17 (2.21)	4.24 (0.47)	4.01 (0.29)	287.21 (20.37)	4,178.38 (451.04)	812.09 (98.44)	3,264.54 (213.45)	437.72 (45.97)	29.42 (7.77)	8,255.01 (643.61)	29,82 (1,54)
Alj	27.11 (1.73)	2.92 (0.36)	2.94 (0.29)	312.97 (25.12)	4,333.84 (430.3)	919.09 (102.87)	4,142.99 (237.77)	398.63 (44.84)	30.25 (3.29)	9,395.93 (571.06)	22,81 (1,60)
Preg	27.55 (2.27)	4.40 (0.44)	1.87 (0.21)	398.73 (53.42)	4,992.9 (757.07)	1,488.69 (284.94)	4,689.17 (504.52)	361.23 (71.48)	39.44 (4.59)	11,170.77 (1,161.66)	25,52 (1,51)
ANOVA (p value)	0.692	0.026*	<0.001*	0.209	0.886	0.016*	0.009*	0.485	0.028*	0.057	0.007*
Tukey HSD ($p < 0.05$)			Alj/Preg Alj/Ref Preg/Ref			Preg/Ref	Preg/Ref		Preg/Ref	Preg/Ref	Alj/Ref

* Statistically significant.

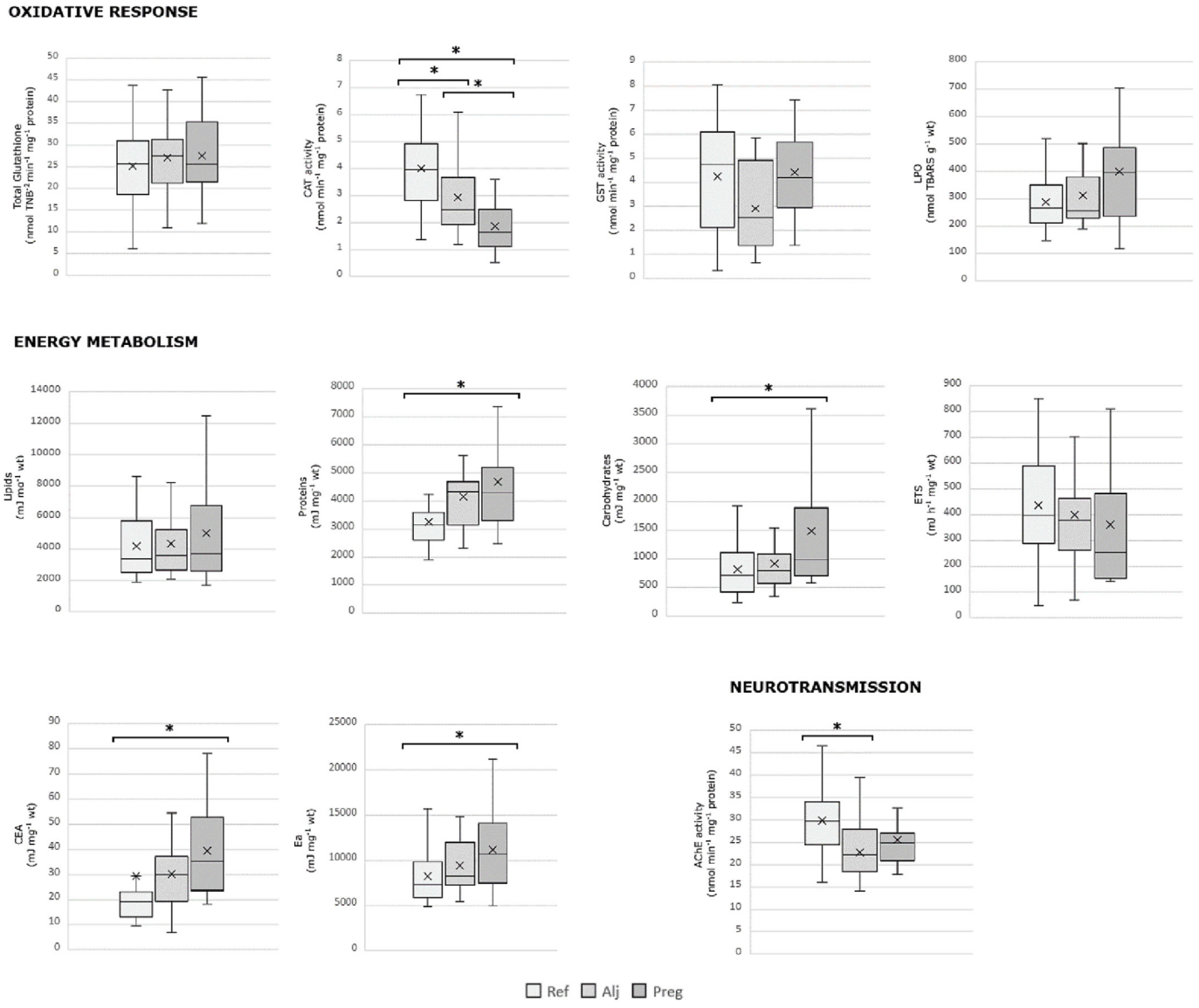

Fig. 1. Box plots of mean, median, 25 % and 75 % quartiles and maximum and minimum values of the measurements of oxidative stress, energy metabolism and neurotransmission parameters estimated from the muscle of shrews from the three sites: Ref – Reference site Alj – Aljustrel mine, Preg – Preguiça mine. Within each box plot: x - mean; line – median. Outside each box plot: minimum and maximum values. Limits of each box plot: the first and third quartiles. Measured parameters were: total glutathione (TG); glutathione S-transferase (GST) activity; catalase (CAT) activity; lipid peroxidation (LPO); lipid, carbohydrate and protein contents; electron transport system (ETS); cellular energy allocation (CEA); available energy reserves (Ea); acetylcholinesterase (AChE) activity. * statistically significant (Tukey HSD test).

