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Circulating concentrations of gonadal and adrenocortical hormones in wild nine-banded armadillos (*Dasypus novemcinctus*)

Abstract: We performed a retrospective analysis of hormone concentrations found in blood sera from culled nine-banded armadillos (*Dasypus novemcinctus*) with the goal of identifying important sources of variation in, and relationships among, circulating levels of testosterone (males), estradiol and progesterone (females), and cortisol (all animals). Testosterone concentrations were greater in adult males than in yearling or juvenile males. Variation in estradiol was limited to significant differences across months, although no pair-wise comparisons between months were significant. Adult females had higher concentrations of progesterone than did juvenile and yearling females. Among adult females, progesterone varied significantly from year-to-year as well as month-to-month, and was positively correlated with body weight. Progesterone in yearling and juvenile females varied significantly from year-to-year, was positively correlated with levels of estradiol, and was higher in animals that were live-caught as opposed to shot. All animals that were live-caught exhibited higher cortisol concentrations than those that were shot. Among shot animals, there were no sex differences in cortisol concentrations but there were for live-caught armadillos. Live-caught adult females exhibited strong positive correlations of cortisol with estradiol and progesterone. Within each sex there was significant month-to-month variation in cortisol, with concentrations the lowest in June and July.

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Introduction

Physiological processes, such as secretions from the endocrine system, are fundamental contributors to the ecology of any species (Adkins-Regan 2005, Cohen et al. 2012, Wingfield 2013). As such, it is important to identify factors that influence variation in hormone concentrations, and how the concentrations of different hormones may covary with one another. In addition, it is especially important to obtain such information from wild animals because it is not always clear to what extent data obtained under precisely controlled laboratory conditions are representative of what occurs in the wild (Calisi and Bentley 2009).

The order Cingulata, which contains the 21 extant species of armadillos, is a group of mammals for which data on hormone concentrations are particularly sparse. Information is available for only five species: *Chaetophractus villosus* Desmarest 1804, *C. vellerosus* Gray 1865 (Luaces et al. 2011), *Tolypeutes matacus* Illiger 1811 (Howell-Stephens 2012, Howell-Stephens et al. 2012, 2013), *Dasypus novemcinctus* Linnaeus 1758 (review in Peppler 2008), and *Zaedyus pichiy* Desmarest 1804 (Superina and Jahn 2009, Superina et al. 2009). In general, most of these studies examined small numbers of captive animals sampled over the course of a year to infer features of the reproductive cycle. Thus, to expand our knowledge of endocrinology in armadillos, we need more expansive studies that examine large numbers of individuals, preferably in the wild, over longer time scales, and analyze multiple types of hormones.

As an attempt at this type of analysis, we exploited the availability of several hundred nine-banded armadillos

(*Dasypus novemcinctus*; hereafter referred to as armadillo) that were culled from three properties in southern Georgia and northern Florida, USA to perform a retrospective study of hormone concentrations found in the blood sera of these animals. Specifically, we measured concentrations of progesterone and estradiol in females, testosterone in males, and cortisol in all animals. Data were analyzed to identify potential sources of variation in each hormone due to age, site, year, month, and, for cortisol, sex. In addition, we investigated possible associations between concentrations of the different hormones (e.g., between cortisol and testosterone), as well as of each hormone with animal body weight. Finally, we compared concentrations of each hormone in animals that were live-caught and killed later *versus* those that were shot immediately, and between animals that tested seropositive for exposure to *Mycobacterium leprae* Hansen 1874, the causative agent in producing leprosy (Truman 2005, 2008) *versus* animals that were seronegative. Our analyses represent one of the largest and most comprehensive field studies of hormone concentrations in any species of armadillo conducted to date, and, as such, lay the foundation for future intra- and interspecific comparisons.

