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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

Alicia Bartolomé [a,*], Rupert Palme [b], Sabine Macho-Maschler [b], Pau Carazo [a], Enrique Font [a]

[a] Ethology lab, Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia. Catedrático José Beltrán Martínez, 2, 46980 Paterna, Valencia, Spain
[b] Experimental Endocrinology, Department of Biological Sciences and Pathobiology, University of Veterinary Medicine, Veterinärplatz, 1, 1210, Vienna, Austria

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ABSTRACT

The assessment of stress-related hormone levels using non-invasive methods has gained popularity in mammal and bird welfare, yet its application in reptiles remains limited. Particularly, the exploration of physiological measures such as faecal corticosterone metabolites (FCMs) for reptilian welfare has scarcely been explored. This study aims to validate two enzyme immunoassays (5α-pregnane-3ß,11ß,21-triol-20-one and 11-oxoaetiocholanolone EIA) for monitoring FCM levels in the European common wall lizard (*Podarcis muralis*). We collected daily faecal samples before (baseline) and after (post-treatment phase) inducing elevated corticosterone levels using transdermal administration of corticosterone (pharmacological treatment) and handling/confinement (biological treatment). We also conducted daily behavioural observations to explore the relationship between stress-related corticosterone changes and behaviour. Although treatments induced significant increases in FCM levels, the effect was much larger in the pharmacological one. Transdermal corticosterone induced a cumulative increase in FCMs over the treatment period, with a higher response observed in females. In contrast, the biological treatment yielded smaller FCM peaks, with no significant sex differences. Overall, 5α-pregnane-3ß,11ß,21-triol-20-one EIA appeared to be more sensitive in detecting these effects. Regarding lizard behaviour, both treatments led to increased hiding and decreased basking compared to baseline. The effects were more pronounced in animals subjected to handling/confinement, despite smaller FCM increases. Our results confirm the suitability of an EIA for monitoring FCMs in both male and female common wall lizards and provide insights into the complexities of using integrated approaches to assess stress, highlighting the need for further research on direct measures to evaluate reptile welfare.

1. Introduction

Public and institutional concern about the well-being of reptiles is increasing, and research on many aspects of their biology in captivity has been accumulating during the last years [1,2]. Despite an increase in reptile welfare research, the information available on this class is still relatively scarce compared to other animal groups in terms of welfare research [3–7]. Although animal welfare is a complex concept to define and measure [8,9], current approaches advocate for an integrative view, where welfare is defined by both the biological functioning and affective experiences of individuals as they cope with their environment [9–12]. This view encompasses both the physical and mental state of the animal [13,14], assessed through a combination of physiological and behavioural indicators [9,12]. Given the variety of available indices, careful selection of the most appropriate ones is crucial [15,16]. However, for reptiles, reliable and validated animal-based measurements of welfare (i.e., measurements that directly assess the animal's physical, physiological, and behavioural state) are scarce, particularly in the case of physiological measurements [17]. Non-invasive techniques to measure stress-related hormonal levels, such as faecal cortisol/corticosterone metabolites (FCMs), have become popular tools to assess the welfare state for mammals (particularly farm animals) and birds, but their use in reptiles is still very limited [18–20].

The stress response is regulated by various systems, among which the

* Corresponding author.
E-mail address: abarca5@uv.es (A. Bartolomé).

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hypothalamic–pituitary–adrenal (HPA) axis is one of the most important. Activation of the HPA axis increases the secretion of steroid hormones called glucocorticoids (corticosterone in reptiles), which is crucial for animals' survival, as it enables them to cope with whatever urgent contingency induced the stress response [21–23]. This response allows free-ranging animals to potentially use the full set of behavioural and physiological changes mediated by the HPA axis to cope with environmental challenges; in contrast, captive animals often live in a restricted environment, unable to escape or act upon potential aversive stimuli, particularly if they live in impoverished environments over which they have little control [24,25]. Consequently, sustained or frequent exposure to stressors can lead to chronic stress, which impairs health and welfare [26,27]. However, glucocorticoid elevation not only occurs in response to aversive stimuli, but also under other circumstances, including courtship, prey hunting, or social interactions [28–30]. Understanding and interpreting the HPA response can be difficult, particularly for taxa such as amphibians and reptiles, for which the amount of information available is scarcer than for other groups [21, 31]. Nonetheless, elevated circulating levels of corticosterone have been associated with stress-induced physiological and behavioural responses in some reptile species, such as conspecific aggression and social dominance [32], handling by humans [33,34], exposure to predator scent [33], or trapping and confinement ([35]; for additional references see Table 4.2 in [21]). Traditionally, corticosterone measurements were carried out using blood samples. In reptiles, plasma corticosterone levels have been studied to evaluate their effects on behaviour (e.g., [32, 36–38]), immune response (e.g., [39–41]), reproduction (e.g., [42–45]), metabolism (e.g., [46–48]) and survival (e.g., [43,49]). However, there are some shortcomings to consider when using blood to assess stress levels as a welfare tool. Blood extraction is a limiting factor in small species, and interpretation of endocrine parameters based on blood measurements can be difficult, as they are affected by periodical effects (such as diurnal or seasonal rhythms; [50,51]) and other spurious fluctuations. In addition, sample collection requires direct handling, which can be stressful and/or dangerous for some animals (e.g., cardiac puncture) [52- 54]. In contrast, measurement of faecal metabolites offers several advantages: collection is arguably easier, it can be done (in the wild or in captivity) with minimal disturbance to the animal, it allows frequent sampling for longer periods of time, and it provides an integrated measure of glucocorticoid levels, reflecting long-term physiological responses more accurately [19,53–56].

Measured concentrations of FCMs depend on multiple factors, such as species-specific glucocorticoid metabolism, sex, sampling methods, and analysis techniques (e.g., [18,54,55,57]). Therefore, it is imperative to validate the technique employed for metabolite measurement (i.e., enzyme immunoassay) prior to its application [19,55,58,59]. Validation can be achieved through different approaches. First, physiological/pharmacological validation involves pharmacological stimulation of an increase or decrease in circulating glucocorticoid levels, allowing for the detection of corresponding changes in the metabolites excreted in faeces. Secondly, biological validation entails subjecting the animal to a relevant known stressful event, such as handling, transportation, social interactions, capture, and/or confinement [59]. The type of stressors used during biological validation may result in considerably lower adrenal responses than those induced through pharmacological means [60]. Furthermore, it is important to supplement the analysis of FCM levels with complementary measurements to enhance our understanding of the observed physiological changes. For instance, combining physiological measurements with behavioural observations allows for a more comprehensive interpretation of the stress response and the impact of aversive stimuli on animals' health and welfare [50,52,61,62]. Nevertheless, it should be noted that the integrated response of physiological and behavioural changes is diverse, and can be influenced by individual, social and/or environmental factors [63–65]. For instance, several studies measuring welfare using behavioural and physiological indexes have found discrepancies or non-analogous responses between the two [66–69], where increases in glucocorticoid production do not necessarily translate to observable changes in behaviour (and vice versa). The nature of the stimuli and the animal's perception may shape these responses according to its functional context [16,65,70]; consequently, these factors should be considered when interpreting different stress-related measurements [16, 28].

To our knowledge, FCMs measurement has been used in 24 different reptilian species, although the methods have been pharmacologically and/or biologically validated in only four species (see [71]): *Crocodylus niloticus* (Crocodylidae: [72]), *Anolis carolinensis* (Dactyloidae: [73]), *Smaug giganteus* (Cordylidae: [74]), and *Trogonophis wiegmanni* (Trogonophidae: [75]). Non-invasive measurement of FCMs has not been validated in any lacertid lizard; it has been used in two previous studies without reported prior validation (*Gallotia galloti*: [76]; *Psammodromus algirus*: [77]).

The purpose of this study was to validate a non-invasive technique for monitoring glucocorticoid levels in European common wall lizards (*Podarcis muralis*). To achieve this, we evaluated the suitability of two enzyme immunoassays (EIAs) to detect changes in faecal corticosterone metabolites (FCMs) following an increase in circulating corticosterone levels. This increase was induced using both pharmacological (transdermal administration of corticosterone) and biological (handling and confinement) treatments. Additionally, we conducted behavioural observations throughout the experiment to explore the relationship between stress-related corticosterone changes and behaviour. We performed two validation experiments: the first one (hereafter referred to as the pilot study) was conducted in August 2021, and the second one (hereafter referred to as the validation experiment) was conducted in September 2022. Although both experiments followed very similar methodologies (see "Experimental design" section in Materials and Methods), the pilot study (in which we only included male lizards) helped to fine-tune the overall schedule of sample collection and behavioural observations for the validation experiment, in which we also included female lizards.

2. Materials and methods

2.1. Species and housing conditions

The European common wall lizard (*P. muralis*) is a heliothermic, diurnal, saxicolous lacertid lizard with a widespread distribution in Europe [78], as well as introduced populations in USA and Canada [79, 80]. This species is extensively used for research in the field and under laboratory conditions [81] and present in some zoos in Europe and elsewhere [82]. For the pilot study, we captured 16 adult male lizards (snout–vent length, SVL ± SE: 60.1 ± 0.64 mm; weight ± SE: 5.1 ± 0.17 g) during July 2021. For validation experiment, we captured eight male (SVL ± SE: 65.9 ± 0.83 mm; weight ± SE: 7.8 ± 0.31 g) and eight female (SVL ± SE: 60.8 ± 0.77 mm; weight ± SE: 5.3 ± 0.13 g) adult lizards during August 2022. All lizards were captured by noosing (i.e., using a pole with a slipknot that tightens around the lizard's neck) in the Eastern Pyrenees, Spain. They were individually held in cloth bags and transported by car to the Ethology laboratory at the University of Valencia the day after capture.