observed in the mean values of TG and LPO, while catalase activities in female muscles were higher than in males. No statistical differences between the sexes were found in AChE activity and muscle parameters of energy metabolism, although apparent differences were observed in mean values for lipids, CEA and Ea (higher in females), and in ETS and carbohydrates content (higher in males) (Table 2 and Fig. 2).

Table 2
Mean values and standard error of the mean (in parenthesis) of the measurements of oxidative stress, energy metabolism and neurotransmission parameters estimated from the muscle of female and male shrews from the Reference site. Also depicted are the p values from the ANOVA analysis (comparison between sexes). See Table 1 for the description of the measured parameters.

	Oxidative response				Energy metabolism						Neurotransmission
	TG	GST activity	CAT activity	LPO	Lipids	Carbohydrates	Proteins	ETS	CEA	Ea	AChE activity
Females	22.31 (2.9)	3.19 (0.49)	4.38 (0.43)	268.55 (23.52)	4,414.13 (682.95)	686.88 (128.48)	3,320.61 (259.13)	394.44 (61.84)	39.22 (13.79)	8,421.62 (868.99)	30,61 (1,71)
Males	28.61 (3.21)	5.5 (0.67)	3.57 (0.35)	309.61 (34.89)	3,895.48 (586.47)	962.34 (144.68)	3,197.24 (366.7)	489.66 (68.47)	17.66 (1.71)	8,055.07 (1,005.36)	28,88 (2,76)
ANOVA (p value)	0.16	0.01*	0.281	0.417	0.854	0.151	0.781	0.229	0.174	0.747	0.588

* Statistically significant.

