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Abstract: The Texas horned lizard (Phrynosoma cornutum) is protected in several states due to its apparently declining numbers; information on its physiology is therefore of interest from both comparative endocrine and applied perspectives. We collected blood samples from free-ranging P. cornutum in Oklahoma from April to September 2005, spanning their complete active period. We determined plasma concentrations of the steroids, progesterone (P), testosterone (T), and corticosterone (CORT) by radioimmunoassay following chromatographic separation and 17β-estradiol (E2) by direct radioimmunoassay. T concentrations in breeding males were significantly higher than in non-breeding males. P showed no significant seasonal variation within either sex. CORT was significantly higher during the egg-laying season compared to breeding and non-breeding seasons for adult females and it was marginally higher in breeding than in non-breeding males (P=0.055). CORT concentrations also significantly increased with handling in non-breeding males and egg-laying females. Perhaps most surprisingly, there were no significant sex differences in plasma concentrations of P and E2. Furthermore, with respect to seasonal differences, plasma E2 concentrations were significantly higher in breeding females than in egg-laying or non-breeding females, and they were significantly higher in breeding than in non-breeding males. During the non-breeding season, yearling males exhibited higher E2 concentrations than adult males; no other

differences between the steroid concentrations of yearlings and adults were detected. In comparison to other vertebrates, the seasonal steroid profile of P. cornutum exhibited both expected and unexpected patterns, and our results illustrate the value of collecting such baseline data as a springboard for appropriate questions for future research.

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Dear Ms. Shapiro:

We were pleased to learn that our manuscript (Ms. No.: GCE-07-52), "Effects of sex, age, and season on plasma steroids in free-ranging Texas horned lizards (Phrynosoma cornutum)" has been favorably reviewed and is still being considered for publication by ***General and Comparative Endocrinology***. We think that GCE is an excellent venue for this work and have carefully considered the reviewers' comments in preparing our revision (for detailed responses, see 'Responses to Reviews').

Our revised manuscript is 30 pages long, including one table and two figures. We hope that you will find it acceptable for publication, but please do not hesitate to contact me if I can provide any additional information. Thank you for the opportunity to have our work published in GCE.

Sincerely,

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Reviewer 1 Comments (*all reviewer comments in italics*)

Major comments

Was a handling effect on sex steroids examined? Given you found one on CORT it would be valuable to check if handling affected sex steroids as has been recorded in other species. If there was an effect on handling of CORT how are you accounting for this when presenting CORT data from your paper?— These data now are presented in the Results LL 250-270 and addressed briefly in Discussion LL 346-359.

Minor comments

Introduction
1. End of first paragraph. Why should our studies of hormone function only be restricted to a species active periods? Hormones also have roles to play in metabolism and survival during torpor and hibernation. — We rewrote this sentence to accurately state what we were measuring in this study, see Introduction LL 37-38.

Methods
2. The number of adults vs immature lizards is not clear. Clarify sample sizes. — Done, see Methods LL 85-86.

3. Lines 85-90 and 152-154. Are you suggesting that because you could not detect the effect of repeated sampling on individuals that you have included the repeated samples in the overall analysis. If this is so, I do not believe this is a valid technique. Repeated samples should be analysed separately by repeated measure ANOVA and not included in the overall analysis as they are not independent in time which is one of the key assumptions of ANOVA. — We clarified how we analyzed these data, see Methods LL 168-175.

4. What was the interval between transmitter attachment and sampling for your study? — We added the interval of transmitter attachment and added results of a correlation analysis of transmitter attachment and sampling time, see Methods LL 92-95.

5. Lines 137-145. What was the correlation between individual E2 results from the two assays? Does this give you confidence to assert that the E2 levels were below the detectable concentration of the assay. — The correlation was $r=0.415$, $p<0.001$. We did not add the results of the correlation in the manuscript, because we stated that we ran all analyses on E2 data from both assays, and the results were the same, see Results LL 230-232.

Results
6. Lines 234-238. I would like to see the results of the regression analysis for males presented with the outlier excluded. — Done, see Results LL 268-270.

Discussion

7. The limitations of using a single sample to assay CORT response to handling should be emphasised in the discussion. It is much preferable to use repeated sampling to assess stress response, although I understand the limitations of sample volume in this species. — We changed the discussion of this section to address this reviewer's concerns, see Discussion LL 364-367.

8. Lines 337-332. I think the authors are overstating their data to suggest the limited sampling here is enough to suggest that radiotransmitters and repeated sampling are not inducing a stress response in the lizards. This is beyond the aims of the paper and the data presented only address this concern in a very limited manner. Indeed the finding that handling induces a CORT response is contradictory to this conclusion. I would suggest this section be deleted from the MS. — We deleted this section as suggested.

9. Lines 336-341. Most endocrinologists are well aware of the roles that "sex specific" hormones play in the opposite sex. I suggest re-writing this paragraph to concentrate on the conclusions of this study. — Done, see Discussion LL 368-373.

Reviewer 2 Comments

Specific comments, including suggestions for improvement

Abstract

LL 8-9: The methods say (L 137) that the final data presented for E2 was obtained without chromatographic separation. — The abstract was corrected to be consistent with the methods, see Abstract LL 8-9.

L 10: This sentence would be better written as 'P showed no significant seasonal variation within either sex' - if that is what you mean. The next sentence on CORT concentrations also needs work to make clear what group is being compared with what. — We rewrote these sentences for clarity, see Abstract LL 10-13.

LL 14-15: This sentence on E2 also needs rewording to make the comparisons clear. Do you mean ' E2 reached higher concentrations in breeding females and males than in non-breeding females, males or egg-laying females'? — We rewrote this sentences for clarity, see Abstract LL 15-18.

Introduction

L 34: the species' (apostrophe misplaced) — Done.

L 49: Better as 'The eggs hatch…' (delete 'will') — Done.

L 50: Better as 'Hatchlings do not become…'— Done.