Lizards were individually caged in 40 × 25 × 30 cm high (pilot study) and 70 cm x 30 cm x 40 cm high terraria (validation experiment; Figure S1). A 40 W lightbulb (Parabolica RP50 Radium, Wipperfürth, Germany) was suspended on one side of the terrarium to create a thermal gradient (ranging from 32 °C under the lamp to 20–25 °C in the cold side during the day). Each terrarium was furnished with a basking stone under the lamp, three shelters positioned along the thermal gradient, a slate tile, and a water dish. The bottom of the terraria was lined with white filter paper to facilitate detection and collection of faecal pellets. The terraria were in a light, humidity, and temperature-controlled room (22°C, 50% humidity, 11 h light:13 h dark). In addition, daylight

fluorescent tubes (F30W Reptistar, Sylvania, Budapest, Hungary; colour temperature 6500K) controlled by high-frequency ballasts were switched on for 2 h (from 11.30 a.m. to 1.30 p.m.) five times per week. Every other day, lizards were fed mealworms (*Tenebrio molitor* larvae), or crickets (*Acheta domesticus*) dusted with vitamins and minerals (JBL Terravit Powder, JBL, Neuhofen, Germany). Water was available ad libitum. Animals were measured and weighed before and after the experiment. All lizards were released back at their capture location after the experiment.

Lizards were captured and kept in the laboratory under research permits SF/024/21 and SF/0187/22 from the "Direcció General de Polítiques Ambientals i Medi Natural", Generalitat de Catalunya, Spain. The procedure was carried out with the approval of the University of Valencia's ethical committee (reference number: A20220614131720) and the "Dirección General de Agricultura, Ganadería y Pesca", Generalitat Valenciana, Spain (authorisation number: 2022 VSC PEA 0185).

2.2. Experimental design

To test whether two different EIAs could detect expected increases in FCM levels, we used two treatments for both the pilot study and the validation experiment. We employed a single-subject design, which minimizes confounding due to individual differences in basal and peak FCM levels [59]. After a two-week acclimation period, we randomly assigned animals to either the pharmacological or the biological treatment group. For the pilot study, we assigned 10 male lizards to the pharmacological treatment and 6 male lizards to the biological treatment. For the validation experiment, we assigned 4 male and 4 female lizards to the pharmacological treatment, and 4 male and 4 female lizards to the biological treatment. Each experiment consisted of a baseline phase, a treatment phase (where lizards were exposed to either the pharmacological or biological treatment), and a post-treatment phase (Fig. 1). The methodology during the pilot study and the validation experiment differed in the total number of days we collected faeces for biologically treated animals (see below) and the behavioural observation schedule (see "Behavioural observations" section). Otherwise, we followed the same methodology for the two experiments.

For the pharmacological treatment, we artificially induced an increase in circulating levels of corticosterone by applying transdermal corticosterone (Sigma C2505) mixed with pure sesame oil (3 μg corticosterone / 1 μl oil) to the backs of the lizards ([83]; modified by [84]). We used the same proportion as with other reptile species of similar size and weight as our study species in which this protocol has been previously applied [73,83,84]. Since complete dilution of the corticosterone crystals proved to be difficult to obtain when mixing them directly with oil, we included a middle step in the preparation of the solution [85]. We diluted 7.5 mg of corticosterone in 750 μl of absolute ethanol (VWR Chemicals). After vortexing, this solution was added to 2.5 ml of sesame oil. We thoroughly mixed the solution again, and left the vial open overnight for ethanol evaporation. We applied a drop of 4.5 μl of the working solution to the backs of the animals (between the shoulder blades) using a pipette. To avoid additional disturbance, we applied the corticosterone solution during the night (around midnight, four to five hours after the lights went off), when lizards were colder and relatively inactive. Also, lower night temperatures reduced the risk of solution evaporation before being absorbed by the animals' skin [84]. We treated the animals with corticosterone for five consecutive nights, starting the last day of baseline. We treated 10 males in the pilot study and four males and four females in the validation experiment. One individual from the pilot study had to be excluded from the analyses due to insufficient faecal samples. Overall, we sampled voided faeces for a total duration of 19 days for both the pilot study and the validation experiment (Fig. 1).

For the biological treatment, we subjected lizards to handling and confinement, mimicking the situation when lizards are captured in the wild and transported to the laboratory. On the last day of the baseline period, we removed the lizards from their enclosures and handled them following standard procedures: we weighed the lizards, took morphometric measurements (SVL, tail length) and then placed the animals in cloth bags used for transportation. We held lizards in the bags for 4–5 h

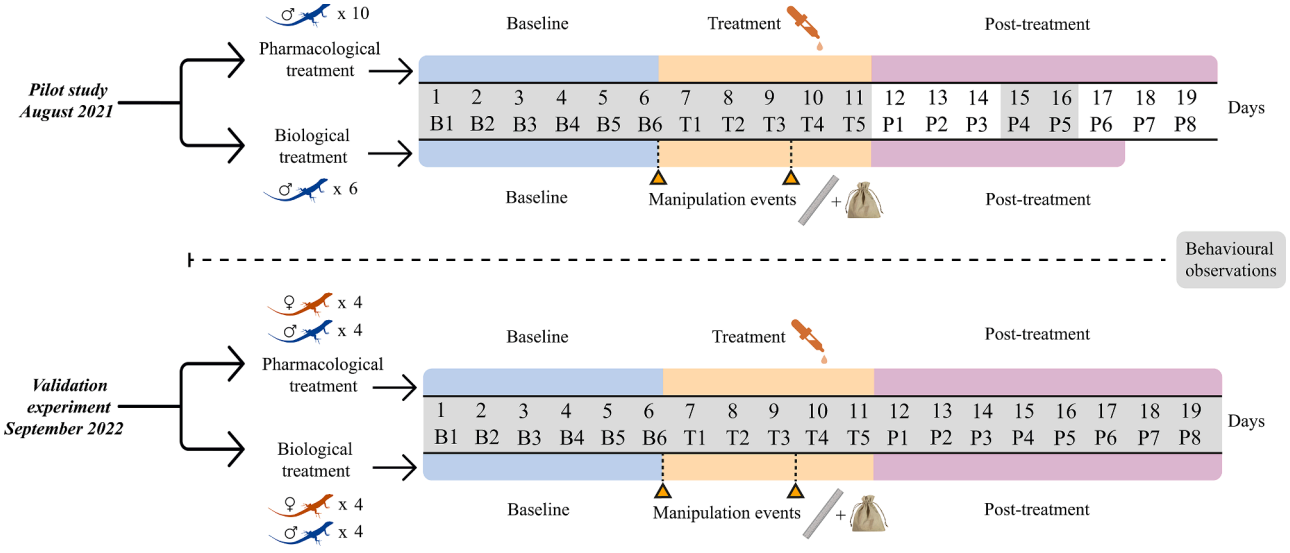

Fig. 1. Schematic overview of the experimental schedule and timeline for the pilot study and validation experiment. The total duration of the experiment spanned 19 days, except for the biologically treated males in the pilot study, for which it lasted 17 days. The experiment consisted of a baseline phase (6 days, blue), a treatment phase (5 days, orange), and a post-treatment phase (6 days for biologically treated males in the pilot study, 8 days for pharmacologically treated males and both treatment groups in the validation experiment, purple). The timeline displays the chronological sequence of natural days, accompanied by corresponding codes referenced in the text for each day within each phase. The baseline phase is denoted by 'B1′ to 'B6′, the treatment phase by 'T1′ to 'T5′, and the post-treatment phase by 'P1′ to 'P8′. In the pharmacological treatment, the treatment days correspond to each of the days following the transdermal application of corticosterone. For the biological treatment, two manipulation events occurred (marked by an orange triangle and dashed line): one in the afternoon/night from day 6 to day 7, and another from day 9 to day 10. "T1" and "T2" correspond to 12 and 36 h after first manipulation, respectively. "T3" corresponds to 60 h after first manipulation, and it is the day in which the second manipulation occurred. "T4" and "T5" correspond to 12 and 36 h after second manipulation, respectively. Faecal samples were collected daily for both experiments, and for all treatments. Behavioural observations were recorded during the days shaded in grey.

Table 1
Partial ethogram of *Podarcis muralis* with empirical descriptions of relevant behaviours. Behaviours were classified as states or events (indicated by a superscript 1).

