Glyphosate exposure alters the faecal corticosterone metabolite profile in a lizard: Validation and application of an enzyme immunoassay in *Podarcis bocagei*

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Abstract

Glyphosate, the active ingredient of world's most widely used herbicide Roundup™, has attracted attention due to its impact on non-target organisms. Recent studies highlight the effects of glyphosate and glyphosate-based herbicides on the hypothalamic-pituitary-adrenal (HPA) axis of vertebrates. Nevertheless, previous studies utilised dosages higher than toxicological reference values (TRVs) suggested by the European Food Safety Authority (EFSA), or formulations of glyphosate, not allowing for clear distinctions of the effects of glyphosate itself. Moreover, its impact on the HPA axis was assessed using blood samples, a technique inappropriate for small animals and for assessing long-term chronic effects. Here we tackled those caveats by analyzing faecal corticosterone metabolites (FCMs) of the wall lizard *Podarcis bocagei* to detect glyphosate-induced effects on the HPA axis. This species provides a promising animal model as reptiles are prevalent in agricultural fields treated with herbicides, reptile-specific TRVs are still lacking, and only five studies have validated immunoassays for measuring FCMs in reptiles. Here we validated the 5α-pregnane-3ß,11ß,21-triol-20-one enzyme immunoassay. We detected a glyphosate-induced moderate increase in FCM levels during the exposure phase, while exposed animals of both sexes displayed an altered mass-depended FCM profile. Our results indicate that glyphosate likely impacts the energy mobilization and management of lizards, at the expense of maintenance, growth and reproduction. Our outcomes support the need for reptile-specific TRVs and the consideration of reptiles in EFSA's risk assessment for glyphosate. In methodological terms, FCM analysis can serve as an effective non-invasive tool to estimate the chronic impacts of glyphosate in animal species.

Keywords: Ecotoxicology, Herbicides, Hypothalamic-pituitary-adrenal axis, Enzymatic immunoassay, Reptiles

1. Introduction

Glyphosate and its potential effects on humans and other non-target organisms has attracted the attention in recent years [1–3]. It was discovered by Dr. Henri Martin in 1950, while its use as an active ingredient in pesticides started in 1974 as part of the broad-spectrum herbicide formulation Roundup™. However, it was the production of genetically modified glyphosate-resistant crops in 1996 that triggered its massive annual production and overuse making it the most popular herbicide worldwide [4]. As a result, glyphosate residues are currently found everywhere, from crops and animal food, to drinking water, rain and air [3]. This increased exposure to glyphosate residues by humans has raised the awareness especially after its classification as "probably carcinogenic" by the World Health Organization (WHO) [5]. Recent studies highlight the potential effects of glyphosate exposure on the central nervous, endocrine, cardiovascular, digestive, reproductive, and immune system of humans [3]. Detected concentration of glyphosate in urine have been correlated with certain diseases. Notably, those studies present correlations and not causations as proper experimental data are lacking [3]. Nevertheless, the potential impact of glyphosate on the gut-brain-microbiome axis and the possible cascading effects on the hypothalamic-pituitary-adrenal (HPA) axis are of legitimate concern [3]. Glyphosate targets the shikimate pathway by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), and subsequently blocking the production of aromatic amino acids, such as tryptophan, tyrosine or phenylalanine [6]. This pathway exists in plants, but also in fungi, bacteria, protozoa and archaea, making glyphosate a potential gut microbiome disruptor.

Several studies conducted in animal models prove how glyphosate and glyphosate-based herbicides (GBH) may alter the corticosterone levels in the blood of non-target species.

Alterations in the gut microbiome due to glyphosate exposure may lead to the activation of the HPA axis by the overexpression of pro-inflammatory cytokines and the elimination of microbial taxa which contribute to the reduction of oxidative stress [1]. Exposure to the GBH Roundup Original® can induce a significant increase in the serum concentration of corticosterone and aldosterone of treated albino rats [7]. The same study reported no significant changes in corticosterone and aldosterone levels after exposure to pure glyphosate holding the adjuvants of the formulation responsible for the observed toxicity. However, that study employed glyphosate dosages above the acceptable daily intake (ADI) for mammals, thus the observed toxicity can be challenged due to overdosing. An increase in plasma corticosterone levels of the Argentinian tegu lizard after the exposure to a cocktail of pesticides (cypermethrin (25%), glyphosate (66.2%) and chlorpyrifos (48%)) has also been reported [8]. In that study, the use of a pesticide mixture was rightfully selected to mimic the practices followed in agricultural fields. Nonetheless the lack of control groups for each pesticide did not allow for discrimination of the impact of each pesticide separately. Moreover, glyphosate was applied as a commercial formulation and not in its pure form. Lastly, Khan et al. [9] reported an increase in plasma cortisol levels after the simultaneous exposure to elevated temperature and a glyphosate formulation in the common carp, while the sole exposure to glyphosate did not increase cortisol levels. Temperature alone induced a significant increase which, however, was of lower magnitude compared to the combined effects of glyphosate and temperature, highlighting how environmental temperature can alter the toxicity of chemicals for ectothermic species. Undoubtedly, the aforementioned studies provide useful evidence on the potential effects of glyphosate and GBH on the HPA axis. However, they either exposed animals to dosages higher than the ADI reference dosage, or to formulations of glyphosate and not to the pure active

ingredient. Thus, the reported toxic effects of glyphosate may either be due to overdosing or to the adjuvants present in formulations. As such the European Food Safety Authority (EFSA) cannot use them as references for the establishment of toxicological reference values (TRVs) like the ADI or the acute reference dose (ARfD). As a result, studies testing biologically relevant dosages are necessary to establish glyphosate TRVs for the non-target species for which such values are still lacking.

