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Assessing Stress Response in Lizards from Agroecosystems with Different Management Practices

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Abstract

Despite the importance of reptiles in agroecosystems, little is known about the effects of agricultural intensification and pesticide use on these animals. We compared antioxidant and haematological biomarkers in the wild Italian wall lizards *Podarcis siculus* from three olive groves representing a gradient of management intensity. Lizards from the conventional grove showed induced antioxidant defences relative to those from the organic field. However, this induction did not avoid the occurrence of oxidative stress in males from intensively managed olive groves, who showed TBARS levels 58%–133% higher than males from the other sites. Haematological responses also suggested increased stress in females from the intensively managed olive groves, with a heterophil-to-lymphocyte ratio 5.3 to 14.8-fold higher than in the other sites. The observed stress responses of lizards along the studied gradient of agricultural management suggest their potential usefulness as non-destructive biomarkers to environmental stressors associated with agricultural intensification.

Keywords Agriculture management · Biochemical markers · Immune defence · Pesticides · Lacertids

Agricultural intensification is recognized as a major threat for wildlife in farmlands (Emmerson et al. 2016; Kehoe et al. 2017). Practices like replacement of natural habitats by crops, or the expansion of monocultures with the consequent removal of natural vegetation patches and field margins, lower habitat quality by reducing food diversity and refuge availability (e.g. Aschwanden et al. 2007; Vickery et al. 2009). In addition, the application of agrochemicals (i.e. pesticides and fertilizers), can also cause toxic effects in wildlife, reducing their fitness and hence their chances to cope with environmental stress (Saaristo et al. 2018). These effects of agricultural intensification have been studied in terrestrial vertebrates like birds or mammals (e.g. Burel et al. 1998; Youngquist et al. 2017; Kirk et al. 2020), but little is known about the effects that pesticides or habitat alterations resulting from agricultural activities can have on reptiles in the wild (Biaggini et al. 2009; Sparling et al. 2010; Amaral et al. 2012a).

The lack of attention received by reptiles, regarding the effects of agricultural intensification is worrisome, given the increased evidence of population declines of reptiles from agricultural areas (de Castro-Expósito et al. 2021). Furthermore, some reptilian species, like lacertid lizards, have potential as indicators of environmental changes associated with agricultural intensification and with pesticide application. Most lizard species have small home ranges and strong site fidelity relative to other vertebrates (Sheldahl and Martins 2000). Moreover, changes in lizard biomass could impact food chains because of the intermediate position that those animals occupy in these chains (Schalk and Cove 2018). In addition, they can be particularly susceptible to

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pesticides, compared to birds and mammals, because they can get exposed through ways that are apparently little relevant to homeothermic vertebrates, including the ingestion of contaminated soils, the dermal uptake from treated soils or after overspray during pesticide applications, or the absorption by eggs which are buried in the soils (Lambert 1997; Russell et al. 1999). Therefore, there is a need to investigate the effects that environmental perturbations associated with agricultural activities, including pesticides, may cause on reptiles in the wild.

Monitoring the effects of environmental stress on wild populations is challenging because of the long time needed to detect population-level effects (e.g. Saha et al. 2018). The use of physiological, non-lethal biomarkers can help in the premature detection of apical effects (e.g. effects on growth, development, reproduction or survival). In the present study, we focus on two types of biomarkers, i.e. oxidative and immune stress biomarkers, that could provide a non-specific indication of environmental stress and that are known to respond to the exposure to environmental pollutants.

The metabolism of pesticides and other environmental pollutants in the organism may produce reactive oxygen species (ROS) that are neutralized by the antioxidant system. Oxidative stress occurs when the production of ROS exceeds the capacity of the antioxidant system to neutralize them. Hence, the non-neutralized ROS can oxidize different macromolecules like cell membrane phospholipids or nucleic acids (Betteridge 2000). This imbalance between ROS and the antioxidant system capacity commonly results from an increased ROS production, as it happens for instance when pesticides or other pollutants are metabolized (Banerjee et al. 2001). In reptiles, signs of increased oxidative stress affecting cell membrane phospholipids were found in Bocage's lizards (*Podarcis bocagei*) from conventional agricultural sites, as compared with lizards from organic farming sites (Amaral et al. 2012a). Also, Mingo et al. (2017) observed increased activity of the antioxidant-related enzyme glutathione reductase in the common wall lizard (*Podarcis muralis*), after application of fungicides or herbicides on German vineyards.

