Fecal adrenal hormone patterns during ovulatory and non-ovulatory reproductive cycles in female veiled chameleons (Chamaeleo calyptratus)

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Research paper

Fecal adrenal hormone patterns during ovulatory and non-ovulatory reproductive cycles in female veiled chameleons (*Chamaeleo calyptratus)*

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ABSTRACT

The relationship between the reproductive (hypothalamic-pituitary-gonadal; HPG) and adrenal (hypothalamic-pituitary-adrenal; HPA) hormone axes is complex and can vary depending on the species and environmental factors affecting an individual. In an effort to understand this relationship in female veiled chameleons (*Chamaeleo calyptratus*), the patterns of fecal metabolites of corticosterone (C), estradiol (E), testosterone (T), and progesterone (P) were analyzed by enzyme immunoassay (EIA) during ovulatory (OC; eggs laid) and non-ovulatory cycles (NOC; no eggs laid). Glucocorticoid (GC) metabolites in the fecal extracts were characterized by HPLC and corticosterone EIA performance was assessed by parallelism, accuracy and precision tests. The results indicated that the assay chosen reliably measured the hormone metabolites present in the fecal extracts. Regular, cyclical hormone metabolite patterns consisting of an E peak followed by peaks of T, P and C in close succession were observed during both ovulatory and non-ovulatory cycles; relative levels of P and C, however, were higher during ovulatory cycles. Corticosterone metabolite levels, in particular, increased throughout vitellogenesis and peaked in late vitellogenesis (in non-ovulatory cycles) or around the time of ovulation, and remained elevated throughout the gravid period, falling just prior to oviposition. The results provide evidence of variation in glucocorticoid production throughout different stages of the reproductive cycle, including a role in the ovulatory process; the physiology, however, remains unclear.

1. Introduction

The relationship between glucocorticoids and reproduction is complex, and highly context-dependent (Moore and Jessop, 2003). While some studies failed to show an increase in plasma corticosterone during the breeding season, such as in bearded dragon lizards; *Pogona barbata* (Amey and Whittier, 2000), and New Zealand common geckos; *Hoplodactylus maculatus* (Girling and Cree, 1995), many reptiles displayed concurrent elevations in plasma corticosterone throughout the breeding season compared to the non-breeding season as observed in side-blotched lizards; *Uta stansburiana* (Wilson and Wingfield, 1992), red-sided garter snakes; *Thamnophis sirtalis parietalis* (Moore et al., 2001), eastern fence lizards; *Sceloporus undulatus* (John-Alder et al., 2002), and Cuban crocodiles; *Crocodylus rhombifer* (Augustine et al., 2020). More specifically, glucocorticoid levels have been shown to vary with the different stages of the reproductive cycle (i.e. previtellogenesis, vitellogenesis, and gravidity), as seen in *U. stansburiana* (Wilson and Wingfield, 1992), desert grassland whiptail lizard; *Cnemidophorus uniparens* (Grassman and Crews, 1990), tuatara; *Sphenodon punctatus* (Tyrrell and Cree, 1998), and the viviparous lizard; *Lacerta vivipara* (Dauphin-Villemant et al., 1990). Therefore, increased glucocorticoid levels can either mediate or be a consequence of normal reproduction (behaviorally and physiologically) (Moore and Jessop, 2003).

Woodley and Moore (2002) found that corticosterone concentration was positively correlated with ovarian weight in vitellogenic tree lizards (*Urosaurus ornatus*) and proposed that it may have a facilitative role in the reproductive cycle of this species. Wilson and Wingfield (1992) demonstrated similar results in side-blotched lizards (*U. stansburiana*), with corticosterone levels increasing with vitellogenesis and remaining elevated during the gravid period. Likewise, Gobbetti et al. (1995)

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showed that adrenal production and plasma corticosterone levels were highest during ovulation in the lizard *Podarcis sicula sicula* and suggested that they may play a role in inducing ovulation in this species. In contrast, Grassman and Crews (1990) found that corticosterone concentrations were highest during pre-vitellogenesis, declined during vitellogenesis, and remained at pre-ovulatory levels during gravidity in the parthenogenetic oviparous whiptail lizard (*C. uniparens*).

Glucocorticoids also increase in response to external stressors as animals respond behaviorally and physiologically to conserve energy and increase their chances of survival (Moore and Jessop, 2003; Millspaugh and Washburn, 2004; Palme, 2018). This often results in acute and severe activation of the hypothalamic–pituitary-adrenal (HPA) axis, which may result in subsequent down-regulation of the hypothalamic-pituitary–gonadal (HPG) axis and reproductive behaviors associated with courtship and mating (Guillette Jr. et al., 1995; Charmandari et al., 2005). This is not, however, a universal phenomenon; in some situations, individuals will also endure stressors (by downregulating the HPA axis) in order to complete reproductive activities (Anderson et al., 2014; Moore and Jessop, 2003).

Reproductive disorders are a common cause of death in captive female reptiles (Cuadrado et al., 2002; Rivera, 2008; Sykes, 2010), such as in veiled chameleons; *Chamaeleo calyptratus* (Kummrow et al., 2010a). In particular, the failure to lay eggs at the expected time based on cycle or breeding history originates from two separate pathological phenomena: 1) follicular atresia, the failure to ovulate (non-ovulatory cycles) and subsequent reabsorption of yolk from follicles, and 2) dystocia, the failure to lay fully formed eggs. Follicular atresia might be a physiological adaptation to changes in environmental conditions including food shortages or captivity (Swingland and Coe, 1978; Kummrow et al., 2010a; Vitousek et al., 2010). Even if considered adaptive, follicular atresia has resulted in the deaths or loss of breeding potential (due to removal of the reproductive tract) in many captive female reptiles (Rivera, 2008; Stacy et al., 2008; Sykes, 2010).

Given that the relationship between glucocorticoid production and ovulation varies from species to species, and even appears to be facilitative or necessary for reproduction in many cases, it is important to establish data on glucocorticoid metabolite excretion throughout the reproductive cycle in each species of interest to determine the role, if any, in follicle maturation, ovulation, and parturition. The objective of this study was to characterize adrenal function at different stages throughout ovulatory (OC; eggs laid) and non-ovulatory (NOC; no eggs laid) reproductive cycles in female veiled chameleons (*C. calyptratus*) and determine if corticosterone concentration is a predictor of ovulatory outcome. This was carried out by means of enzyme immunoassay (EIA) detection of fecal hormone metabolites; corticosterone (C), progesterone (P), estradiol (E), and testosterone (T).