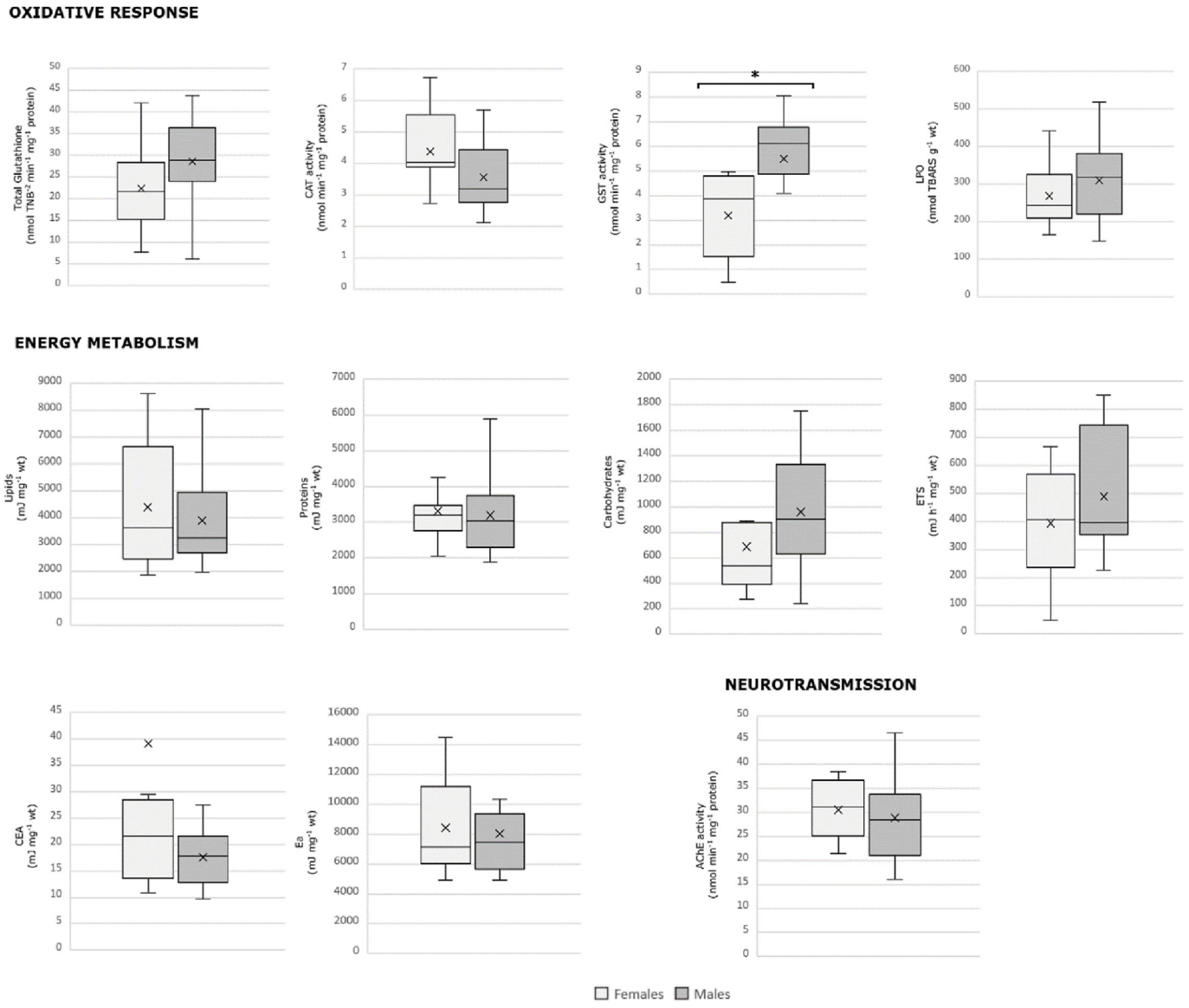

Fig. 2. Box plots of mean, median, 25 % and 75 % quartiles and maximum and minimum values of the measurements of oxidative stress, energy metabolism and neurotransmission parameters estimated from the muscle of female and male shrews from the Reference site. See Fig. 1 for box plot description and of the measured parameters. * statistically significant (ANOVA test).

3.2.2. Females

Statistically significant differences were found in carbohydrates content ($F_{(2,32)} = 5598$, $p = 0.008$) and CAT activity ($F_{(2,32)} = 9342$, $p < 0.001$), which were respectively higher and lower in the mine females, especially in the Preguiça population (Table 3 and Fig. 3). Other energy metabolism parameters also tended to be higher in mine females than in females from the reference site, such as mean muscle protein content and Ea, but the energy consumed by the ETS was lower. A contrasting pattern was observed

Table 3
Mean values and standard error of the mean (in parenthesis) of the measurements of oxidative stress, energy metabolism and neurotransmission parameters estimated from the muscle of female shrews from the three sites: Ref – Reference site Alj – Aljustrel mine, Preg – Preguiça mine. Also depicted are the p values from the ANOVA analysis (comparison among sites) and the statistically significant pairwise comparisons obtained from the Tukey HSD test. See Table 1 for the description of the measured parameters.

	Oxidative response				Energy metabolism						Neurotransmission
	TG	GST activity	CAT activity	LPO	Lipids	Carbohydrates	Proteins	ETS	CEA	Ea	AChE activity
Ref	22.31 (2.9)	3.19 (0.49)	4.38 (0.43)	268.55 (23.52)	4,414.13 (682.95)	686.88 (128.48)	3,320.61 (259.13)	394.44 (61.84)	39.22 (13.79)	8,421.62 (868.99)	30,61 (1,71)
Alj	25.67 (2.01)	2.79 (0.51)	2.91 (0.35)	312.0 (36.17)	4,431.43 (643.72)	913.70 (135.81)	3,743.73 (260.47)	387.99 (54.03)	28.58 (3.87)	9,088.87 (815.12)	24,29 (2,14)
Preg	24.15 (2.49)	4.28 (0.58)	1.92 (0.34)	350.92 (60.17)	4,046.12 (795.95)	1,618.9 (357.68)	3,956.67 (412.48)	316.71 (75.01)	37.09 (5.87)	9,621.69 (1,248.99)	26,15 (2,55)
ANOVA (p value)	0.604	0.164	<0.001*	0.569	0.709	0.008*	0.35	0.632	0.482	0.727	0.097
Tukey HSD ($p < 0.05$)			Preg/Ref			Preg/Ref					

* Statistically significant.