L 51: 'however, this could vary…': What does 'this' refer to? The age at maturity? If so, is it always greater than or equal to 19 months, as stated in the preceding phrase (i.e. never less than 19 months)? — We reworded this sentence to make it clear what we meant by 'age at maturity,' see Introduction LL 54-55.

L 54: 'and, as a result, the species is listed' (insert words in italics) — Done.

L 61: 'for any species': of lizard? Of vertebrates generally? (define the group). — Done.

L 85: provide an indication of the body mass of yearlings and adults. This is relevant for assessing the significance of the blood volume removed from these small animals. — Done, see Methods LL 112-114.

L 95-97: My initial reaction to this method of blood sampling is 'ouch'. I know incising the tail has sometimes been used before in small lizards, but I wonder why the authors did not use a hypodermic needle, which in my experience is feasible for lizards of about or only slightly larger than the size of the adults studied here. Surely this would be less painful and have less risk of introducing infection? I think it is important that the authors comment on any evidence they may have on the individuals' recovery from the incision, and also on whether this is still the best method to use in future studies of this species of concern. Other researchers are likely to be guided by your procedures, so it's important to indicate whether or not this is still considered best practice and what evidence is available to support this. — We added more information here to specifically explain how we took blood samples from individuals and information on the observation of recovery of those individuals. Additionally, we stated the alternative method of blood sampling and why we chose the method we did, see Methods LL 100-108.

L 98: Add here the average and maximum time to completion of blood sampling. — Done, see Results LL 250-252.

L 101: It's important to define what you mean by females being 'gravid' or not. This word simply means heavy and is not sufficient on its own to define reproductive condition. Were you able to distinguish females in vitellogenic or preovulatory condition from those that were carrying oviducal eggs, as well as from those that were post-partum? If the first two of these conditions could not reliably be distinguished, this reduces the value of the results (e.g. LL 199-206) because it would mean that 'breeding' females are a mixture of pre-ovulatory and post-ovulatory conditions, and these are likely to have very different hormonal profiles. — We defined what we meant by 'gravid,' see Methods L 114.

L 129: Essential to add here the specific antibodies used and the cross-reactivities of them with other steroids. — We added cross-reactivity of E2, because this was the only steroid in which we measured concentrations using a direct assay, see Methods LL 153-154.

L 135: These inter-assay CVs (31-65%) are very high: I would be hoping for values less than 25%. I think some comment on the significance of this high variation is needed. We have to hope that the randomization of samples among assays was sufficient for this variation not to have

contributed to the differences detected between reproductive groups. — We addressed this issue, see Methods LL 148-149.

L 156-157: is a 'one way ANOVA on ranks' the same as the non-parametric Kruskal-Wallis test (which is a name that may be more familiar to readers)? — It is not a Kruskal-Wallis test, but is a suitable way to analyze these data.

Results

LL 178-183: This first paragraph of the results makes no reference to where the data are presented. — We added the Figure number to these results, see Results LL 199-205.

L 188: Could the difference in plasma CORT concentrations between yearling females and males be due to a difference in the mean time to obtain the blood sample? I recommend adding the mean time to sample (± SE) for each group in Table 1. — Done

LL 207-215: Adult males had elevated concentrations of E2 in some samples, a point the authors take up further in the discussion (LL 255 ff) as being of note. However, concentrations of T were also at the high end of what one expects in lizards (>100 ng/ml), and T and E2 were correlated in males overall. These observations raise the question of whether the apparently high E2 in some males was a result of cross-reactivity with T in the E2 assay. I do not have enough information or expertise to judge this, but I think it is an important point for someone with more knowledge of steroid biochemistry to comment on. — This is a good point, and one that we've addressed by adding the cross-reactivities of the E2 antibody with other steroids - see LL 153-154. Our E2 antibody is highly specific for estradiol-17B, and it has a cross-reactivity with T of < 0.01%. This – along with the fact that the E2 results were the same when we ran the column assays (which isolates E2 from the other steroids) – make it very probable that the E2 results are real and not an artifact of the assay.

LL 228-229: Always present means ±SE. — Done

LL 229-238: It's hard to grasp the significance of some of the data sets showing no relationship between CORT and time to sample, because we don't know what the dispersion of data points is for time to sample. I suggest adding the 'overall' data sets to Figures 1 and 2, using a different symbol, so that we can see the overall dispersion of data for both CORT and sampling times. — We added the overall data sets to Figures 2a and 2b.

L 257: Podarcis? (sp.) — Done

LL 297-309: I'd like to see the relationship between CORT and reproductive condition in female reptiles taken further. There should be enough published data for sea turtles and for tuatara (e.g. Tyrrell and Cree 1998: GCE 110 97-108; Cree & Tyrrell 2001: In Perspective in Comparative Endocrinology: Unity and Diversity. 14th International Congress of Comparative Endocrinology, Sorrento. Italy. Goos, HJ et al. eds. Monduzzi Editore 433-441) to look specifically at the relationship with nesting behaviour (as opposed to breeding or 'gravidity' in

general). — We addressed the relationship between CORT and nesting behavior, see Discussion LL 338-342.

LL 329-330: the authors say that in the current study, the stress from resampling and carrying radiotransmitters was largely non-detectable. However, no data are presented, and it's not clear what we can read into the word 'largely'. Unless the authors present data on these points (which would add to the study) I feel this statement should be taken out. — We removed this statement.

L 335: 'for both sexes' - of reptiles? Or some other group? — We clarified the group, see Discussion L 369.

LL 337-339: 'and therefore is not measured in both sexes': this is too sweeping - I can think of studies where E2 (as mentioned earlier in the discussion) and P have been measured in male reptiles, and T in female reptiles. The possibility of 'non-traditional' functions is already recognised. — We removed this statement.