Category	Behaviour	Description
Orientation and locomotion	Body/head movement[1]	Adjustment of body posture not associated with locomotion, (e.g., turn on its axis, wag the tail, head movement).
	Walking (site change)	Movement of the animal in enclosure, characteristically relatively slow (i.e., walking/climbing resulting in a change in the previous place of sighting).
	Running	Fast-paced movement of the animal around the enclosure.
General behaviour	Basking	Motionless and immediately under the heating source, with ventral side in contact with the surface.
	Hiding	Not visible to the observer (i.e., inside the shelter or under the substrate).
	Head out	Motionless inside the shelter but fully or partially sticking its head out (not beyond forelimbs).
	Perching	Motionless and visible to the observer (either fully exposed or with the body sticking out beyond the forelimbs from a shelter) from an elevated posture (head and body elevated, forelegs partially or fully extended).
	Resting	Motionless and visible to the observer (either fully exposed or with the body sticking out beyond the forelimbs from a shelter), usually with eyes closed and with ventral side in contact with the surface (and not basking).
	Foraging	Foraging behaviour including actively chasing a prey or biting/restraining prey.
	Drinking	Drinking water.
	Defecating	Extrusion of faecal material.
	Cloacal drag[1]	Pressing the cloaca to the substrate while slowing moving the body forward.
Abnormal behaviour	Interaction with transparent boundaries	Stereotyped interaction of the lizard with the limits of the terrarium consisting of: a) scratching/clawing the walls repeatedly with its forelegs, as if attempting to climb or dig-out and/or b) pressing its snout or trying to bite the walls of the terrarium.

[1] Event behaviours.

in a humidity and temperature-controlled dark room, simulating transportation to the laboratory. We then released them into their enclosures during the night. On the third day after the initial manipulation event, we again gently handled the animals, placed them in bags and kept them there for 2 h before releasing them back into their respective terraria. We treated six males in the pilot study, and four males and four females in the validation experiment. One individual from the pilot study had to be excluded from the analyses due to insufficient number of faecal samples collected. Overall, we sampled voided faeces for a total duration of 17 days for the pilot study and 19 days for the validation experiment (Fig. 1).

2.3. Faecal corticosterone metabolites (FCMs)

We checked the terraria for fresh faeces four times a day (at 10.35 a.m., 11.55 a.m., 2.30 p.m., and 3.30 p.m.) throughout the experiments. Faecal pellets were collected using tweezers that were cleaned with alcohol between each collection to avoid cross-contamination, placed into zip-lock plastic bags, and frozen to −20°C immediately after collection to avoid degradation by intestinal bacteria, which can affect the structure and stability of corticosterone metabolites [54]. As all samples were processed at the same time (October-November 2022), samples from the pilot study were kept frozen for a year, but samples from the validation only remained frozen for a month before being analysed.

To extract the metabolites, we first thawed and weighed all samples. A minimum of 10 mg is recommended for accurate steroid measurement [73,86]. Very small faecal samples can give disproportionally high FCM concentrations [18], so pellets weighing less than 5 mg were excluded from the experiment. Faecal samples weighing from 5 mg to 10 mg were pooled with samples of the same individual within the same treatment (either from the previous or next day, or from the same day if available). Those weighing $>$ 10 mg were processed individually. Faecal samples were transferred to Eppendorf tubes and weighed after removal of any crystalized uric acid remains; a total of 1 ml of 60% methanol (methanol: water, 60:40) was added per 50 mg of wet faeces (and the proportionate volume for samples weighing less than 50 mg). The suspended samples were then vortexed for 15–20 min and centrifuged (9000 g; 15 min). Supernatant was then collected, diluted 1 + 9 with assay buffer and stored frozen in microtubes. Metabolites are stable in methanol and the same extract was used to perform both EIA assays.

We measured FCMs using two group-specific EIAs: 5α-pregnane-3ß,11ß,21-triol-20-one ([87]; hereafter referred to as 37e), and 11-oxoaetiocholanolone ([88]; hereafter referred to as 72T), which have been successfully validated for other reptiles [72–74]. We used 10 µl aliquots of the diluted samples' extract for the 72T assay and 25 µl aliquots per sample for the 37e assay. Assays were performed using microtiter plates following the EIA procedure described by Möstl et al. [88] and Touma et al. [87]. The sensitivity of the 72T and 37e EIAs were 8 and 2 ng/g, respectively. Intra- and inter-assay CVs were always below 10% and 13%.

2.4. Behavioural observations

We performed behavioural observations throughout the validation experiment (Fig. 1). We built a partial ethogram (Table 1) from the published literature on this species and from our own observations. The ethogram includes behaviours relevant for welfare assessment [17, 89–91] that are frequently observed (excluding social interactions) in this species in the wild and in captivity. Observations during the validation experiment started at 9.35 a.m., shortly after the lights were turned on (9.15 a.m.), and continued until 1.30 p.m. Behaviour was recorded by an observer (AB) using scan sampling, in which all lizards were observed at 20-minute intervals. We used an instantaneous recording rule, noting the behaviour occurring at the exact moment of each scan (Martin & Bateson, 2021). This approach provided data on behaviour occurrences across all individuals. Observations were made from behind a hide to allow unobtrusive observation of the animals, thus minimizing observer impact on lizard behaviour [92]. Faeces were collected daily while behaviour was being recorded. Sample collection required the observer to inspect the cages, potentially disturbing the lizards' behaviour. To mitigate this, the interval between scans was doubled (40 min) after we checked for faeces, allowing animals to recover from the perturbation. For the pilot study, we collected behavioural data throughout all days of the baseline and treatment phases, but only for two days of the post-treatment phase (P4 and P5, Fig. 1). Observations started at 9.35 a.m. and continued until 12.35 p.m. using the same methodology as in the validation, with intervals of 30 min between scans. In the pilot experiment, we only performed behavioural observations of lizards under the pharmacological treatment.

2.5. Data analysis

To interpret the results of the EIA analysis, we calculated the following metrics (Table 2 and Table S1): 1) median FCM concentrations of all samples available during baseline (pre-treatment), treatment and post-treatment phases; 2) peak levels after start of treatment and the day within phase in which peak occurred; 3) x-fold increase of peak levels

Table 2
Summary of results from both experiments (including pharmacological and biological treatments) and EIA tests (37e and 72T). During the pilot study, we included nine male individuals in the pharmacological treatment and five male individuals in the biological. During the validation experiment, we included four male and four female individuals for both treatments.

	Treatment	Sex	Test	Baseline[a]	Treatment[b]	Post[c]	Peak after treatment[d]	Fold increase[e]	Overall peak[f]	Minimum value[g]	Last value[h]
Pilot study August 2021	Pharmacological	M	37e	99	481	179	881	9.0	881	68	173
			72T	315	706	360	1163	4.9	1163	161	441
	Biological	M	37e	57	99	160	265	4.7	265	44	143
			72T	253	388	523	809	2.6	809	181	523
Validation experiment September 2022	Pharmacological	F	37e	214	3184	909	5794	25.3	5794	143	648
			72T	555	2745	1715	4951	8.5	4951	391	1275
		M	37e	185	731	456	2087	13.9	2087	104	270
			72T	435	972	793	1829	3.3	1830	254	550
	Biological	F	37e	155	232	158	427	2.6	426	84	192
			72T	462	579	547	1043	1.8	1093	250	719
		M	37e	154	175	218	310	1.8	310	83	207
			72T	405	621	621	832	2.2	832	210	566

[a] median of baseline (pre-treatment) levels in ng/g faeces.
[b] median of levels in ng/g faeces during treatment.
[c] median of post-treatment levels in ng/g faeces.
[d] peak levels after start of treatment (ng/g).
[e] x-fold increase of peak levels above median baseline concentrations.
[f] overall peak levels (ng/g).
[g] minimum value (ng/g) recorded, and day within phase in which it occurred.
[h] concentration levels at the end of the experiment (last sample collected).

above median baseline concentrations; 4) overall peak levels and the day within phase in which peak occurred; 5) minimum value of FCM levels and day within phase in which it occurred; and 6) FCM levels at the end of the experiment (last sample collected). Peak values were defined as the highest FCM concentration observed among samples. In the biological treatment, medians of the treatment phase included all samples from the two days after manipulation plus the day of the second manipulation (five days of treatment in total).

For statistical analyses we used R version 4.2.2 [93]. We used linear mixed models (LMM) with lizard identity as a random factor to deal with unbalanced design (due to missing data for some lizards on some days) and repeated measurements on the same individuals [94]. To evaluate the effects of each treatment on FCM levels, we fitted separate models using the package lme4 [95]. For the validation experiment, we included "phase" ("baseline", "treatment", and "post-treatment") and "sex" ("male" and "female") as fixed factors, and the interaction between the two. For the pilot study, we only included "phase" as a fixed factor, as all individuals were male. For the biological treatment, we used a different approach to better reflect the experimental design. To assess the effect of each manipulation event on FCM levels, we created a variable called "subphase" which included seven levels: "baseline", "T1", "T2", "T3", "T4", "T5" (treatment days 1 to 5, Fig. 1), and "post treatment". For the validation experiment, "T3" was eliminated from the analyses as only one sample was retrieved during that day. We conducted Tukey's post-hoc tests for pairwise comparisons using the package emmeans [96], with p-values adjusted for family-wise error rate due to multiple comparisons. We log transformed the data of FCM levels in order to meet normality and homoscedasticity assumptions. Model selection was done using stepwise elimination of non-significant terms [94]. Since there were significant interactions, we used ANOVA type III to compute p-values ($\alpha = 0.05$). Reported p-values refer to the final model without non-significant interactions.