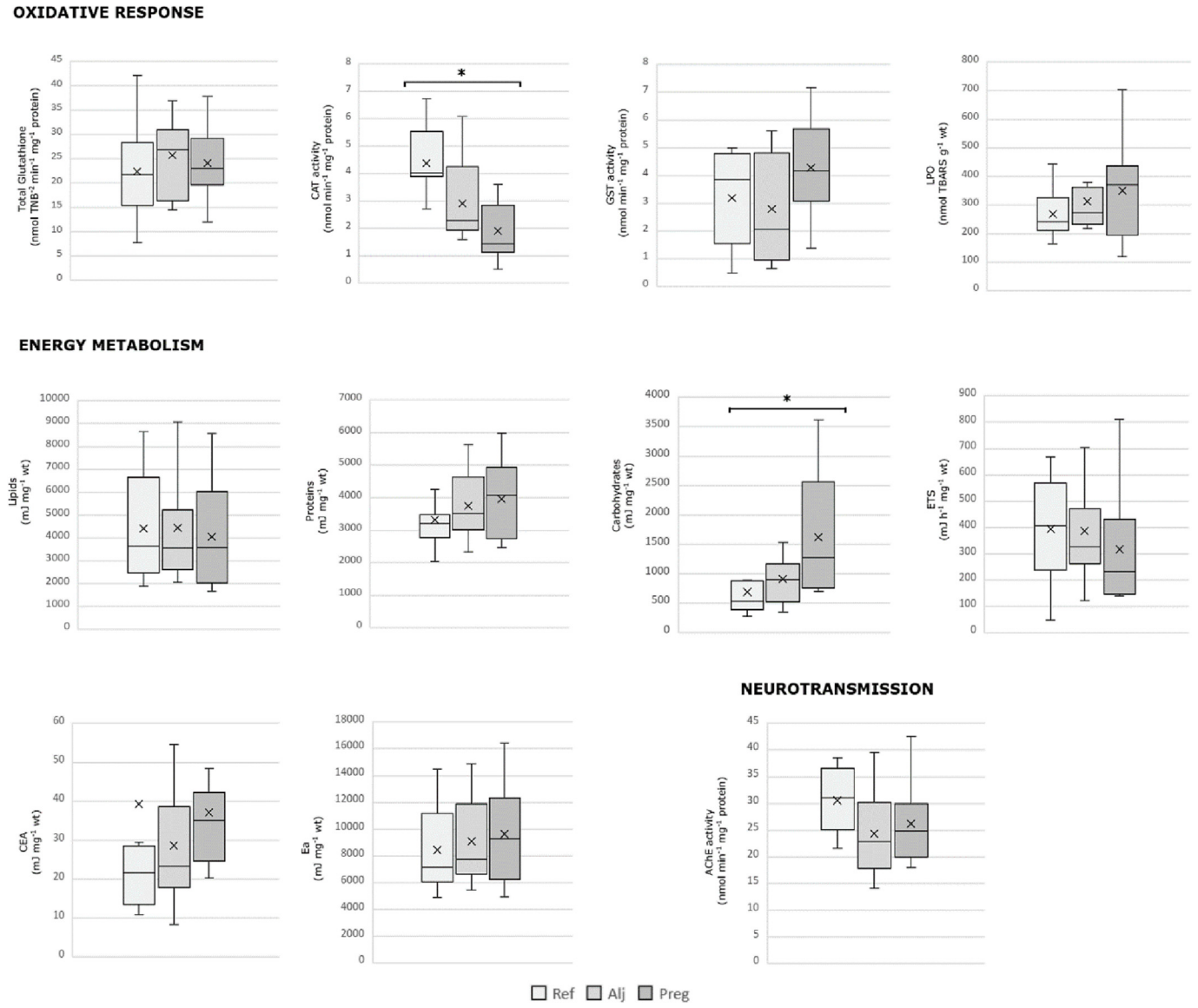

Fig. 3. Box plots of mean, median, 25 % and 75 % quartiles and maximum and minimum values of the measurements of oxidative stress, energy metabolism and neurotransmission parameters estimated from the muscle of female shrews from the three sites: Ref – Reference site Alj – Aljustrel mine, Preg – Preguiça mine. See Fig. 1 for box plot description and of the measured parameters. * statistically significant (Tukey HSD test).

in the mean values of muscle GST activity, which were higher in the Preguiça females and lower in Aljustrel compared to the reference site. Mean values of AChE activity tended to be lower in the female shrews from both mines.

3.2.3. Males

Compared with males from the Reference site, males from the mine populations had lower mean values of GST and CAT activities ($F_{(2,24)}$ = 3635, p = 0.042 and $F_{(2,24)}$ = 6391, p = 0.006, respectively; Table 4

Table 4
Mean values and standard error of the mean (in parenthesis) of the measurements of oxidative stress, energy metabolism and neurotransmission parameters estimated from the muscle of male shrews from the three sites: Ref – Reference site Alj – Aljustrel mine, Preg – Preguiça mine. Also depicted are the p values from the ANOVA analysis (comparison among sites) and the statistically significant pairwise comparisons obtained from the Tukey HSD test. See Table 1 for the description of the measured parameters.

	Oxidative response				Energy metabolism						Neurotransmission
	TG	GST activity	CAT activity	LPO	Lipids	Carbohydrates	Proteins	ETS	CEA	Ea	AChE
Ref	28.61	5.5	3.57	309.61	3,895.48	962.34	3,197.24	489.66	17.66	8,055.07	28,88
	(3.21)	(0.67)	(0.35)	(34.89)	(586.47)	(144.68)	(366.7)	(68.47)	(1.71)	(1,005.36)	(2,76)
Alj	29.84	3.13	3.07	320.46	4,304.45	939.31	4,646.19	413.67	33.40	9,889.95	20,85
	(3.17)	(0.59)	(0.55)	(38.91)	(618.11)	(181.72)	(428.17)	(86.35)	(6.3)	(885.48)	(2,65)
Preg	31.92	4.56	1.79	460.19	6,210.19	1,321.28	5,630.97	418.47	42.45	13,162.44	24,70
	(3.64)	(0.73)	(0.25)	(94.76)	(1,322.91)	(486.15)	(948.71)	(136.29)	(7.67)	(1,965.1)	(1,32)
ANOVA (p value)	0.799	0.042*	0.006*	0.282	0.265	0.739	0.02*	0.49	0.01*	0.031*	0.082
Tukey HSD (p < 0.05)		Alj/Ref	Preg/Ref				Preg/Ref		Preg/Ref	Preg/Ref	

* Statistically significant.

and Fig. 4). Like females, Aljustrel males had the lowest mean value of GST activity, while CAT activity was lower in Preguiça. Regarding energy metabolism parameters, males from the mines (especially from Preguiça) had higher mean values in protein content ($F_{(2,24)} = 4635$, $p = 0.02$), cellular energy allocation ($F_{(2,24)} = 5586$, $p = 0.01$) and energy available ($F_{(2,24)} = 4049$, $p = 0.031$), than males from the Reference site. Lipid content also tended to be higher in males from the mines, while ETS activity was lower. Mean values of AChE activity were lower in males from the mines compared to the Reference males, as observed for females from the mines.