Another caveat in the existing literature regarding the effects of glyphosate and GBH on the HPA axis stands in the techniques used for the determination of glucocorticoids. Most studies opt for blood samples, which have been proven to be limited especially for stressors that induce long-term chronic effects [10]. As blood collection is an invasive technique, both the handling and the needling can act as an extra stressor, resulting in the elevation of glucocorticoids in the blood which may confound or even mask the effect of the toxicant [11–13]. This is especially true for small animals like lizards for which handling acts as an acute stressor [14]. After handling, glucocorticoids in the blood usually spike within two to three minutes thus the time window of sample acquisition to capture the baseline glucocorticoid levels is narrow [11]. Moreover, glucocorticoid assessment from blood samples depicts a snapshot view of the HPA axis activity by the time of sample acquisition. However, when determining the sub-lethal, long-term effects where stress response potentially occurs after the bioaccumulation of the toxicant such time window is difficult to define and the direction (increase or decrease) of the glucocorticoids becomes unpredictable [15]. Lastly, repeated blood sampling can damage the vascular beds and is not recommended for long-term monitoring of the HPA axis activity of small animals [11,16]

In this context, the use of faecal samples for determining faecal corticosterone metabolites (FCMs) can serve as an effective alternative. This is a non-invasive technique which does not require animal handling. It can capture the effect of chronic stressors providing the profile of FCM fluctuations of an individual over time as it allows for frequent serial sampling. As such, each animal serves as its own control [10]. Moreover, faeces are considered more appropriate for assessing baseline or chronic HPA activity, since short episodic variations in plasma glucocorticoids are not portrayed in FCM levels [17]. FCM analysis has been applied in research areas, such as ethology, ecology, animal conservation and welfare, as well as biomedicine. Important prerequisites for the proper application of this method are 1) that samples are collected quickly enough after each defecation event, 2) are cold-stored immediately after collection and 3) the assay used for the determination of FCMs is analytically, physiologically and/ or biologically validated for each new focal species that is being investigated [10]. Most studies that have applied an FCM analysis have focused on mammals and birds while other groups like reptiles are underrepresented [10]. Notably, FCMs have been estimated for approximately 25 reptilian species, but a proper physiological and/or biological validation of the assays used have been reported for only five species [14]. This reveals an essential gap in the existing literature regarding the correct use of FCMs for addressing the effects of stressors on reptiles. To our knowledge, no previous study has attempted to assess the effects of glyphosate on animals by analyzing FCM levels after exposure to the toxicant. As glyphosate or GBH can impact reptiles' DNA [18], reproductive system [19,20], metabolism [21], gut microbiome [22] and the levels of corticosterone in their blood [8], the use of FCMs could be used as a tool to understand if pure glyphosate can trigger the activation of the HPA axis.

The purpose of this study was twofold. On the one hand, we aimed to validate the 5α-pregnane-3ß,11ß,21-triol-20-one enzyme immunoassay (hereafter 37e EIA) for FCM detection in *Podarcis bocagei.* This immunoassay has been recently validated for the closely related species *P. muralis* [14], but, as stated above, FCM immunoassays should be validated for each new focal species that is being investigated. On the other hand, we investigated whether exposure to pure glyphosate can induce alterations in the FCM levels of exposed lizards. We hypothesized that glyphosate exposed animals would present altered FCM levels during the period of exposure to glyphosate due to the activation of the HPA axis, the potential alterations in the gut microbiome of the lizards or both.

2. Materials and Methods

2.1. Experimental animals

In March 2025, we captured by noose eighty-three *P. bocagei* individuals. No previous application of pesticides has been reported at the sampling site (Vila do Conde, NW Portugal; 41.356099, -8.740022, 10 m a.s.l.). All lizards were subsequently transported and housed at CIBIO facilities in Vairão (about 7 km distant), where we measured their Snout-Vent Length (SVL; ±0.01 mm) and body mass (±0.0001 g) with digital callipers and a precision balance (Sartorius M-Pact AX224, Sartorius AG, Goettingen, Germany), respectively. We only used lizards of SVL more than 45 mm as this is considered the threshold for reproductive maturity for *P. bocagei* [23]. Moreover, we visually assessed all individuals for injuries, malformations, missing fingers and the status of their tail (fully or partly regenerated or intact). Eventually, our dataset consisted of 71 adult individuals (36 females and 35 males). Out of those we randomly selected three males and four females that formed the group for the validation of the 37e EIA. As a result, the experimental group consisted of 32 adult males and 32 adult females.

To ensure proper housing conditions that minimizes external stress and probability of infections, each animal was housed separately, with paper substrate that was replaced regularly [24]. Paper was also placed on one side of the terrarium to prevent eye-contact between individuals which could result in aggression and thus elevated stress. Sterilized ceramic bricks were used as basking spots. Infrared lamps were used during the day (from 9:00 a.m. to 7:00 p.m.) to provide heat for natural thermoregulation. The maximum room temperature detected by dataloggers (RuuviTag Pro, Ruuvi Innovations Ltd) was 25 ± 1° C, while during the night it gradually dropped to 18 ± 1° C and remained stable thanks to a circulating air-conditioning system. We opted for these temperature regimes as they represent air temperature conditions of an average warm day in April in Vila do Conde [25]. Water was checked daily and provided *ad-libitum*. Lizards were allowed to acclimate in laboratory conditions for at least twenty-three days before the collection of faeces for FCM analysis. During the acclimation period two mealworms (*Tenebrio molitor* larvae) were offered as food every other day.

The individuals of the experimental group were split into two sub-groups, a control sub-group receiving 0 mg/ kg bw (milligrams per kilogram of body weight) and a treatment sub-group receiving 0.5 mg/ kg bw with 32 individuals each. The chemical component used was pure glyphosate (96% N-(Phosphonomethyl)glycine, Sigma-Aldrich, CAS-No: 1071-83-6). As dosages are always proportional to the body mass of an animal, we ensured that each group consisted of individuals with similar body masses. To achieve this, animals were grouped by sex and were categorized into eight body mass quartiles. Subsequently animals from each quartile were randomly assigned as control or treatment. This stratified randomization ensured that both sub-groups consisted of animals of similar body masses. During the experiment body mass was monitored every seven days (every Friday).

