

ASSESSMENT OF THE STATE OF THE TERRESTRIAL ENVIRONMENT IN SKIKDA REGION (ALGERIA) USING ENZYMATIC ACTIVITY AND ENERGY RESERVE CONTENTS OF LAND SNAILS, *CANTAREUS ASPERSUS*

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ABSTRACT: Environmental pollution is one of the most significant issues in industrial regions. Land snails are commonly used as suitable organisms to monitor environmental pollution. Therefore, the present study aims to investigate the effects of anthropogenic pollution on biochemical biomarkers in land snails, *Cantareus aspersus*. Snails were obtained from four sites of northeastern Algeria, namely El-Hadaiek, Azzaba, Ben-Azzouz (Skikda city) and El-Kala (El-Taref city). The sites of Skikda city were selected in this study because of their proximity to industrial factories, while the El-Kala site was used as a reference site (control site) for its far location from pollution sources. Moreover, the enzymatic activities of glutathione S-transferase (GST) and acetylcholinesterase (AChE), and the three energy reserves (glycogens, lipids and proteins) were measured in the digestive gland of snails collected from each site during the winter and spring seasons. The results revealed a significant variation in the AChE activity and energy reserves between sites and seasons, along with significant correlations between the enzymatic and non-enzymatic biomarkers. The highest activity of GST and total proteins were observed during spring in snails of Azzaba site, identified as the closest site to potential pollution sources. In addition, AChE activity was significantly decreased during spring, as well as depletion of lipid and glycogen contents with the highest values was recorded in the site of Azzaba. Conclusively, the land snails, *C. aspersus* are proven as sentinel organisms effectively used in biomonitoring of terrestrial environment in Skikda region.

Keywords: Pollution, *Cantareus aspersus*, Biomarkers, Energy reserves, Oxidative stress, Skikda city.

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INTRODUCTION

Skikda city is located in the Northeast of Algeria (36° 52' 34 N, 6° 54' 33 E) at 510 km from Algiers (capital of Algeria). It is an excellent industrial and economic center in the country and the capital place of petrochemical products production in the northeastern part of Algeria. Among its most towns, Azzaba which had previously included a mercury factory, but it has been closed over several years due to its serious effect on human health (Megueddem *et al.*, 2004; Benhamza *et al.*, 2008; Seklaoui *et al.*, 2016) and environmental quality (Alioua *et al.*, 2004; Kahoul *et al.*, 2014). Also, Skikda city includes many manufacturing industries, like the processing industry (wood, cork, plastic, and others), metal industry; building materials, mines and quarries, canning, agro-food industry, and textile industry. Despite the important economic role of these industries, they can cause a lot of mischiefs in city dwellers and on the city itself. As a result, Skikda city is threatened by environmental pollution (Fadel *et al.*, 2016; Zeghdoudi *et*

al., 2019), especially aquatic pollution (Kehal *et al.*, 2004; Boutefnouchet *et al.*, 2005).

The land snails, *Cantareus aspersus* (Syn: *Helix aspersa*) is the prevalent and widespread gastropod species in northeast Algeria (Larbaa and Soltani, 2013; Douafer and Soltani, 2014), and are used as bioindicators of metallic and organic pollution of soil (Gimbert *et al.*, 2006). Owing to its easy accumulation of pollutants, including heavy metals in its body (Viard *et al.*, 2004; Jordaens *et al.*, 2006). Thus, land snails have been suggested as sentinel organisms to biomonitor environmental pollution (Regoli *et al.*, 2006; Mleiki *et al.*, 2017). Whilst the accumulation of pollutants in snail tissues affects their physiology (Berger and Dallinger, 1993; Gomot *et al.*, 2000) and damages the antioxidant activities of many target organs (Ismert *et al.*, 2002). The antioxidants can effectively remove or convert reactive oxygen species (ROS) into non-harmful metabolites, and maintain the cell function and metabolism (Mates, 2000; Li *et al.*, 2003; Zhang *et al.*, 2008). The hazards of the environmental contamination on the organism's bodies

are basically assessed by determining the changes in some enzyme markers, in particular acetylcholinesterase (AChE) and glutathione-S-transferase (GST) which are often used as chemical pollution biomarkers. In addition, the alterations of glycogen, proteins, and lipids levels in tissue are used as good biomarkers in evaluating the pollution impact on the organism's health (Drobne *et al.*, 2008; Dutra *et al.*, 2009; Sancho *et al.*, 2009; Javed and Usmani, 2014; Sifi and Soltani, 2018).