2. Materials and methods

All procedures involving animals were approved by the Toronto Zoo Animal Care and Research Committee and the University of Guelph Animal Care Committee. The experiments were conducted in accordance with the requirements of the Animals for Research Act of Ontario and the recommendations of the Canadian Council for Animal Care. All chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) unless otherwise stated.

2.1. Animals

Data from two cohorts of female veiled chameleons (*C. calyptratus*) were included in this study (2008; n = 5 and 2012; n = 28). Both cohorts were purchased from the same commercial reptile breeder as 2–3 week old hatchlings, housed individually in identical containers and maintained under the same parameters (Kummrow, 2009); including a constant 12 h light:dark cycle, with temperature and humidity maintained between 18 and 30 °C, and 20–80%, respectively. The lighting included a F40 T12/BL/RS black light (Standard Products Inc., Saint Laurent, QC, Canada) as UV source and a Vita-lite lamp (Duro-Test Corp., Fairfield, NJ, USA) for general lighting. The animals were misted with warm water for 20–30 mins twice daily. The diet consisted of mineral and vitamin (Toronto Zoo reptile supplement mix) enriched crickets (*Acheta domesticus*), lettuce, and wax worms (*Achroia grisella)*, given three days per week. Animal weights were recorded weekly for the entire study period. Reproductive stages (i.e. previtellogenic with primordial follicles, vitellogenic with follicular growth) and egg production status (i.e. pre- versus post-ovulatory) were determined by transabdominal ultrasound bi-weekly as described in a previous study (Pimm et al., 2015) in the 2012 cohort animals, whereas reproductive cycles were determined as OC or NOC by presence or absence of oviposition, respectively, in the 2008 cohort animals.

2.2. Fecal collection

Fecal samples were collected daily from all animals prior to morning and evening misting, within 15 h of defecation, with forceps that were rinsed in 80% methanol:water between collections from different animals to avoid cross contamination. Individual fecal samples were placed in labeled plastic bags (EJ Bags, Scarborough, ON, Canada) and stored at −20 °C until the extraction procedure was performed. Samples were processed for extraction within 6 months after collection and analyzed for hormone metabolite levels immediately thereafter.

2.3. Fecal steroid extraction

The fecal steroid extraction procedure for fecal metabolites of corticosterone (C), estradiol (E), testosterone (T), and progesterone (P) was carried out using a protocol previously described by Kummrow et al. (2011) and Pimm et al., 2015. Fecal samples collected over 4 days were pooled together for analysis and dried at 75 °C for four hours in order to eliminate any effects of water content within the feces, after which any enclosure substrate and uric acid were scraped off. The feces were crushed and weighed in glass scintillation vials (Fisher Scientific, Ottawa, ON, Canada), after which 80% methanol:distilled water was added proportionately for 0.04 g/ml (samples weighing between 0.03 and 0.22 g). The extraction mixture was manually homogenized and mixed at room temperature on a rotator at 3 rpm overnight (16–18 h). Samples were then centrifuged and the supernatant (fecal extract) was decanted into a clean glass scintillation vial and stored at −20 °C until analysis.

2.4. Enzyme immunoassays (EIA)

2.4.1. Reproductive hormone metabolites

Values for fecal reproductive hormone metabolites (E, P and T) reported herein were previously published (Kummrow et al., 2010a, 2010b; Pimm et al., 2015). Briefly, antisera and horseradish peroxidase conjugates from C. Munro, University of California, Davis, CA, USA were used. Standards used were β-estradiol (Sigma E8875; 39 pg/ml – 10,000 pg/ml), testosterone (Steraloids Inc., Newport, RI, USA; A6950; 48 pg/ml – 12,500 pg/ml), and progesterone (Sigma P0130; 15.6 pg/ml – 4000 pg/ml). Fecal hormone levels are presented as mass/g dry weight.

2.4.2. Corticosterone metabolites

Fecal C metabolites were quantified using EIA methods previously described (Augustine et al., 2020). Antisera were diluted as follows: goat anti-rabbit IgG (GARG) polyclonal antibody (Sigma-Aldrich, Canada), 0.25 μg/well; and C (polyclonal CJM006, provided by C. Munro, University of California, Davis, CA, USA), 1:200,000. The cross-reactivities of C were: corticosterone, 100.0; 11-deoxycorticosterone, 14.3; tetrahydrocorticosterone, 0.9; progesterone, 2.7; testosterone, 0.6; cortisol, 0.2; prednisolone, 0.1; prednisone, cortisone and estradiol 17β, <0.01; 11-deoxycortisol, 0.0 (Metrione and Harder, 2011; Watson et al., 2013).

C HRP conjugate (provided by C. Munro, University of California, Davis, CA, USA) was diluted 1:1,000,000 and C standards (Steraloids Q1550) ranged from 39 pg/ml – 10,000 pg/ml. Inter-assay controls consisted of laboratory stocks of pooled fecal extracts obtained from female spotted-necked otters (*Hydrictis maculicollis*) that were run at 25% and 65% binding. Fecal extracts were diluted 10-fold in EIA buffer (0.1 mM sodium phosphate buffer, pH 7.0, containing 9 g of NaCl and 1 g of BSA per liter) for hormone analysis.

Microtiter plates (Nunc Maxisorp, VWR, Mississauga, ON, Canada) were coated with GARG polyclonal antibody (Sigma-Aldrich Canada) diluted in coating buffer (50 mM bicarbonate buffer, pH 9.6) at 0.25 μg/well. After overnight incubation at room temperature in the dark, plates were washed with 0.05% Tween 20, 0.15 M NaCl solution using a Bio-Tek ELx 405VR microplate washer (BioTek Instruments, Winooski, VT) and blocked with 250 μl EIA buffer per well for minimum 1 h at room temperature. Soon after, 50 μl of fecal extracts, standards, and controls diluted in EIA buffer were added in duplicates followed by 100 μl of horseradish peroxidase conjugate and 100 μl C antiserum diluted in EIA buffer. Plates were incubated overnight in the dark at room temperature. On the third day, the plates were washed and 200 μl of substrate solution (0.5 ml of 4 mg/ml tetramethylbenzidine in dimethylsulphoxide and 0.1 ml of 0.176 M H_2O_2 diluted in 22 ml of 0.01 M $C_2H\ 3NaO_2{\cdot}3H_2O$, pH 5.0) was added. After 30 min in the dark at room temperature, color development was stopped with 50 μl H_2SO_4 (1.8 M). Absorbance was measured at 450 nm using a spectrophotometer (MRX microplate reader, Dynex Technologies, Chantilly, VA).