2.2 Glyphosate treatment

Administration of pure glyphosate occurred through the food with the aim of achieving a "food-chain" contamination. To ensure equivalent feeding of animals within similar body masses the *Tenebrio* larvae were split into four groups depending on their weight following Limnios et al.[21]. Lizards belonging to quartiles 1 and 2 were given larvae of weight that ranged between [0.04-0.06g), quartiles 3 and 4 got [0.06 - 0.07g), quartiles 5 and 6 got [0.07-0.09g) and quartiles 7 and 8 got [0.09-0.11g]. Each *T. molitor* larva was measured prior to the injection, to keep track of the accumulated food consumption for each lizard and was subsequently injected with 5 µl of nominal solutions (control: 0 mg/ kg bw; treatment: 0.5 mg/ kg bw) and offered to the lizards. Dosages of 0.5 mg/kg bw are considered as acceptable daily intake (ADI) for mammals [26]. Since no reference ADI is available for reptiles, we opted for the ADI of mammals as it has been proven to induce an increase in the metabolism of *P. bocagei* [21]. One contaminated larva was offered every second day. The nominal solutions were always prepared the same morning immediately prior to the injection of the larvae. The *Tenebrio* larvae were then injected and offered to the lizards. All individuals accepted the injected larvae almost immediately; thus, no force-feeding was needed. This ensured accurate pesticide levels and effective dosing of our animals without inducing any stress due to handling. The exposure phase lasted for 21 days. Results from our previous studies already highlighted that 21 days are enough to detect the effects of glyphosate on the metabolism of *P. bocagei* [21], thus we opted for the same exposure period.

2.3 Faecal sample collection

The collection of faecal samples lasted from the 7th of April until the 23rd of May 2025. This time window allowed us to collect a total of 1627 faecal pellets to determine individual

baseline, during-treatment and post-treatment FCM levels. The baseline phase lasted between seven and fifteen days. The glyphosate exposure phase lasted 21 days for all individuals. The post treatment phase lasted between 11 and 19 days. The terraria were checked daily for fresh faeces every two hours from 09:00 to 17:00 throughout the experiments. We used tweezers cleaned with alcohol between each collection to avoid cross-contamination, the crystallized uric acid was immediately removed, and the remaining sample was placed into 2ml Eppendorf tubes. All samples were then frozen to -20° C immediately after collection in order to avoid further degradation by intestinal bacteria, as it has been demonstrated that they may affect the structure and stability of corticosterone metabolites [17].

2.4 Enzyme immunoassay validation

To pharmacologically validate the 37e EIA we followed a similar approach as described in Bartolomé et al.[14] for the closely related species *P. muralis*. A mix of corticosterone (Sigma C2505) and pure sesame oil (3 µg corticosterone / 1 µl oil) was applied to the back of the lizards. For the preparation of the corticosterone solution, corticosterone was first diluted in absolute ethanol (7.5 mg of corticosterone in 750 µl of absolute ethanol), vortexed and added to 2.5 ml of sesame oil [14]. Lastly, this solution was vortexed again and the vial was left open overnight for the efficient evaporation of ethanol. A drop of 4.5 µl of the corticosterone solution was applied to the back of the lizards between their shoulder blades with the use of a pipette. The transdermal administration occurred during the night to avoid further disturbance of the animal, while lower temperatures allowed for better absorption of the corticosterone by the skin since the evaporation of the solution is minimized [27]. Corticosterone administration occurred for five consecutive nights, starting the last day of baseline. We treated four females and three males. One male had

to be excluded from the final analysis as it died after the first day of corticosterone administration.

2.5 Faecal corticosterone metabolite (FCM) analysis

The analysis of all faecal samples took place at the lab of experimental endocrinology of the University of Veterinary Medicine of Vienna in November-December 2025 under the supervision of RP and SMM. For extraction of the glucocorticoid metabolites samples were defrosted and weighed. Pellets that weighed less than 5 mg or that were very dry were excluded from the analysis as they can yield erroneously high FCM concentrations [28]. Even though a minimum of 10 mg is recommended for accurate steroid measurement [29] we explicitly avoided pooling samples that weighed between 5 to 10 mg to avoid reducing sample size. Thus, all samples weighing more than 5 mg were processed individually. Subsequently, the faecal samples were diluted in 60% methanol (at a rate of 1 ml per 50 mg of wet faeces), vortexed for 15 minutes and centrifuged (9000 g; 15 min). Following the centrifugation, the supernatant was collected and diluted 1 + 9 with assay buffer and stored frozen in microtubes. FCMs were measured in duplicate by using the 5α-pregnane-3ß,11ß,21-triol-20-one (37e) EIA [30]. Assays were performed on microtiter plates [30,31]. The sensitivity of the 37e EIA was 2 ng/g. Intra- and inter-assay CVs were always below 10% and 13% [14]. The faecal pellets of a few animals that died during the experimental phase were analysed but were excluded from statistical analysis as their FCM levels were found to be extremely high a few days before they died. This highlights the effectiveness of FCM analysis on the monitoring of the condition of animals in lab conditions.

2.6 Statistical analysis

To validate the 37e EIA we defined the categorical variable "phase" that consisted of three levels/subphases (baseline, administration, post-administration). To test the effect of corticosterone on the FCM levels of the lizards we fitted generalized linear mixed effects models ("glmer()" function from lme4 package in R; [32] with a Gamma error distribution and a log link function. FCM levels was the response variable, while phase (categorical with three levels: baseline, administration, post-administration), sex (categorical with two levels: male, female), and their interaction were included as predictors. Since each individual was measured repeatedly, and not all lizards had data for each day, individual ID was included as random factor. We conducted Tukey's post-hoc tests for pairwise comparisons between the different subphases using the function "emmeans()" from the package emmeans [33].