In vertebrates, the family of cholinesterases is divided into two groups: acetylcholinesterase and butyrylcholinesterase. Acetylcholinesterase group is involved in the mechanisms of transmission of nervous influx through the body. Also, its inhibition by many neurotoxicants leads to the accumulation of a chemical mediator, acetylcholine, in the synaptic cleft, which thereby maintains a permanent transmission of nerve impulses, and subsequently leads to muscle tetany and death of the organism (Bocquené *et al.*, 1995; Bainy, 2000). Butyrylcholinesterase (BChE) is identified at high concentrations in several organs and tissues, including plasma, liver, lung, intestine, smooth muscle, skin, brain and heart (Massoulie *et al.*, 1993; Cokugras, 2003), however, its physiological function is still unknown (Nicolet *et al.*, 2003). In addition, BChE is a detoxifying enzyme able to trap many xenobiotics before they reach their target sites (Massoulie *et al.*, 1993; Cokugras, 2003; Nicolet *et al.*, 2003) and protect AChE against anticholinesterase substances (Whitaker, 1986). Nevertheless, most invertebrates possess one basic type of cholinesterases (AChEs) (Massoulie *et al.* 1993) having two different roles: role in the transmission of nervous influx, and analogous role to that of BuChE in vertebrates (Shi *et al.*, 2012). The enzymatic activity of AChEs has been determined in a digestive gland of the land snail, *Achatina fulica* Bowdich (Manna, 1979; Zaitseva and Kuznetsova, 2008), *Eobania vermiculata* (Itziou and Dimitriadis, 2011) and *Cantareus apertus* (Mleiki *et al.*, 2015).

Furthermore, glutathione S-transferases (GST) represent a family of multifunctional enzymes (Georges and Buchanan, 1990), involved in many physiological processes, including the detoxification of many xenobiotics (Habig *et al.*, 1974; Lauterburg and Mitchel, 1981) and protection of cell against oxidative stress (Halliwell and Gutteridge, 2007). Indeed, these enzymes catalyse the addition reaction between the xenobiotics and the -SH group of glutathione, generating consequently more hydrophilic substrates by neutralization of the electrophilic sites of xenobiotics (Hayes and Pulford, 1995; Baussant *et al.*, 2009). The utility of GST as biomarkers of chemical pollution in land snails has been well documented (El-Shenawy *et al.*, 2012; Grara *et al.*, 2012; El-Gendy *et al.*, 2019).

Although environmental pollution harmfully affects organism metabolism, in addition to many other

effects, pollution monitoring is a necessary part in terms of quantifying pollutant amounts and pollution detection problems. *C. aspersus* has been commonly used as sentinel species for monitoring metallic pollution (Larba and Soltani, 2014) and soil quality (Bairi *et al.*, 2020) in several sites of Northeast Algeria. Seasonal responses of two biomarkers (GSH and AChE) in this land snail and its relationship with the soil physic-chemical factors were performed in five sites (National Park of El Kala, EL Bouni, Sidi Amar, Nechmaya and Guelma) of Northeast Algeria (Douafer *et al.*, 2020). Since there is no corresponding study has been conducted in Skikda region. Therefore, the present study was undertaken to assess the utility of *C. aspersus* as a bioindicator in environmental pollution monitoring in some regions of this city. Our hypothesis is that land snails living near to the pollution sources and, exposed directly (inhaling industrial air pollutants) and indirectly (eating the contaminated plant) to a wide variety of pollutants can regulate their antioxidant enzyme activities and energy reserves for protection against the contamination, and therefore, these animals can be important sentinel organisms in a program of terrestrial pollution biomonitoring. To test this hypothesis, we would determine the activity of two biomarker enzymes (AChE and GST), and the content of three biomolecules (carbohydrate, protein, and lipid) in the digestive gland of land snail collected from three sites of Skikda city and El-kala which used as reference. The selected biomarkers were determined in two seasons (winter and spring) to provide sufficient understanding regarding the effect of seasonal variations on the antioxidant defense system and energy reserves in terrestrial snails.

MATERIALS AND METHODS

Sample collection: Live samples of the land snails, *Cantareus aspersus* were collected during two seasons (winter and spring) in 2017 from four sites, among which three are located in Skikda city (northeast Algeria) (El-Hadaiek (36° 49' 32" N, 6° 53' 18" E, Azzaba (36° 43' 60" N, 7° 06' 00" E) and Ben-Azzouz (36° 51' 40" N, 7° 17' 33" E)), and were selected according to their proximity to industrial units and other potential pollution sources, including the previous factory of mercury of Azzaba, and cement factory of Hadjar-soud region (about 54km from Skikda city). Meanwhile, the fourth sampling site is El Kala (36° 53' 48.55" N, 8° 26' 36.80" E) located in E-Taraf city (northeast Algeria) and was used as a control site due to its far location from motorized traffic and other anthropogenic sources of metal contamination. The selected study sites were characterized by a Mediterranean climate with a hot, dry summer and a mild, rainy winter (Remenieras, 1972). In response to these environmental factors, the snails enter in a state of inactivity, aestivation and hibernation, respectively,

during hot and cold periods, accompanied by a set of biological modifications to resist environmental stress (Storey, 2002; Reuner *et al.*, 2008). In addition, in this climate (Mediterranean climate), the reproduction of snails is at the end of spring or the beginning of summer. The seasonal temperatures of each site are listed in Table 1. The adult specimens of *C. aspersus* were collected in winter during the hibernation and in spring after the

resumption of their biological activity, and then were transported to the laboratory. Whilst, 12 specimens (weight 12.85 ± 0.9 g, shell diameter 28.50 ± 1.5 mm) from each site were rapidly killed by deep freezing in liquid nitrogen (Ismert *et al.*, 2001) and the digestive glands were dissected, weighed, frozen and stored at -20 C.