For behavioural data, we calculated the rate of each behaviour per day and individual by dividing the number of occurrences of each behaviour by the number of times the animals were observed each day. Subsequently, we calculated the relative change from baseline for each behaviour using the following formula:

$$(\text{Behaviour rate during treatment} - \text{Behaviour rate during baseline}) / \text{Behaviour rate during baseline}$$

A positive or negative result indicates the proportion by which the rate increased or decreased compared to the baseline, respectively.

3. Results

3.1. Defaecation rate

In the pilot study, a total of 181 samples were collected from 16 male lizards. Defaecation rate ranged from 0.41 to 0.86 faecal sample per individual per day, with an overall average defaecation rate of 0.59 ± 0.15 per day (mean ± SD). In the validation experiment, a total of 231 samples were collected from 8 male and 8 female lizards. Defaecation rate ranged from 0.52 to 1 faecal sample per individual per day, with an overall average defaecation rate of 0.76 ± 0.16 per day. It was not possible to collect samples from all the lizards on a daily basis. Not all samples collected were included in the analyses. Samples too small (weighing less than 5 mg, see Methods) were excluded. In the pilot study we had to exclude 20 samples, resulting in a total of 161 samples available for analysis. In the validation experiment, we had to exclude 34 samples, leaving a total of 197 samples for analysis.

3.2. Pharmacological treatment

Baseline FCM levels of pharmacologically treated animals showed large inter-individual variations for both EIAs (for details see Table S1). In the validation experiment, peak increases were larger using the 37e assay compared to the 72T. For the 37e assay, a median 25.3-fold increase for females and a 13.9-fold increase for males was detected (Table 2). Respective increases for the 72T EIA were 8.5-fold and 3.3-fold (Table 2). In the pilot study, the increase was lower: a median 9-fold increase for the 37e and 4.9-fold increase for the 72T EIA (Table 2). Despite these differences, both assays responded similarly for both sexes (Figs. 2 and 3). Peak FCM concentrations were reached during different days for each individual (Table S1), but overall, the peak was seen during day five of treatment (the last day of transdermal corticosterone administration) for the pilot (Figure S2) and the validation (Fig. 4), except for FCM concentrations detected using the 37e assay in the validation experiment, in which the overall peak was reached on day four of treatment (Fig. 4).

We statistically analysed FCMs elevation for both assays. For the validation experiment, we found a significant interaction between sex

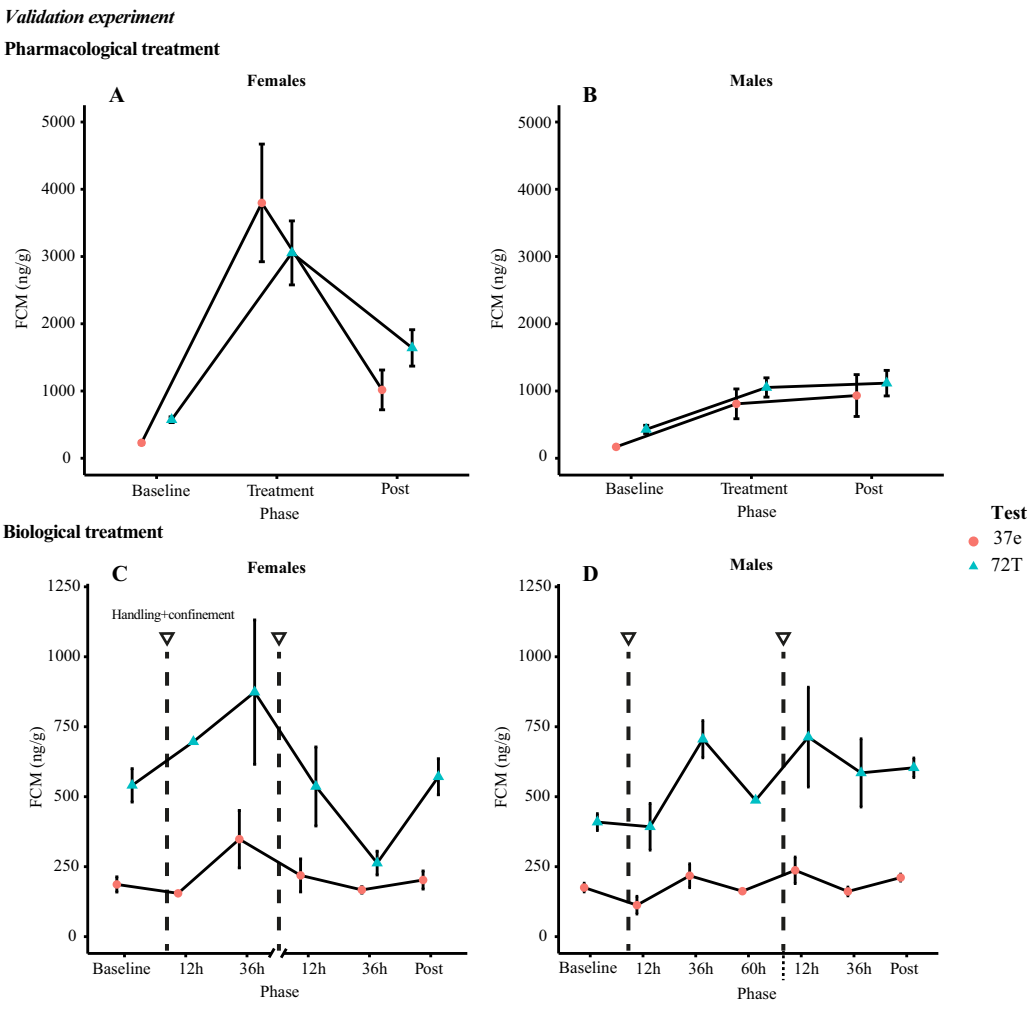

Fig. 2. Mean (± SE) FCM (faecal corticosterone metabolite) levels (ng/g faeces) during the validation experiment for both EIAs (pink circles: 37e; blue triangles: 72T). Top figures show FCM concentrations for females (panel A, $N = 4$) and males (panel B, $N = 4$) in the pharmacological treatment during baseline (6 days), treatment (5 days) and post-treatment ("post", 8 days) phase. Bottom figures show FCM concentrations for females (panel C, $N = 4$) and males (panel D, $N = 4$) in the biological treatment. Dashed lines represent the moment of manipulation (handling and confinement), after which the daily mean concentrations are represented for the two following days of each manipulation, expressed in hours after handling (the manipulation was done during the night, around 12 h prior to first collection of faeces the following day). The second manipulation was performed the night of the third day after the first manipulation (60 h). No samples were collected for the 60h period for females. Baseline for the biological treatment was 6 days, and post-treatment ("post") was 8 days.

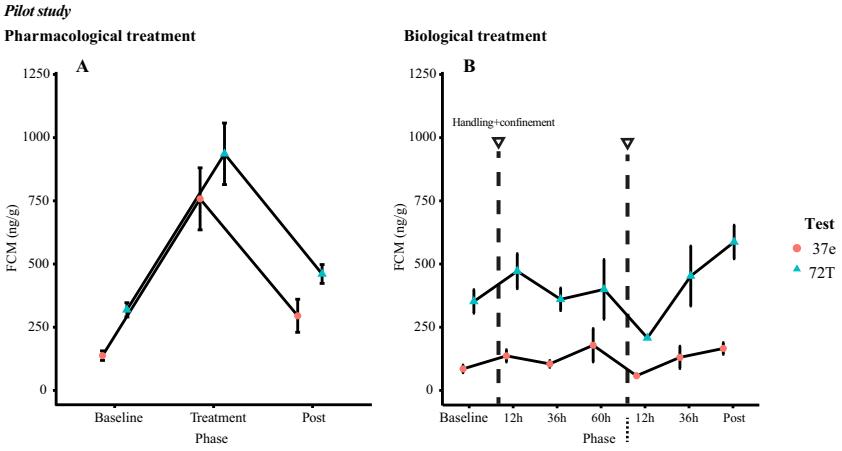

Fig. 3. Mean FCM levels (ng/g faeces) during the pilot study for both EIAs (pink circles: 37e; blue triangles: 72T). Panel A shows FCM concentration for all animals included in the analysis ($N = 9$ males) in the pharmacological treatment during baseline (6 days), treatment (5 days) and post-treatment ("post", 6 days). Panel B shows FCM concentration for all animals included in the analysis ($N = 5$) in the biological treatment. Dashed lines represent the moment of manipulation (handling and confinement), after which daily mean concentrations are represented for the two following days of each manipulation, expressed in hours after handling.