Fig 1: make it clear in the caption that 'breeding', 'egg-laying' and 'non-breeding' are defined by the month of sampling, rather than the reproductive condition of individual females (if that is the case). For example, does the 'egg laying' group contain both females with oviducal eggs that are not nesting, females that are nesting and females that are post-partum? It would greatly add to the value of the study to discriminate more clearly among females in different reproductive conditions. — We defined the breeding, egg-laying, and non-breeding periods by including the months of sampling, see Figure 1 caption.

Fig 1: it would be helpful if the results of post-hoc tests were indicated with lower case letters, to avoid confusion with the upper case letters used to identify different graphs. — Done.

Effects of sex, age, and season on plasma steroids in free-ranging Texas horned lizards (*Phrynosoma cornutum*)

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Abstract

The Texas horned lizard (*Phrynosoma cornutum*) is protected in several states due to its apparently declining numbers; information on its physiology is therefore of interest from both comparative endocrine and applied perspectives. We collected blood samples from free-ranging *P. cornutum* in Oklahoma from April to September 2005, spanning their complete active period. We determined plasma concentrations of the steroids, progesterone (P), testosterone (T), and corticosterone (CORT) by radioimmunoassay following chromatographic separation and 17β-estradiol (E2) by direct radioimmunoassay. T concentrations in breeding males were significantly higher than in non-breeding males. P showed no significant seasonal variation within either sex. CORT was significantly higher during the egg-laying season compared to breeding and non-breeding seasons for adult females and it was marginally higher in breeding than in non-breeding males (P=0.055). CORT concentrations also significantly increased with handling in non-breeding males and egg-laying females. Perhaps most surprisingly, there were no significant sex differences in plasma concentrations of P and E2. Furthermore, with respect to seasonal differences, plasma E2 concentrations were significantly higher in breeding females than in egg-laying or non-breeding females, and they were significantly higher in breeding than in non-breeding males. During the non-breeding season, yearling males exhibited higher E2 concentrations than adult males; no other differences between the steroid concentrations of yearlings and adults were detected. In comparison to other vertebrates, the seasonal steroid profile of *P. cornutum* exhibited both expected and unexpected patterns, and our results illustrate the value of collecting such baseline data as a springboard for appropriate questions for future research.

Keywords: Testosterone, Estradiol, Progesterone, Corticosterone, Texas horned lizard, Reptile, Stress

1. Introduction

Temporal changes in plasma hormones, particularly steroids, frequently interact with changes in physiology, ecology, and behavior. For example, plasma testosterone concentrations often exhibit positive correlations with home-range size (e.g., Denardo and Sinervo, 1994) and agonistic behavior in male lizards (e.g., Fox, 1983). Although such generalities are useful, it is important to keep in mind that plasma steroids can vary in their effects on different species as well (Bern, 1990). For example, male red-sided garter snakes (*Thamnophis sirtalis parietalis*) exhibit peak testosterone concentrations the season before mating (Crews, 1984; Krohmer, 2004). Thus to understand the potential effects of hormones for any given species, it remains useful to first collect data on endogenous steroid concentrations.

We documented the steroid profile of the Texas horned lizard (*Phrynosoma cornutum*) in Oklahoma during its active period from April to September. This species ranges from the south-central United States to northern Mexico and has an adult size of 70-120 mm snout-vent length, with two occipital horns that point upward and dorsally-located spines (Sherbrooke, 2003). The reproductive strategy of *P. cornutum* has been described as late maturing with one large clutch per year (Ballinger, 1974; Pianka and Parker, 1975; Tinkle et al., 1970), although *P. cornutum*, in areas of southern Texas, can have two clutches per year (Burrow, 2000; Kazmaier, unpub). Breeding occurs after individuals emerge from hibernation in late March to early April through

May, when adult males and females are found with mature spermatids and yolked follicles, respectively (Ballinger, 1974; Howard, 1974). The non-breeding period occurs after breeding and continues until individuals enter hibernation in late September to early October (Endriss et al., 2007; Sherbrooke, 2003). Adult males, which are not territorial, increase their movements and cover a greater area during the breeding season, when they are searching for mates (Stark et al., 2005). Females have been reported to oviposit primarily in June with an average clutch size of 17 eggs (Endriss et al., 2007). The eggs hatch 49-68 days later, depending on weather conditions (Endriss et al., 2007). Hatchlings do not become reproductively active until the second summer following their birth when they are at least 19 months of age (Sherbrooke, 2003).

The population size of Texas horned lizards apparently has declined over the last several decades and, as a result, is listed as a *Species of Special Concern* in many states, including Oklahoma (Carpenter et al., 1993). In spite of broad interest in and need for information about *P. cornutum*, surprisingly little is known about its physiology. Most papers published on this species focus on behavior (e.g., Cahn, 1926; e.g., Milne and Milne, 1950; Sherbrooke and Middendorf, 2004) and ecology (e.g., Burrow et al., 2001; Donaldson et al., 1994; Fair and Henke, 1997); none have been published on endocrinology. On a larger scale, considering published endocrine studies across vertebrate species, few have comprehensively described steroid profiles for yearlings and adults of both sexes.

The objective of this study was to document plasma concentrations of 17β-estradiol (E2), progesterone (P), testosterone (T), and corticosterone (CORT) in adult and yearling Texas horned lizards. Specifically, we addressed the following questions: (1) Do steroid concentrations differ by sex or age?; (2) Do steroid concentrations differ between reproductive and non-reproductive periods?; and (3) Does brief capture and handling affect CORT concentration?

This descriptive study should contribute to development of an effective conservation management program; endocrine data on gonadal steroids serve as a foundation for further research, and data on glucocorticoid concentrations can reveal how a species is responding to environmental stressors and human disturbance (Cockrem, 2005).