Table 1: seasonal temperatures (C°) recorded in the different studied sites.

	El-Hadaik	Azzaba	Ben-Azzouz	El-Kala
Winter	9.1	9.5	11.5	11.9
Spring	18.2	20.0	20.0	19.9

Enzymatic activity assays

Acetylcholinesterase activity: AChE activity was determined according to the method of Ellman *et al.* (1961) as previously described (Zaidi and Soltani, 2010). In brief, samples of digestive gland tissue from each site were homogenized with 1ml solution composed of 38 mg ethylene glycol tetra acetic acid (EGTA), 1 ml Triton X-100 %, 5.845 g NaCl, and 80 ml Tris buffer (0.01 M, pH 7), then the homogenate was centrifuged at 5000 rpm for 5 min, and the supernatant was used for enzyme activity determination. AChE activity was measured at 412 nm every 4 min for a period of 20 min using 5,5'-dithiobis-2-nitrobenzoic acid (DNTB), Tris buffer (0.1 M, pH 7) and acetylthiocholine iodide as substrate.

Glutathione-S-transferase activity: The glutathione-S-transferase activity was analyzed in the snail digestive gland by the method of Habig and Jakoby (1981) as previously described in Zaidi and Soltani (2011). Briefly, the digestive gland was individually homogenized in buffer phosphate (0.1 M, pH 6) and centrifuged at 1300 rpm for 30 min, and the supernatant was kept for the enzymatic determination. GST activity was determined by the addition of supernatant to the mixture GSH-CDNB in phosphate buffer (0.1 M, pH 7) using 1-chloro-2,4 dinitrobenzene (CDNB) as a substrate. Changes in absorbance were measured every 1 min for 5 min at 340 nm.

Biochemical assays: Carbohydrate, lipid and protein contents were extracted from the digestive gland following the procedure of Shibko *et al.* (1966). Each sample was individually homogenized in 1 ml of trichloroacetic acid (20%) and centrifuged at 5000 g for 10 min. The supernatant was used for the carbohydrates determination in accordance with the method of Duchateau and Florkin (1959) using anthrone as a reagent and glucose as a standard (Cerning-Beroard, 1975). Absorbance was measured at 620 nm. The obtained pellet was mixed with ether and chloroform (1V/1V) and centrifuged at 5000 g for 10 min. The resulted supernatant was used to estimate the lipid contents based

on the method of Goldsworthy *et al.* (1972) using sulphophosphovanillin as a reagent and Sunflower oil (contains more than 99% of triglyceride) as a standard (Ghorab, 2016). Absorbance was read at 530 nm. The resulted pellet was resuspended in 1ml de NaOH (0.1 N), and was used to determining total protein contents according to Bradford (1976) assay using Coomassie brilliant blue (G 250, Merck) as reagent and bovine serum albumin (BSA) as a standard (Dauvillier, 1998). Absorbance was measured at 595 nm. All biochemical assays were expressed as $\mu\text{g}/\text{mg}$ wet weight tissue.

Statistical analysis: All data are given as mean $\pm$ standard deviation (SD). The normality and homogeneity of variances were respectively tested by Levine's and Kolmogorov-Smirnov tests. Data were analyzed with one and two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Statistical tests were conducted using Minitab Software (Version 17, State College, and Penn, USA) where $p < 0.05$ was considered statistically significant.

RESULTS

Changes in the enzymatic activities: As shown in figures 1 and 2, the enzymatic activity of Acetylcholinesterase (AChE) in *C. aspersus* digestive gland from the polluted sites of Skikda city was significantly lower than that of snails collected from the reference site (El Kala) showing the highest AChE activity (8.039 ± 0.973 pM/min/mg protein, and 6.900 ± 0.874 pM/min/mg protein, in winter and spring, respectively). Besides, the lowest AChE activity was observed in the site of Azzaba during winter (4.299 ± 0.415 pM/min/mg protein) and spring (3.620 ± 0.313 pM/min/mg protein). Two-way ANOVA (site, season) revealed significant effects of site ($F_{3, 88} = 237.07$; $p < 0.001$), season ($F_{1, 88} = 151.59$; $p < 0.001$) and season-site interaction ($F_{3, 88} = 5.71$; $p = 0.001$). Tukey's test showed a significant difference ($p < 0.001$) in AChE activity in all the studied sites (El-Hadaiek, Azzaba, Ben-

Azzouz and El-Kala) between winter and spring. Hence, the enzymatic activity of AChE activity is overall, high in winter compared to that noticed in spring and differs between sites in the following order: Azzaba < El-hadaiek < Ben-Azzouz < El-Kala. Furthermore, the GST activities were significantly increased in the snails collected from the three sites of Skikda city compared to that of snails collected from the control reference site (El Kala). The higher values of GST activity were found in Azzaba in winter (1.530 ± 0.235 nM/min/mg protein), and in spring (1.729 ± 0.256 nM/min/mg protein). Two-way ANOVA

revealed a significant effects of site ($F_{3, 88} = 44.13$; $P < 0.001$) on GST activity, since the season ($F_{1, 88} = 3.51$; $P > 0.05$) and site-season interaction ($F_{3, 88} = 1.45$, $p > 0.05$) show no significant effect. On top of that, Tukey's test showed no significant change ($p > 0.05$) in the enzymatic activity of GST between the two seasons in all selected sites. Of note, the GST activity was found to be higher in spring than that seen in winter, and is different between sites in the following order: Azzaba > El-hadaiek > Ben-Azzouz > El-Kala.