The environmental stress suffered by organisms can also result in the alteration of the immune system parameters. The total amount of white blood cells (WBC) is an indicator of the constitutive immune response and can be used to determine the individuals' response to the stress associated with an exposure to pollutants (Soltanian et al. 2018). In particular, the ratio between the two main types of WBC, heterophils and lymphocytes (H/L) was observed to grow in birds subject to different physiological stress (Gross and Siegel 1983). Although this was in principle related to the increasing number of heterophils that occur as part of acute inflammatory responses following infection, further investigations showed that increased H/L ratios happened also under nutritional, parenteral or environmental stress (Maxwell 1993). After the initial evidence with birds, H/L ratio has also been used as an indicator of environmental stress in reptiles (Canfield 1998; Mader 2000; Sacchi et al. 2011).

We hypothesize that increased intensity of agricultural management, reflected in part in an increased use of pesticides, can affect the health of lizards inhabiting agricultural sites, and that this effect can be identified throughout the evaluation of biomarkers associated with the function of the antioxidant and immune systems.

To test this hypothesis, we conducted a field survey to assess the influence of different agricultural management practices on biomarkers measured in Italian wall lizards (*Podarcis siculus*). The study system consisted of four fields representing a gradient of agricultural intensification, from organic olive groves with minimum management to intensively managed olive grove and vineyard fields. In parallel to agricultural intensification, this system also entailed a gradient in the use of pesticides. *Podarcis siculus* was chosen as the model species because of its ubiquitous presence in both altered and unaltered ecosystems in the region.

Materials and Methods

The study was performed at Villa Montepaldi Agricultural Estate in Tuscany, Central Italy (43° 40′ E, 11° 8′ N), during June 2015. Four sites, including three olive groves and one vineyard, were surveyed. The four sites differed in habitat structure and management (Table 1), and were separated from each other between 0.2 and 1 km (Fig. 1):

(1) An organic olive grove that has been maintained free from management practices and pesticide applications since 2012. This grove shows a dense understory with abundant grass and bushes.
(2) A conventional olive grove with an intermediate degree of management intensity, consisting of the application of copper-based fungicides two to three times per year, between spring and autumn, and of organophosphate insecticides up to twice per year, in summer or early autumn. This management system has been maintained in this grove since 1992. The field shows an herbaceous soil cover.
(3) A conventional olive grove subject, since 2008, to an intensive management, including the application of fungicides and insecticides as in the previous case, and the application of broad-spectrum herbicides. This grove is irrigated and subject to ploughing, and there is almost no vegetation cover in the soil.
(4) A vineyard that has been intensively managed since 1991, on which broad-spectrum herbicides are applied in spring, followed by frequent applications of different

Table 1 List of pesticides presented by classes (i.e. fungicides, herbicides, insecticides), as well as of other agricultural practices being applied in each of the four study plots

Site	Fungicide	Herbicide	Insecticide	Other practices
Organic olive grove	None	None	None	None
Intermediate management olive grove	Copper	None	Dimethoate	Mowing
Intensive management olive grove	Copper	Glyphosate flazasolfuron	Dimethoate	Ploughing Irrigation
Intensively managed vineyard	Ametoctradin, copper, cyflufenamide, dimetomorph, fluopicolide, fluopyram Fosetyl-aluminium, meptyldinocap, metiram, metrapenone, potassium phosphonate, pyrimetanil, sulphur, tebuconazole, zoxamide	Glyphosate flazasolfuron	Chlorpyrifos	Mowing