Materials and methods

Study species

Nine-banded armadillos are medium-sized (adult body weight ~4 kg), burrowing mammals that are normally active at night, during which time they forage for various soil invertebrates (McBee and Baker 1982, Loughry and McDonough 2013). Except during the breeding season, adults are typically solitary and asocial. In the United States, the reproductive cycle begins with mating, with the highest levels of breeding activity normally observed in June and July (Jacobs 1979, McDonough 1997, Loughry and McDonough 2013). During this time, females ovulate a single egg (Enders 1960). After fertilization, there is a variable period of delayed implantation (Talmage et al. 1954, Storrs et al. 1988, Peppler 2008) that usually ends sometime in late fall (Hamlett 1932, Talmage et al. 1954, Enders 1966). Gestation lasts ~134 days with birth normally occurring in March–April (Hamlett 1932, Enders 1966). Young remain belowground nursing from the mother for about 40 days; they first emerge from natal burrows sometime between May and July (McDonough et al. 1998, Loughry and McDonough 2013).

Sample collection

Armadillos were collected from three locations in Georgia and Florida between 2003 and 2006 as part of an experiment to eliminate nest predators of northern bobwhite (*Colinus virginianus* Linnaeus 1758; see McDonough et al. 2007, Jarvis et al. 2013). Armadillos were exterminated from the eastern portion of Pinebloom Plantation, near Albany, Georgia and Pebble Hill Plantation, near Thomasville, Georgia in 2003. Subsequently, this experiment was repeated over the next 3 years (2004–2006) on the western portion of Pinebloom Plantation, and at Tall Timbers Research Station, located near Tallahassee, Florida. See Table 1 for a summary of the number of animals collected at each site.

Armadillos at all sites were either live-trapped (n=441) and then killed by gunshot within several hours

Table 1 Breakdown of the number of nine-banded armadillos of each age and sex category collected at each site in this study.

Site	Juveniles		Yearlings		Adults	
	Females	Males	Females	Males	Females	Males
Pebble Hill (2003)	2	5	2	2	5	4
Pinebloom East (2003)	1	0	2	0	9	6
Pinebloom West						
2004	4	2	1	1	15	15
2005	0	2	1	2	13	13
2006	1	1	1	0	9	10
Tall Timbers						
2004	5	4	1	3	42	42
2005	2	14	3	2	37	45
2006	4	7	4	8	59	46

In addition to what is provided above, across all sites there were 19 animals for which sex but not age was known, three animals for which age but not sex was known, and one animal lacking information about both sex and age. See Figures 1 and 2 for sample sizes by month.

of capture, or shot on sight immediately while active above ground (n=36) by technicians working for the United States Department of Agriculture. In a few rare cases, animals were caught in leg hold (n=2) or Connibear (n=1) traps. Armadillos were collected between 1 March to 30 September of each year (see McDonough et al. 2007 for further details). At death, blood was immediately collected via cardiac puncture, placed into Vacutainer tubes and allowed to clot. Serum was subsequently removed, placed in O-ring screw cap vials and stored frozen at -20°C until analyzed. As part of another study, a portion of the serum from each animal was screened for the presence of antibodies against *Mycobacterium leprae* following the protocols of Truman et al. (1986). Prior to disposal, each animal was weighed, and weights were used to assign individuals to the following age categories: <2 kg = juveniles (young of the year); 2–3 kg = yearlings; and >3 kg = adults (Loughry and McDonough 1996; see also Jarvis et al. 2013).

Hormone assays

The serum concentration of all hormones was determined by enzyme immunoassay (EIA). In all cases, EIAs were biochemically validated by demonstrating (1) parallelism between binding inhibition curves of serum extract dilutions, and (2) significant recovery (>90%) of exogenous hormone added to serum. For each hormone, intra- and inter-assay coefficients of variation were <10% and 15%, respectively. Biological validation of each assay was assessed by the extent to which patterns of variation in each hormone fit expected predictions (described below).

Serum progestagen was measured using commercial progesterone EIA (Arbor Assays, Ann Arbor, MI, USA). According to the manufacturer, cross-reactivity of the progesterone antibody was: progesterone 100%, 3β-hydroxy-progesterone 172%, 11β-hydroxy-progesterone 188%, 11α-hydroxy-progesterone 147%, 5α-dihydroprogesterone 7%, pregnenolone 5.9%, 3α-hydroxy-progesterone 2.7%, corticosterone <0.1%, and androstenedione <0.1%. Parallelism between binding inhibition curves used serum extract dilutions from 1:10–1:320; recovery of exogenous progestagen added to serum extracts used a dilution of 1:500 with a resulting relationship of $\hat{y}$ =1.0899x+61.756 (r^2 =0.99). From the parallelism data, the appropriate dilution at 50% binding was prepared for progesterone (range: 1:2–1:500). Assay sensitivity was 50 pg/ml.