2.5. Corticosterone metabolites assay performance

2.5.1. Fecal extraction efficiency

Efficiency of the extraction procedure for C was analyzed through recovery of exogenous C added to the fecal samples before extraction. Five pooled fecal samples (0.2 g each) were spiked with either C standard or no hormone. The samples were mixed and extracted as described previously. The percent efficiency was calculated using the following formula: amount observed/amount expected × 100%; where amount observed is the value obtained from the spiked sample minus background and amount expected is the calculated amount of C standard added. The percent efficiency is presented as mean ± s.e.m.

2.5.2. EIA parallelism

Parallel displacement between the standard curve and serial dilutions of fecal extract was used as an indirect measure of assay specificity. A representative pooled sample of fecal extracts was serially diluted two-fold between 1:2 to 1:512 in assay buffer and run on the C assay alongside the standard curve. The graph was plotted as log(relative dose) vs. percent antibody bound and sample dilution was selected based on 50% binding of the pooled sample curve.

2.5.3. Precision

To assess repeatability of results, calculation of intra- and inter-assay coefficients of variation (CV) was evaluated. Intra-assay CV's were consistently monitored on each plate in real time by examining the CV of each duplicate run on the plate. Only values from duplicates with $< 10\%$ CV were recorded as data. Intra-assay CV's were further evaluated using a pooled extract at 50% binding loaded in different spots on the plate, and this was repeated three times. Inter-assay CV's were evaluated using fecal extract controls (25% and 65% binding) loaded in duplicate on each plate.

2.5.4. Hormone recovery

To examine possible interference of components within the extract binding to the antibody, recovery of a known amount of C was calculated. A pooled sample of fecal extracts diluted to the usual range for unknown samples was used. To 100 μl of pooled extract, 100 μl of increasing concentrations of C standard were added in the range used for the standard curve. The diluted pool was assayed alone to determine endogenous hormone levels. The percent recovery was calculated using the following formula: amount observed / amount expected × 100%; where amount observed is the value obtained in the spiked sample and amount expected is the calculated amount of C standard added plus the amount of endogenous hormone in the unspiked sample. The percent recovery is presented as the mean ± s.e.m. of all concentrations assayed. The graphs were plotted as hormone added vs. hormone recovered.

2.6. High performance liquid chromatography (HPLC)

HPLC characterization of reproductive hormone (E, P and T) metabolites (number and relative proportions) in fecal extracts was conducted according to the method of Walker et al. (2002), and previously published (Kummrow et al., 2011). Novel to this study, fecal glucocorticoid metabolites (FGM) were characterized using the same method as follows: fecal extracts ($n \geq 5$) consisting of elevated levels of FGM were selected for analysis and pooled. Immediately prior to HPLC analysis, the samples were resuspended in 100 μl of acetonitrile and 90 μl were injected onto the HPLC system. Analysis was performed using a Waters Alliance 2695 HPLC separation system connected to a computer with Empower 2 software (Waters Corp., Mississauga, ON, Canada) for data collection and processing. The analytical column used was a Nova-Pak C_{18} column (150 mm × 3.9 mm I.D., 4 μm; Waters Corp., Dublin, Ireland), connected with a Security Guard C_{18} guard column (4 mm × 3.0 mm I.D.; Phenomenex, Torrance, CA, USA). Twenty-one reference steroid hormone standards (Steraloids Inc., Newport, RI, USA) and the hormone samples were eluted at room temperature using a gradient of acetonitrile and water (28–90) over 45 min at a flow rate of 0.7 ml/min. Absorbance was recorded at 254 nm for FGM. Eluent fractions were collected every minute using a Waters Fraction Collector II (Waters Corp., Mississauga, ON, Canada). The fractions (700 μl) were dried down under flow of nitrogen gas, resuspended in 350 μl of EIA buffer and assayed in duplicate on the corticosterone EIA to determine immunoreactivity.

2.7. Data amalgamation and statistical analysis

Statistical analyses were performed using R 3.5.1 (R Core Team, Vienna, Austria) and alpha values for all analyses were set to 0.05. Baseline concentrations of E, P, T and C were determined on an individual basis using methods described by Kummrow et al. (2011). In brief, baseline values were calculated from samples collected from 5 months of age until the completion of at least two reproductive cycles. An iterative process was used in which values exceeding the mean + 1.5 s.d. were removed. The mean was then recalculated and the process repeated until no values exceeded the mean + 1.5 s.d. The final calculated mean was considered the baseline value. Significant peaks were defined as one or more values (C), or two or more (E, P or T) above baseline + 1.5 s.d. The peak maximum value was calculated as the difference between the highest measurement of the peak and the baseline concentration, measured in ng of hormone metabolite (E, P, T or C) per g dry fecal matter. Outlying peak concentrations were identified as those exceeding 3 s.d. from the mean and were removed from subsequent analyses ($n = 1$ reproductive cycle of $n = 1$ individual).

Hormone metabolite values were graphed against time, recognizing the fact that each measurement represented a sample pooled over four days and that hormones appear in the feces up to four days after their presence in the blood based on previous gut retention studies in the chameleon (Kummrow et al., 2011).

Maximum hormone peak values were compared between ovulatory (OC) and non-ovulatory cycles (NOC) of chameleons using a generalized linear mixed-effect model with binomial distribution in the R package "LME4" (Bates et al., 2015). Here, only individuals which had experienced at least one ovulatory cycle were included, to permit within-individual comparisons and to correct for zero-inflation. Furthermore,

only cycles containing peak concentrations of all hormones of interest (P, C, T, and E) were included in the analysis to ensure that estimates of the effects of each steroid on ovulatory outcomes were dependent upon known concentrations of each other steroid (n = 9 OC, and n = 13 NOC cycles included in final analyses). In our model, ovulatory outcome (OC or NOC) was used as the response variable, and concentrations of each steroid were included as fixed effect predictors. All hormone concentrations were scaled between 0 and 1 to aid in model convergence and to reduce heteroskedasticity of residuals across steroids. Interactive effects of each steroid on ovulatory outcome were not considered, owing to a small sample size and relatively low statistical power in estimation of interaction terms when compared to fixed effects (Aiken et al., 1991). To account for variation in ovulatory outcome explained by sampling year and individual identity, year and individual ID were included as random intercepts in our model. Collinearity between individual hormone concentrations was not detected in our data set (variance inflation factors < 2.0) as assessed by in the R package "Car" (Fox and Weisberg, 2018).