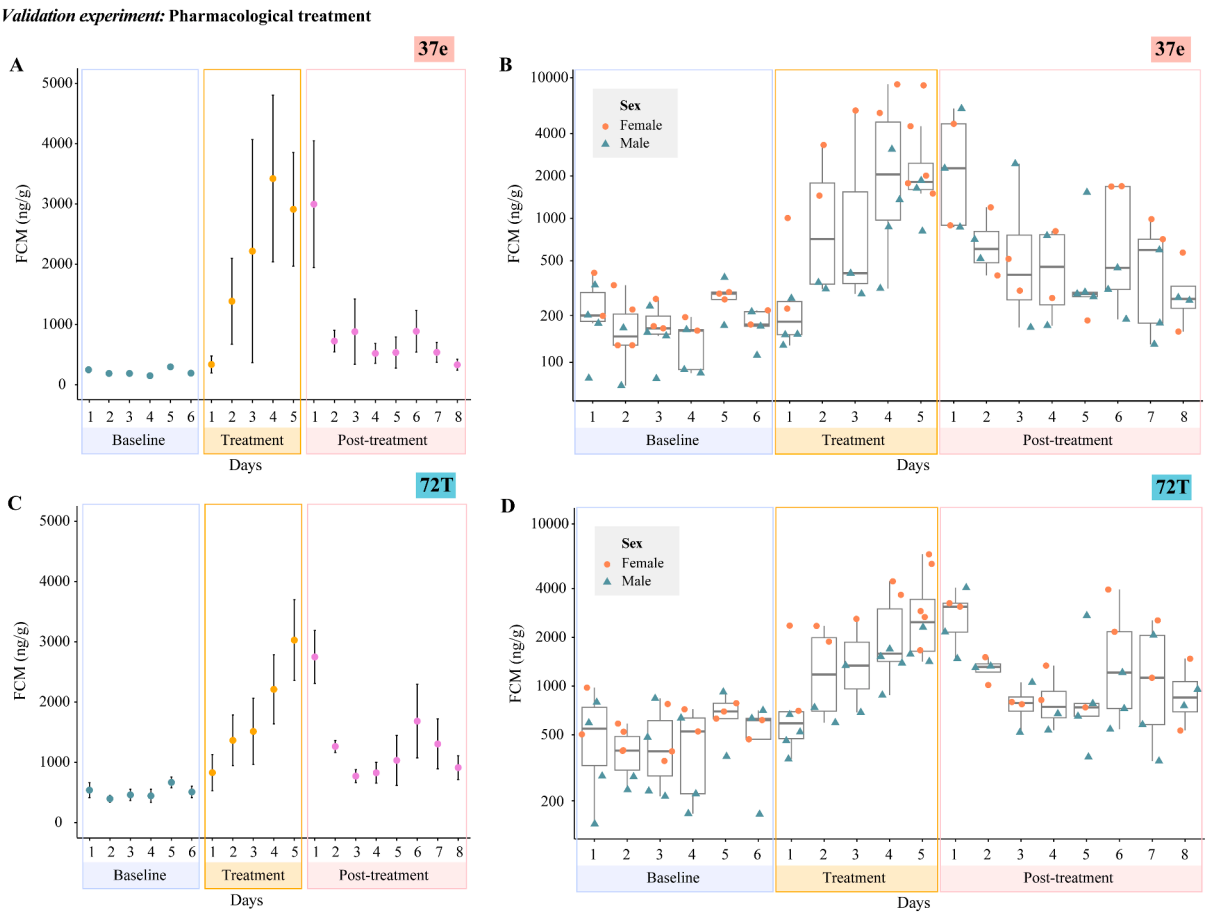

Fig. 4. Comparison of FCM levels (ng/g faeces) of the pharmacological treatment measured using the 37e EIA (A, B) and 72T EIA (C, D) during the validation experiment. Panels A and C show mean (± SE) FCM concentration per day throughout the whole experiment. Panels B and D show boxplots of FCM concentrations per day (orange circles: samples from females; blue triangles: samples from males). The shaded area depicts the period in which corticosterone was transdermally administered. Note that in B and D the y-axes tick labels are not equidistant due to logarithmic scaling to prevent overlap.

and phase (37e: $\chi^2 = 11.68$, p-value = 0.0029; 72T: $\chi^2 = 6.28$, p-value = 0.043), so we fitted separate models for each sex (Table 3). We found a significant effect of phase in both assays for males (37e: $\chi^2 = 23.76$, p-value <0.001; 72T: $\chi^2 = 28.04$, p-value <0.001) and for females (37e: $\chi^2 = 67.31$, p-value <0.001; 72T: $\chi^2 = 65.89$, p-value <0.001) on FCM levels (ng/g). Similarly, we found a significant effect of phase in the pilot study for both assays (37e: $\chi^2 = 108.4$, p-value <0.001; 72T: $\chi^2 = 67.53$, p-value <0.001) (Table 4). To examine which phases significantly differed in FCM concentration, we performed post-hoc pairwise comparisons. All phases significantly differed from each other in the case of females for both assays (37e and 72T) and experiments (validation and pilot) (Figs. 2 and 3; Table S2A and Table S3A). In the case of males, we found significant differences among all phases for both assays in the pilot (Fig. 3; Table S3A) but not between treatment and post-treatment

Table 3
Output from LMMs for the validation experiment. A) Results from LMMs evaluating the effect of "phase" (baseline, treatment and post-treatment) and its interaction with "sex" on FCM levels for both EIAs (37e and 72T) for the pharmacological treatment. B) Results from LMMs evaluating the effect of "subphase" and its interaction with "sex" on FCM levels for both EIAs (37e and 72T) for the biological treatment. Statistics for non-significant interactions are included at the point of their deletion from the model. The variable called "subphase" included seven levels: "baseline", "T1", "T2", "T3", "T4", "T5" (treatment days 1 to 5, Figure 1), and "post treatment". Treatment day "T3" was eliminated from the analyses as only one sample was retrieved during that day. All models include the identity of the lizard as a random factor. Significant factors are highlighted in bold ($p < 0.05$).

A) Pharmacological treatment		37e			72T		
Model	Term	χ^2	Df	p-value	χ^2	Df	p-value
*FCM concentration (log transformed) ~ phase*sex*	Phase*Sex	11.68	2	**0.003**	6.28	2	0.043
	Phase	64.04	2	**< 0.001**	55.14	2	**< 0.001**
	Sex	0.66	1	0.418	3.52	1	0.061
FCM concentration of males (log transformed) ~ phase	Phase	23.76	2	**< 0.001**	28.04	2	**< 0.001**
FCM concentration of females (log transformed) ~ phase	Phase	67.31	2	**< 0.001**	65.89	2	**< 0.001**
B) Biological treatment		37e			72T		
Model	Term	χ^2	Df	p-value	χ^2	Df	p-value
*FCM concentration (log transformed) ~ subphase*sex*	Subphase*Sex	3.64	5	0.604	8.166	5	0.147
	Subphase	15.90	5	**0.007**	18.52	5	**0.002**
	Sex	0.06	1	0.811	0.007	1	0.934

Table 4
Output from LMMs for the pilot study. A) Results from LMMs evaluating the effect of "phase" (baseline, treatment and post-treatment) on FCM levels for both EIAs (37e and 72T) for the pharmacological treatment. B) Results from LMMs evaluating the effect of "subphase" on FCM levels for both EIAs (37e and 72T) for the biological treatment. The variable called "subphase" included seven levels: "baseline", "T1", "T2", "T3", "T4", "T5" (treatment days 1 to 5, Fig. 1), and "post treatment". All models include the identity of the lizard as a random factor. Significant factors are highlighted in bold ($p < 0.05$). We only included "subphase" in the models as a fixed factor, as all individuals were male.

A) Pharmacological treatment		37e			72T		
Model	Term	χ^2	Df	p-value	χ^2	Df	p-value
FCM concentration (log transformed) ~ phase	Phase	108.40	2	< **0.001**	67.53	2	< **0.001**
B) Biological treatment		**37e**			**72T**		
Model	Term	χ^2	Df	p-value	χ^2	Df	p-value
FCM concentration (log transformed) ~ subphase	Subphase	17.58	6	**0.007**	9.84	6	0.131

in the validation experiment (Fig. 2; Table S2A).

3.3. Biological treatment

Baseline FCM levels of biologically treated animals also showed large inter-individual variations in both EIAs (for details see Table S1). In the validation experiment, peak increases were very similar for both tests and sexes: a median of 2.6-fold increase in females vs 1.8-fold increase in males for the 37e assay; for 72T, females showed a median of 1.8-fold increase vs 2.2-fold increase in males (Table 2). In contrast, in the pilot study, the increase was comparatively higher: a median of 4.7-fold for the 37e and 2.6-fold for the 72T EIA (Table 2). Although peak increases were generally higher with the 37e assay, FCM concentrations were higher using the 72T EIA (Figs. 2 and 3). Different individuals reached FCM peak concentrations in different days (Table S1). Overall, peaks during the validation experiment were reached on the second day of treatment (day 8 of the experiment, 36 h after the first manipulation event), for both assays (Fig. 5). On the other hand, in the pilot study, peaks were observed during day one post-treatment (day 12 of the experiment, 60 h after the second manipulation event) for the 37e assay (Figure S3). Regarding 72T EIA, peaks were reached during day two post-treatment ("P2"), but only one sample was collected during that day (day 13 of the experiment, Figure S3).

For the validation experiment, we found a significant effect of subphase (37e: $\chi^2 = 15.9$, p-value = 0.007; 72T: $\chi^2 = 18.52$, p-value = 0.002) but not sex (37e: $\chi^2 = 0.06$, p-value = 0.81; 72T: $\chi^2 = 0.007$, p-value = 0.93) on FCM levels (ng/g) (Table 3). Post-hoc pairwise comparisons detected that, in the case of the 37e assay, the only significant difference was between "T1" and "T2", i.e., between 12 h and 36 h after the first manipulation event (Figure 5; Table S2B). Conversely, the only significant difference using the 72T assay was between "baseline" and "T2", i.e., between the baseline phase and 36 h after the first manipulation event (Figure 5; Table S2B). In the pilot study we found a significant effect of subphase, but only when using the 37e assay ($\chi2 = 17.58$, p-value = 0.007) and not the 72T ($\chi2 = 9.84$, p-value = 0.13) (Table 4). Post-hoc pairwise comparisons only revealed a significant difference between "baseline" and "post" phases (Figure S3; Table S3B).