2. Methods

2.1 Sampling methods

We collected data on free-ranging male and female *P. cornutum* during sampling trips occurring every two weeks from 15 April to 18 September 2005. All work was conducted as approved by the Oklahoma State University Institutional Animal Care and Use Committee (Animal Care and Use Protocol No. AS059). Individuals were sampled from two sites, one located at Tinker Air Force Base in Oklahoma County, OK (35˚24'58"N and 97˚24'41"W) and the other in Payne County, OK (36˚06'30"N and 97˚01'30"W). We collected data from adults (females [n = 43] with snout-to-vent length [SVL] >60 mm; males [n = 30] with SVL >55mm) throughout the sampling period. We only sampled yearlings (males [n=6] and females [n = 7] with SVL between 51-59 mm and 49-54 mm, respectively) beginning 10 July 2005 because prior to this period their body mass was insufficient to acquire a sufficient volume of blood. A small subset of individuals (n = 17) was resampled once; resampling occurred at least eight weeks after the first sample. Also, a subset of individuals (n = 20) sampled from Tinker Air Force Base (AFB) had been previously fixed with radiotransmitters as part of a research project on horned lizard ecology (Endriss et al., 2007). Duration of radiotransmitter attachment ranged from 15 to

483 days. There was no correlation between CORT concentration and duration of radiotransmitter attachment (r=0.466, *P*=0.080). Additionally, all animals with radiotransmitters were distributed between the sexes and breeding and non-breeding periods. Data were analyzed to examine the effects of radiotransmitters and resampling on steroid concentrations and none were found (see below). Sampling occurred between 0800 and 1300 hours to minimize the potential impact of diel variation on steroid concentrations.

Individuals were found by visual scan or radiotelemetry. Once located, we noted their behavior (e.g., basking, feeding, or mating), and then collected them by hand. Immediately following capture, a blood sample ($\leq$ 75 µl) was collected by making a < 5 mm longitudinal incision on the ventromedial side of the tail using a sterile scalpel and inserting a heparinized capillary tube to draw the blood (Middendorf et al., 2001). Animals with radiotransmitters were monitored for infection. No infections were detected and these animals, as well as others caught following previous capture and blood collection, were completely healed within eight weeks; most probably much sooner. We chose this method of blood collection over others (e.g., venipuncture of the caudal vein with a needle with syringe; Brown, 1999) because in our experience it minimized blood collection time.

Using a stopwatch, we recorded time from initial sighting to the completion of blood collection ("sampling time.") Samples were transferred to plastic centrifuge tubes and stored on ice until they were returned to the laboratory. We also noted SVL of each individual to the nearest mm using a ruler and mass to the nearest 0.1g using an Ohaus Scout Pro scale. Mean body masses for adult males and females were 15.2 and 20.3 g, respectively and for yearling males and females, 9.2 and 9.4 g, respectively. Females were noted as having oviductal eggs

115 ("gravid") or not by palpation of the abdomen. All lizards were toe-clipped for individual
116 identification (if they had not been previously) and then released where they were found.
117
118 *2.2 Laboratory methods*
119
120 Blood was centrifuged at 3500 rpm for 5 minutes; the plasma fraction was removed,
121 measured to the nearest µl with a Hamilton syringe and then kept frozen at -20˚ C until analysis
122 for steroid content.
123 We measured plasma concentrations of E2, P, T, and CORT by radioimmunoassay
124 following ether extraction and chromatographic separation (Wingfield and Farner, 1975). Once
125 samples were thawed, 5-20 µl of plasma (recorded to the nearest µl for each sample) was placed
126 into individual culture tubes and 0.5 ml ddH_2O was added to provide a sufficient volume for
127 extraction. Samples were then equilibrated overnight at 4˚ C with 2000 dpm each of tritiated E2,
128 P, T, and CORT (Cat. Nos. NET317, NET381, NET370, NET399, respectively, from Perkin
129 Elmer Life Sciences, Inc.) for individual recovery determination. The following day, samples
130 were extracted twice with 3 ml diethyl ether, then dried in a 37˚C water bath under nitrogen gas.
131 They were then reconstituted in 500 µl of a mixture of ethylene glycol in isooctane in preparation
132 for column chromatography.
133 Samples were further purified and isolated by column chromatography. Columns
134 consisted of diatomaceous earth (Celite, Sigma) with a Celite:ethylene glycol: propylene glycol
135 upper phase (6:1.5:1.5 m:v:v) and a Celite:ddH2O (3:1 m:v) lower phase. Neutral lipids were
136 eluted with 2.0 ml isooctane and discarded. P, T, E2, and CORT fractions were eluted with 4.5
137 ml 10%, 4.5 ml 20%, 4.0 ml 40%, and 4.5 ml 50% ethyl acetate in isooctane, respectively, and

collected in test tubes. Samples were then dried in a 37˚C water bath under nitrogen gas, resuspended in 0.5 ml assay buffer, and held overnight at 4˚ C.

The next day, we performed competitive binding radioimmunoassays using the appropriate tritiated steroid tracers and E2, P, CORT, and T antisera (Cat. Nos. 7010-2650 from Biogenesis, P0130 and C8784 from Sigma, and T3003 from Wien Laboratories, respectively). Standard curves and samples were run in triplicate and duplicate, respectively. Standard curves were run from 500 to 0.98 ρg for E2, P, and T and 1000 to 1.95 ρg for CORT. Four to six aliquots from a standard pool, treated the same as the samples above, also were run in each assay for estimation of assay precision. Samples were randomized over four assays with average intra-assay coefficients of variation (CV's) for E2, P, T, and CORT of 18, 13, 16, and 13% and inter-assay CV's of 31%, 40%, 65%, and 25%, respectively. Although these inter-assay CV's are quite high, the potential effect of assay variation on our data was mitigated by sample randomization. Average recoveries for E2, P, T, and CORT were 75%, 61%, 71%, and 53%, respectively.