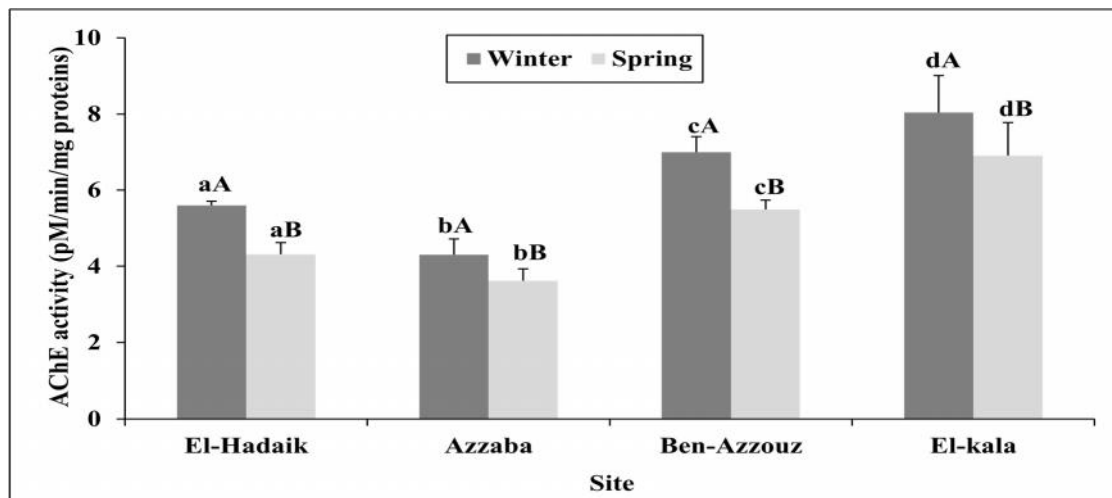


Figure 1. Acetylcholinesterase activity (pM/min/mg protein) in the digestive gland of *C. aspersus* collected from different sites during winter and spring (2017) ($m \pm SD$, $n=12$). Analysis of variance (ANOVA) followed by Tukey's test. The lowercase letters compare the values of the same season between the sites and the uppercase letters compare the values of the same site between seasons.

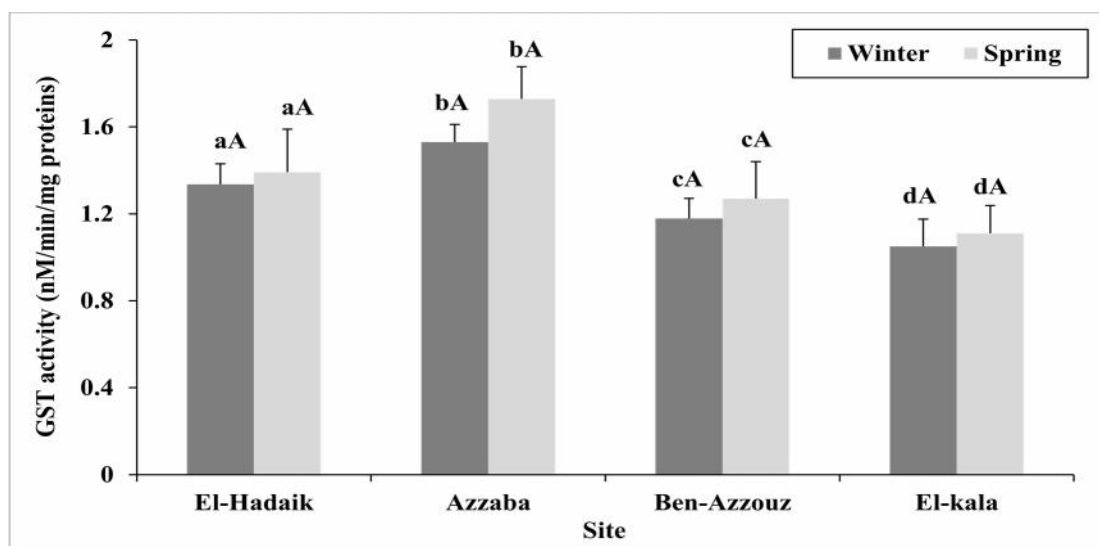


Figure 2. GST activity (nM/min/mg proteins) in the digestive gland of *C. aspersus* collected from different sites during winter and spring (2017) ($m \pm SD$, $n=12$). Analysis of variance (ANOVA) followed by Tukey's test. For each season mean values followed by the same letter are not significantly different from each other at $p > 0.05$). The lowercase letters compare the values of the same season between the sites and the uppercase letters compare the values of the same site between seasons.