3.3. Comparative analyses

In most parameters, the mine animals responded to heavy metal pollution in similar ways compared to the Reference population (Table 5). The main difference was the intensity of the responses, which, except for AChE activity, were often higher in the shrews of Preguiça than in Aljustrel. Both females and males from the mine populations also tended to respond in the same direction, i.e., a similar trend. An exception was the values of GST activity which, when compared to Reference females, were higher in Preguiça females and lower in Aljustrel females, while males from both mines had lower values than males from the Reference site. This difference stems from the physiological discrepancy between females and males of this species in GST muscle activity (3.19 and 5.5 nmol/min/mg protein, respectively), which was not observed in the mine animals. In the Reference population, the values of energy metabolism parameters also tended to differ between the sexes, especially Ea, which was higher in females than in males. This resulted from the higher lipid content in females (the most energetic nutrient) while male muscles had a higher carbohydrate content (less energetic nutrient) (Table 2). This difference was not observed in the mine animals where males had higher Ea values compared to females, most noticeable in Preguiça (Tables 3 and 4). Furthermore, although Ea increased in both males and females of the mine populations, the sources of energy reserves differed between them: females increased proportionally in muscle carbohydrate content, while males increased in protein (Fig. 5).

These different responses to heavy metal pollution between females and males may help explain the major differences observed between mining populations and reference: females increased in carbohydrate content, while males showed large increases in protein content, Ea and CEA. On the other hand, both sexes showed a decrease in catalase activity and cholinergic neurotransmission in the skeletal muscle.

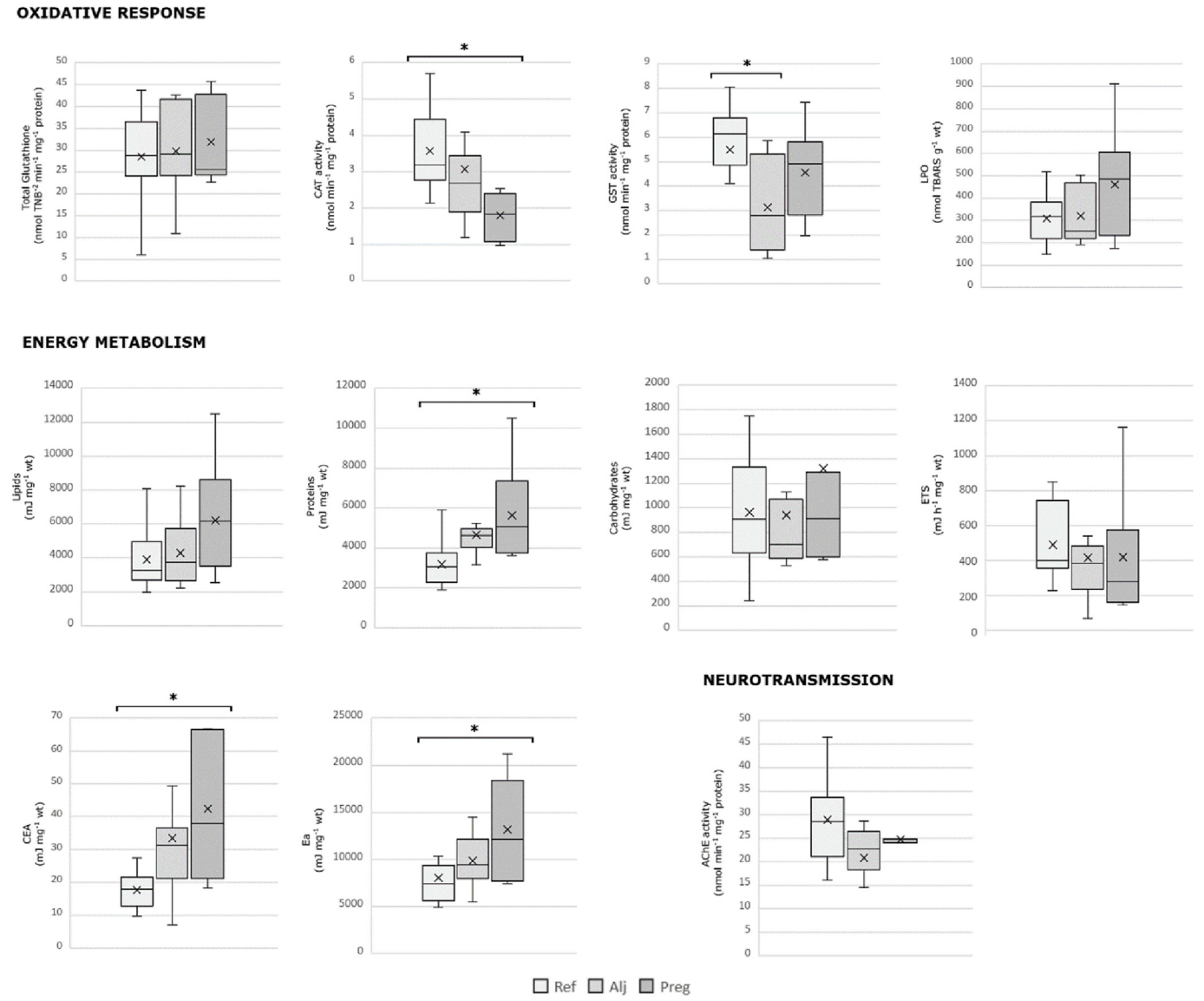

Fig. 4. Box plots of mean, median, 25 % and 75 % quartiles and maximum and minimum values of the measurements of oxidative stress, energy metabolism and neurotransmission parameters estimated from the muscle of male shrews from the three sites: Ref – Reference site Alj – Aljustrel mine, Preg – Preguiça mine. See Fig. 1 for box plot description and of the measured parameters. * statistically significant (Tukey HSD test).