Because 21% of the samples contained non-detectable E2, we ran a single direct assay for plasma E2 in an attempt to increase recovery and subsequent detection. Our E2 antibody is highly specific (cross-reactivity with E2 = 100%, estrone = 14%, estriol = 5%, T and other steroids = < 0.01%). This assay was performed as described above except that the chromatographic isolation step was eliminated after the extraction; ether extracts were dried and suspended in 0.5 ml assay buffer. Intra-assay variation for E2 was 22% and average recovery was increased to 99%, but 21% of samples again were undetectable. Therefore, for analyses these samples were assigned the minimum detectable dose and corrected for individual recovery and initial sample volume. Because inter-assay variation was eliminated and recoveries were

160 higher than for the assays using column chromatography, E2 data reported below are from this
161 direct assay (although the results did not differ regardless of which assay was used).
162
163 *2.3 Statistical analysis*
164
165 We organized sample periods into three seasons: breeding (April to May); egg-laying
166 (females in June); and non-breeding (July to September for females and June to September for
167 males). These seasons were based on reports from the literature (Ballinger, 1974; Endriss et al.,
168 2007; Howard, 1974) as well as our own personal observations in the field. We used preliminary
169 analyses to compare hormone concentrations between (1) individuals that were resampled and
170 those that were not during the non-breeding season (the only season during which second
171 samples were obtained); (2) individuals from different study sites; and (3) individuals with and
172 without radiotransmitters. We used two-way ANOVAs on ranked data, separately for each sex
173 with season, the factor of concern (i.e., resampling, transmitters, or site) and the interaction as
174 effects. Because there were no effects of resampling, transmitters, or site on steroid
175 concentrations (ANOVA; all $P>0.05$), data were combined for subsequent analyses.
176 Steroid data neither met assumptions of normality (Kolmogorov-Smirnov, $P<0.05$) nor
177 homogeneity of variance (Levene, $P<0.05$) even after transformations, so one-way ANOVA on
178 ranks was used unless noted otherwise. To examine sex differences within seasons, steroid
179 concentrations were compared between the adult sexes separately within the breeding and non-
180 breeding seasons. The same comparison was made between sexes of yearlings during just the
181 non-breeding season. Concentrations of each steroid for adults of each sex were separately
182 analyzed with season (breeding, egg-laying, and non-breeding for females; and breeding and

183 non-breeding for males) as the main effect. We also compared steroid concentrations between
184 gravid and non-gravid females overall and within just the egg-laying season. Differences
185 between adult and yearling steroid concentrations from the non-breeding season were analyzed
186 within each sex with age as the main effect.
187 We used regression analysis to examine the relationship between plasma steroid
188 concentrations and sampling time. Separate analyses were performed on males and females
189 overall and within each season. Additionally, we used Spearman correlation analysis to examine
190 the relationship between T and E2 in adult males.
191 Tukey's *a posteriori* tests on ranks were performed when the overall ANOVA
192 significance level of 0.05 was reached (Sokal and Rohlf, 1995). All statistical analyses were
193 done using SAS Version 9.1 (SAS Institute, 2003).
194
195 **3. Results**
196
197 *3.1 Sex differences in plasma steroid concentrations*
198
199 There were no differences between breeding males and females for E2 (Figure 1;
200 $F_{1,24}$=0.40, *P*=0.531), P (Figure 1; $F_{1,33}$=2.40, *P*=0.131), or CORT (Figure 1; $F_{1,33}$=3.26,
201 *P*=0.080). However, breeding males had higher plasma T concentrations than breeding females
202 (Figure 1; $F_{1,31}$=44.57, *P*<0.001). Also, there were no differences between non-breeding males
203 and females for E2 (Figure 1; $F_{1,57}$=0.10, *P*=0.751), P (Figure 1; $F_{1,71}$=0.18, *P*=0.669), or CORT
204 (Figure 1; $F_{1,71}$=3.19, *P*=0.078). However, non-breeding males had higher plasma T
205 concentrations than non-breeding females (Figure 1; $F_{1,69}$=99.62, *P*<0.001).

206 Plasma steroid concentrations also were analyzed by sex for yearlings, but these analyses
207 were limited to the non-breeding season, the only time period in which yearlings were sampled.
208 No differences in P ($F_{1,11}$=0.12, *P*=0.737) or T ($F_{1,11}$=3.21, *P*=0.101) were found; however,
209 yearling males had higher E2 concentrations than yearling females (Table 1; $F_{1,7}$=9.74,
210 *P*=0.017), and yearling females had higher CORT concentrations than yearling males (Table 1;
211 $F_{1,11}$=23.40, *P*=0.001).
212
213 *3.2 Seasonal effects on plasma steroid concentrations for adults*
214
215 Adult female E2 concentrations were higher during breeding than either egg-laying or
216 non-breeding periods (Figure 1; $F_{2,34}$=13.39, *P*<0.001). Plasma P and T concentrations for adult
217 females did not differ among the reproductive seasons (Figure 1; $F_{2,40}$=0.97, *P*=0.389 and
218 $F_{2,40}$=2.33, *P*=0.110, respectively). There was seasonal variation in plasma CORT concentrations
219 (Figure 1; $F_{2,40}$=3.50, *P*=0.040) overall with peak plasma CORT concentrations during egg-
220 laying, although Tukey's test could not isolate the seasonal difference.
221 Differences in steroid concentrations between gravid and non-gravid females were
222 analyzed across all time periods and within the egg-laying period. No differences were found for
223 E2, P, and T plasma concentrations between gravid and non-gravid females across all time
224 periods ($F_{1,35}$=0.01, *P*=0.9334; $F_{1,41}$=0.00, *P*=0.984; and $F_{1,41}$=3.99, *P*=0.052 respectively), but
225 gravid females had a higher ($F_{1,41}$=13.91, *P*<0.001) mean CORT concentration (80.92 ng/ml)
226 than non-gravid females (13.76 ng/ml). CORT concentrations also varied ($F_{1,5}$=15.00, *P*=0.0117)
227 within the egg-laying season between gravid and non-gravid females, but were not different for
228 E2 ($F_{1,4}$=0.38, *P*=0.573), P ($F_{1,5}$=0.45, *P*=0.530), or T ($F_{1,5}$=1.80, *P*=0.238).