To test our hypothesis that glyphosate exposure can increase FCM levels of exposed lizards we defined the categorical variable "phase" in a similar way as described above which again consisted of three levels/subphases (baseline, glyphosate-exposure, post-glyphosate-exposure). Subsequently, we fitted linear mixed effects models ("lmer()" function from lme4 package in R; [32]. The continuous response variable was FCM levels ($\log_{10}$ transformed), while body mass (continuous), treatments (categorical factor with two levels; control vs glyphosate treated), sex (categorical factor with two levels; male vs female) and phase (categorical factor with three levels; baseline, glyphosate-exposure, post-glyphosate-exposure) were used as predictors. The initial global model included all interactions between the predictors. Individual ID was included as random factor in a similar manner as above.

For all modelling processes, we ensured normality of all continuous variables. A backward stepwise model selection approach was applied selecting the model with the lowest corrected AIC for low sample sizes (AICc), while likelihood ratio test was performed to

determine which model provided the best fit (function "lrtest()" from lmtest package; [34]. All model diagnostics were performed with functions from DHARMa package [35]; Figures S1 & S2). To examine differences between phases within each group we conducted post-hoc pairwise comparisons ("pairs()" function from emmeans package in R [33]. Both unadjusted p-values and p-values adjusted using the Tukey HSD method are presented. We decided to also report unadjusted p-values as we were mostly interested in the shift between FCM baseline levels and exposure levels of the glyphosate exposed animals. Effect sizes for pairwise differences were calculated as Cohen's d using the model's residual standard deviation to provide a standardized measure of the magnitude of change. All statistical analyses were performed under the R software, version 4.5.0 [36].

3. Results

We successfully validated the 37e EIA as it efficiently captured the increase of the FCM levels after the transdermal administration of corticosterone (Figure 1 & 2). The simplest model included phase, sex, and random effect of individual IDs as predictors. FCM concentrations were higher during the administration phase ($\beta = 0.96 \pm 0.23$, $z = 4.26$, $p < 0.001$) and post-administration phase ($\beta = 0.62 \pm 0.22$, $z = 2.85$, $p = 0.004$), while the effect of sex was non-significant ($\beta = 0.61 \pm 0.33$, $z = 1.85$, $p = 0.064$). More specifically, the transdermal administration of corticosterone resulted in a 2.6-fold increase in FCM levels of treated animals (Tukey test = 0.96 ± 0.23, $z = 4.26$, $p < 0.001$). Post-administration levels were also significantly higher than baseline (Tukey test = 0.62 ± 0.22, $z = 2.85$, $p = 0.012$) even if lower (~1.9-fold increase) than during administration. No significant difference was detected between administration and post-administration phases (Tukey test = -0.34 ± 0.28, $z = -1.22$, $p = 0.44$).

We tested our hypothesis that glyphosate could alter FCM levels of glyphosate exposed animals with the selected model including body mass, treatments, sex, phase, all two-way interactions between treatment and the rest of the predictors, the two-way interaction between sex and mass, the three-way interaction between treatment, sex and mass, and individual ID as random factor. Log-transformed FCM levels were significantly different between the two groups ($F_{1,39.44}$= 10.42, p = 0.003) and between sexes ($F_{1,39.35}$= 4.31, p = 0.044), while the effect of body mass was non-significant ($F_{1,38.77}$= 3.55, p = 0.067). A significant interaction between treatment and body mass was detected ($F_{1,38.77}$ = 11.43, p = 0.002), with FCM levels for glyphosate exposed animals decreasing with increasing body mass (coefficient level effects; β = -0.415 ± 0.150, t = -2.77, p = 0.009). In contrast, the interaction between treatment and phase was non-significant even if marginally ($F_{2,471.42}$ = 2.84, p = 0.059) at the term level. However, inspection of model coefficients indicated that treatment was associated with higher FCM levels during the exposure phase relative to baseline (β = 0.131 ± 0.057, t = 2.31, p = 0.021), whereas no difference was detected during the post-exposure phase (β = 0.064 ± 0.062, t = 1.03, p = 0.305; Figure 3). Similarly, the interaction between treatment and sex ($F_{1,39.35}$ = 1.04, p = 0.315), the interaction between sex and mass ($F_{1,38.66}$ = 2.89, p = 0.097) as well as the three-way interaction between treatment, sex, and mass ($F_{1,38.66}$ = 2.62, p = 0.113) were not statistically significant at the term level. Nevertheless, examination of coefficient-level effects revealed significant interactions between treatment and mass (β = −0.415 ± 0.150, t = −2.766, p = 0.009), and sex and mass (β = −0.275 ± 0.123, t = −2.235, p = 0.032), indicating that the relationship between body mass and FCM levels differed across treatment groups and between sexes. (Figure 4). Supplementary Tables 1 & 2 present the results of our linear mixed effect model at the term and coefficient level respectively.

Pairwise post-hoc comparisons revealed an increase in predicted faecal corticosterone metabolite levels between baseline and exposure phase (estimate = 0.10 ± 0.04). This comparison yielded a raw p-value of 0.022, however, following Tukey penalization, the p-value became 0.057, indicating that with a slightly bigger sample size the increase in predicted FCM levels would be statistically significant even after Tukey adjustment (Table 1). This is also supported by the Cohen's d results which represented a medium effect size (Cohen's d = 0.41), with the 95% confidence interval not crossing zero, thus favouring an increase in FCM levels between baseline and glyphosate exposure phases [0.05, 0.77; Figure 5]. Conversely, the Control group showed a non-significant decrease trend during the same period (d = -0.13, p = 0.66; Table 3, Figure 5). These patterns suggest that glyphosate exposure may be associated with elevated FCM levels, though the statistical significance of this specific transition is marginal when controlling for multiple comparisons.