This information was kindly provided by the field managers

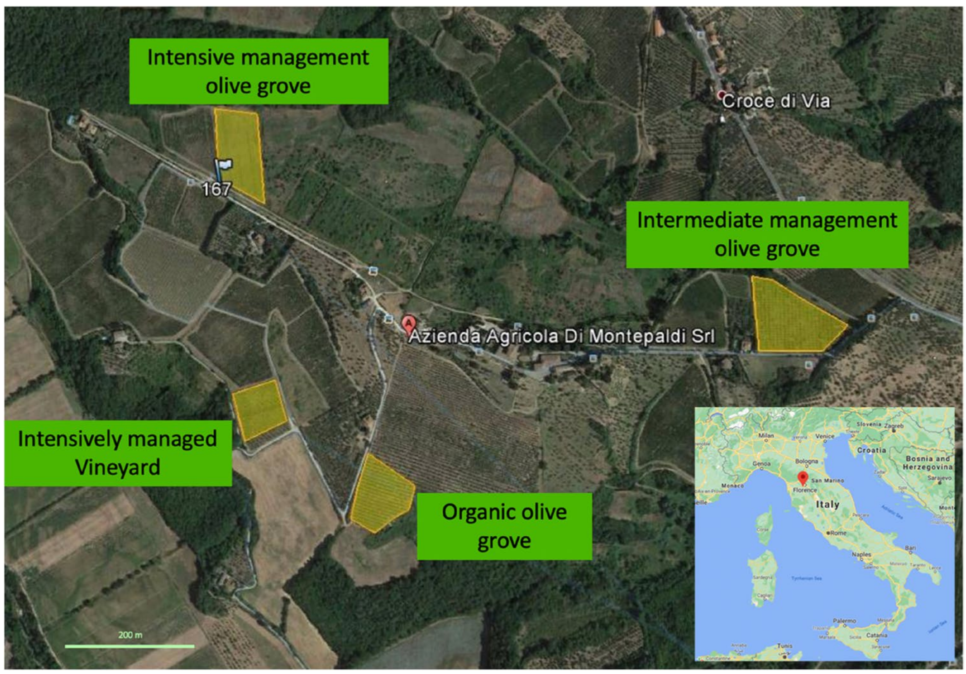

Fig. 1 Location of the four study sites within the Villa Montepaldi Agricultural Estate in Tuscany (central Italy). Map Data ©2021 GeoBasis-DE/DKG (©2009), Google, Inst. Geogr. Nacional. Reproduced according to https://about.google/brand-resource-center/products-and-services/geo-guidelines/

fungicides during the leaf development stages in late spring and summer (up to eight applications per year). In this vineyard, herbaceous vegetation grows under the grapevines, while the lanes between plant rows present bare soils.

Samplings were performed on four consecutive days (one day per study site), always between 8:00 and 12:00 h in the morning. Weather conditions were similar during the four sampling days, and optimal for lizard sampling (i.e. sunny, windless, with temperatures rising from 19 to 27°C during the sampling period). A total of 25 adult lizards were collected by noosing: 6 (3 males, 3 females) from the organic olive grove, 4 (3 males, 1 female) from the olive grove with the intermediate management, 6 (4 males, 2 females) from the olive grove with intensive management, and 9 (5 males, 4 females) from the vineyard. We recorded gender, snout-vent length (SVL, to the nearest 0.1 mm, measured with a calliper) and body mass (to the nearest 0.001 g, measured with a digital scale) of each captured lizard. Body condition indexes were calculated as the residuals from the linear regression between the natural logarithms of mass and SVL. Two samples were collected from each lizard: (i) tail tip (about 1 cm) used for antioxidant and oxidative stress biomarker analysis; (ii) blood from the caudal vein, used for the haematological analysis. Individuals were released immediately after sample collection. The biopsied tail tip was used as a mark to avoid animal recapture.