Adult male E2 concentrations varied between reproductive seasons with a higher mean plasma E2 concentration during the breeding season, regardless of whether analyzed by direct assay (Figure 1; $F_{1,20}$=79.76, P<0.001) or following column chromatography ($F_{1,28}$=9.35, P=0.005). P concentrations did not differ between breeding and non-breeding adult males (Figure 1; $F_{1,28}$=3.21, P=0.084). Plasma T concentrations were higher during the breeding season for adult males (Figure 1; $F_{1,26}$=7.16, P=0.013). Plasma concentrations of T and E2 for adult males were correlated (r=0.727, P<0.001). Plasma CORT concentrations tended to be higher during the breeding season for adult males compared to the non-breeding season (Figure 1; $F_{1,28}$=4.01, P=0.055), but this difference was not significant at the 0.05 level.

3.3 Differences in steroid concentrations between adults and yearlings

No age-related difference in plasma concentrations of E2 ($F_{1,21}$=0.32, P=0.577), P ($F_{1,74}$=1.74, P=0.199), T ($F_{2,20}$=1.24, P=0.151), or CORT ($F_{1,24}$=0.02, P=0.889) was evident for females during the non-breeding season. There were also no differences between adult and yearling males during the non-breeding season for P ($F_{1,16}$=0.30, P=0.590), T ($F_{1,16}$=0.13, P=0.720), and CORT ($F_{1,16}$=3.21, P=0.092). In contrast, yearling males had a higher mean E2 concentration than adult males ($F_{1,11}$=25.21, P<0.001). Steroid concentrations for yearlings are listed in Table 1.

3.4 Handling time and plasma steroid concentrations

Average sampling times for adult females and males were 4.8 ± 0.73 and 5.6 ± 1.03 minutes, respectively. Maximum sampling times for adult females and males were 13.43 and 14.33 minutes, respectively. There was no relationship between sampling time and E2, P, T, or CORT for adult females analyzed overall ($F_{1,41}$=0.64, *P*=0.427, $F_{1,48}$=0.00, *P*=0.961, $F_{1,48}$=0.19, *P*=0.667, and $F_{1,41}$=1.02, *P*=0.319, respectively), during breeding ($F_{1,12}$=0.68, *P*=0.426, $F_{1,15}$=0.03, *P*=0.867, $F_{1,15}$=0.07, *P*=0.795, and $F_{1,15}$=0.65, *P*=0.432, respectively), or during non-breeding ($F_{1,21}$=2.02, *P*=0.170, $F_{1,24}$=0.22, *P*=0.646, $F_{1,24}$=0.66, *P*=0.426, and $F_{1,17}$=0.23, *P*=0.637, respectively). There was no relationship between sampling time and E2, P, or T during the egg-laying season ($F_{1,4}$=0.04, *P*=0.845, $F_{1,5}$=0.08, *P*=0.784, $F_{1,5}$=0.23, *P*=0.655, respectively). In contrast, CORT concentrations increased with increased sampling time during the egg-laying season for females (Figure 2; $F_{1,5}$=9.66, *P*=0.027).

There was no relationship between sampling time and E2, P, T, or CORT for males analyzed overall ($F_{1,23}$=1.28, *P*=0.270, $F_{1,34}$=0.87, *P*=0.357, $F_{1,32}$=0.90, *P*=0.349, and $F_{1,28}$=3.50, *P*=0.072, respectively) or during breeding ($F_{1,10}$=0.37, *P*=0.5563, $F_{1,16}$=0.27, *P*=0.611, $F_{1,14}$=0.65, *P*=0.433, and $F_{1,16}$=0.51, *P*=0.486, respecitively). There was no relationship between sampling time and E2, P, and T during non-breeding ($F_{1,11}$=1.48, *P*=0..250, $F_{1,16}$=0.14, *P*=0.712, and $F_{1,16}$=0.16, *P*=0.693, respectively), but there was a significant correlation for CORT (Figure 2; $F_{1,10}$=18.29, *P*=0.002), although the extreme leverage of a single datum corresponding to a lizard with a long sampling time biases the regression, as indicated by removal of that datum ($F_{1,9}$=0.97, *P*=0.35).

4. Discussion

The seasonal steroid profile of *P. cornutum* exhibited several traditional and non-traditional hormone-behavior relationships. E2 concentrations typically elevate in female lizards during the breeding season when vitellogenesis is occurring and reach peak concentrations just before ovulation when vitellogenic follicles are large (Alberts et al., 2004; Carnevali et al., 1991; Edwards and Jones, 2001a; Radder et al., 2001). The E2 profile of female *P. cornutum* suggests that a similar pattern is occurring with this species based on higher concentrations during the breeding season. Howard (1974) and Ballinger (1974) reported that adult female *P. cornutum* were found with yolked follicles from April to May, a finding supported by the elevated E2 concentrations that we found during the same period. Another phrynosomatid species, *P. coronatum blainvillei*, also exhibited a peak concentration of E2 during the breeding season (Alberts et al., 2004). These higher concentrations also may be indicative of the role E2 plays in adult female lizards to enhance sexual receptivity (Gans and Crews, 1992; Whittier and Tokarz, 1992).

E2 concentrations also were higher during the breeding season in adult male *P. cornutum* and were not different from adult females overall. Several bird species (Adkins-Regan et al., 1990; Saldanha and Schlinger, 1997; Watson et al., 1990) and at least two squamates, *Podarcis sicula sicula* (Ando et al., 1992; Cardone et al., 1998) and *Tiliqua nigrolutea* (Edwards and Jones, 2001b) have exhibited elevated E2 concentrations in adult males. However, the role that plasma estrogens play in male reproduction in *P. cornutum* or these other species has not been clearly defined. Estrogens regulate Leydig cell and Sertoli cell development, descent of the testes, and control viability, apoptosis, and acrosome biogenesis of germ cells in mammals (Hess, 2003; O'Donnell et al., 2001). Additionally, Russo et al. (2005) have shown that estrogens

play a role in proliferation and survival of germ cells in *P. s. sicula* (Chieffi et al., 2002; Russo et al., 2005).