Changes in energy reserve contents: The results of glycogen contents showed a significant difference ($P < 0.001$) between the sampling sites of Skikda city and the control site (El Kala). The lowest values were recorded in Azzaba during the two seasons, while the highest values were observed in El-Kala site in winter ($1.015 \pm 0.136 \mu\text{g. mg}^{-1} \text{ ww}$) and spring ($1.136 \pm 0.143 \mu\text{g. mg}^{-1} \text{ ww}$). The two-way ANOVA revealed a significant effect of sites ($F_{3, 88} = 120.62$; $P < 0.001$) and season-site interaction ($F_{3, 88} = 5.60$; $P < 0.001$), and no significant effect of season ($F_{1, 88} = 1.89$; $P = 173$) in all the study sites (Table 2). Pairwise comparisons (Tukey test) revealed a significant difference ($p < 0.05$) between the two seasons in the site of Ben-Azzouz and El-Kala, and no significant difference ($p > 0.05$) in the others sites. The lipid contents were significantly ($P < 0.01$) decreased

in samples of all study sites of Skikda city as compared with that of El Kala. This decrease was significantly marked in the site of Azzaba with levels of $0.537 \pm 0.084 \mu\text{g. mg}^{-1} \text{ ww}$, and $0.502 \pm 0.080 \mu\text{g. mg}^{-1} \text{ ww}$, respectively, in winter and spring. Further, the highest contents of lipid were found in El-Kala site in winter ($0.883 \pm 0.192 \mu\text{g. mg}^{-1} \text{ ww}$) and spring ($0.714 \pm 0.136 \mu\text{g. mg}^{-1} \text{ ww}$). The contents of lipids were higher in winter than spring. This result was confirmed by two-way ANOVA test, exhibiting a significant effect of site ($F_{3, 88} = 31.29$; $P < 0.001$) and season ($F_{1, 88} = 17.11$; $P < 0.001$) (Table 3). Moreover, Tukey's test showed a significant change ($p < 0.05$) in lipid contents between the two seasons in El-Hadaik, Ben-Azzouz and El-Kala, excluding Azzaba site ($p = 0.313$).

Table 2. Glycogen content ($\mu\text{g. mg}^{-1} \text{ ww}$) in the digestive gland of *C. aspersus* collected from different sites during winter and spring of 2017) ($m \pm \text{SD}$, $n=12$). Analysis of variance (ANOVA) followed by Tukey's test, mean values followed by the same letter are not significantly different from each other at $p > 0.05$.

	El-Hadaik	Azzaba	Ben-Azzouz	El-Kala
Winter	$0.634 \pm 0.119 \text{ aA}$	$0.561 \pm 0.088 \text{ aA}$	$0.793 \pm 0.085 \text{ bA}$	$1.015 \pm 0.136 \text{ cA}$
Spring	$0.546 \pm 0.088 \text{ aA}$	$0.516 \pm 0.084 \text{ aA}$	$0.684 \pm 0.098 \text{ bB}$	$1.136 \pm 0.143 \text{ cB}$

The lowercase letters compare the averages of the same season between the sites.

The uppercase letters compare the values of the same site between seasons.

Table 3. Total lipids ($\mu\text{g. mg}^{-1} \text{ ww}$) in the digestive gland of *C. aspersus* collected from different sites during winter and spring (2017) ($m \pm \text{SD}$, $n=12$). Analysis of variance (ANOVA) followed by Tukey's test, mean values followed by the same letter are not significantly different from each other at $p > 0.05$.

	El-Hadaiek	Azzaba	Ben-Azzouz	El-Kala
Winter	$0.627 \pm 0.056 \text{ aA}$	$0.537 \pm 0.084 \text{ aA}$	$0.657 \pm 0.044 \text{ aA}$	$0.883 \pm 0.192 \text{ bA}$
Spring	$0.567 \pm 0.068 \text{ aB}$	$0.502 \pm 0.080 \text{ aA}$	$0.571 \pm 0.081 \text{ aB}$	$0.714 \pm 0.136 \text{ bB}$

The lowercase letters compare the averages of the same season between the sites.

The uppercase letters compare the values of the same site between seasons.

In the present study, a significant increase in total protein levels was observed in the digestive gland of snails collected from the three sites of Skikda city as compared with those of snails collected from a reference site (El Kala). Total protein contents were significantly higher in Azzaba in winter ($2.191 \pm 0.189 \mu\text{g. mg}^{-1} \text{ ww}$) and spring ($2.480 \pm 0.279 \mu\text{g. mg}^{-1} \text{ ww}$). The two-way ANOVA revealed a significant effect of site ($F_{3, 88} =$

77.57 ; $P < 0.001$) and season ($F_{1, 88} = 21.44$; $P < 0.001$), along with no significant effect of season-site interaction ($F_{3, 88} = 2.02$; $P > 0.05$) (Table 4). Tukey's test showed a significant change ($p < 0.05$) in total protein contents between the two seasons in El-Hadaik, Ben-Azzouz, and Azzaba, since El-Kala site showed no significant change in this parameter ($p > 0.05$).

