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The Use of Tail as a Minimal-Invasive Method to Detect a Large Set of Biochemical Responses in the Italian Wall Lizard *Podarcis siculus* (Rafinesque, 1810)

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Abstract: Conventional methods to analyze biochemical processes related to contaminant toxicity usually require the sacrifice of animals to collect tissues and organs. However, for ethical reasons and especially for endangered species, non- or minimal-invasive methods should be preferred. Among vertebrates, reptiles show a general decline worldwide and therefore the use of non- or minimal-invasive methods to measure some biochemical processes in these animals are encouraged. It is well known that most lizards use a common safety behavior implying the natural loss of tail in the case of predation events. Therefore, if common analyses testing contaminant toxicity could be performed in tail tissue, this method, not implying the sacrifice of the animals, could be considered as a good minimal-invasive method. The aim of this study is to test on wild Italian wall lizard *Podarcis siculus* the use of tail to detect a large set of biomarkers including oxidative stress (TOSCAROO, TOSCAOH, CAT, tGSH, MDA), biotransformation processes (EROD, GSTs) and neurotoxicity (AChE, BChE). All the biochemical responses, excluding EROD and MDA, resulted to be analytically detectable in tail tissues of *P. siculus*, although the mean values obtained with this minimal-invasive method were significantly lower than those obtained with invasive one.

Keywords: field study; reptiles; biomarker

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1. Introduction

Biomarkers are usually considered as biochemical, physiological or histological indicators of exposure to or effects of chemicals [1]. Moreover, biomarkers represent a useful method for gaining insight into the mechanisms causing observed effects of chemical compounds on whole-organism performance [2] and are widely used in environmental monitoring programs. However, most of biomarkers commonly used in these assessments require invasive methods, involving analysis in tissues and organs, such as liver and brain, after animal sacrifice, e.g., [3–6]. Moreover, the blood cells, accompanied by plasma or serum, are often used to assess animal health and a wide range of biomarkers [3]. Although blood sampling is considered a non-lethal procedure, in some cases it was performed by cardiac puncture, which could lead to death of the animal [7,8]. Instead of these methods, non-invasive or non-destructive biomarker should be preferred beyond ethical considerations in the cases where the number of animals available at a site is limited, the study involves an endangered species, or sequential samples from the same individual may be required for time course studies [3,9]. In the case of endangered vertebrate species, as in the case of species of this study, authorizations and permissions for scientific purposes are often required in a conservation framework. EU legislation regarding protection of animals used for scientific purposes is strict, even more so for strictly protected species listed under

Annex IV of the Habitat Directive [10,11]. Therefore, the implementation of less invasive techniques is encouraged, especially in the case of protected and endangered species.

Many reptile species, about 21%, are classified as threatened by IUCN [12], and among them, the Italian wall lizard *Podarcis siculus* (Rafinesque, 1810) belonging to the Lacertidae family, is listed on Annex II of the Bern Convention and Appendix IV of the EU Habitats Directive 92/43/CEE. It has a wide distribution in Europe, and it is quite abundant along the Italian peninsula; its habitat includes open fields, walls and areas extending up to 1800 m; it has a reduced mobility and has an important role in the food chain working as a link between invertebrates and higher predators. Most studies concerning biomarker analyses on *Podarcis* spp. involve destructive methods [5,13–17], and few studies exist involving non- or minimal-invasive ones. In general, most of non-destructive or minimal-invasive methods used to measure biological alterations in organisms consist of collection of blood by vein, saliva, urines, feces, part of tissue such as fur, skin, hair [3]. In recent decades, increasing attention has been paid to the use of non- or minimal-invasive methods for biomarkers in lizards. Some studies reported micronuclei frequency (MN) and comet assay performed in blood samples collected from the caudal vein of lizards, i.e., [16,18]; in several studies, serum was used as a target tissue to analyze butyrylcholinesterase (BChE) on *Gallotia gallotia* [19–22]; in other studies, BChE together with acetylcholinesterase (AChE), superoxide dismutase (SOD) and glutathione-S-transferases (GSTs) were measured in the tails of the spiny lizard *Sceloporus* spp. [23]. On the Italian wall lizard *P. siculus*, beyond a study (previously mentioned) on MN in blood sample taken by subcaudal vein [16,24], the suitability of saliva samples was evidenced as a non-invasive method to measure some enzymatic activities such as GSTs, AChE and glutathione reductase (GR), while [25] investigating some hematological parameters in white blood cells and antioxidant biomarkers such as SOD, glutathione peroxidase (GPX), total glutathione content (tGSH) and thiobarbituric acid reactive substances (TBARs) in the tail of this species. Nevertheless, although collection of tail samples of this species represents a good minimal-invasive method, only a small set of biomarkers was analyzed so far in this tissue. It is well known that in many species of lizard, tails could be obtained after a "voluntary" loss (caudal autotomy), as response to attempted predation [26]. The fracture typically happens just ahead of the segment where the lizard is caught or at most no more than three fracture planes anterior to it [27]; however, some lizards can also shed their tail without physical contact between the tail and the external stimulus [28]. The autotomy capacity of *P. siculus* was also attested by a previous study [29]. The detachment of the tail enables the lizard to escape from a predator that has seized it by this appendage. Additionally, the tail may serve as a distraction with spontaneous writhing or wriggling movements, diverting the predator's attention and facilitating the lizard's escape [26].