Tail tips were kept refrigerated until arrival to the laboratory within one hour after sampling, and then stored at − 80°C until they were processed. Samples were homogenized (0.1 g) in 1 mL phosphate buffer with 0.02 M EDTA (pH 7.4). The activity of the antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD) was analysed according to Paglia and Valentine (1967) and Woolliams et al. (1983), using RANSEL and RANDOX commercial kits (RANDOX Laboratories, Crumlin, UK), respectively. Enzymatic activities were calculated relative to the protein content in the sample, which was measured spectrophotometrically following the method by Bradford (1976). The total concentration of glutathione (GSH), the main endogenous antioxidant peptide of the organism, was analysed in the tail tissue homogenate after protein precipitation with 10% trichloroacetic acid. GSH concentrations were calculated spectrophotometrically using the protocol by Griffith (1980). The concentration of thiobarbituric acid reactive substances (TBARS) was measured in the tail tissue homogenate as an indicator of the oxidative damage caused by ROS to cell membranes. We quantified TBARS spectrophotometrically, following Reglero et al. (2009). Blood samples were used right after collection to prepare smears, which were air dried at the collection site. Smears were then stained with May–Grünwald/Giemsa and analysed using a light microscope (Olympus BX41) at × 100 oil immersion following the protocol by Canfield (1998). In each blood smear, the different WBC types were counted and the H/L ratio was calculated.

To analyse the factors influencing the variability of each parameter measured in lizards (biometric or biomarkers), we ran generalized linear models (GzLM) with site and gender as categorical factors. For the biomarker analyses, we also included body condition as a covariate in the models. All dependent variables but GSH followed a normal distribution (checked through Kolmogorov-Smirnoff test), although for SOD, WBC and H/L ratio normality was achieved only after logarithmic transformation. The GzLM conducted to test normally distributed dependent variables were adjusted to a linear distribution, while the GzLM used to test GSH was adjusted to a gamma distribution. In all models, we initially tested the influence of all considered factors, covariates and their interactions, and those terms that did not significantly explain the variability in the response were subsequently excluded from the model in a backwards selection procedure. Best-fit models were selected according to the Akaike's Information Criterion (Vittinghoff et al. 2012). When the site was a significant factor in the final model, Least Significant Difference post-hoc tests were performed. Statistical analyses were run using the software STATISTICA 8.0 for Windows.

Results and Discussion

Lizard body condition significantly varied among sites and a close to significant interaction between site and gender was detected (Table 2). Males from the organic olive grove showed the best body condition (Fig. 2), compared to all other sites. Exposure to pesticides can lower body condition due to the allocation of energy reserves to detoxification metabolism (Johnston et al. 2014). However, the better body condition of lizards from the less managed sites could also be related to a higher resource availability (Amo et al. 2007). In our study system, sites with a less intensive management provided a more diverse vegetation structure, which has been shown to favour the occurrence of reptiles because of the increased resource variability (Pulsford et al. 2017).

Lizards sampled in the conventional olive groves showed higher levels of GSH than those from the organic site (Table 2; Fig. 3a). The levels of SOD did not vary in relation to management practices, but a significant difference in the activity of GPx was observed among sites (Table 2).

Table 2 Selected generalized linear models to explain the influence of site, gender and individual body condition on the variability of the analysed biomarkers in *Podarcis siculus* from an organic olive grove, an olive grove with intermediate management intensity, an intensively managed olive grove, and a vineyard

Response variables	Source of variation	Wald χ^2	df	Significance
Body condition	(Intersection)	8198.794	1	< 0.001
	Site	8.488	3	0.037
	Gender	2.908	1	0.088
	Site * Gender	7.634	3	0.054
TBARS	(Intersection)	269.004	1	< 0.001
	Site	4.595	3	0.204
	Gender	2.712	1	0.100
	Site * Gender	11.324	3	0.010
GPx	(Intersection)	2.751	1	0.097
	Site	9.234	3	0.026
	Body condition	8.807	1	0.003
	Site * condition	10.422	3	0.015
SOD[a]	(Intersection)	288.040	1	< 0.001
	Site	1.191	3	0.755
GSH	(Intersection)	279.516	1	< 0.001
	Site	22.185	3	<0.001
WBC[a]	(Intersection)	1884.539	1	< 0.001
	Site	1.586	3	0.633
H/L ratio[a]	(Intersection)	48.961	1	< 0.001
	Site	8.411	3	0.038
	Gender	5.285	1	0.022
	Site * Gender	11.640	3	0.009