3.4. Behavioural observations

We selected the relevant behaviours for analysis based on their frequency (infrequent behaviours were discarded; Table 1) and relevance for welfare assessment [17,89–91,97]: "interaction with transparent boundaries" (ITB), "walking", "hiding", "head out", "basking", and "perching".

We observed an increase in the rate of "hiding" over baseline in the pilot study (only males) and for all animals in the validation experiment except for pharmacologically treated males (Fig. 6). The increase in "hiding" after pharmacological treatment was similar for males in the pilot study ($n = 10$; mean change from baseline ± SE; treatment phase: 0.27 ± 0.19; post-treatment phase: 0.65 ± 0.38) and females in the validation experiment ($n = 4$; treatment phase: 0.28 ± 0.16; post-treatment phase: 0.58 ± 0.2). Notably, the increase in "hiding" was more pronounced in females from the biological treatment, particularly during the treatment phase (treatment phase: 1.37 ± 0.38; post-treatment phase: 0.52 ± 0.19).

In relation to "basking", the most pronounced change compared to baseline occurred in females subjected to the biological treatment during the treatment phase ($n = 4$; treatment phase: −0.48 ± 0.14; post-treatment phase: −0.14 ± 0.09). The rate of change in "basking" was similar for males in the pilot study ($n = 10$; treatment phase: −0.14 ± 0.04; post-treatment phase: 0.14 ± 0.2) and females in the validation experiment ($n = 4$; treatment phase: −0.14 ± 0.1; post-treatment phase: −0.23 ± 0.12), particularly during the treatment phase.

Finally, "interaction with transparent boundaries" (ITB) was more affected for females under pharmacological ($n = 4$; treatment phase: 0.38 ± 0.24; post-treatment phase: 0.16 ± 0.22) and biological treatments ($n = 4$; treatment phase: −0.23 ± 0.09; post-treatment phase: −0.2 ± 0.09) than males (Fig. 5). The relative changes from baseline for the remaining behaviours are depicted in Figure S4.

4. Discussion

In our study, we assessed the suitability of two EIAs for the measurement of FCMs in European common wall lizards. Both tests detected significant increases of metabolite concentration after a pharmacological (transdermal corticosterone administration) and a biological treatment (handling and confinement). The 37e assay (5α-pregnane-3ß,11ß,21-triol-20-one) resulted in a greater sensitivity in detecting increases over baseline compared to the 72T (11-oxoaetiocholanolone) EIA, particularly with the pharmacological treatment (Table 2). For the biological treatment, the increase in FCM levels over baseline levels was generally slightly larger using the 37e assay, except for the males in the validation experiment. Overall, the 37e EIA seems more suitable to monitor changes in FCM levels in *P. muralis*.

4.1. Pharmacological and biological validation

Both of our experiments followed a repeated-measures design, therefore FCMs elevation was compared with the baseline. We observed a marked variability in baseline levels (Table S1), in accordance with most studies investigating FCMs [19,59,64]. For instance, in reptiles, Martín et al. [75] performed a validation experiment in the amphisbaenian species *T. wiegmanni*, and observed basal concentrations of FCMs ranging from 50 to 600 ng/g of dry faeces, with some samples reaching levels as high as 2850 ng/g. Borgmans et al. [73] performed a validation experiment in an anole lizard (*A. carolinensis*) and reported FCMs ranging from 12 to 147 ng/g. In our study, FCM levels varied from 53 ng/g to 317 ng/g when measured using the 37e assay, and from 184 ng/g to 801 ng/g using the 72T EIA (Table S1).

Compared to median baseline values, FCM levels significantly increased during pharmacological treatment in both experiments and sexes, particularly in females (up to 25.3 times with 37e EIA in the validation experiment). We observed a cumulative effect of corticosterone administration, which was already evident after initial administration, but peaked during the last two days of treatment. As expected, FCM elevation was lower for animals under the biological treatment [60], with fold increases up to 4.7 in males from the pilot experiment,

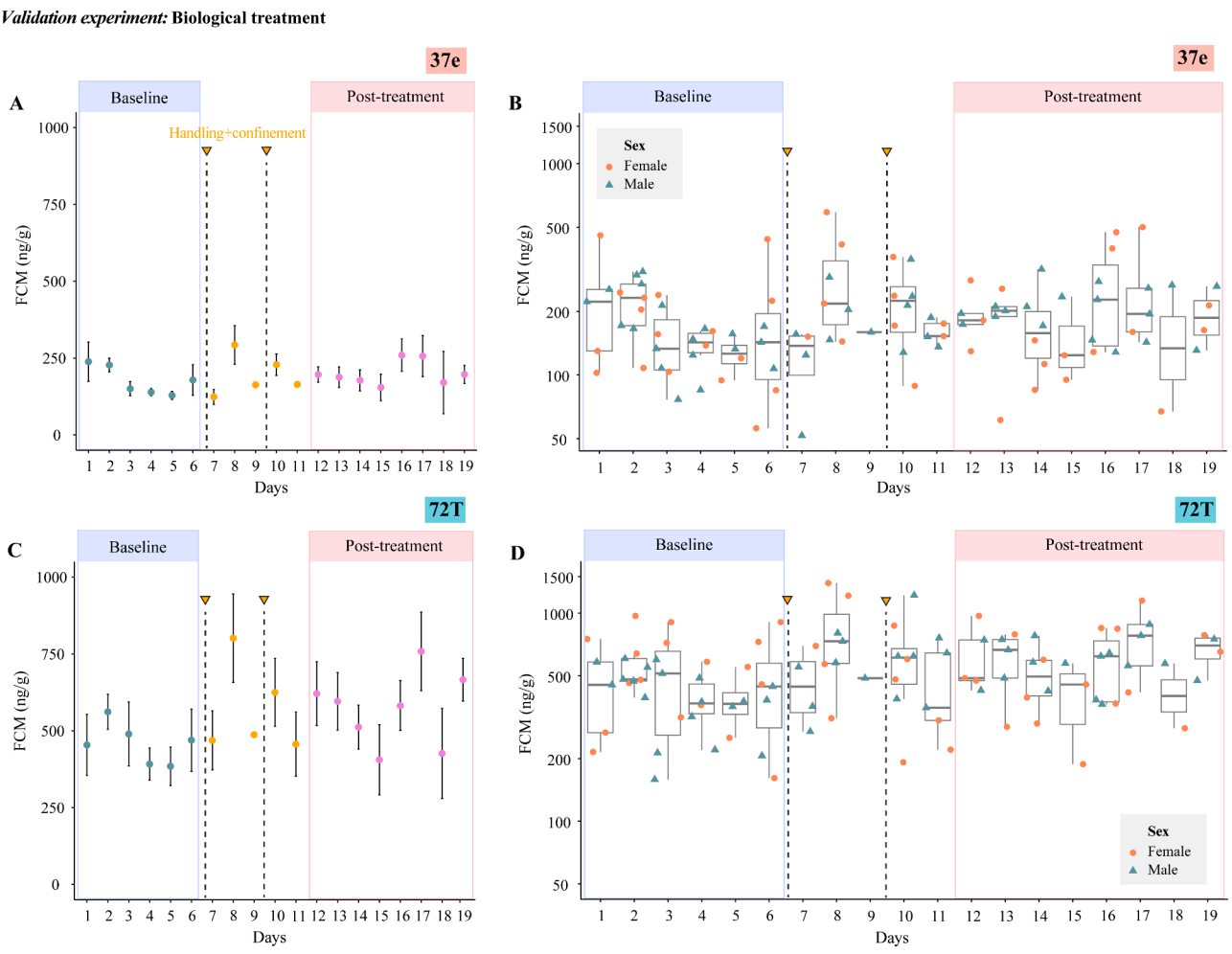

Fig. 5. Comparison of FCM levels (ng/g faeces) of the biological treatment measured by 37e (A, B) and 72T EIA (C, D) during the validation experiment. Panels A and C show mean (± SE) FCM concentrations per day throughout the whole experiment. Panels B and D show boxplots of FCM concentrations per day (orange circles: samples from females; blue triangles: samples from males). Blue shaded area includes the baseline period (6 days), and red shaded area includes post-treatment period (8 days). Dashed lines represent the moment of manipulation (handling and confinement). Days seven and eight correspond to 12 h and 36 h after the first manipulation event, respectively. Days 10 and 11 correspond to 12 h and 36 h after the second manipulation event, respectively. Second manipulation was performed the night of day 9 of the experiment (60 h after first manipulation). Note that in B and D y-axes tick labels are not equidistant due to logarithmic scaling to prevent overlap.

and slightly lower during the validation experiment (1.8 and 2.6-fold increases in males and females, respectively, using 37e). We did not observe significant sex differences in animals subject to handling and confinement.