Tail regeneration is a common adaptation among many lizard species, although not all lizards possess this ability. Once the tail is detached, the lizard initiates a spontaneous regenerative program. The time required for complete tail regeneration depends on factors such as the lizard's age, available nutrition and environmental factors [30]. The growth rate for tail regeneration also varies according to environmental conditions and fluctuates with daily temperature and different seasons. Additionally, the level of amputation plays a role, with higher growth rates observed in proximal tail losses [31]. Different lizard species exhibit varying rates of tail regeneration. For example, in *P. siculus*, the maximum growth rate occurs between 2.5 and 3.5 weeks after amputation under summer conditions, while in *Anolis carolinensis*, the growth rate is 1.5 mm per day from 14 to 28 days after amputation at a constant 32 °C but drops to 0.15 mm per day at 21 °C from 28 to 45 days after amputation [31]. On the other hand, in *Teira dugesii,* it regenerates rapidly, with a maximum growth rate of 2.6 mm per day during the fifth week after autotomy and a 90% recovery of the original tail length after 12 weeks [32].

The number of possible regenerations, although varying with species, seems unlimited as long as the lizard is alive, but repeated autotomy can lead to reduced regeneration rates due to the significant energy and resources required for the process [32]. The aim

of this study is to assess the detectability in *P. siculus* of a large set of biomarkers, including antioxidant biomarkers, biomarkers of neurotoxicity and of biotransformation, using minimal-invasive methods involving analyses in tails. To achieve this aim, specimens of wall lizard were collected from fields, and biomarkers were analyzed in different tissues using the two methods: an "invasive method" using conventional target tissues/organs (liver and brain) obtained after animal sacrifice and a "minimal-invasive" method using tail and blood obtained by alive specimens. Moreover, BChE conventionally measured in serum was measured in tails to test this tissue as an alternative minimal-invasive method.

The set of analyses of this study consists of biomarkers of the neuronal system, such as AChE and BChE activities; biomarkers of antioxidant systems, such as catalase activity (CAT), total antioxidant capability toward hydroxyl and peroxyl radicals (TOSCA $HO^{\cdot}$, TOSCA $ROO^{\cdot}$), tGSH and the level of the lipid peroxidation product malondialdehyde (MDA); biomarkers of the biotransformation system, such as ethoxyresorufin-o-deethylase activity (EROD) and GSTs, involved in phase I and II of the process, respectively. The selected biomarkers were chosen considering previous studies using minimal invasive methods on *Podarcis* spp. and other lizard species [19,22–25], adding other biomarkers usually analyzed with invasive methods on *Podarcis* spp. [5,15–17,33,34].

The detectability of these biochemical responses was tested in tail and in conventional tissues (liver, brain, serum). Values obtained by conventional methods and using lizard tail were compared.