Testosterone (HRP) ligands and polyclonal antiserum (R156/7; provided by C. Munro, Davis, CA, USA) were used at dilutions of 1:30,000 and 1:10,000, respectively (Santymire and Armstrong 2010). Cross-reactivity of the testosterone antibody was previously published (Santymire and Armstrong 2010). Parallelism between binding inhibition curves used serum extract dilutions ranging from 1:16–1:8192; recovery of exogenous testosterone added to serum extracts used a dilution of 1:2500 with a resulting relationship of $\hat{y}$ =1.1549x (r^2=0.99). From the parallelism data, the appropriate dilution at 50% binding for testosterone was 1:130. Assay sensitivity was 2.3 pg/well.

Estradiol polyclonal antiserum and HRP (R0008; provided by C. Munro) were used at 1:10,000 and 1:95,000 dilutions, respectively. Antiserum cross-reactivity was: estradiol 17β 100%, estrone 0.73%; estrone sulfate, progesterone, testosterone, cortisol, and corticosterone were all <0.01%. Parallelism between binding inhibition curves used serum extract dilutions from 1:8 to 1:512; recovery of exogenous estradiol added to serum extracts used a dilution of 1:200 with a resulting relationship of $\hat{y}$ =1.035x–9.44 (r^2=0.98). From the parallelism results, the appropriate dilution at 50% binding was 1:125. Assay sensitivity was 3.9 pg/well.

Cortisol polyclonal antiserum and HRP (R4866; provided by C. Munro) were used at 1:8500 and 1:20,000 dilutions, respectively (Loeding et al. 2011). Cross-reactivity to the cortisol antiserum was: cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5%, corticosterone 0.7%, deoxycorticosterone 0.3%, 21-deoxycortisone 0.5%, 11-deoxycortisol 0.2%, progesterone 0.2%, 17α-hydroxyprogesterone 0.2%; pregnenolone, 17α-hydroxypregnenolone, anderostenedione, testosterone, androsterone, dehydroepiandrosterone, dehydroisoandrosterone-3-sulfate, aldosterone, estradiol-17β, estrone, estriol, spironolactone, and cholesterol were all <0.1% (Loeding et al. 2011). Parallelism between binding inhibition curves used serum extract dilutions ranging from 1:32–1:4096; recovery of exogenous cortisol added to serum extracts used a dilution of 1:200 with a resulting relationship of $\hat{y}$ =1.002x (r^2=0.97). From the parallelism results, the appropriate dilution (1:25–1:500) at 50% binding was prepared. Assay sensitivity was 3.9 pg/well.

Predictions

The retrospective nature of our study means that by necessity most of our results are descriptive, rather than representing tests of specific hypotheses. Nonetheless, in many cases we were able to make predictions about expected outcomes. Regarding sex hormones, we expected adults

to have higher concentrations of testosterone and progesterone than younger animals (juveniles and yearlings) because increases in both hormones are associated with the onset of reproductive maturity during the second year of life (Peppler et al. 1986, Peppler 2008). In contrast, there is little evidence of age differences in estradiol concentrations (Peppler 2008). Therefore, no age effect was predicted. For all sex hormones there was no obvious basis for predicting differences between sites, years, capture methods, or between seropositive and seronegative armadillos. Regarding the latter, testosterone can suppress the immune system (Roberts et al. 2004), thus making individuals more susceptible to infection. Consequently, it might seem reasonable to predict that concentrations of testosterone would be higher in seropositive than in seronegative males. However, it is important to note that the serology tests employed to determine infection status (Truman et al. 1986) only indicated exposure to *Mycobacterium leprae*, not necessarily manifestation of disease. It is possible that the immunosuppressive effects of testosterone would make it more likely that seropositive males would eventually develop leprosy, but we did not have the data available to test this hypothesis.