Table 5
Summary table depicting the direction and amount of change in the mean values of the measurements of oxidative stress, energy metabolism and neurotransmission parameters estimated from the muscle of shrews from each mine site compared to shrews from the Reference site. Gray cells indicate statistically significant pairwise comparisons (Tukey HSD test).

	ALL		FEMALES		MALES	
	PREGUIÇA	ALJUSTREL	PREGUIÇA	ALJUSTREL	PREGUIÇA	ALJUSTREL
OXIDATIVE RESPONSE						
GST activity	≈	↓↓	↑↑	↓	↓	↓↓
CAT activity	↓↓↓	↓	↓↓↓	↓↓	↓↓	↓
TG	≈	≈	≈	↑	↑	≈
LPO	↑↑	≈	↑↑	↑	↑↑	≈
ENERGY METABOLISM						
CARBOHYDRATES	↑↑↑↑	↑	↑↑↑↑↑	↑↑	↑↑	≈
PROTEINS	↑↑	↑	↑	↑	↑↑↑↑	↑↑
LIPIDS	↑	≈	≈	≈	↑↑↑	↑
Ea	↑↑	↑	↑	≈	↑↑↑	↑
ETS	↓	≈	↓	↓	↓	↓
CEA	↑↑	≈	≈	↓	↑↑↑↑↑	↑↑↑↑
NEUROTRANSMISSION						
AChE activity	↓	↓	↓	↓	↓	↓

[a] Legend: ≈ mine mean value is within ± 10 % of Reference site mean value; ↑ mine mean value is within ± 10 - 30 % of Reference site mean value; ↑↑ mine mean value is within ± 30 - 50 % of Reference site mean value; ↑↑↑ mine mean value is within ± 50 - 70 % of Reference site mean value; ↑↑↑↑ mine mean value is within ± 70 - 90 % of Reference site mean value; ↑↑↑↑↑ mine mean value is within ± 90 - 120 % of Reference site mean value; ↑↑↑↑↑↑ mine mean value is within ± 120 - 150 % of Reference site mean value.

4. Discussion

Muscle biomarkers (energy metabolism, oxidative response, and neurotransmission) showed differences between shrews from two heavy metal polluted sites and shrews from an unpolluted location indicating a poor health condition of the environmentally exposed organisms. This suggests that chronic exposure to heavy metals develop physiological adaptations in skeletal muscle helping the liver (i.e. all organism) to cope with the adverse effects of metal exposure. The results also show that between the mining specimens, stronger parameter responses were often observed in animals from Preguiça compared to Aljustrel, which can be explained by the distinct metal composition, intensity of metal extraction, landscape, and time since mining labor between these two mines (Quina et al., 2019). Despite a different impact on the adaptive process of shrews to heavy metal pollution, this adaptation seems to involve the activation of the same metabolic and stress response pathways, because the two mine populations of shrews showed a similar directional tendency in the response of the parameters.

The aerobic energy metabolism from the mine animals decreased the electron transport system (ETS) activity concomitant with an increase of energy reserves and total available energy. In addition, cholinergic activity was also reduced in the mine animals, suggesting an impairment in neurotransmission at the neuromuscular junction that could be a proxy for less active mine animals. Therefore, muscle metabolism seems to be decreased in populations of shrews inhabiting metal-polluted sites near mining areas. In shrews, energy reserves are greatly allocated to three major regulated functions (Genoud, 1988): homeostasis (involving the metabolic rate and temperature regulation), energy budget (considering, e.g., body mass, activity rate, energy saving mechanisms, foraging), and reproduction (e.g., size of the litter). Interference with this physiological balance can lead to profound ecological effects, with energetically costly traits such as locomotion, in good agreement with the decreased activity of acetylcholinesterase (e.g., foraging for feeding and home range dispersion, predation risk), thermogenesis (decreased ETS activity might compromise the body temperature), but also the reproductive success (not assessed in this current study) being potentially compromised in animals from poor quality environments such as mining areas. Changes in the contractile capacity of skeletal muscle in mine animals may result not only from the observed impaired cholinergic neurotransmission but also from the decreased electron transport system (ETS) activity, since in this species aerobic energy metabolism in skeletal muscle is prominent (mostly composed of fast-oxidative fibers) (Peters et al., 1999). Interestingly, we had previously found that the *Cytb* gene, which encodes a component of the OXPHOS pathway (complex III), is under purifying selection in the mine populations (Quina et al., 2021). This conservation of protein sequence could be related to the observed decrease in ETS function in these animals, but further studies should be done to test this possibility. In addition, some of the above-mentioned traits differ between the sexes, for example, territory defense and fecundity (Bouteiller-Reuter and Perrin, 2005), which could help explain (some of) the sex differences found in *C. russula*, both in mine and reference animals. Different quality and quantity in energy reserves (e.g., proportionally higher muscle carbohydrate and protein content in, respectively, mine