4. Discussion

Understanding how stressors, like pesticides, affect species in agricultural environments is key to assess the condition and fate of their populations in those human-modified environments [37]. Here, we validated for the first time the 37e (5α-pregnane-3ß,11ß,21-triol-20-one) EIA for the lizard *P. bocagei*, a species common in agricultural fields treated with pesticides. The transdermal administration of corticosterone yielded significantly higher FCM levels during and after the corticosterone exposure compared to baseline levels (Figures 1 & 2). Bartolomé et al.[14] reported similar results for the congeneric species *P. muralis.* We also proved that pure glyphosate can moderately alter the FCM levels of lizards when contamination occurs through food at dosages of 0.5 mg/ kg bw every second day (Figures 3 & 5). Even though

this dose is considered as ADI for mammals (EFSA, 2023) it was sufficient to alter the FCM levels of lizards at a non-daily exposure, highlighting the need for the development of reptile-specific TRVs. Moreover, our results suggest that the effect of glyphosate on the FCM levels of lizards might be mass and sex dependent with the heavier females experiencing the biggest shift in their FCM levels (Figure 4).

The 37e EIA effectively captured the expected sex-dependent difference on the FCM levels of the non-exposed animals (Figure 4). The female lizards of the control group exhibited higher FCM values compared to males of the same group, while both sexes of the control group showed a positive relationship between FCM levels and body mass. Studies on mammals, mostly on rats [38], but also on reptiles and amphibians [39] indicate that males and females often display differences in the magnitude of activation of the HPA axis. When exposed to acute stress females usually display a more pronounced neuroendocrine response compared to males with higher levels of corticosterone and adrenocorticotropin hormone [38], although this pattern can be reversed in reptiles, like in the case of breeding marine turtles [39–41]. However, in our case the observed differences in FCM levels between males and females of the control group are more likely to be explained by the functional cross-talk between the HPA axis and the hypothalamic-pituitary-gonadal (HPG) axis [42]. More specifically, the activity of the HPA axis can be influenced by the levels of gonadal hormones, like oestradiol, during the oestrous cycle [38]. Considering that our experiments took place from March until May, thus overlapping with the reproductive season of our focal species [23,43,44], the observed differences in FCM levels between non-exposed female and male lizards are possibly due to the influence of gonadal hormones on the activity of the HPA axis. In fact, female lizards undergo steeper hormonal fluctuations in their reproductive cycles compered to males, hence are more affected by stressors

towards the end of the breeding season [45,46]. Moreover, larger females of our focal species lay more eggs, thus the pronounced positive trend between FCM levels and body mass of control females might be attributed to higher hormonal fluctuations due to the higher reproductive output. Interestingly, the glyphosate exposed animals presented a completely different pattern in FCM levels. Both sexes displayed a reversed, negative relationship with body mass, while females had lower FCM levels compared to males. Thus, glyphosate exposure can have an influence on the overall FCM profile of lizards for both sexes. This may have demographic repercussions for the populations of our species as the heavier females responsible for laying a high amount of eggs are affected the most.

Glyphosate significantly increased FCM levels of lizards during the exposure phase compared to baseline and post-exposure levels. The baseline FCM levels of control and glyphosate-treated animals significantly differed at the beginning of the experiment. Nevertheless, inter-individual variability in baseline FCM levels has been previously reported in lizards [14] and appears to be the norm rather than the exception [47]. Despite the initial baseline differences, the treated group showed an increase in their FCM levels after exposure to glyphosate (exposure phase). Notably, after the termination of the exposure to glyphosate their FCM levels reduced, approaching similar levels to baseline showcasing that the impact of glyphosate on the FCM levels of lizards might be reversible. On the contrary, the control group did not show such a tendency, with FCM levels slightly dropping during the exposure phase. No significant differences could be observed in the FCM levels of control animals between the different phases, while the small reduction in FCM levels could be due to habituation to the housing conditions and the experimental procedure [48].

The altered FCM levels due to glyphosate exposure cannot be attributed to a single mechanism. The different ways with which glyphosate can affect the physiology of reptiles, together with the limitations that derive from assessing corticosterone metabolite levels from faecal samples lead to two potential mechanisms for interpreting our results. The first mechanism is the activation of the HPA axis after exposure to glyphosate which could result in elevated FCM levels during the exposure phase, while also alter the sex-dependent relationship between FCM levels and mass. Previous studies have highlighted the potential of GBH or other pesticides as triggers of the HPA axis [7–9], as those chemicals increased blood corticosterone levels compared to baseline. Even if those studies did not find significant effects of pure glyphosate on blood corticosterone levels here we demonstrated that pure glyphosate at a dosage of 0.5 mg/ kg bw (ADI for mammals; [26] is sufficient to increase FCM levels of lizards at a non-daily exposure scenario. The activation of the HPA axis eventually leads to the secretion of glucocorticoids which result in the mobilization of energy reserves to overcome a stressful situation [49]. We have previously demonstrated that the exact same exposure scenario to pure glyphosate can elevate the maintenance metabolism of our focal species [21], supporting that glyphosate can possibly trigger the activation of HPA axis. This highlights the effectiveness of FCM analysis as a proxy for capturing the chronic impact of glyphosate on lizards, which can reasonably be extended to other animal species.

The second mechanism we propose derives from the potential effects of glyphosate on the metabolism of glucocorticoids, by affecting the liver or the gut microbiome of reptiles. Glyphosate can impact the liver [50] and also alter the gut microbiome composition of non-target species like the cases of the green sea turtle *Chelonia mydas* or the snail *Pomacea canaliculata* [22,51]. These alterations in the liver and gut microbiome might eventually interfere with the

metabolism of glucocorticoids which naturally happens in the liver and in the gut [10]. Thus, the observed differences in FCM levels found here could also be the result of altered liver functioning and/ or gut microbiome composition induced by glyphosate exposure. It was not in the scope of this study to assess the impacts of glyphosate on the liver or gut microbiome of *P. bocagei* lizards. However, we encourage further research on the gut microbiome of lizards as it may serve as a potential biomarker [52,53] and help elucidate the mechanisms underlying the observed differences in the FCM levels of glyphosate exposed animals. Lastly, we cannot exclude the possibility of simultaneous action of both mechanisms, since glyphosate-induced alterations on the gut microbiome can lead to the activation of the HPA axis through the overexpression of pro-inflammatory cytokines and the elimination of microbial taxa involved in reducing oxidative stress [1].