Elevated E2 concentrations in males may be directly related to aromatase activity. It has been demonstrated that aromatase gene expression in male rats is regulated by T, so an increase in T causes an increase in aromatase and as a result, E2 concentrations also increase (Bourguiba et al., 2003; Genissel et al., 2001). Given the positive correlation of T and E2 in adult male *P. cornutum*, it is possible that the elevated E2 concentrations are due to increased aromatase activity. However, yearling males have high E2 concentrations when T concentrations are low. Elevated E2 in yearling males may be due in part to its established role in sexual differentiation, in which E2 is involved in organization of the sexually dimorphic nucleus of the preoptic area of the brain, which controls male sexual behavior (Baum, 2003; Cross and Roselli, 1999; Norris, 1997; Watson et al., 1990). Without measuring testis size, sperm number, or aromatase activity, we cannot accurately predict which of the above established hypotheses fits best for *P. cornutum* or what other function E2 could have in this species.

Unlike E2, there was no difference in P concentrations among reproductive time periods in adult females. In female lizards, P is partly responsible for oviductal egg development (Norris, 1997); therefore, P concentrations typically peak during egg development and remain elevated until oviposition (Radder et al., 2001; Whittier and Tokarz, 1992). Blood samples may have been taken early in egg development, so P was not at peak concentrations. Another possibility is that P may not play a role in oviductal egg development in female *P. cornutum*.

Adult male *P. cornutum* had similar P concentrations as adult females, suggesting that P has an important function in adult males. However, this result is difficult to interpret. P influences male sexual behavior in various species and may be required for its activation

(Andersen and Tufik, 2006). For example, P can recover sexual behavior in male *Anolis carolinensis* from loss of T when individuals are castrated (Young et al., 1991). Although T has a more potent effect on male reproductive behavior, P may influence male sexual behavior by regulating androgen receptors (Crews et al., 1996; Phelps et al., 1998; Sakata et al., 2003). Testosterone concentrations were highest in adult breeding male *P. cornutum* as seen in other phrynosomatid lizards (Alberts et al., 2004; Arslan et al., 1978; McKinney and Marion, 1985; Tokarz et al., 1997). If P mediates reproductive behavior similar to T, P concentrations also should be elevated during the breeding season in males, however there was not a significant difference in P concentrations between breeding and non-breeding males (although there was that tendency).

The energy-mobilization hypothesis states that glucocorticoid concentrations will be highest during times of the year that are energetically demanding, such as periods of gravidity (Romero, 2002), because glucocorticoids aid in energy allocation by enhancing gluconeogenesis (Norris, 1997). The seasonal peaks in CORT concentrations for adult male and female *P. cornutum* exhibited patterns seen in other lizard species and corresponded to extra energetic needs of the individuals during those time periods. Like *P. cornutum*, the phrynosomatid lizards *Uta stansburiana* (Wilson and Wingfield, 1994) and *P. coronatum blainvillei* (Alberts et al., 2004) had peak CORT concentrations during breeding and egg-laying in adult females and during breeding for adult males. CORT also reached peak concentrations when female tree lizards (*Urosaurus ornatus*) were gravid (Woodley and Moore, 2002). The relative peak in CORT concentrations during the egg-laying period may also be due to nesting at this time as is suggested by Tyrell and Cree (1998) in *Sphenodon punctatus*. Adult females dug multiple nests to find a suitable place to oviposit. They also guarded the nests for at least 24 hours post-

342 excavation. These stressors may be inducing the additional CORT response. Although seasonal
343 differences in CORT concentrations for adult females could not be isolated by Tukey's test, we
344 consider the statistically significant overall seasonal effect to be biologically significant for the
345 reasons mentioned above.

346 The handling stress of acquiring blood samples and CORT concentrations exhibited
347 relationships seen in other vertebrates. However, handling stress did not increase CORT
348 concentrations in breeding male *P. cornutum* as it did in non-breeding males. Elevated CORT
349 reduces aggression and courtship in males through its reciprocal relationship with T, i.e., when
350 CORT concentrations are increased, T concentrations are depressed (Denardo and Licht, 1993;
351 Knapp and Moore, 1997; Manzo et al., 1994; Moore and Miller, 1984; Tokarz, 1987; Woodley
352 and Moore, 1999). The mechanisms of inhibition can occur at any level of the hypothalamo-
353 pituitary-gonad (HPG) axis (Rivier and Rivest, 1991). Male *P. cornutum* may be modulating the
354 adrenocortical response by suppressing the hypothalamo-pituitary-adrenal (HPA) axis during the
355 breeding season to prevent a fitness loss of decreased reproduction as is seen in birds and other
356 lizards (see reviews, Moore and Jessop, 2003; Wingfield and Sapolsky, 2003). Unlike breeding
357 males, egg-laying females exhibited a significant increase in CORT concentrations as handling
358 time increased. CORT levels were already increased during this period, thus the HPA axis was
359 already primed and sensitive to an acute stressor, i.e., handling stress.