Table 4. Total protein ($\mu\text{g. mg}^{-1} \text{ ww}$) in the digestive gland of *C. aspersus* collected from different sites during winter and spring (2017) ($m \pm \text{SD}$, $n=12$). Analysis of variance (ANOVA) followed by Tukey's test, mean values followed by the same letter are not significantly different from each other at ($p > 0.05$).

	El-Hadaiek	Azzaba	Ben-Azzouz	El-Kala
Winter	$1.983 \pm 0.171 \text{ aA}$	$2.191 \pm 0.189 \text{ bA}$	$1.736 \pm 0.136 \text{ cA}$	$1.596 \pm 0.127 \text{ cA}$
Spring	$2.184 \pm 0.185 \text{ aB}$	$2.480 \pm 0.279 \text{ bB}$	$1.856 \pm 0.127 \text{ cB}$	$1.648 \pm 0.128 \text{ cA}$

The lowercase letters compare the averages of the same season between the sites

The uppercase letters compare the values of the same site between seasons

Correlation between the enzyme activities (AChE and GST) and the energy reserve contents (glycogen, lipids and protein) in *C. aspersus*: In table 5, statistical analyses of the correlation between the enzyme activities and the energy reserve contents in *C. aspersus* digestive gland recorded in all study sites showed a highly significant negative correlation between AChE activity and protein contents ($p < 0.001$), and between GST activity and the contents of glycogen and lipids in all sites

($p < 0.001$). Meanwhile, a highly significant correlation between GST activity and protein contents ($p < 0.001$), and between AChE activity and the lipid contents ($p < 0.001$) were observed in all sites. Besides, we noted a highly significant correlation between AChE activity and glycogen content ($p < 0.001$) in Azzaba and Ben-Azzouz. Also, AChE activity was significantly ($p < 0.01$) correlated with glycogen content in El-Hadaik site.

Table 5. Relationship between the AChE and GST activities and the glycogen, lipids and proteins content in *C. aspersus*. (R = coefficient of correlation, p = significance level).

Enzyme/site		El-Hadaiek		Azzaba		Ben-Azzouz		El-Kala	
		AChE	GST	AChE	GST	AChE	GST	AChE	GST
Glycogen	R	0.597	-0.823	0.753	-0.826	0.723	-0.723	0.277	-0.747
	P	0.002	0.000	0.000	0.000	0.000	0.000	0.190	0.000
lipids	R	0.634	-0.813	0.693	-0.761	0.780	-0.819	0.827	-0.798
	P	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
proteins	R	-0.661	0.859	-0.842	0.956	-0.703	0.829	-0.790	0.954
	P	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

DISCUSSION

Mollusks, owing to their typical properties, including wide environmental distribution, easy sampling, stress tolerance and ability to accumulate contaminants are mainly used in environmental risk and pollution assessment (Laskowski and Hopkin, 1996; Beeby and Richmond, 2002; Regoli *et al.*, 2005), such as land snails are considered as an effective biomonitor organism (Anim *et al.*, 2011; Itziou and Dimitriadis, 2011; Vega *et al.*, 2012; Radwan *et al.*, 2020). The validity of land snails, *Cantareus aspersus* as bioindicator organisms of terrestrial pollution in the region of Skikda was assessed by determination of a battery of digestive gland enzymatic and non-enzymatic biomarkers. Hence, the digestive gland was selected, in this study, as the main pollutant-target organ for the biochemical evaluation due to its ability to accumulate various pollutants, and its effective involvement in detoxification processes (Berandah *et al.*, 2010). This organ possesses an efficient antioxidant status and exhibits a model of free radical injury (Ramos-Vasconcelos *et al.*, 2005). The use of biochemical alterations of gastropods digestive glands as biomarkers of xenobiotics contamination have been investigated (Snyman *et al.*, 2005; Radwan *et al.*, 2008; Attia *et al.*, 2021)

In the present study, some biomarkers in the digestive gland of these snails, including enzymatic biomarker of neurotoxicity and immunotoxicity (AChE), enzymatic antioxidant (GST), non-enzymatic antioxidants (glycogen, total lipid and total protein) were

determined. Previous studies have identified AChE as a biomarker of chemical exposure such as pesticides (Coeurdassier *et al.*, 2002), and heavy metals (Dallinger *et al.*, 2004; de Vaufléury, 2015). The enzymatic activity of AChE of snails collected from the three sites of Skikda city is lower than that of the control site (El-Kala), indicating thus the presence of pollution in the investigated areas. In addition, AChE activity was found to be significantly lower in snails of Azzaba as compared to the other sites. The site of Azzaba is known for its proximity to potential pollution sources (factory of mercury) and thus is classified as the most polluted site. Furthermore, the site of Ben-Azzouz is located near the cement factory of Hadjar-Soud, generating contamination by the cement dust. Cement dust of Hadjar-Soud constitutes numerous types of metal oxides including calcium oxide (CaO), silicon oxide SiO₂, aluminum trioxide Al₂O₃, ferric oxide Fe₂O₃, and other components (Mekti, 2018). In accordance with this result, the decrease in AChE activity was reported in the head of *Cantareus aspersus* collected from the same north-east Algerian sites compared to snails of the reference site (El Kala). This is likely explained by the metal pollution with high concentrations of Fe, Al₂O₃, MgO and FeO (Douafer *et al.*, 2020) and Fe, Mn, Pb, and Cd (Larba and Soltani, 2014). Bairi *et al.* (2020) have reported inhibition of AChE activity in *C. aspersus* collected from the site of El Hadjar. In our study, the inhibition of AChE activity observed in snail's digestive gland from the polluted site may be due to the neurotoxic or immunotoxic effects, or both, of pollutants against this land snail (Mleiki *et al.*, 2015). Zaitseva and Kuznetsova (2008) reported that the