Continuing with sex hormones, results from previous laboratory studies led us to expect substantial seasonal (i.e., month-to-month) variation in both estradiol and progesterone concentrations, but not testosterone (Peppler and Stone 1981, Peppler 2008). The concentration of circulating progesterone has been shown to correlate positively with body weight in female armadillos (Truman et al. 1991). Consequently, we expected to find a similar relationship. However, we could make no clear predictions about the relationship between body weight and any of the other hormones. Likewise, although there should be associations between the secretion of one hormone with another (e.g., Baird and Hews 2007, Veronesi et al. 2010), we had no rationale for making any specific predictions about possible correlations between the circulating concentrations of different steroid hormones in armadillos.

Predictions regarding cortisol are complicated by the fact that the vast majority of animals were live-caught in traps and later euthanized, as opposed to being shot on sight immediately while active above ground (see below). Thus, although it seems obvious to expect higher cortisol concentrations in live-caught armadillos *versus* animals that were shot, the inflated values obtained from live-caught animals may obscure (or exaggerate) differences in baseline concentrations. For example, finding an age difference in cortisol concentrations among live-caught animals could reflect that adults are generally more stressed than juveniles (perhaps because of events associated with reproduction), or it could be the case that adults and juveniles do not differ in baseline concentrations but adults respond more strongly to the stress of being confined in a trap. Given these difficulties, we made no predictions about expected differences in cortisol concentrations. However, because we had no reason to suspect that responses to live capture were biased in any particular direction, we did examine the same set of influences on cortisol concentrations as was done for each sex hormone. Still, we caution that these analyses are preliminary and will require confirmation, perhaps using non-invasive techniques (e.g., Howell-Stephens et al. 2012) that will allow clear comparisons between baseline concentrations of cortisol.

Statistical analyses

Data on hormone concentrations were examined using JMP 8.0. For each sex hormone, we initially used an ANOVA to examine differences due to age. If age differences were present, we then performed all subsequent analyses separately for each age group. If not, then data were pooled across age classes. For cortisol, we used a two-way ANOVA to identify possible age or sex differences. For all hormones, subsequent analyses entailed using ANOVAs to determine if there were differences in concentrations between sites, across months, and, for the Pinebloom West and Tall Timbers sites, across years. We also used ANOVAs to examine whether hormone concentrations differed between animals that were live-caught *versus* shot, and for seropositive *versus* seronegative individuals. Where relevant, Tukey-Kramer HSD tests were used for post-hoc pair-wise comparisons. Finally, we used linear regressions to investigate possible associations between the concentrations of different hormones (i.e., each sex hormone with cortisol and, for females, estradiol with progesterone), and of each hormone with animal body weight. In what follows, means are reported±1 SE.

Results

Testosterone

Nine males could not be classified to an age group because of missing body weight data. These individuals were excluded from the analyses reported here, but the pattern of results was unaffected by their inclusion. As expected, pooling across all sites and all years, we found substantial and significant differences in serum testosterone concentrations between adult males and juvenile or yearling

males (Table 2). Because of these findings, we conducted all further analyses separately for adult males and yearling/juvenile males.

There was no evidence of any significant sources of variation in serum testosterone concentrations among adult males (Table 3; see also Figure 1). Likewise, there was no significant relationship between the circulating concentration of testosterone and body weight (r=0.11, $F_{1,175}$=2.05, p=0.15). Similar findings were obtained for yearling and juvenile males (Table 3; correlation between testosterone concentration and body weight, r=-0.16, $F_{1,52}$=1.38, p=0.25). Note that we could not examine differences due to capture method in juveniles and yearlings because virtually all individuals were live-caught (51/53).