2. Materials and Methods

2.1. Sampling Sites and Species

Wild lizards were collected in hazelnuts (pesticides free) in the same area of the province of Viterbo, in Latium (central Italy), during summer 2018. A total of 31 specimens of *P. siculus* were captured by noose or hand in field and transported in a bag in darkness to the ISPRA laboratory, where they were maintained in a terrarium at room temperature. Once in the laboratory, each animal was labelled, measured for snout-vent length (SVL, 0.01 cm), weighed (0.01 g) and left in the terrarium until they lost their tails after induction by gently pinching the base of the tail [29]. Blood samples were obtained from subcaudal vein. Following this collection, all captured animals were euthanized through cervical dislocation and dissected to retrieve brain and liver. The number of animals sacrificed was defined according to the Italian Ministry of Environment authorization (MATTM Protocol n. 0013659 of 21 June 2018).

2.2. Biochemical Analyses

Samples of liver, brain and tail were immediately treated with liquid nitrogen and stored at −80 °C in suitable tubes. Blood samples were withdrawn by subcaudal vein using a sterile insulin syringe, previously heparin-initiated, and stored at 4 °C until analysis. Frozen tissue of liver, brain and tail were thawed on ice. Samples for AChE, GSTs, EROD, CAT), total antioxidant capability (TOSC) were homogenized (1:10 ratio w/v for liver and brain, 1:5 ratio for tail) in 0.1 M Tris-HCl buffer (pH 7.6) 0.25 M sucrose and 1 mM EDTA and centrifuged at 10,000× g for 20 min at 4 °C [5]. Those for tGSH analysis were homogenized 1:5 (w/v) in 5% sulfosalicylic acid and 4 mM EDTA, let to deproteinize on ice for 45 min and centrifuged at 37,000× g for 15 min at 4 °C; those for MDA were homogenized 1:5 (w/v) in 20 mM Tris–HCl buffer (pH 7.4) and centrifuged at 3000× g for 20 min. Each cellular extract (fraction S10, S37, S3) was frozen at −80 °C until analysis. The blood samples for BChE analysis were immediately centrifuged at 10,000× g for 10 min at 4 °C to retrieve the serum. The enzymatic activities (except BChE) were measured according to procedure reported in [16,17]. AChE activity was assessed using spectrophotometric analysis by [35] method, lightly modified; CAT activity was quantified by spectrophotometry as in [36], modified according to [37]; TOSC assays for peroxyl radicals (ROO·) and hydroxyl radicals (HO·) were performed using the gas chromatographic method of [38]; tGSH was assessed according to the enzymatic method of [39]; levels of MDA were evaluated spectrophotometrically

by [40] method; GSTs activities were determined spectrophotometrically by [41] method, slightly modified according to [37]; the inducibility of cytochrome P450 was measured in terms of EROD activity according to [42] method; serum BChE activity was quantified colorimetrically according to [35] with adjustments to optimize assay conditions [21]. Before the substrate (butyrylthiocholine iodide) at a concentration of 2 mM was added, samples were preincubated for 3 min with 5,5-dithiobis-2-nitrobenzoic acid (0.3 mM final concentration) in 25 mM Tris-HCl, 1 mM CaCl2 (pH 7.6). The reaction was monitored at 25 °C, over a 3 min period at 410 nm. The activity was expressed as nmol/min/mg prot.

2.3. Statistical Analyses

Results from analyses using invasive methods were compared with those using minimal invasive methods. All biochemical data were tested for normal distribution (Shapiro Wilk test) and homogeneity of variances (Cochran C test). When these assumptions were met, the parametric *t*-test was performed, whereas, when data did not follow a normal distribution, the nonparametric Mann–Whitney U test was carried out. All data were processed using the software package "Statistica® v. 12" [43] with a 0.05 *p*-level.

3. Results

Overall, 31 lizards were captured (ratio male:female = 1.3), with a mean weight of 7.75 ± 2.18 g and a mean length (SVL) of 8.90 ± 0.51 cm.