activity of AChE in digestive gland tissue was found in the intraepithelial nerve plexus of the gut and the digestive gland and in the nerve fibers and subepithelial neurons located in muscle cells. It was also detected in the haemocytes of blood vessels and connective tissue (Moore, 1991). Similar to our result, a decrease of AChE activity was also observed in the digestive tract of *C. aspersus* treated with ferric oxide (Besnaci *et al.*, 2019), mercury (Leomanni *et al.*, 2016), and pesticides (Radwan and Mohamed, 2013).

GST is an important stress marker in heavy-metals exposed land snails (El-Shenawy *et al.*, 2012; Abdel-Halim *et al.*, 2013). In the present study, GST activity of snails collected from the polluted sites of Skikda city was significantly increased in comparison with other snails of the reference site (El-Kala). The increase of GST activity refers to the organism's adaptation to the increased pollution stress (Astani *et al.*, 2012). Hence, the increase of GST activity could be due to the pollutants-inducing the activation of the natural antioxidant defense systems (Elia *et al.*, 2007). Our result coincides with that of Larba and Soltani (2014) who reported that GST activity was significantly increased in the digestive gland of the land snails *C. aspersus* collected from several sites in Northeast Algeria. An activation of GST has been also reported in this species collected from the same sites exposed to metallic pollution (Bairi *et al.*, 2020; Abdel-Halim *et al.*, 2013). Also, induction of GST was observed in the digestive gland of the land snails *Eobania vermiculata* collected from the metal-polluted areas (De Souza *et al.*, 2015).

The measure of energy reserves is of potential interest for assessing the health quality of terrestrial and aquatic ecosystems (El-Shenawy *et al.*, 2012; Bislmi *et al.*, 2013; Sifi and Soltani, 2018). The findings showed also a significant difference between the study sites in the levels of glycogen, lipids, and proteins of the land snail digestive gland. Furthermore, the glycogen and lipid levels were significantly declined in snails of Azzaba site when compared with those of the reference site (El-Kala). Carbohydrates are the first and immediate source of energy in living organisms (Umminger, 1977), and are importantly involved in detoxification, biotransformation, and excretion of toxicants (Wiens and Gilbert, 1967). Noteworthy, the stress condition can lead to a decrease in the carbohydrate reserves to meet the energy demands (Arasta *et al.*, 1996). Also, the results revealed a decrease in the level of carbohydrates of the digestive gland in snails collected from polluted sites as compared to the reference site (El Kala). Depletion of glycogen in this study is likely due to its mobilization to satisfy the energy demand during stressful conditions. This may result in the direct use of this compound for energy production (Ribeiro *et al.*, 2001) or from an increased rate of glycogenolysis (Radwan *et al.*, 2008). Among the overall carbohydrate molecules, especially glycogen and lipids

are mainly utilized to overcome stress. Lipids serve as a highly reduced storage form of energy and play an important role in the structure of cell membranes and intracellular organelles (Kandil *et al.*, 2009). In addition, total lipids in animals from Azzaba site were significantly lower than those found in animals of the reference site (El-Kala). This result might be explained by a drastic depletion of glycogen storage in the snail digestive gland, where lipid contents may be used for energy production (Radwan *et al.*, 2008). Also, the decrease of total lipid may be due to the increased enzymatic activity of lipase involved in the breakdown of lipid (Merad *et al.*, 2020) or reduction in synthesizing capacity of lipid (Abd-El-All, 2004)). It is reported that during chronic stress, lipid constitutes another source of energy offered to tissues when needed after carbohydrates (Moussard, 1999). Moreover, proteins are not only essential in tissue building and repair but also can, during chronic periods of stress, supply energy in metabolic activities and biochemical reactions. Our findings revealed significant differences in the total proteins (TP) between the study sites. TP content was significantly increased in the digestive gland of snails collected from Azzaba site as compared to El-kala (reference site). The increase in total protein level noticed in the present study could be attributed to the increase of protein synthesis to supply the increased energy requirement for body use. El-Shenawy *et al.* (2012) reported an increase in energy demand in the snail digestive gland under stress conditions to detoxify the toxicants and to overcome the induced stress. It is reported that Carbohydrates and lipid contents are limited in snail and the corresponding alternative source of energy to satisfy the increased energy demand in proteins (Radwan *et al.*, 2008). Moreover, the increase in total protein amount of the snail digestive gland may be partly due to the destruction of cell organelles of snails collected from polluted sites that may lead to the inhabitation of protein synthesis (Eissa *et al.*, 2002). Similarly to our results, depletions in glycogen and lipid contents with an increase in proteins were also observed in land snails after exposure to metal dust collected at the level of the iron and steel complex of EL-Hadjar (Eastern Algeria) (Grara *et al.*, 2012), pesticides (Aït Hamlet *et al.*, 2012) and Weatfert fertilizer (Attia *et al.*, 2021). On the other hand, El-Shenawy *et al.* (2012) observed an increase in the level of lipid and decrease in the protein content in the digestive gland of *E. vermiculata* snails collected from polluted areas as compared to non-polluted areas. The decrease in total protein reserves may be explained by the imbalance between the rate of protein synthesis and degradation.