Within each cycle the time between the maximum peaks of C and each quantified hormone (E, P, T) was measured in days. We tested whether OC or NOC cycles can be predicted by time-lags between C and individual hormone peaks differed using a general linear model with binomial distribution, in base R (R Core Team, Vienna, Austria). Here, ovulatory outcome was again used as the binomial response variable and the times between peaks of C and all other quantified hormones (i.e.; C-P, C-T, and C-E; days) were included as fixed effect predictors. Both individual ID and year were initially included as random intercepts to account for individual and year-based variability. As each explained zero variance, however, they were subsequently excluded from the final model. Again, only individuals who had experienced at least one ovulatory cycle were included in analyses, and cycles that did not include all hormones of interest were excluded. Finally, peak-to-peak time-lags that exceeded 3 s.d. from mean values were identified as outliers, and consequentially removed (n = 1 reproductive cycle from n = 1 individual). Our final model included 9 OC and 13 NOC cycles from 9 chameleons. All model estimates are reported ± standard errors of the means (s.e.m.), and significant coefficients are accompanied by estimated effect sizes (here, Cohen's D, or "D"; Cohen, 2013).

Parallelism and hormone recovery were assessed using Microsoft Excel. For parallelism, the values within the linear range of the curve were subject to linear regression analysis using log(relative dose) vs percent antibody binding of the standard and sample dilution curves independently. The probability of a significant difference between the resulting slopes was determined, where $p < 0.05$ indicates a significant difference between the slopes (Soper, 2021). Hormone recovery data were plotted as hormone added vs. hormone recovered and assessed by linear regression analysis.

3. Results

Reproductive cycles were categorized as either OC (eggs laid) or NOC (no eggs laid). A total of 9 females, 4 from the 2012 cohort and 5 from the 2008 cohort, achieved at least one OC and laid eggs, as well as at least one NOC. One female (Chameleon 3) achieved two OCs (Table 1). The remaining 24 females never laid eggs but experienced at least two NOCs throughout the course of the study.

3.1. Corticosterone assay performance

Extraction of exogenous C resulted in fecal extraction efficiency of 96.6 + 4.2% (mean + s.e.m.). There was no significant difference between the slopes of the standard curve and sample serial dilutions ($t = 1.19$, $p = 0.28$, $df = 6$; Fig. 1). The intra-assay CV at 50% binding was 5.6% and inter-assay CV's were 17.0% and 15.0% at 25% and 65% binding, respectively. The recovery of known concentrations of C was 85.4 + 4.4%. The measured hormone concentrations in the spiked samples correlated with the expected concentrations ($r = 0.99$, $p < 0.01$) (presented in supplementary material Fig. S1).

3.2. HPLC

Immunoreactivity of the HPLC fractions on the corticosterone EIA revealed a peak between 11 and 12 min, corresponding with the elution time for the corticosterone standard (11.5 min; Fig. 2). Two less polar peaks were also detected, one at 24–26 min which corresponds to eluted standards 21-deoxycorticosterone, androstenedione and 17α-hydroxyprogesterone, and a second at 28–30 min which does not have a corresponding standard. An immunoreactive peak was also recorded in the fractions containing conjugated steroids collected within the first 5 min of elution from the column.

Table 1
Hormone peak maximum and baseline values for ovulatory and non-ovulatory cycles of egg-laying animals. Peak maximum values and calculated baseline values for fecal corticosterone (C), estradiol (E), progesterone (P) and testosterone (T) metabolites during ovulatory (OC) and non-ovulatory (NOC) cycles of 10 veiled chameleons. All values are in ng/g dried feces. Effect sizes (Cohen's D, or "D") are given significant effects ($p < 0.05$; indicated by asterisks, "*"). Daggers (†) indicate outlier cycles that were removed from analyses and mean calculations.

Animal	Cycle	Corticosterone C			Estradiol E			Progesterone P			Testosterone T		
		OC	NOC	baseline	OC	NOC	baseline	OC	NOC	baseline	OC	NOC	baseline
1	1	401	150	158	1057	523	256	36,404	2704	1442	3018	834	467
	2		153			942			6920			929	
2	1	579	172	108	1155	328	296	9369	2389	631	2350	999	209
	2		241			437			1349			938	
3	1	427	538	211	945	1108	319	12,928	2379	747	678	1264	165
	2	552	229		667	797		11,442	1885		624	833	
4	1	†707	250	168	†1405	581	468	†1116230	7559	1680	†2725	847	395
	2		370			1184			12,834			1160	
5	1	281	97	156	1417	643	227	42,582	3316	1311	1259	640	347
6	1	382	141	65	925	814	179	7570	1105	147	1892	410	230
7	1	673	444	92	949	1517	73	22,883	1736	309	1234	1890	183
8	1	299	94	95	997	515	112	6360	817	326	321	546	199
9	1	296	277	145	1050	412	141	9347	1368	318	399	569	268
Average		460	243		1057	772		27,518	3797		1450	888	
Estimate		176.99			−35.38			210.12			−43.67		
s.e.m.		29.05			22.89			40.05			25.26		
z-value		6.09			1.55			5.25			1.73		
p-value		<0.001*			0.12			<0.001*			0.08		
D		>1						>1					

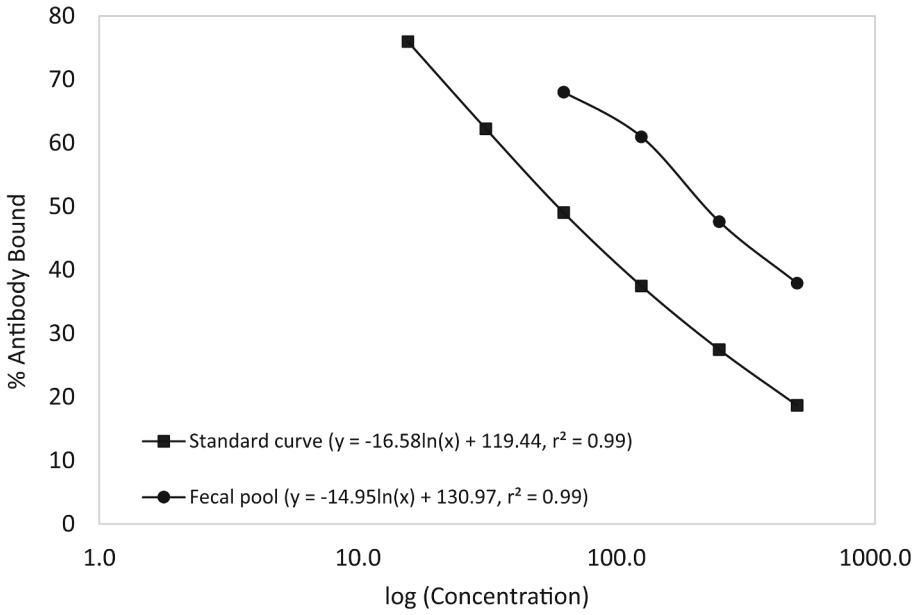

Fig. 1. Parallelism for serial dilutions of pooled fecal extracts of female veiled chameleons against the standard curve.