The mean values of biochemical responses analyzed in tails and serum (minimal invasive method) or liver and brain (invasive method) of lizards are reported in Table 1. Comparisons between biomarker values recorded in tail and those analyzed in tissues collected using invasive methods are reported in Figure 1.

Minimal-invasive methods employing lizard tails allowed the detection of enzymes activities related to neurotoxicity such as AChE, as well as enzymes and molecules associated with the antioxidant system such as CAT, TOSCA HO, TOSC ROO, tGSH or in the biotransformation system such as GSTs; however, EROD and MDA were not detected in tail tissue. When the values of biomarkers measured by the minimal-invasive method were compared with the values obtained with invasive methods, the former always had a significantly lower result ($p < 0.05$). BChE was detectable both in serum and in tail (both minimal-invasive methods), with a significantly higher value in serum than in tail.

Table 1. Comparison of biomarker results between tail and other tissues: u.m. = unit of measure; Tis. = tissue; n= number of replicates; s.d.= standard deviation; Min = minimum value; Max = maximum value; M–W U test = Mann–Whitney U test. B = brain; L = liver; S = serum; T = tail; Stat. test = statistical test.

Analysis (u.m.)	Tis.	n	Mean	s.d.	Min	Max	Tis.	n	Mean	s.d.	Min	Max	*p*-Level
AChE (nmol/min/mg prot)	B	31	26.32	6.74	13.92	36.92	T	30	31.55	9.18	16.09	49.05	0.014
CAT (µmol/min/mg prot)	L	21	76.67	19.87	35.75	104.22	T	21	11.98	3.23	7.97	18.44	0.000
tGSH (µmol/g)	L	10	2.91	0.88	1.48	3.80	T	10	0.25	0.04	0.20	0.31	0.000
MDA (nmol/g)	L	8	94.73	8.00	85.52	108.72	T	n.d.					
TOSCA HO (GSHeq/g tis.)	L	12	1779.24	397.10	1226.61	2512.47	T	12	465.54	134.78	303.54	730.19	0.000
TOSCA ROO (GSHeq/g tis.)	L	29	868.82	265.14	460.37	1504.58	T	30	212.66	61.04	115.73	407.52	0.000
GSTs (nmol/min/mg prot)	L	31	635.56	250.05	236.46	1264.67	T	30	39.29	10.24	22.79	64.94	0.000
EROD (pmol/min/mg prot)	L	24	14.20	7.98	6.70	35.73	T	n.d.					
BChE (nmol/min/mg prot)	S	28	202.82	56.51	93.74	325.45	T	17	25.88	10.19	13.84	45.12	0.000