Similar glucocorticoid elevations have been reported in studies measuring plasma corticosterone levels. For instance, in *Zootoca vivipara* [84] and *Urosaurus ornatus* [83] transdermal administration of corticosterone at the same concentration as in our study resulted in 5 to 13-fold increases. Also, injection of corticosterone in western fence lizards (*Sceloporus occidentalis*) led to a substantial 26-fold increase in plasma corticosterone. In *U. ornatus*, confinement in bags for up to 4 h led to 6-fold increases [34]. Although research has demonstrated a correlation between plasma glucocorticoid increases following a stressful event and corresponding elevations in faecal glucocorticoid metabolite levels ([55, 59]; in reptiles: [72,98]), it should be noted that these fluctuations of corticosterone in blood are of shorter duration compared to the duration reflected in faecal samples, and do not represent an integrated measure of corticosterone levels [20]. However, comparing our results with other studies measuring corticosterone directly in faeces may be challenging, given that the number of studies that have used validated FCMs techniques in reptiles is very low: 66% of the papers dealing with FCMs have not been validated [71].

In the validation experiment with the amphisbaenian *T. wiegmanni*, FCM levels were observed to be 327 times higher than initial levels, after 12 days of corticosterone administration (ratio of 1 μg corticosterone / 3 ml oil) [75]. The same technique of transdermal corticosterone administration was employed with *A. carolinensis*, a species of similar size and weight to our study species. After five days of corticosterone administration (at the same concentration as in our study) peak concentration values were reported to be 7 to 144 times higher than baseline levels, with a median of 27 [73]. Finally, in the sungazer lizard (*S. giganteus*), an ACTH challenge involving an injection of synthetic adrenocorticotropic hormone resulted in a 5.5-fold increase using the 37e assay, and a 2.2-fold increase using the 72T assay [74].

Overall, the FCMs fold increases from baseline observed in our experiment agree with findings from other studies where animals were subjected to similar treatments involving administration of exogenous corticosterone, and confinement, restraint, and/or handling. In our results, the elevation of FCMs for females undergoing pharmacological treatment raises the question of whether corticosterone metabolite concentrations exceed the species' natural range, i.e., if we were administering pharmacological doses of exogenous corticosterone to them. Some studies consider doses of corticosterone or cortisol as pharmacological if they induce greater elevations than those observed

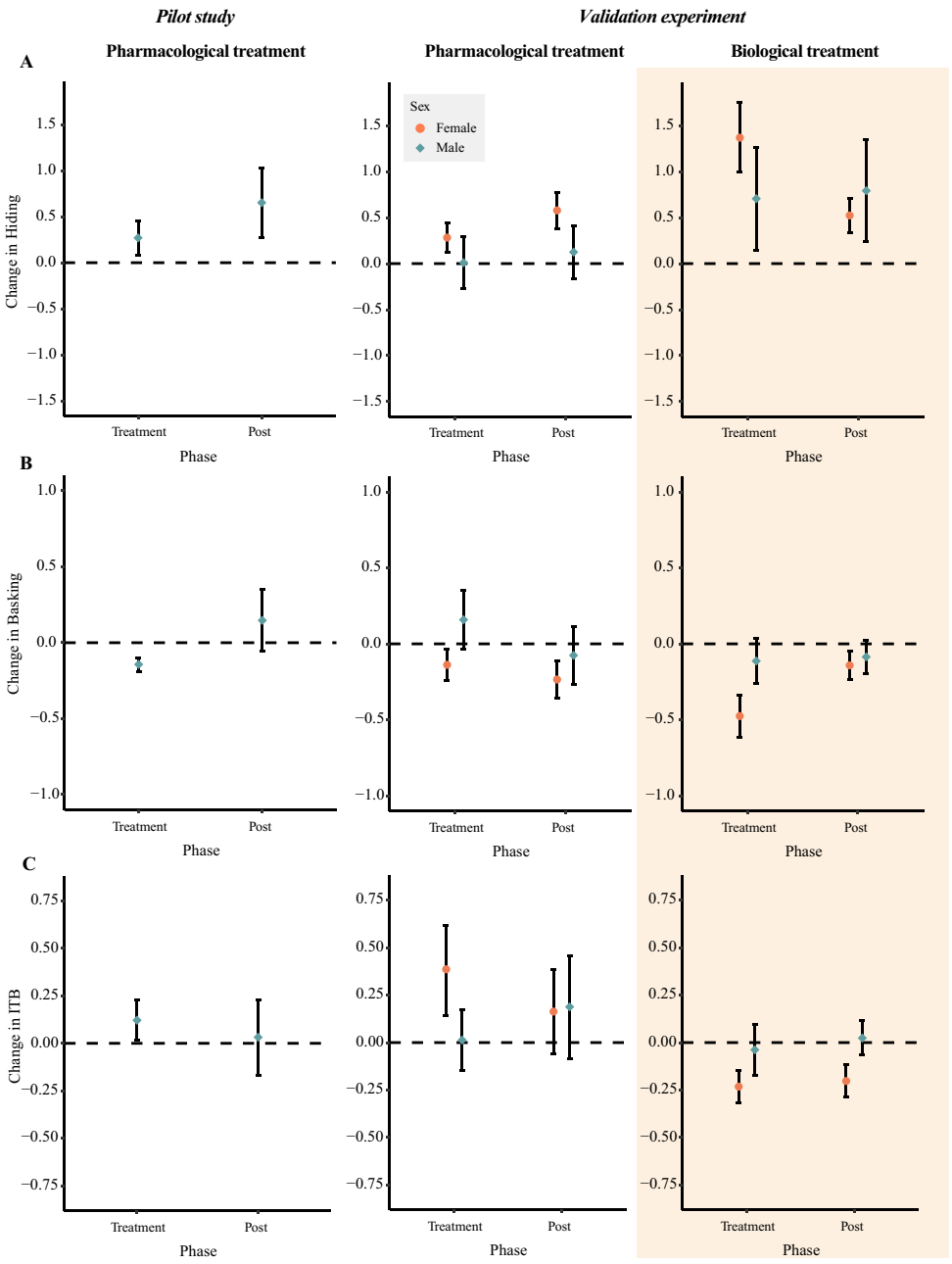

Fig. 6. Change in rate (relative to baseline) for "hiding" (A), "basking" (B) and "interaction with transparent boundaries" (ITB, C). Datapoints show the mean proportion of change over baseline (± SE). Values above the dashed line mean positive changes over baseline relative frequencies of a given behaviour while values below the dashed line mean negative changes. In the pilot experiment, behavioural observations were only recorded under the pharmacological treatment.

with a stressor such as an ACTH challenge (e.g., [99]) or if they exceed the range of glucocorticoid concentration seen in the wild [46]. The relative increase in corticosterone-treated females fell within the upper range of variation observed in studies of similar-sized species employing the same technique and corticosterone concentration (measured in blood), and other authors considered changes in glucocorticoid to be within the physiological range of their respective study species [37,38,40,84,83,100]. However, due to the limited experimental evidence and lack of information on corticosterone variability in reptiles, establishing the distinction between physiological and pharmacological doses can be difficult. To determine whether the doses administered in our study are pharmacological, it would be necessary to gather species-specific information regarding the normal range of corticosterone levels in faecal samples, as well as the observed increases following natural stressful events. Additionally, a study could be conducted to assess the impact of the administered dose on corticosterone levels (e.g., [46,48]).

To properly interpret the results of our validation experiment, it is important to consider several factors. First, the relative concentration of corticosterone differed between females and males due to their size difference. As males were larger, females received a higher relative dose of corticosterone per unit mass, which could potentially yield higher elevations of FCM levels. However, males from the pilot study and females from the validation experiment had similar weights (pilot males: 5.1 ± 0.17 g; validation females: 5.3 ± 0.13 g) and, yet, pharmacologically treated males from both experiments exhibited corticosterone elevation within a similar range (Fig. 2B and Fig. 3A), suggesting that sex differences in glucocorticoid stress response in our study are likely not solely attributable to the weight disparity, but to differences in adrenal activity and/or in the metabolism and excretion of glucocorticoids between males and females [33,101,102].

4.2. Low defaecation rates in reptiles

Another important factor to consider when interpreting FCM levels is defaecation rate [72,74]. Infrequent defaecation has been reported in other validation studies in reptiles (e.g., [72,73]), and was an issue in our case. It is also important to investigate whether the cumulative effect of increased stress on faecal metabolites is reflected in the faeces even when there is no immediate defaecation after the stressor event. For instance, if an individual did not defecate the day after the stressor (or corticosterone administration), would the next voided sample still reflect a transient increase in circulating levels? What is the maximum interval at which the increase in corticosterone is reflected in FCMs? For instance, the peak of excretion in pigs intravenously injected with ^{14}C-steroids was delayed due to a lack of defaecation [103]. Since this depends on factors ranging from the individual to the species (for instance, species' gut passage time, [54]), it is important to consider defaecation rate in future studies.