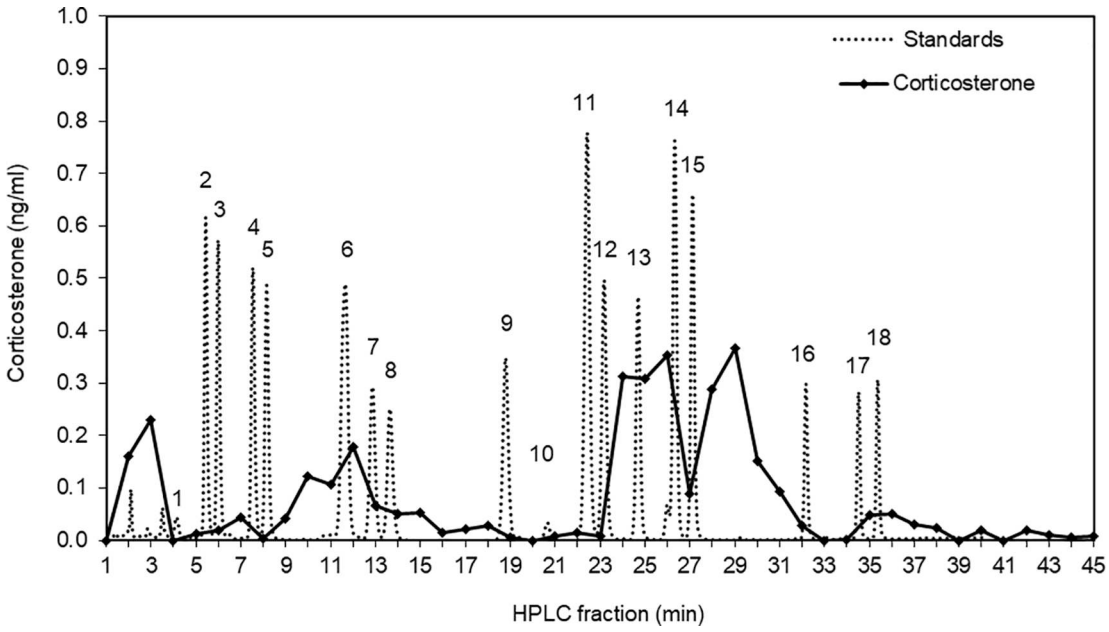

Fig. 2. Immunoreactivity of fractions collected following high-performance liquid chromatography (HPLC) separation of pooled fecal extracts from female veiled chameleons, as determined by corticosterone enzyme immunoassay (solid line) and compared to elution times of 21 purified hormone standards (dashed line); 1: aldosterone, 2:cortisol, 3: cortisone, 4: 11β-hydroxytestosterone, 5: 11-ketotestosterone, 6: corticosterone. 7: 11β-hydroxyandrostenedione, 8: 11-ketoandrostenedione, 9: 11-deoxycorticosterone, 10: 19-nortestosterone, 11: estradiol, 12: 17,20β-dihydroxy-4-pregnen-3-one, 13: 19-norandrostenedione, 14: testosterone, 15: 11β-hydroxyprogesterone 16: estrone, 17: androstenedione, 18: 17α-hydroxyprogesterone, 19: 20a-dihydroxyprogesterone, 20: 20B-dihydroxyprogesterone, 21: progesterone.

3.3. Adrenal and reproductive activity throughout the reproductive cycle

Peak levels of C and P significantly predicted ovulatory outcome, with peak C and P positively correlating with OC cycle (P: $p < 0.001$, $D > 1$; C: $p < 0.001$, $D > 1$; Table 1; Fig. 3). Neither peak T, nor peak E were predictive of the ovulatory outcome (T; $p = 0.08$: E; $p = 0.12$; Table 1; Fig. 3).

Hormone profiles indicated a cyclical pattern of C, consistent with patterns observed in the reproductive hormone metabolites (Fig. 4, supplementary material Fig. S2). Levels of C began to increase during early-mid vitellogenesis, peaked in late vitellogenesis (when follicles reached mature size), or within the vitellogenic/gravid period overlap (the transition from follicle to egg), and remained elevated until just prior to oviposition (in OCs).

In NOCs, C levels returned to baseline following the peak in late vitellogenesis. In a small number of animals, significant peaks in C and T occurred during the early vitellogenic phase, but this trend did not correlate with reproductive events as depicted by ultrasound data nor was it repeated in subsequent cycles (Fig. 4, Chameleon 9).

A cycle consisted of complexes of hormone peaks, in which a peak in E was followed by distinct peaks in C, P and T, occurring in close proximity to each other. Interestingly, the mean number of days