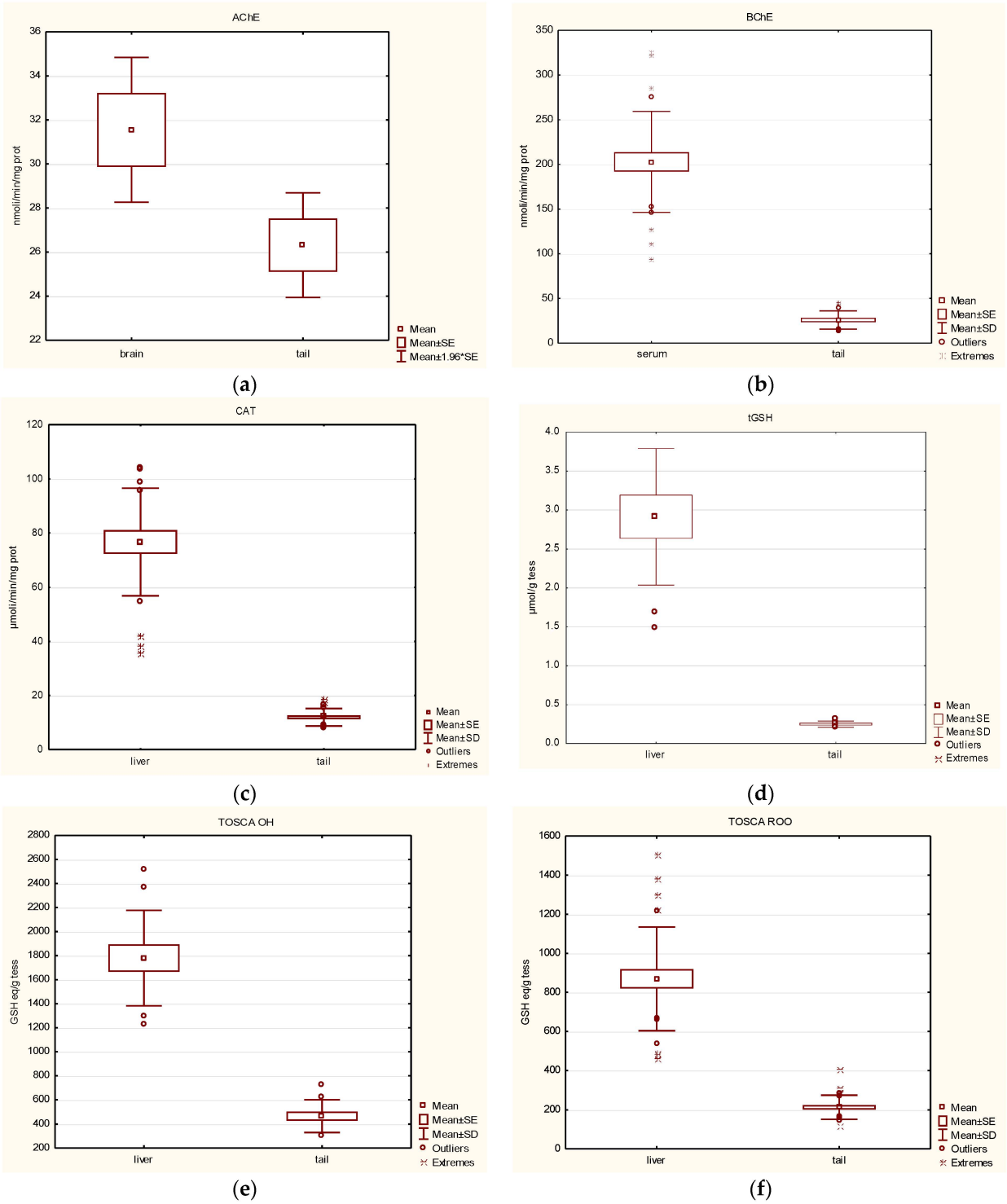

Figure 1. Comparison of biomarker analyses in different tissues of *P. siculus*. Mean value (and standard deviation/error) of biochemical responses. (**a**) Analysis of AChE in brain and tail. (**b**) Analysis of BChE in serum and tail. (**c**) Analysis of CAT in liver and tail. (**d**) Analysis of tGSH in liver and tail. (**e**,**f**) Analysis of TOSCA HO and TOSCA ROO in liver and tail.

4. Discussion

The aim of this study was to assess the detectability of a large set (N = 9) of conventional biochemical responses related to oxidative stress, neurotoxicity and biotransformation processes in tail of the Italian wall lizard *P. siculus*, as a minimal-invasive method.