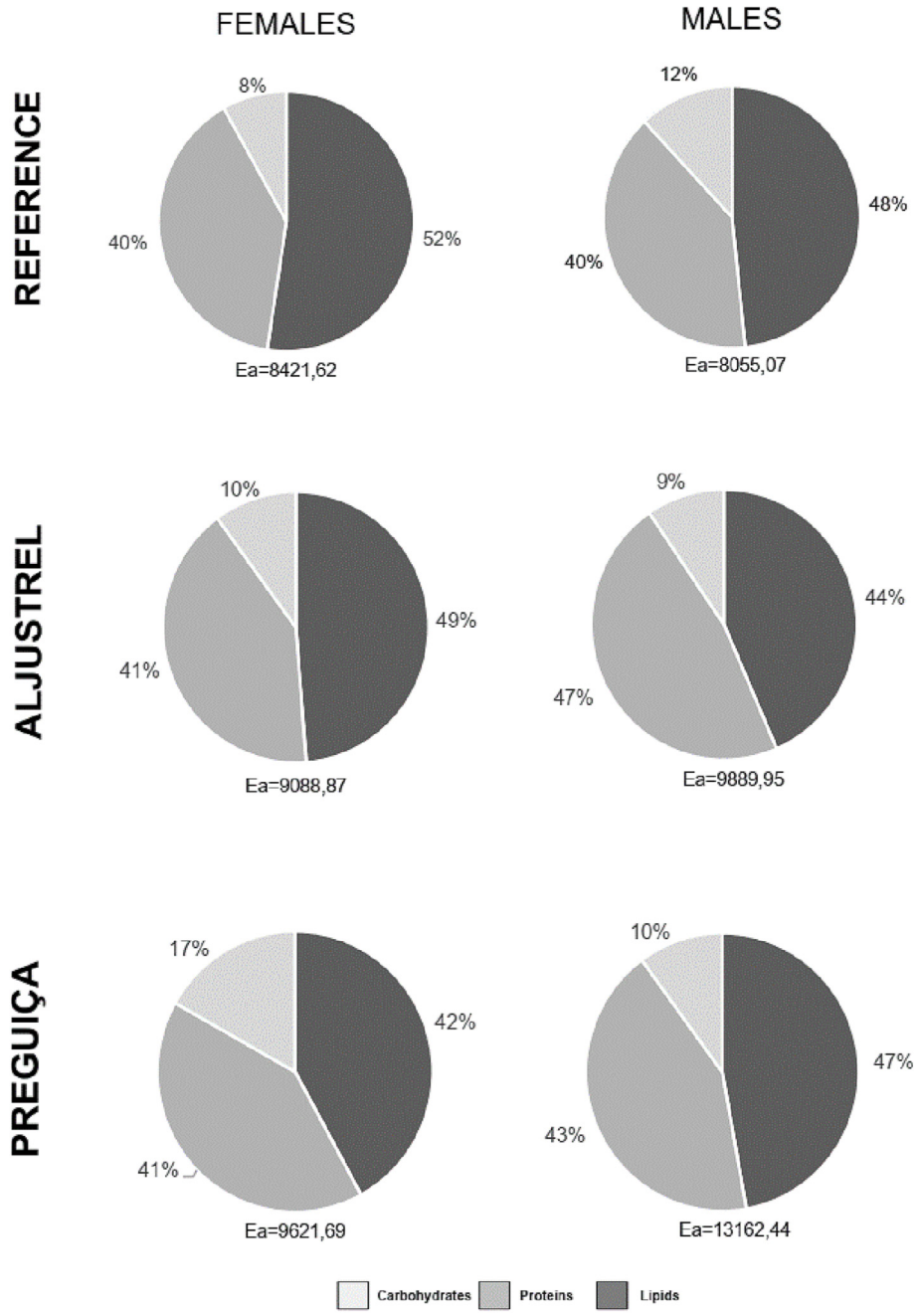

Fig. 5. Pie charts depicting the fractions of Ea (Energy available) corresponding to lipids, proteins and carbohydrates, estimated from the muscle of female and male shrews from the three sites. Available energy reserves are measured in mJ mg^{-1} wt.

females and males) may also be crucial for survival, with adaptation to a polluted environment most likely selecting for necessary adjustments in the balance of energy expenditure and intake (see e.g., (Das et al., 2020)).