360 Baseline data on glucocorticoid concentrations can serve as good indicators of habitat
361 quality and how species respond to environmental stressors; therefore, they are important tools in
362 developing a conservation management program for threatened species (Cockrem, 2005).
363 Furthermore, these data can be utilized as diagnostic tools to test effects of human disturbance
364 from research, such as radiotransmitters, on study individuals (Cockrem, 2005). It is difficult to

365 draw conclusions from the data presented here, given a single blood collection. A future study
366 collecting multiple samples over a period of stress could be done to further examine the role of
367 stress and breeding in this apparently declining species.
368 The patterns in steroid concentrations exhibited in this study indicate the need for more
369 research in the field of comparative endocrinology. Few published data exist for all of these
370 steroids for both sexes among reptiles; none exist for *P. cornutum*. Given breeding males have
371 higher E2 concentrations and there is no sex differences with regards to P concentrations, *P.*
372 *cornutum* may be a good model for understanding the "non-traditional" functions of these
373 steroids.
374
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383
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Figure 1. Mean plasma (A) 17β-estradiol concentration, (B) progesterone concentration, (C) testosterone concentration, and (D) corticosterone concentration, (±1SE) for adult Texas horned lizards (*Phrynosoma cornutum*)during breeding (April to May); egg-laying (females in June); and non-breeding (July to September for females and June to September for males) analyzed within sex. Bars with different letters or an asterisk (*) denote significant differences within sexes following rank-based ANOVAs and subsequent rank-based multiple comparison tests. Sample sizes are listed above or within bars.

Figure 2. Relationship of sampling time and corticosterone concentrations in Texas horned lizards (*Phrynosoma cornutum*). Graph (A) shows the regression for adult males during the non-breeding season (n=19), indicated by closed circles. Open circles indicate data from adult, breeding males. Graph (B) shows the regression for adult females during the egg-laying season (n=7), indicated by closed circles. Open circles indicate data from adult, breeding and non-breeding females. R^2 and P-values are shown for each regression

Table 1

Mean plasma steroid concentrations by season and mean sampling times for yearling Texas horned lizards of each sex during the non-breeding season

	FEMALES MEAN ± 1SE (ng/ml)	N	MALES MEAN ± 1SE (ng/ml)	N
E2	0.033 ± 0.026	6	0.393 ± 0.172	3
P	1.15 ± 0.225	7	1.133 ± 0.444	6
T	4.21 ± 2.852	7	13.858 ± 4.568	6
CORT	8.817 ± 1.868	7	2.843 ± 0.337	6
Mean Sampling Time (min)	2.95 ± 1.12		3.77 ± 1.54	

Figure 1

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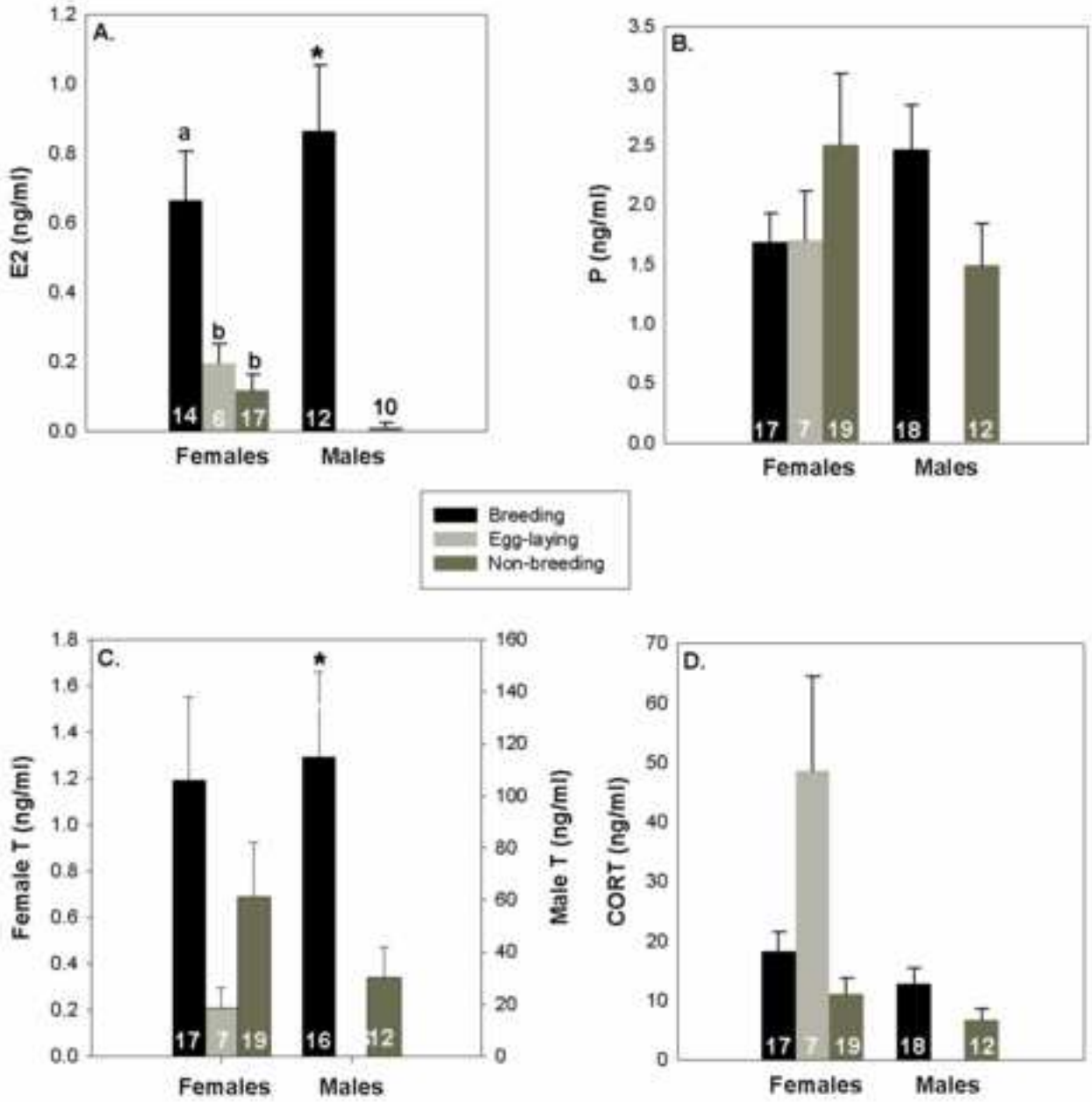

Figure 2

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