Collectively, our findings indicate that exposure to glyphosate at dosages considered as acceptable for mammals can alter the FCM profile of lizards even at a non-daily scenario. An increase in FCM levels was observed only during the exposure phase, the natural FCM differences between sexes were weakened, while the glyphosate-exposed group displayed a reversed relationship between FCM levels and body mass. In contrast to previous studies, which failed to find an influence of pure glyphosate on the glucocorticoid profile of other species assessed from blood samples [7,9], here we demonstrated how the non-invasive FCM analysis can serve as a valuable tool to uncover the potential chronic impacts of pure glyphosate on lizards. Certainly, this technique did not allow disentangling if glyphosate-induced differences at the FCM levels stem from the activation of the HPA axis, or changes of the metabolism of glucocorticoids caused by effects on the liver or gut microbiome, or both. Considering the current knowledge gap that our study addressed, future research could tackle our limitations and

explore if pure glyphosate can alter the gut microbiome of lizards. For the moment, the results of this study, together with our previous findings on the effects of glyphosate on the metabolism of *P. bocagei* [21], already suggest that pure glyphosate may impact the energy mobilization and management of lizards, especially in the heaviest female individuals. This higher energy demand for overcoming the stress derived from glyphosate exposure may undermine maintenance, growth and reproduction, as energy is possibly devoted to detoxification. Nevertheless, as we did not directly measure such effects, we wish to restrain ourselves from making overspeculations. The existing literature already highlights that glyphosate or GBH have the potential to alter a wide range of aspects of the physiology of reptiles [19–22]. Undoubtedly, the outcomes of this study compliment the existing evidence on the effects of glyphosate on lizards and greatly support the need for reptile-specific TRVs [54] and their consideration in EFSA's risk assessment for glyphosate. They also underline the power of FCM analysis as an effective tool to capture the chronic impacts of glyphosate or other pesticides in animal species.

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Author contributions

AL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review and editing

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Ethical approval

The present study was carried out according to the ethical principles of the license (LICENÇA Nº 456 / 2024 / CAPT) for the capture, handling, transport and collection of samples of wildlife specimens in the territory of continental Portugal under decree law No, 140/99 of April 24, as amended by decree-law No, 49/2005 of February 24, and by decree-law No. 156-A/2013 of November 8, and decree-law No. 38/2021 of May 31.

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Data availability statement

The datasets generated and analyzed during the current study are available online on Figshare: 10.6084/m9.figshare.32922782

Reference list

[1] Barnett JA, Bandy ML, Gibson DL. Is the Use of Glyphosate in Modern Agriculture Resulting in Increased Neuropsychiatric Conditions Through Modulation of the Gut-brain-microbiome Axis? Front Nutr 2022;9:827384. https://doi.org/10.3389/fnut.2022.827384.

[2] Gill JPK, Sethi N, Mohan A, Datta S, Girdhar M. Glyphosate toxicity for animals. Environ Chem Lett 2018;16:401–26. https://doi.org/10.1007/s10311-017-0689-0.

[3] Walsh L, Hill C, Ross RP. Impact of glyphosate (Roundup^{TM}) on the composition and functionality of the gut microbiome. Gut Microbes 2023;15:2263935. https://doi.org/10.1080/19490976.2023.2263935.

[4] Benbrook CM. Trends in glyphosate herbicide use in the United States and globally. Environ Sci Eur 2016;28:3. https://doi.org/10.1186/s12302-016-0070-0.

[5] Williams GM, Aardema M, Acquavella J, Berry SC, Brusick D, Burns MM, De Camargo JLV, Garabrant D, Greim HA, Kier LD, Kirkland DJ, Marsh G, Solomon KR, Sorahan T, Roberts A, Weed DL. A review of the carcinogenic potential of glyphosate by four independent expert panels and comparison to the IARC assessment. Crit Rev Toxicol 2016;46:3–20. https://doi.org/10.1080/10408444.2016.1214677.

[6] Helander M, Saloniemi I, Saikkonen K. Glyphosate in northern ecosystems. Trends Plant Sci 2012;17:569–74. https://doi.org/10.1016/j.tplants.2012.05.008.

[7] Owagboriaye F, Dedeke G, Ademolu K, Olujimi O, Aladesida A, Adeleke M. Comparative studies on endogenic stress hormones, antioxidant, biochemical and hematological status of metabolic disturbance in albino rat exposed to roundup herbicide and its active ingredient glyphosate. Environ Sci Pollut Res 2019;26:14502–12. https://doi.org/10.1007/s11356-019-04759-1.

[8] Mestre AP, Amavet PS, Vanzetti AI, Moleón MaS, Parachú Marcó MaV, Poletta GL, Siroski PA. Effects of cypermethrin (pyrethroid), glyphosate and chlorpyrifos (organophosphorus) on the endocrine and immune system of *Salvator merianae* (Argentine tegu). Ecotoxicol Environ Saf 2019;169:61–7. https://doi.org/10.1016/j.ecoenv.2018.10.057.

[9] Khan I, Islam FU, Khan NU, Ali H, Attaullah M, Khan MI. Cumulative effect of chronic thermal stress and glyphosate based herbicide exposure on blood parameters and cortisol levels of common carp (*Cyprinus carpio*). Ecotoxicology 2025;34:760–8. https://doi.org/10.1007/s10646-025-02875-y.

[10] Palme R. Non-invasive measurement of glucocorticoids: Advances and problems. Physiol Behav 2019;199:229–43. https://doi.org/10.1016/j.physbeh.2018.11.021.

[11] Carbajal A, Serres-Corral P, Olvera-Maneu S, López-Béjar M. Non-invasive measurement of glucocorticoids: The reptile perspective. J Zool 2024;323:87–96. https://doi.org/10.1111/jzo.13157.

[12] Langkilde T, Shine R. How much stress do researchers inflict on their study animals? A case study using a scincid lizard, *Eulamprus heatwolei*. J Exp Biol 2006;209:1035–43. https://doi.org/10.1242/jeb.02112.