[a]Model run on log-transformed variable

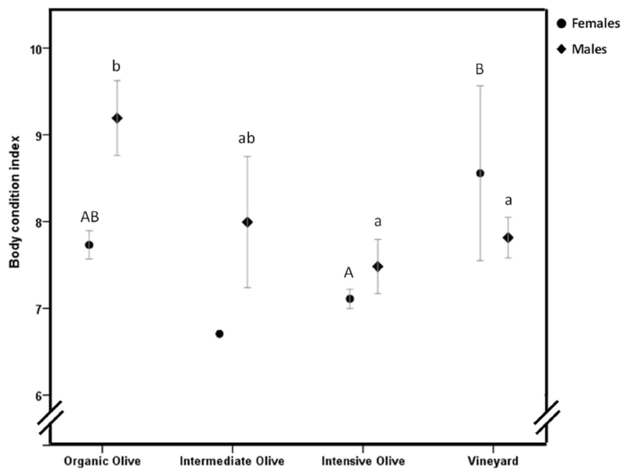

Fig. 2 Mean (± SE) body condition indexes of *Podarcis siculus* as a function of the sampling site and the gender. Different upper-case and lower-case letters define significantly different ($p < 0.05$) values between sites for the female and male subgroups, respectively

Individuals from the olive grove with an intermediate management intensity showed lower GPx activity than those from the other sites (Fig. 3b). The activity of GPx was also dependent on body condition (Table 2). We found a significant interaction between site and gender in the occurrence of oxidative damage (Table 2; Fig. 3c). Males from the intensively managed olive grove showed higher TBARS levels than males from the other sites, while no difference among sites in TBARS levels was observed in females.

Increased GSH in lizards from the conventional olive grove relative to those from the organic one could be due to the activation of the antioxidant system in response to the stress induced by management. Amaral et al. (2012a) found that Bocage's lizards (*P. bocagei*) exposed to pesticides showed increased GSH activity compared to non-exposed individuals. Regarding GPx, the observed differences among sites would indicate an enacted antioxidant activity in response to excess ROS (Bannister and Calabrese 1987). The observed relationship between the activity of GPx and body condition suggests that the capacity of lizards to boost this antioxidant mechanism against ROS is higher in animals with better condition. Therefore, lizards from intensively managed fields, who might be at risk of suffering oxidative stress because of pesticide exposure, would have their antioxidant capacity compromised because of their reduced body condition. This hypothesis can be partially confirmed by the observed increase of TBARS, which are direct indicators of a status of oxidative stress (Lefèvre et al. 1998), in males from the intensively managed olive grove. The gender-based difference in the variation of TBARS across study sites could be partially explained by the metabolic costs associated with the mating season, during which the study was performed. During this season, males display nuptial parades consisting of bright colours and have increased levels of circulating hormones to enhance their aggressiveness towards other males, all of which entails increased energetic costs (Sacchi et al. 2011). This can cause a trade-off in the allocation of energetic reserves, leading to decreased metabolic energy to be spent in detoxification and compromising males' ability to cope with oxidative stress. In this context, the application of the dynamic energy budget (DEB) theory could serve to predict how the energy investment, associated with pesticide metabolism and detoxification, would affect organisms' apical functions like growth or reproduction (Accolla et al. 2020). DEB models have been used to estimate how pesticide exposure causes these effects in different taxa (Jager 2020), although, to the best of our knowledge, this is an unexplored field in reptiles.

While oxidative damage, indicated by the measured TBARS levels, increased with agricultural management in olive groves, lizards from vineyards did not show evidence of this effect. Oxidative stress may originate from chemical exposure, but also from other factors like radiations, starvation, infections, or diet (Kaur et al. 2014). In this line, an important part of antioxidant molecules, used to counteract the action of ROS (and to prevent TBARS formation), is obtained from the diet (e.g. vitamins and carotenoids, Benzie 2003). Unfortunately, there are no published data about the antioxidant composition in the diet of lizards inhabiting vineyards or olive groves. Biaggini and Corti (2021) reviewed the diversity of arthropods, the main food source for *P. siculus*, in agricultural fields across Italy; although they reported a lower diversity in Tuscan vineyards than in Sardinian olive groves, no directly comparable data from the same region and month were available to compare these two types of agricultural uses. If exposure to pesticides were assumed as a possible reason for increased oxidative stress in sampled lizards, an explanation for the low TBARS values in vineyard lizards could be that oxidative stress is mostly associated with insecticide use, which in vineyards is occasional. However, there is little information on how different pesticide classes can cause antioxidant or oxidative stress responses in lizards. The only study evaluating the antioxidant responses of lacertids after exposure to insecticides (i.e. chlorpyrifos, one of the insecticides used in the surveyed conventional olive groves) found no evidence for alteration of the antioxidant system in *P. bocagei* (Amaral et al. 2012b).