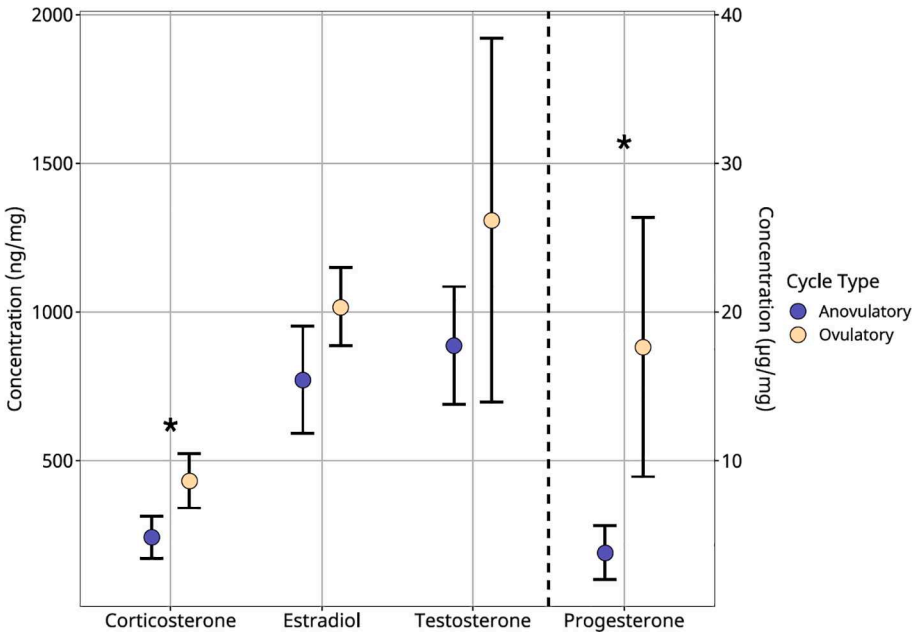

Fig. 3. Mean maximum peak hormone values during ovulatory and non-ovulatory cycles of female veiled chameleons. Calculated mean maximum peak values for fecal metabolites of corticosterone (C), testosterone (T), estradiol (E) and progesterone (P) during ovulatory (9 cycles) and non-ovulatory cycles (13 cycles) based on 9 female veiled chameleons. Open circles represent means for ovulatory cycles, and closed circles represent means for non-ovulatory cycles. Whiskers represent ± 95% confidence intervals around mean estimates, and asterisks mark significant differences ($p < 0.05$) according to a generalized linear mixed-effect model. Means for T, E, and C are scaled according to the left-hand axis (ng/mg), while mean P is scaled according to the right-hand axis (µg/mg).

between C maximum peak values and each other hormone peak (E, P, T), did not significantly differ between OC (n = 9) and NOC cycles (n = 13) (Table 2).

4. Discussion

Corticosterone enzyme immunoassay performance was assessed using parallelism, recovery and precision tests, which showed that the chosen EIA reliably measured GC metabolites present in the fecal extracts without being influenced by substrate components. Furthermore, immunoreactivity of fecal extract fractions separated by HPLC demonstrated the presence of corticosterone metabolites in the excreta from this species. While physiological validation was not possible during this study, Scheun et al. (2018) confirmed the presence of fecal C metabolites following ACTH administration in a lizard species (*Smaug giganteus*). Furthermore, a recent study in crocodiles (Augustine et al., 2020) showed significant and biologically relevant changes in fecal corticosterone concentrations using the same assay as that chosen for this study.

This study demonstrated a significant elevation in fecal corticosterone and progesterone metabolite levels before oviposition in female veiled chameleons. Increased fecal progesterone metabolite levels preceding oviposition have been previously published for this species (Kummrow et al., 2010a, 2010b), and here we demonstrate a concurrent increase in fecal corticosterone metabolite levels. Kummrow et al. (2010a) proposed that ovulation occurred as the result of the decreasing E to P ratio and that progesterone peaked during mid-gravidity and fell prior to oviposition. It is logical to assume that this dramatic increase in progesterone following ovulation is the result of corpora lutea formation on the ovaries, and that progesterone produced therein is responsible for the maintenance of the gravid state (Callard et al., 1972; Shanbhag et al., 2001). In this study, significantly higher C values around the time of ovulation were predictive of ovulatory cycles. However, since maximum peaks in C and the transition from follicle to egg (the vitellogenic-gravid overlap phase) occurred in the timing between ultrasound exams, it remains unclear whether the C surge facilitated or occurred as a result of the ovulatory process. The facilitative role of C with oviposition has repeatedly been reported in reptiles (Anderson et al., 2014; Dauphin-Villemant et al., 1990; Liu et al., 2020; Radder et al., 2008) but its role with regard to ovulation is less clear and has not been specifically investigated.

The results of the current study indicate a close relationship between the timing of peaks in P, T and C (within 3 days of each other). Although not directly related to gravidity, a correlation between plasma P and C was described in sexually mature river turtles during the nesting season, when values of both P and C were elevated (Freneau et al., 2017). Additionally, exogenous supplementation of C in pre-vitellogenic and vitellogenic female tree lizards (*U. ornatus*) led to elevated circulating P compared to sham-treated females, suggesting a causal correlation, the source of which was not investigated (French et al., 2007). A correlation between T and C has been reported in nesting loggerhead turtles, *Caretta caretta* (Whittier et al., 1997) and related to female ovarian development. Schramm et al. (1999) also described a weak statistical correlation in Galápagos tortoises, *Geochelone nigra*, during the mating season, with levels of both T and C remaining elevated until first eggs were laid. Contrary to levels of P and C, the results of our study did not indicate a significant difference in circulating levels of T between ovulatory and non-ovulatory cycles. Therefore, although it is possible that there is an interaction between these three hormones around the time of ovulation, conclusive evidence requires further study in this species.

We also describe a cyclical pattern in C levels which varied according to reproductive stage in female veiled chameleons. This is consistent with other studies reporting a cyclical pattern of corticosterone throughout the reproductive cycle in other reptile species (Dauphin-Villemant et al., 1990; Grassman and Crews, 1990; Wilson and Wingfield, 1992). Based on our data, we identified the following pattern: C levels rose during mid-late vitellogenesis, peaked during the vitellogenic/gravid overlap phase when follicles had reached mature size, and remained elevated until just prior to oviposition. Similar patterns were observed throughout the reproductive cycles of female side-blotched lizards, *U. stansburiana* (Wilson and Wingfield, 1992); in which corticosterone production increased throughout the vitellogenic phase and remained elevated in gravid individuals until just prior to oviposition. Additionally, Dauphine-Villemant et al. (1990) observed low circulating levels of corticosterone during the vitellogenic phase of the viviparous lizard *L. vivipara*, which gradually increased during the first half of gestation, peaked significantly in late gestation, then fell abruptly prior to parturition. In contrast, Grassman and Crews (1990) found that plasma levels of corticosterone were highest during the previtellogenic period, decreased during vitellogenesis when estradiol levels started to increase, and remained low during the gravid period of *C. uniparens*.