[13] Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 2011;166:869–87. https://doi.org/10.1007/s00442-011-1943-y.

[14] Bartolomé A, Palme R, Macho-Maschler S, Carazo P, Font E. Validation of two enzyme immunoassays for non-invasive glucocorticoid measurement in a lacertid lizard (*Podarcis muralis*): Effects of pharmacological and biological stimuli on faecal corticosterone

metabolites and behaviour. Physiol Behav 2025;290:114751. https://doi.org/10.1016/j.physbeh.2024.114751.

[15] Dickens MJ, Romero LM. A consensus endocrine profile for chronically stressed wild animals does not exist. Gen Comp Endocrinol 2013;191:177–89. https://doi.org/10.1016/j.ygcen.2013.06.014.

[16] Kersey DC, Dehnhard M. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. Gen Comp Endocrinol 2014;203:296–306. https://doi.org/10.1016/j.ygcen.2014.04.022.

[17] Palme R, Rettenbacher S, Touma C, El-Bahr SM, Möstl E. Stress Hormones in Mammals and Birds: Comparative Aspects Regarding Metabolism, Excretion, and Noninvasive Measurement in Fecal Samples. Ann N Y Acad Sci 2005;1040:162–71. https://doi.org/10.1196/annals.1327.021.

[18] Schaumburg LG, Siroski PA, Poletta GL, Mudry MD. Genotoxicity induced by Roundup® (Glyphosate) in tegu lizard (*Salvator merianae*) embryos. Pestic Biochem Physiol 2016;130:71–8. https://doi.org/10.1016/j.pestbp.2015.11.009.

[19] Rosati L, Chianese T, De Gregorio V, Verderame M, Raggio A, Motta CM, Scudiero R. Glyphosate Interference in Follicular Organization in the Wall Lizard *Podarcis siculus*. Int J Mol Sci 2023;24:7363. https://doi.org/10.3390/ijms24087363.

[20] Verderame M, Chianese T, Rosati L, Scudiero R. Molecular and Histological Effects of Glyphosate on Testicular Tissue of the Lizard *Podarcis siculus*. Int J Mol Sci 2022;23:4850. https://doi.org/10.3390/ijms23094850.

[21] Limnios A, Boratyński Z, Enriquez-Urzelai U, Pafilis P, Charles S, Simbula G, Carretero MA. Combined effects of glyphosate and ambient temperature on the energy management

of a lizard: Evidence from a controlled exposure study. Environ Res 2026;300:124415. https://doi.org/10.1016/j.envres.2026.124415.

[22] Kittle RP, McDermid KJ, Muehlstein L, Balazs GH. Effects of glyphosate herbicide on the gastrointestinal microflora of Hawaiian green turtles (*Chelonia mydas*) Linnaeus. Mar Pollut Bull 2018;127:170–4. https://doi.org/10.1016/j.marpolbul.2017.11.030.

[23] Carretero M, Ribeiro R, Barbosa D, Sá-Sousa P, Harris DJ. Spermatogenesis in two Iberian Podarcis lizards: relationships with male traits. Anim Biol 2006;56:1–12. https://doi.org/10.1163/157075606775904759.

[24] Vosjoli P de. Designing Environments for Captive Amphibians and Reptiles. Veterinary Clin North Am Exot Anim Pract 1999;2:43–68. https://doi.org/https://doi.org/10.1016/S1094-9194(17)30139-1.

[25] Weather Underground. Monthly Weather History for Vila Nova Da Telha (LPPR) –April 2025 2025. https://www.wunderground.com/history/monthly/pt/vila-nova-da-telha/LPPR/date/2025-4 (accessed July 4, 2026).

[26] European Food Safety Authority (EFSA), Álvarez F, Arena M, Auteri D, Binaglia M, Castoldi AF, Chiusolo A, Crivellente F, Egsmose M, Fait G, Ferilli F, Gouliarmou V, Nogareda LH, Ippolito A, Istace F, Jarrah S, Kardassi D, Kienzler A, Lanzoni A, Lava R, Linguadoca A, Lythgo C, Mangas I, Padovani L, Panzarea M, Parra Morte JM, Rizzuto S, Romac A, Rortais A, Serafimova R, Sharp R, Szentes C, Terron A, Theobald A, Tiramani M, Vianello G, Villamar-Bouza L. Peer review of the pesticide risk assessment of the active substance glyphosate. EFSA J 2023;21. https://doi.org/10.2903/j.efsa.2023.8164.

[27] Meylan S, Dufty AM, Clobert J. The effect of transdermal corticosterone application on plasma corticosterone levels in pregnant *Lacerta vivipara*. Comp Biochem Physiol A Mol Integr Physiol 2003;134:497–503. https://doi.org/10.1016/S1095-6433(02)00343-4.

[28] Millspaugh JJ, Washburn BE. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. Gen Comp Endocrinol 2004;138:189–99. https://doi.org/10.1016/j.ygcen.2004.07.002.

[29] Borgmans G, Palme R, Sannen A, Vervaecke H, Van Damme R. The effect of environmental provisioning on stress levels in captive green anole (*Anolis carolinensis*). Anim Welf 2018;27:35–46. https://doi.org/10.7120/09627286.27.1.035.

[30] Touma C, Sachser N, Möstl E, Palme R. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and faeces of mice. Gen Comp Endocrinol 2003;130:267–78. https://doi.org/10.1016/S0016-6480(02)00620-2.

[31] Möstl E, Maggs JL, Schrötter G, Besenfelder U, Palme R. Measurement of Cortisol Metabolites in Faeces of Ruminants. Vet Res Commun 2002;26:127–39. https://doi.org/10.1023/A:1014095618125.

[32] Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw 2015;67:1–48. https://doi.org/10.18637/jss.v067.i01.

[33] Lenth RV. emmeans: Estimated Marginal Means, aka Least-Squares Means. 2025. https://doi.org/10.32614/CRAN.package.emmeans.