An overall decrease in detoxification capacity and enzymatic antioxidant response (GST and CAT activities, respectively), which may be associated with higher level of lipid damage (LPO), was observed in the shrew populations from both mines. Given the results from other research on the effects of metals on biomarkers of oxidative stress assessed in different classes of organisms, gender and/or tissues, changes in these biomarkers from the skeletal muscle were not surprising. These results are in good agreement with the research hypothesis showing that tissue damage and genotoxic effects previously observed (Marques et al., 2007; Sánchez-Chardi et al., 2008) were indicative of metal redistribution through the body of shrews. This is clearly shown in the current study, because skeletal muscle of shrews demonstrated an oxidative stress condition. Nevertheless, the deleterious effects observed in muscle of shrews allow organisms to survive in polluted sites due to changes in detoxification, antioxidant capacity and cellular energy allocation. For example, a study performed with populations of chironomids inhabiting sites historically polluted by metals has shown constitutional higher levels of total glutathione, metallothioneins, and lipid peroxidation, as well as increased activity of the electron transport system compared to organisms from reference sites (Pedrosa et al., 2017). Female sharks were shown to accumulate more environmental mercury in their muscles than males, and present higher antioxidant defenses as well as higher levels of LPO (Rodrigues et al., 2022). Metallothionein and metal levels in liver and kidneys measured in several small mammal species were shown to be influenced by factors such as species, age, and sex (Fritsch et al., 2010). Also, previous studies performed on shrews from Preguiça and Aljustrel mines using liver biomarkers showed lower GST activity in animals from Aljustrel and

a tendency towards higher values of GST activity in shrews from Preguiça, compared to animals from the reference site, which could mean that the liver might be a confounding factor when interpreting the effects of metals on organisms historically exposed to metals (Marques et al., 2007; Sánchez-Chardi et al., 2008). This fact also shows that when liver detoxification is overwhelmed, the redistribution of pollutants leads to their accumulation in secondary target organs. In the particular case of the large skeletal muscle proportion compared to liver, makes this secondary target tissue an ideal sink for pollutants because lower detoxification rates would lead to a great accumulation, but the larger mass enables the accumulated levels of pollutants to remain tolerable in skeletal muscle. However, this "tolerance" does not mean that at the organismal level the activity of exposed shrews (less active), reproduction (low reproductive success) or even health (increased frequency of tumors) would not be compromised, but at higher levels of biological organization it seems those populations persist in mining areas. Previous research work conducted with polycyclic aromatic compounds in fish has shown that when liver detoxification is exceeded, redistribution of the parental and metabolized compounds can occur, which start to accumulate in other tissues such as muscle (Almeida et al., 2012). Also, the authors show that the levels of polycyclic aromatic compounds determined in liver and bile lack significant correlation with the biomarkers assessed in those tissues, but not when assessed in skeletal muscle and brain (Almeida et al., 2012).

Our results suggest that the survival of shrews over generations inhabiting sites polluted by metals depends on the muscle detoxification processes of metals accumulated in this particular tissue. Even presenting low detoxification and antioxidant capacity, the higher proportion of body muscle would be a factor of "dilution" of metals inside an organism and the possibility of their continuous metabolization without damaging the tissue. In fact, the liver of the mine shrews showed signs of neoplasia that could be an indication of damage due to metal detoxification incapacity, and which did not correlate with liver metal concentrations (Sánchez-Chardi et al., 2008). Histopathological alterations in liver and kidney have also been observed in other small species inhabiting mining areas (Shahsavari et al., 2019). Changes in the function of major organs can have a profound effect on the survival of the organism and, consequently, the overall fitness of the population. In the specific case of skeletal muscle, impaired mitochondrial and neuromuscular function due to higher levels of oxidative stress can lead to changes in physical performance (Jang et al., 2010), with implications for foraging and predator avoidance, for example. As such, behavioral studies of these wild shrews could be a good follow-up to the current one. Population-level effects have already been found through a genetic study in these same specimens (Quina et al., 2021), providing evidence that heavy metal pollution has a relatively rapid impact on the evolution of this small mammal species.

Thus, it is shown that in *Crocidura russula* heavy metal pollution can lead to physiological alterations that include organs other than the liver, namely skeletal muscle. For biomonitoring purposes, it would be desirable to include the use of other organs besides the primary target organ for detoxification. Since these changes may have resulted from an impaired detoxifying capacity of the liver and could potentially lead to significant ecological effects for this highly active species, the multibiomarker responses of the muscle of shrews is not only an indication of liver exceeding capacity, but also the metabolic trigger that allows rapid adaptation, evolution and persistence of populations facing moderate stress induced by metals.

5. Conclusions

The skeletal muscle of shrews inhabiting metal-polluted sites near mining areas showed decreased energy consumption, increased energy reserves and total available energy. Moreover, muscle of shrews from mining areas are facing an oxidative stress condition due to decreased detoxification and antioxidant processes. Decreased acetylcholinesterase activity in muscle of shrews of mining areas potentially brings about significant ecological effects for this highly active species. Overall, the physiological changes in *Crocidura russula* show that skeletal muscle may serve as a backup sink organ allowing rapid species adaptation and evolution. From an ecological point of view, muscle participation on detoxification and serving as a sink enables the organisms to survive in a moderate pollution scenario. Our results also highlight the importance of determining biomarkers in secondary organs because the observed responses might reflect the incapacity of liver to deal with pollutants in the environment. This would help our interpretation of biomonitoring data obtained with liver showing absence of correlation between levels of metals and effects on organisms.

CRediT authorship contribution statement

ASQ and CG conceived and designed the study. Specimens were previously collected by MLM. Experimental work and statistical analyses were performed by ASQ and ACMR. Research funding was acquired by CG, MLM and AMVMS. The first draft of the paper was written by ASQ and CG. All authors commented on previous versions of the paper. All authors have read and agreed to the final version of the paper.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare no conflict of interest.

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