The elevated corticosterone levels following ovulation may facilitate

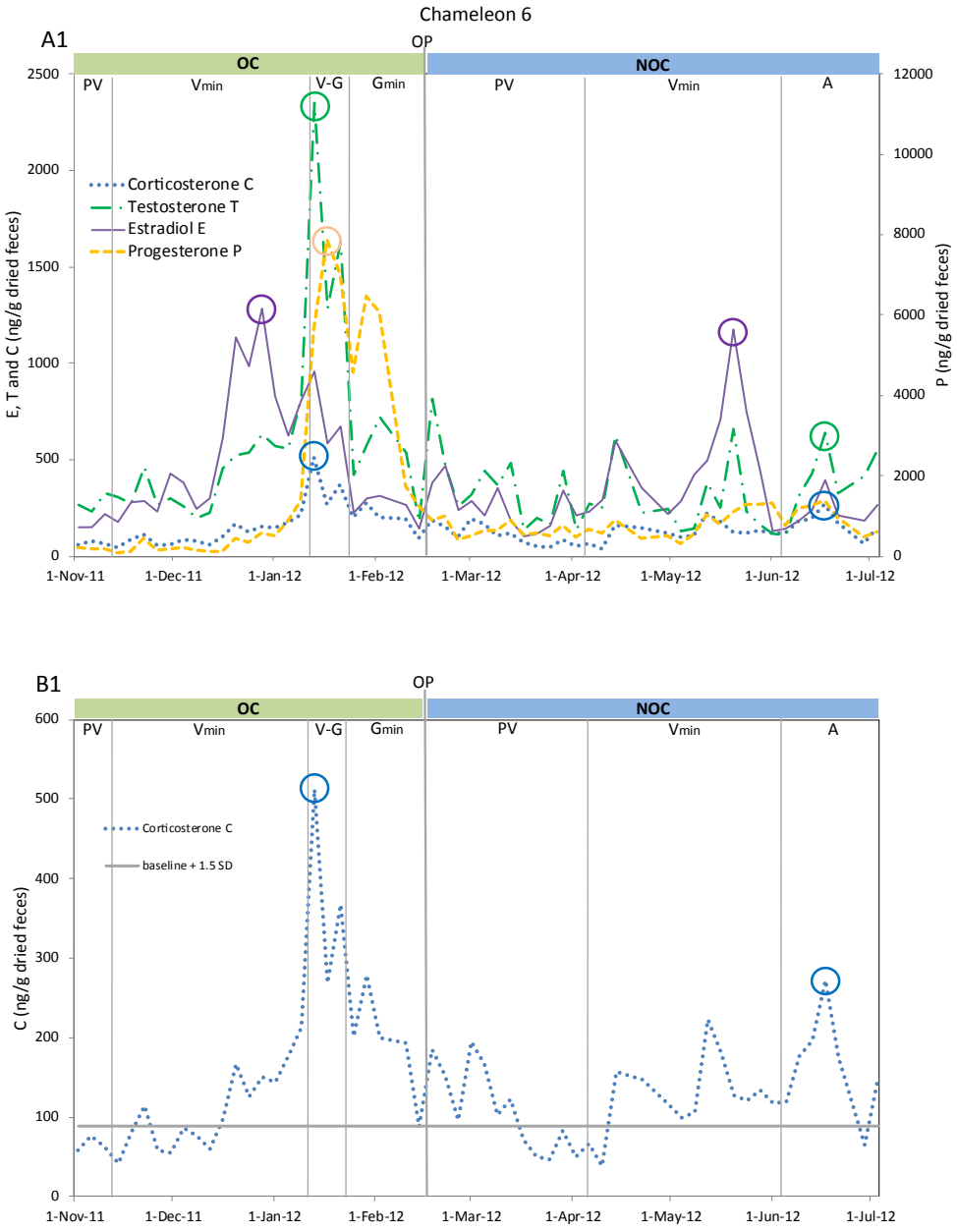

Fig. 4. Fecal hormone metabolite patterns of two female veiled chameleons with ovulatory and non-ovulatory cycles. Figures A1/B1 represent animal 6 and A2/B2 animal 9 in Table 1. Examples of the relationships between fecal metabolite patterns of estradiol (E), progesterone (P), testosterone (T) and corticosterone (C) (A1, A2), and C patterns relative to baseline values + 1.5 s.d. (B1, B2), plotted against time for two animals (A; 6 and B; 9) that had both ovulatory (OC) and non-ovulatory (NOC) cycles. Stages of the reproductive cycle are identified at the top of the graph: PV = pre-vitellogenic, Vmin = minimal duration of vitellogenesis, V-G = vitellogenesis/gravid overlap including the event of ovulation, Gmin = minimal duration of gravid stage, OP = oviposition, A = atretic. Blue dotted line = C; green dash-dotted = T; yellow dashed = P; purple solid = E. Maximum peak hormone values associated with reproductive stages are indicated with a circle.

the natural retention of eggs during the gravid phase in order to prevent early expulsion. Dauphine-Villemant et al. (1990) found that parturition was significantly advanced in adrenalectomized female *L. vivipara*, and significantly delayed in animals treated with exogenous corticosterone in late gestation. Additionally, Shanbhag et al. (2001) speculate that prolonged egg retention observed in *Calotes versicolor* could have been accomplished by the secretion of progesterone and corticosterone by the adrenals under "stressful" conditions, which in turn inhibit the oviductal contractions associated with oviposition.

In some species, plasma corticosterone levels are elevated during the breeding season compared to the non-breeding season, but variations over time (months) or between different stages of the reproductive cycle do not occur. This has been reported in common geckos; *Hoplodactylus maculates* (Girling and Cree, 1995), and American alligators; *Alligator mississippiensis* (Guillette Jr et al., 1997). In contrast, there are species that do not display elevated levels during the breeding season, such as *P. barbata* (Amey and Whittier, 2000).

Adrenocortical activity, and subsequently, glucocorticoid levels can vary in a particular animal with age, reproductive status, sex, diet, and season (Woodley and Moore, 2002; Moore and Jessop, 2003; Millspaugh and Washburn, 2004; Palme, 2018). In the current study, all of the animals were female, sampled at the same age, and maintained under identical conditions, eliminating any age, sex, diet and seasonal effects. Therefore, an assessment of variation in glucocorticoid levels could be made between the various stages of the reproductive cycle, and more specifically, between OCs and NOCs without interference from those confounding factors.