Regarding the immunological biomarkers, we found significant effects of both site and gender, as well as their interaction, on the H/L ratio (Table 2). Females from the intensively managed olive grove and males from the olive grove with intermediate management showed significantly higher H/L ratios than lizards from the other sites (Fig. 3d).

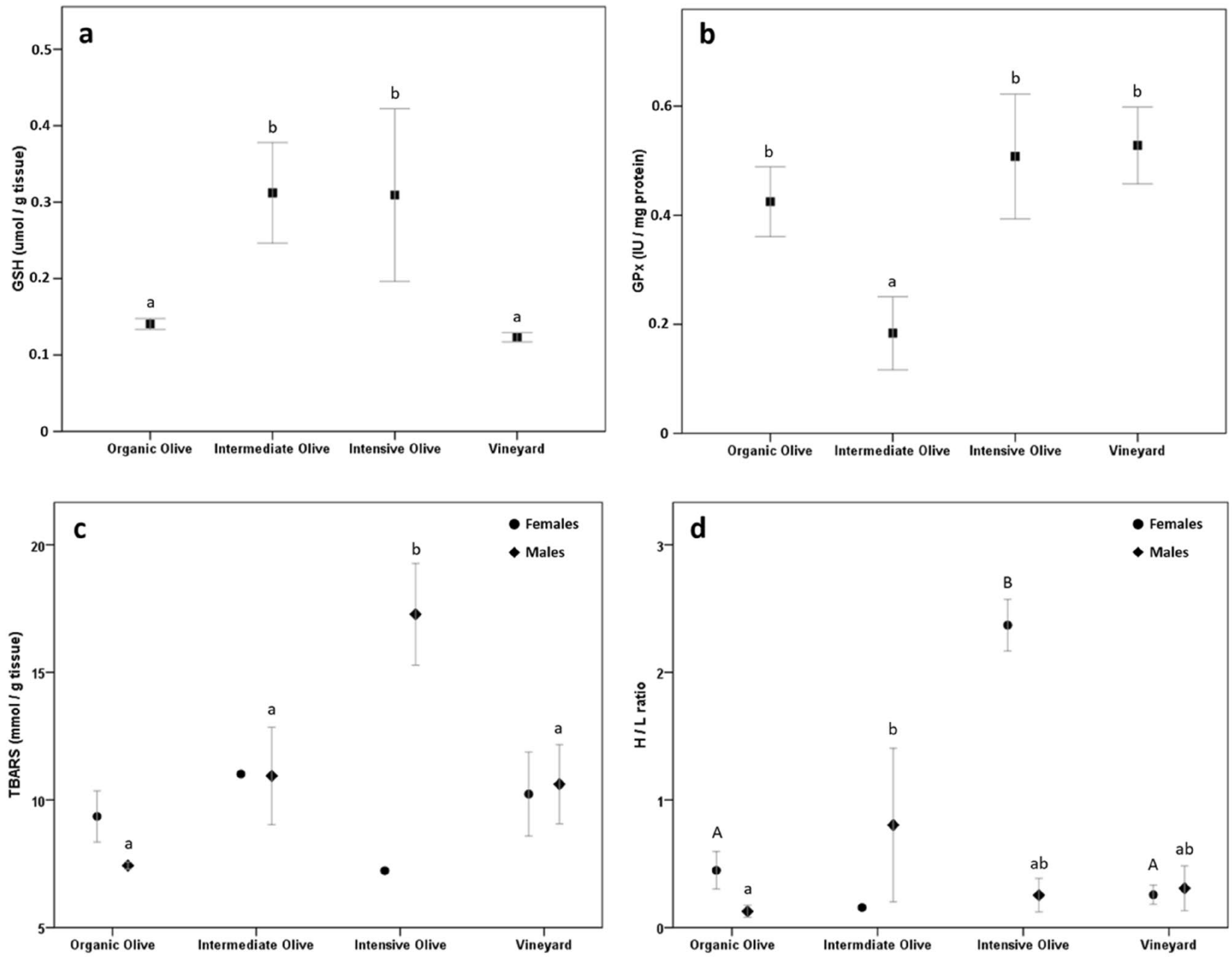

Fig. 3 Mean (± SE) values of **a** glutathione (GSH), **b** glutathione peroxidase (GPx), **c** TBARS in biopsied lizard tails, and **d** heterophil/lymphocyte (H/L) ratio in peripheral blood as a function of the site and, when significantly interacting with the site, also as a function of the gender in *Podarcis siculus*. Different letters define significantly different values ($p < 0.05$) between sites

An increased H/L ratio can be caused by the growing number of heterophils, the primary phagocytic cells in reptiles, related to acute inflammatory responses (Mader 2000), but can also reflect a suppression of lymphocyte proliferation caused by environmental stress. Stressed organisms can experience a rise in corticosterone plasma levels that reduces the responsiveness of the immune system (Davis et al. 2008). However, while increased plasma corticosterone represents a quick response to stress, H/L ratio in ectotherms may take several hours to rise, hence it is not affected by capture stress (Case et al. 2005). The influence of impoverished environments as a factor contributing to increased H/L ratio in reptiles was demonstrated experimentally by Case et al. (2005) with eastern box turtles (*Terrapene carolina*), but its applicability to wild populations has hardly been accomplished. Smyth et al. (2014) found no difference in H/L ratios of sleepy lizard (*Tiliqua rugosa*) populations from two agricultural sites with different levels of habitat fragmentation, whereas French et al. (2008) measured a decreased H/L ratio in the tree lizards (*Urosaurus ornatus*) from urban sites compared to those from non-urban sites. Our results would suggest that increased management in olive groves would constitute a source of stress for *P. siculus*. In this context, although some confounding factors like concurrent inflammatory responses may influence the H/L ratio, the absence of differences among sites in WBC counts provides additional support to that conclusion.