In our study, overall mean defaecation rate per lizard per day was 0.59 in the pilot study and 0.76 in the validation experiment. Defaecation rates of 0.18 and 0.24 have been reported in Nile crocodiles (*C. niloticus*; [72]), and three-toed box turtles (*Terrapene carolina triunguis*; [104]), respectively. In our study, we cannot know if missing samples are due to lack of defaecation or to loss of the sample, i.e., animals defaecated outside of the period in which we searched for samples. The period during which we collected the samples corresponds to the more active period of our species [105–107]. Daily activity of *P. muralis* is usually higher in the mornings and early afternoon (9.00 a.m. to 4.00 p.m.), although activity patterns are temperature and weather dependent, so their activity period can change to a bimodal pattern in hot weather conditions, as animals appear less active during the hottest hours of the day [106,107]. We checked the lizard's cages four times a day during their activity period, but some samples might have been lost if voided during the afternoon. Reduced defaecation due to captivity-induced decrease in food intake [90,108] can also have contributed to low defaecation rates. Faeces production varies with food consumption, daily activity patterns, metabolism, and other factors [105,109,110], which can vary greatly among individuals [111]. The information on defaecation rates in *P. muralis* is scarce. In a field study, Avery and Perkins [112] recounted faeces found in the home range of their study population; they recorded mean daily defaecation rates ranging from 0.39 to 0.81, but their estimation was that they were finding approximately 40% of the faeces that the animals actually produced, given that under favourable environmental conditions (high food consumption) lizards were recorded to produce up to two faeces per day [112]. Although some of our lizards defaecated more than once a day, we did not retrieve daily samples from all of them (i.e., we have missing data samples for some days for all of the lizards in the experiment). Nonetheless, all our experimental animals gained weight during the experiment (average percentage weight increase was 11.5% and 8% for females and males, respectively, after the experiments ended). One possible way to overcome the problem of low defaecation rates in reptiles and other species with infrequent defaecation is to collect samples over longer periods of time [72,73], although for wild-caught species such as ours this could add the confounding factor of time spent in captivity, which can increase glucocorticoid levels [75,113].

4.3. Behaviour

Our behavioural results show some trends of reduced thermoregulatory behaviour and increased avoidance behaviour in response to both an artificial elevation of corticosterone and handling and confinement of the lizards; in particular, the behaviour that exhibited the largest change from baseline was "hiding". This behaviour increased in all groups of animals except for pharmacologically treated males during the validation experiment. As a result of increased time spent in shelters, we also observed a decrease in thermoregulatory behaviour, i.e., "basking." Other studies have shown that artificially elevated corticosterone levels lead to increased hiding behaviour, which can be considered an avoidance response (e.g., [114–116]). For example, male tree lizards (*U. ornatus*) with corticosterone dermal patches hid for longer periods of time and exhibited faster anti-predatory responses during simulated encounters with predators than control-patched animals [115]. In our experiment, this change was more pronounced in animals undergoing the biological treatment during the validation experiment. It is worth noting that our experimental animals increased the rate of "hiding" by delaying their daily emergence from shelters, contrary to what was observed in common lizards (*Z. vivipara*), where lizards with experimentally elevated corticosterone levels emerged from their shelters earlier than control lizards [37]. Since we only observed lizards in the mornings, it remains unclear whether the animals' overall daily activity patterns changed or simply shifted to later hours as a result of delayed emergence from shelters.

In an experiment with juveniles of *P. muralis*, basking behaviour was reduced due to an increase in locomotor activity, both in laboratory conditions and in the field [36]. This contrasts with studies that report an increase in "basking" after corticosterone treatment in other lizard species [48,100]. In our experiment, locomotor activity (walking around the enclosure) increased during the pharmacological treatment for males in the pilot study, but we did not observe the same effect for males and females in the validation experiment. Another important behaviour in captive reptiles, particularly from the welfare perspective, is "ITB" (a.k.a., escape attempt or scratching). Although small, the changes from baseline regarding "ITB" seem to follow opposite trends in our two treatments. During the validation experiment, the rate change of "ITB" is more marked in females, increasing in the pharmacological treatment and decreasing in the biological treatment. This decrease is probably due to the fact that biologically treated animals spent a large part of their mornings hiding. However, the increase in "ITB" for animals treated with corticosterone is in line with other studies in which corticosterone levels were artificially elevated either transdermally [36,117] or using implants [118].

Overall, our findings could contribute to the development of comprehensive welfare assessment in captive reptiles. The use of physiological welfare measurements in reptiles is scant compared to other groups, reflecting a marked taxonomic bias that favours primarily mammals and birds (e.g., [4,5,119,120]). It is important to study a wider array of welfare measurements beyond indirect ones (i.e., resource-based measurements, such as enclosure dimensions, shelter, nutrition, and other resources provided to the animals, [17]) as well as to validate new techniques to assess captive reptiles' welfare. Our results offer some information on the potential divergence between physiological and behavioural stress responses (e.g., [67,121,122]), but must be taken as preliminary. We also observed substantial inter-individual variability in FCM levels during both the biological and pharmacological treatments. Although this was expected [19], it may be a confounding factor that should be considered when interpreting the results along with other measures [16]. Future studies should apply more intensive methods for recording behaviour—such as combining various sampling methodologies [92,123]—to further explore the interplay between physiological and behavioural responses at both species and individual levels in response to specific, potentially stress-inducing events.

Specifically, we observed a greater increase in hiding behaviour following handling and confinement (biological treatment) compared to corticosterone administration (pharmacological treatment). However, the levels of FCMs showed a much higher elevation in the pharmacological treatment compared to the biological treatment group (Table 2). Different types of stimuli can trigger various endocrine, behavioural, immune, and neural changes [124], and behavioural responses do not always exhibit a linear relationship with glucocorticoid production [125]. In our case, handling and confinement can be considered psychological stressors (e.g., fear) [126,127], with a clear valence and

functional context [17,90,91,124]. In contrast, the pharmacological treatment directly affects HPA axis function, without a clear threatening stimulus and associated functional context. Thus, the disparity between the physiological and behavioural responses to both treatments could be due to perception of the stressor in the biological treatment as a threat. In this case, it could be argued that our biological treatment simulated a predation event, similarly to what has been observed in other animals [128–130], where rapid behavioural responses such as fleeing or hiding are critical for survival [124,131]. In contrast, the pharmacological treatment may simulate a situation where animals experience repeated events which effectively increase their corticosterone production without perceiving a threat. Additionally, the manipulation in the biological treatment results in an acute and sporadic increase in corticosterone levels reflected in a single peak after the manipulation event, whereas, in the pharmacological treatment, we see a steady increase in FCMs during the five days of treatment, as administration of exogenous corticosterone was done daily. Green iguanas (*Iguana iguana*) exposed to two potentially stressful situations (handling/restraint and removal of climbing structures), differ in their behavioural response, although they show similar elevations of FCM levels [132]. The authors argue that the behavioural changes during the periods of climb deprivation correspond to chronic stress, whereas handling and restraining may have elicited a transitory response (acute stress). Similarly, short periods of handling in blue-tongued skinks (*Tiliqua scincoides*) and in ball pythons (*Python regius*) does not seem to produce chronic stress, but restrain in a container resulted in an acute response in corticosterone elevation for pythons [133]. This interplay among physiology, behaviour, and other factors relevant to the stress response (neuroendocrinology and immune responses; [124]), as well as their implications in terms of emotional arousal, constitute longstanding topics in stress research [134–136]. The perception of physiological stimuli, rather than the perception of the threat, and its interoceptive effect on the animal's emotional response have been the subject of debate throughout the last century, and different theories have been put forward [134]. Research in human and in animal models highlight the importance of context in modulating the effect of physiological signals in the behavioural response (e.g., [137]), along with other factors. For instance, the effect of glucocorticoids involves intricate interactions between the adrenal response and factors such as age, body condition, reproductive status, maternal effects, or environmental characteristics, influencing the behavioural response (in reptiles: [37,38,41,139,138]).

4.4. Conclusion

The results of the present study successfully validate a non-invasive technique (FCMs measurement) for assessing adrenocortical activity in the lacertid lizard *P. muralis* in response to transdermal corticosterone administration, and handling and confinement. In addition, our study provides preliminary data into the complex relationship between glucocorticoid levels and behaviour for this species. The behavioural changes observed, such as increased hiding and decreased basking behaviour, suggest that the animals are responding to perceived threats and engaging in avoidance or anti-predatory behaviours [140,141]. The observed disparity between physiological and behavioural stress responses highlights the importance of considering the animals' perception of the stimuli. Behavioural changes are an integral component of the stress response and encompass alterations that are critical for the survival of the animal [27,142]. While our study validates the suitability of two EIAs for measuring faecal corticosterone metabolites in European common wall lizards, our behavioural findings underscore the intricate interaction between behaviour and physiology, and highlight how interpreting physiological indices (e.g., corticosterone measurements) alone can be insufficient from the perspective of animal welfare [16,28,61,62]. Understanding the integrated response to stress and specific stressors is of utmost importance in captive settings. Both physiological and behavioural changes serve as direct measures (animal-based) of stress; however, limited information is available regarding these aspects in most reptile species [17,21]. Therefore, there is a need to establish comprehensive approaches that encompass both physiological and behavioural dimensions to assess and promote the well-being of animals in captivity.

CRediT authorship contribution statement

Alicia Bartolomé: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rupert Palme:** Writing – review & editing, Supervision, Resources, Investigation. **Sabine Macho-Maschler:** Writing – review & editing, Resources, Investigation. **Pau Carazo:** Writing – review & editing, Supervision, Resources. **Enrique Font:** Writing – review & editing, Supervision, Resources, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2024.114751.

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