This study demonstrated that biochemical responses such as AChE, GSTs, CAT, TOSCA HO and ROO and tGSH, usually measured in tissues of *P. siculus* obtained with invasive methods (liver and brain), are also detectable in tail (minimal-invasive method), although with different range of values. BChE was detectable with both tested minimal-invasive methods (i.e., blood serum from subcaudal vein and tail) with different range of values. On the contrary, biochemical responses such as EROD and MDA, usually measured in liver, were not detectable in the tail. The detectability of CAT activity, glutathione level and the total antioxidant capability signal (TOSC ROO and HO assay) confirms the presence of the antioxidant system to counteract the excess of free radicals in the case of oxidative stress in the cells of tail of *P. siculus*, in agreement with results of [25] on SOD, GPX and tGSH. In general, organisms require an antioxidant system, consisting of both enzymatic and nonenzymatic components, to maintain a balance in the concentration of oxidative species. However, when the antioxidant system is unable to regulate the levels of pro-oxidant substances, oxidative stress levels may increase. Among the environmental factors the ultraviolet radiation is an important oxidative stressor for reptiles which, as ectotherms, spend extended periods basking in the sunlight to regulate their body temperature, making them more susceptible to damage from solar radiation [44]. This is just only one of several reasons why the presence of the antioxidant system is fundamental in lizard tissue, including the skin of lizard tail. Then, the absence of MDA in the tail of lizards of this study could suggest that product of lipid peroxidation (related to oxidative stress) is not present in this tissue, even if enzymatic and nonenzymatic compounds of the antioxidant system are present. However, the study by [25] performing the TBAR analyses in lizard tails, a less specific analysis of peroxidation products, suggested that other peroxidation products, excluded MDA, could be present in this tissue. Indeed, MDA is just one of several end products produced during the decomposition of lipid peroxidation products [45]. The absence of EROD activity signal in the tails is likely to be related to the main location of cytochrome p450, the endoplasmic reticulum membrane of liver cells, where the metabolic activities occur. Therefore, this result is what we expected to find. Instead, the presence of GSTs activity in the tail, as already found in other lizard such as *Sceloporus* spp. [23], suggests their crucial role in the tail. Indeed, GSTs are key phase II detoxification enzymes primarily located in the cytosol. Besides their role in catalyzing the conjugation of electrophilic substrates with glutathione (GSH), these enzymes are also involved in various other functions. They can decrease lipid hydroperoxides by means of their Se-independent glutathione-peroxidase activities and can also detoxify LPO end products, having a crucial role in protecting against oxidative damage and peroxidative products of DNA [46,47].

Another interesting result is that both "B" esterases such as AChE and BChE, are also detectable in lizard tails, demonstrating the presence of these enzymes in the peripheral nervous system (tail) as well as in the central nervous one and blood, respectively. Our findings on the Italian wall lizards agree with what was found for other vertebrates. Most of studies on vertebrates are on humans and rats and demonstrate that these two groups of enzymes are usually found in a diverse range of tissues including brain, liver, muscle and blood. AChE is generally thought to be present at its highest amounts in nervous tissue, while BChE in liver and serum [48]. Indeed, BChEs are synthesized and released into blood by the liver, but they can also be present in adipose tissue, small intestine and smooth muscle human cells [49]. Our results demonstrate the presence of these enzymes in muscle of tail, beyond in serum, of the Italian wall lizards, with the highest values in blood as expected. The two cholinesterases (AChE and BChE) are probably located at the neuromuscular junction of lizard muscular cells, as well as for other vertebrates [50]. Indeed, the physiological role of AChE in the neuromuscular junction is well known; in nicotinic cholinergic synapses, it is believed that AChE terminates impulse transmission by rapid hydrolysis of the neurotransmitter acetylcholine [50]. On the contrary, the precise physiological role of BChE at the neuromuscular junction still remains unclear. The currently prevailing hypothesis suggests that BChE acts as a poison scavenger, protecting AChE from inactivation [51]; it is thought to have a shielding function by

sequestering circulating organophosphorus compounds, thereby reducing their toxic effect on brain AChE [22]. Moreover, it is proposed to have a role in growth and development and to function as a scavenger of cholinergic toxins in addition to serving as an auxiliary element in synaptic transmission [52]. Therefore, BChE may have a natural physiological support function, with a backup role when AChE activity is compromised or absent [53].

In conclusion, performing biomarker analyses in tail of the lizard *P. siculus*, together with blood from subcaudal vein, is an effective minimal-invasive method allowing for the detection of a large set of biochemical responses related to oxidative stress (CAT, GSTs, TOSCA, tGSH) and neurotoxicity (AChE, BChE), which could potentially be employed to assess the effects of the exposure to environmental contaminants. Moreover, since the organisms in this study were collected from pesticide-free hazelnuts (possibly serving as good control sites), the results could be valuable in establishing the baseline levels of these biochemical responses in the tail of *P. siculus*.

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