The conclusions from the present study are clearly limited by the low number of sampled animals and fields. However, the scarcity of published evaluations about the potential influence of agricultural habitat management on wild reptiles can make this a relevant contribution and stimulate future studies. Assuming that this was a preliminary approach to evaluate the effects of agricultural management on *P. siculus*, we established the sampling protocol to minimize the influence of other factors. As explained above, spatial heterogeneity beyond the own characteristics of each field was limited by sampling four nearby fields representing a gradient in agricultural intensification. Temporal heterogeneity was minimized by sampling within four consecutive days with similar, stable meteorological conditions. Finally, genetic heterogeneity was reduced by sampling a species within which no different lineages or major genetic differences have been described from central Italy (Capula and Ceccarelli 2003; Senczuk et al. 2017). However, several studies point to the effect that agricultural habitats have as barriers for dispersion of lizards, becoming potential sources of habitat fragmentation and genetic isolation (e.g. Driscoll 2004). If sampled populations, especially those inhabiting the intensively managed sites, may be affected by genetic isolation should be considered when interpreting the observed differences in responses among the sampled sites.

Our results indicate that different agricultural managements may influence the antioxidant metabolism and the haematological response in lizards, with increased oxidative stress and H/L ratio in *P. siculus* from conventional olive groves where pesticides are applied. However, to distinguish the influence of pesticide applications from other field-associated factors (e.g. resource availability, habitat structure), field studies must characterize as many environmental factors as possible and include pesticide measurement data, for which frequent, repeated measures over time may be necessary. These multi-factor approaches would also contribute to a better interpretation of field study results from the perspective of regulatory risk assessment of pesticides, which currently does not contemplate the influence of additional stressors on the risk posed by pesticides. In this context, an integrated environmental risk assessment, considering interactions among pesticides, organisms and their environments has been recommended (Topping et al. 2020). With this purpose the combination of ecological observations on the use of agricultural habitats by lizards, with the analysis of different stress indicators could provide information on the real susceptibility of these animals to pesticides.

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