[34] Zeileis A, Hothorn T. Diagnostic Checking in Regression Relationships. R News 2002;2:7–10.

[35] Hartig F. DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. 2024. https://doi.org/10.32614/CRAN.package.DHARMa.

[36] R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2025.

[37] Mingo V, Lötters S, Wagner N. Risk of pesticide exposure for reptile species in the European Union. Environ Pollut 2016;215:164–9. https://doi.org/10.1016/j.envpol.2016.05.011.

[38] Heck AL, Handa RJ. Sex differences in the hypothalamic–pituitary–adrenal axis' response to stress: an important role for gonadal hormones. Neuropsychopharmacology 2019;44:45–58. https://doi.org/10.1038/s41386-018-0167-9.

[39] Moore IT, Jessop TS. Stress, reproduction, and adrenocortical modulation in amphibians and reptiles. Horm Behav 2003;43:39–47. https://doi.org/10.1016/S0018-506X(02)00038-7.

[40] Jessop TS. Modulation of the adrenocortical stress response in marine turtles (Cheloniidae): evidence for a hormonal tactic maximizing maternal reproductive investment. J Zool 2001;254:57–65. https://doi.org/10.1017/S0952836901000553.

[41] Valverde RA, Owens DW, MacKenzie DS, Amoss MS. Basal and stress-induced corticosterone levels in olive ridley sea turtles (*Lepidochelys olivacea*) in relation to their mass nesting behavior. J Exp Zool 1999;284:652–62. https://doi.org/10.1002/(SICI)1097-010X(19991101)284:6%3C652::AID-JEZ7%3E3.0.CO;2-U.

[42] Viau V. Functional Cross-Talk Between the Hypothalamic-Pituitary-Gonadal and -Adrenal Axes. J Neuroendocrinol 2002;14:506–13. https://doi.org/10.1046/j.1365-2826.2002.00798.x.

[43] Galán P. Reproductive ecology of the lacertid lizard *Podarcis bocagei*. Ecography 1997;20:197–209. https://doi.org/10.1111/j.1600-0587.1997.tb00362.x.

[44] Galán P. Reproductive and fat body cycles of the lacertid lizard *Podarcis bocagei*. Herpetol J 1996;6:20–5.

[45] Carretero MA. Reproductive cycles in Mediterranean lacertids: plasticity and constraints. Mainl Insul Lizards Mediterrean Perspect-Monogr Sci 8 2006:1000–22.

[46] Megía-Palma R, Arregui L, Pozo I, Žagar A, Serén N, Carretero MA, Merino S. Geographic patterns of stress in insular lizards reveal anthropogenic and climatic signatures. Sci Total Environ 2020;749:141655. https://doi.org/10.1016/j.scitotenv.2020.141655.

[47] Galeotti P, Pellitteri-Rosa D, Sacchi R, Gentilli A, Pupin F, Rubolini D, Fasola M. Sex-, morph- and size-specific susceptibility to stress measured by haematological variables in captive common wall lizard *Podarcis muralis*. Comp Biochem Physiol A Mol Integr Physiol 2010;157:354–63. https://doi.org/10.1016/j.cbpa.2010.08.005.

[48] Babb JA, Masini CV, Day HEW, Campeau S. Habituation of hypothalamic–pituitary–adrenocortical axis hormones to repeated homotypic stress and subsequent heterotypic stressor exposure in male and female rats. Stress 2014;17:224–34. https://doi.org/10.3109/10253890.2014.905534.

[49] Sapolsky RM, Romero LM, Munck AU. How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions*. Endocr Rev 2000;21:55–89. https://doi.org/10.1210/edrv.21.1.0389.

[50] Verderame M, Scudiero R. How Glyphosate Impairs Liver Condition in the Field Lizard *Podarcis siculus* (Rafinesque-Schmaltz, 1810): Histological and Molecular Evidence. BioMed Res Int 2019;2019:1–13. https://doi.org/10.1155/2019/4746283.

[51] Bao Y, He X, Zhai Y, Shen W, Jing M, Liu Y, Yang H, Chen L. Effects of glyphosate-based herbicide on gut microbes and hepatopancreatic metabolism in *Pomacea canaliculata*. Ecotoxicol Environ Saf 2024;280:116549. https://doi.org/10.1016/j.ecoenv.2024.116549.

[52] Littleford-Colquhoun BL, Weyrich LS, Jackson N, Frere CH. City life alters the gut microbiome and stable isotope profiling of the eastern water dragon (*Intellagama lesueurii*). Mol Ecol 2019;28:4592–607. https://doi.org/10.1111/mec.15240.

[53] Vasconcelos DS, Harris DJ, Damas-Moreira I, Pereira A, Xavier R. Factors shaping the gut microbiome of five species of lizards from different habitats. PeerJ 2023;11:e15146. https://doi.org/10.7717/peerj.15146.

[54] Ortiz-Santaliestra ME, Maia JP, Egea-Serrano A, Lopes I. Validity of fish, birds and mammals as surrogates for amphibians and reptiles in pesticide toxicity assessment. Ecotoxicology 2018;27:819–33. https://doi.org/10.1007/s10646-018-1911-y.

643 **Table 1. Pairwise post-hoc comparisons between baseline, exposure and post-exposure**
644 **phase for control and glyphosate treated animals. Both unadjusted p-values (raw) and p-**
645 **values adjusted using the Tukey HSD method are presented.**

	Estimates	*SE*	*df*	*t-ratio*	*p-value (raw)*	*p-value (Tukey)*
Control						
exposure - baseline	-0.032	0.037	472.827	-0.860	0.390	0.666
post - baseline	-0.003	0.041	473.400	-0.074	0.941	0.997
post - exposure	0.029	0.034	469.511	0.841	0.401	0.678
Glyphosate Treated						
exposure - baseline	0.100	0.044	476.148	2.297	**0.022**	**0.057**
post - baseline	0.061	0.047	478.766	1.288	0.198	0.403
post - exposure	-0.039	0.038	468.988	-1.041	0.298	0.551

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