When characterizing hormones extracted from feces, it is possible to encounter native, as well as metabolized forms of the hormone resulting from digestion (Touma and Palme, 2005). Evaluation of the HPLC fractions on the corticosterone assay revealed three immunoreactive peaks at different polarities, including a peak that co-eluted with corticosterone, similar to previous studies reporting multiple immunoreactive fractions using a comparable polyclonal antibody (Bamberg

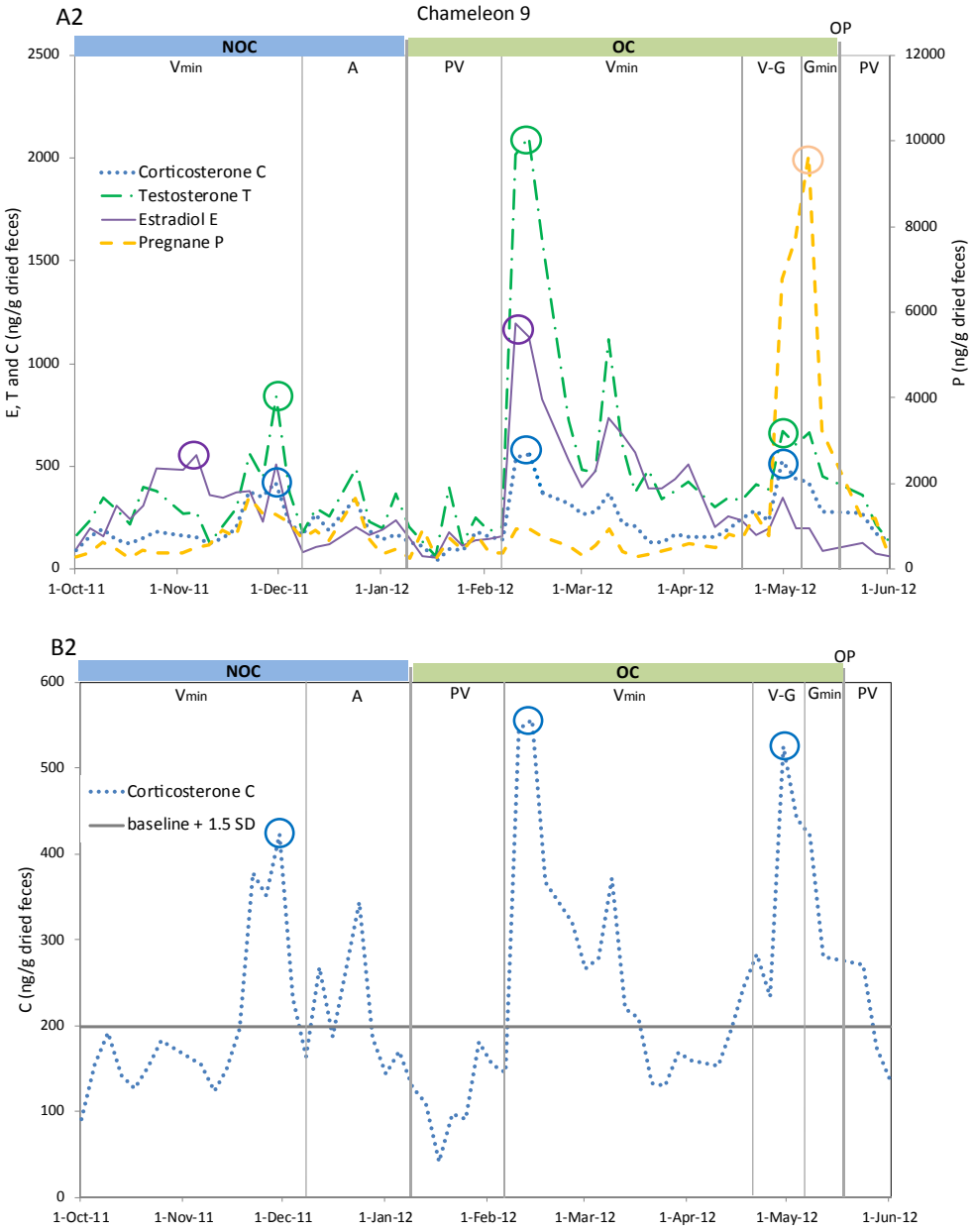

Fig. 4. (*continued*).

et al., 2001; Dehnhard et al., 2003; Ludwig et al., 2013). The antibody used in this study (CJM006; raised against corticosterone-3-CMO) also cross-reacts with 11-deoxycorticosterone (14.3%), a precursor for corticosterone. The immunoreactive fractions between 26 and 28 min co-eluted with several standards, including 21-deoxycorticosterone, also a precursor for corticosterone and structurally akin to 11-deoxycorticosterone. Interestingly, 21-deoxycorticosterone, also known as 11β-hydroxyprogesterone, is a potent inhibitor of corticosterone-inactivating enzyme, 11β-hydroxysteroid dehydrogenase in rat kidney (Souness and Morris, 1996), an enzyme that has also been identified in mammalian placenta (Yang, 1997). Further investigation would be required to identify the immunoreactive metabolites that did not co-elute with a known standard.

Altogether, the above studies indicate that the relationship between glucocorticoid production and reproductive activity is species-specific, and thus cannot be universally defined in reptiles. Additionally, the source of glucocorticoid production and secretion during periods of reproductive activity remains unclear. To this point, it has yet to be determined whether glucocorticoid levels are elevated as a result of the stress associated with reproduction, or if it is necessary to facilitate reproductive processes, limiting the direct clinical application of these findings (e.g. administration of exogenous corticosterone to increase the rate of ovulation). To address these questions, visualization of the reproductive tract should be done more frequently to delineate the stages surrounding ovulation more precisely when comparing to levels of the individual hormones.

5. Conclusion

Cyclical patterns of fecal corticosterone metabolites that occur throughout the reproductive cycle of female veiled chameleons were characterized, revealing a difference in corticosterone concentrations

Table 2
Days between maximum peak hormone values for egg-laying animals (n = 9 chameleons, n = 9 OC cycles, and n = 13 NOC cycles). Mean number of days between maximum peak values for fecal metabolites of corticosterone (C) and progesterone (P), estradiol (E) and testosterone (T) during the ovulatory (OC) and non-ovulatory (NOC) cycles of female veiled chameleons. Negative numbers indicate that the maximum peak of the second hormone preceded the first. Estimates, standard errors around estimates, and z values from a binomial GLMM with days between maximum peaks predicting ovulatory outcome (OC/NOC) are included. $p < 0.05$ is significant.

	C-P	E-C	T-C
OC	−0.89	26.20	0.89
NOC	−0.62	17.80	0.31
Estimate	0.01	0.06	0.07
s.e.m.	0.08	0.04	0.08
z-value	−0.10	1.45	0.82
p-value	0.92	0.15	0.41

between OCs and NOCs. Further investigation into the mechanisms that trigger and inhibit ovulation in this species is still needed, as are methods of inducing ovulation in animals that failed to do so, and whether corticosterone plays an active role in these processes. The knowledge gained from this model species could be instrumental to the understanding of normal and pathological reproductive events in other lizard species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2021.113822.

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