Our results showed a positive correlation between AChE activity and metabolism of glycogen and lipids, and between GST activity and protein content, while, a negative correlation was noted between AChE

and protein, and between GST and the amount of glycogen and lipid. Importantly, the digestive gland of the land snails is the principal organ of pollutants detoxification, requiring high energy, and hence the energy content would be reduced in this organ.

It is reported that in land snails *C. aspersus* seasonal variations can influence the capacity of the antioxidant defense system (Ramos-Vasconcelos *et al.*, 2005) and energy reserves (Silva *et al.*, 2012). Further, the AChE and carbohydrate and lipid levels were decreased, since protein contents in the digestive gland of *C. aspersus* collected in spring compared to winter were increased. In addition, we noted no significant difference in the GST activity between the two seasons. This may be attributed to the climate change between seasons (winter and spring), physiological conditions (hibernation aestivation), and reproductive cycle. Of note, these sites are characterized by a Mediterranean climate (Remenieras, 1972). The precipitation (humidity) and temperature are important environmental climatic factors (Ramade, 2003) able to affect the species distribution (Faurie *et al.*, 2006; Somero, 2010) and vital functions of organisms by resulting in changes in biochemical and physiological processes (Somero, 1995; Kingsolver and Huey, 2008). Several studies (Dellali *et al.* 2001; Singh *et al.*, 2009; Itziou and Dimitriadis, 2012) have suggested that AChE activity is related to seasonal variability and extreme physiological conditions such as hibernation and aestivation. Inhibition of AChE activity in *C. aspersus* has been demonstrated in spring as compared to winter (Bairi *et al.*, 2020; Douafer *et al.*, 2020). Snails can regulate their defense system seasonally in response to environmental factors such as temperature and humidity (Nowakowska *et al.*, 2010; Staikou *et al.*, 2016). In contradiction of our results, Bairi *et al.* (2020) and Larba and Soltani, (2014) have reported a seasonal variation in GST activity with an increase of GST activity in the spring and summer compared to winter. Energy reserves are considered as biomarkers of stressful xenobiotic exposure (Iagadic *et al.*, 1994), but their storage and variation are closely linked to environmental factors (Rosa *et al.*, 2005). In contrast to our results, the highest values of glycogen and lipid were found in the digestive gland of *Megalobulimus oblongus* in spring compared to those found in winter (Da Silva and Zancan, 1994). These authors suggest that during hibernation, glycogen in tissues constitutes the major source of energy, while the amount of lipid in the digestive gland (hepatopancreas) remains constant in autumn and winter. The changes in the biochemical composition of the digestive gland are related to the reproductive cycle. Reductions of reserves are noted during spring, due likely to the mobilization of these reserves for reproduction and aestivation (Da Silva and Zancan, 1994). The results of this study may be explained by increasing or decreasing the proximity to

pollution sources (factory of mercury and cement factory of Hadjar-Soud).

Conclusion: This study provides original data on some enzymatic and non-enzymatic biomarkers in the digestive gland of land snails, *Cantareus aspersus*, collected from three sites in Skikda city, and El Kala town (Taref city, northeast Algeria) during winter and spring seasons. The results showed an alteration in the enzymatic activity of AChE and GST, and the energy reserves (glycogen, lipids and proteins) of snails from the sites located in the proximity of pollution sources as compared to the reference site (El Kala). A significant variation between seasons was noted in the levels of all measured biomarkers, except the GST activity. Also, the biological data suggest that Azzaba is identified as the most contaminated site because the study site is located near the factory of mercury which has been closed over several years. Further, *C. aspersus* is considered an efficient bioindicator of environmental pollution in Skikda region. These species can reduce oxidative damage by developing a defense system leading to remove or transform ROS into less toxic metabolites. In addition, changes in activities of AChE and GST and energy reserves can be used as environmental biomarkers in terrestrial animals exposed to cement dust, metals, and other contaminant in field condition. For all these reasons, we recommended the use of snail *C. aspersus* as a sentinel organism of environmental pollution by chemical pollutants that might be present in the Skikda region. Other studies must be conducted in this region to complete the present findings like the analysis of metallic elements in soil and tissues of snails and the determination of histological and molecular effects of pollutants in this species.

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