Table 2 Comparisons of age differences in the serum concentrations (ng/ml) of sex hormones in wild nine-banded armadillos.

	n	Mean±SE	ANOVA results
Testosterone			
Juvenile males	35	63.74±18.94[a]	$F_{2,234}$=8.22
Yearling males	18	43.52±26.41[a]	p=0.0004
Adult males	181	128.20±8.33[b]	
Estradiol			
Juvenile females	19	167.63±47.96[a]	$F_{2,220}$=1.68
Yearling females	15	319.73±122.90[a]	p=0.19
Adult females	189	216.06±99.19[a]	
Progesterone			
Juvenile females	19	37.75±12.82[a]	$F_{2,220}$=7.74
Yearling females	15	36.89±14.43[a]	p=0.0006
Adult females	189	78.89±4.07[b]	

For each hormone, data were pooled across all sites. Shared superscripts indicate means that did not differ from one another (p>0.05) using pair-wise Tukey-Kramer HSD tests.

Estradiol

We found no evidence of age differences in the concentration of estradiol (Table 2). In addition to the data reported in Table 2, 10 females could not be assigned to an age category because of missing body weight data. However, because we found no age differences in estradiol concentrations, where possible we included these females of unknown age in the remaining analyses.

Just as with testosterone in males, most comparisons of circulating estradiol concentrations yielded no significant differences (Table 3). There was also no significant relationship between estradiol concentrations and body weight (r=0.03, $F_{1,211}$=0.24, p=0.63). However, there was significant variation across months (Table 3; see also Figure 2), although no pair-wise comparisons between months were significant with Tukey-Kramer HSD tests. To the extent that month-to-month variation in estradiol might be linked with reproductive events, it is possible that the inclusion of data from yearlings, juveniles, and females of unknown age might lessen such differences. To investigate this possibility, we redid the analysis using just the data from adult females and found no significant monthly variation ($F_{6,182}$=2.01, p=0.07; see Figure 2).

Progesterone

Unlike estradiol, concentrations of progesterone differed significantly among age groups, with adult females having significantly higher values than juvenile and yearling females (Table 2). Because of this result, all further analyses were conducted separately for adult females and

Table 3 Summary of ANOVA comparisons examining variation in the circulating concentrations of testosterone, estradiol, progesterone and cortisol in wild nine-banded armadillos that was due to site, year (Tall Timbers and Pinebloom West sites only), month, leprosy infection status (seropositive or seronegative) and capture method (live-caught in a trap vs. shot on sight).

Comparison	Testosterone (adult males)	Testosterone (juvenile and yearling males)	Estradiol (all females)	Progesterone (adult females)	Progesterone (juvenile and yearling females)	Cortisol (all males)	Cortisol (all females)
Site	$F_{3,177}$=1.03 p=0.38	$F_{3,49}$=1.03 p=0.36	$F_{3,229}$=2.19 p=0.09	$F_{3,185}$=1.30 p=0.28	$F_{3,30}$=0.67 p=0.58	$F_{3,224}$=0.91 p=0.44	$F_{3,206}$=0.96 p=0.41
Year	$F_{2,168}$=1.01 p=0.37	$F_{2,50}$=0.47 p=0.70	$F_{2,207}$=0.60 p=0.55	$F_{2,172}$=3.36 p=0.04	$F_{2,24}$=3.38 p=0.051	$F_{2,209}$=0.86 p=0.43	$F_{2,187}$=1.76 p=0.18
Month	$F_{6,174}$=0.93 p=0.47	$F_{6,46}$=1.18 p=0.33	$F_{6,226}$=2.29 p=0.036	$F_{6,182}$=4.96 p=0.0001	$F_{5,28}$=0.93 p=0.48	$F_{6,221}$=3.54 p=0.002	$F_{6,203}$=14.88 p<0.0001
Leprosy infection status	$F_{1,179}$=1.97 p=0.16	$F_{1,51}$=0.04 p=0.85	$F_{1,231}$=1.00 p=0.32	$F_{1,187}$=0.01 p=0.92	$F_{1,32}$=0.69 p=0.69	$F_{1,226}$=1.39 p=0.24	$F_{1,208}$=2.26 p=0.13
Capture method	$F_{1,177}$=3.03 p=0.08	na	$F_{1,230}$=1.81 p=0.18	$F_{1,186}$=0.006 p=0.94	$F_{1,32}$=6.07 p=0.019	$F_{1,239}$=16.01 p<0.0001	$F_{1,230}$=28.33 p<0.0001

For cortisol, except for capture method, all other comparisons used data from only live-caught animals.

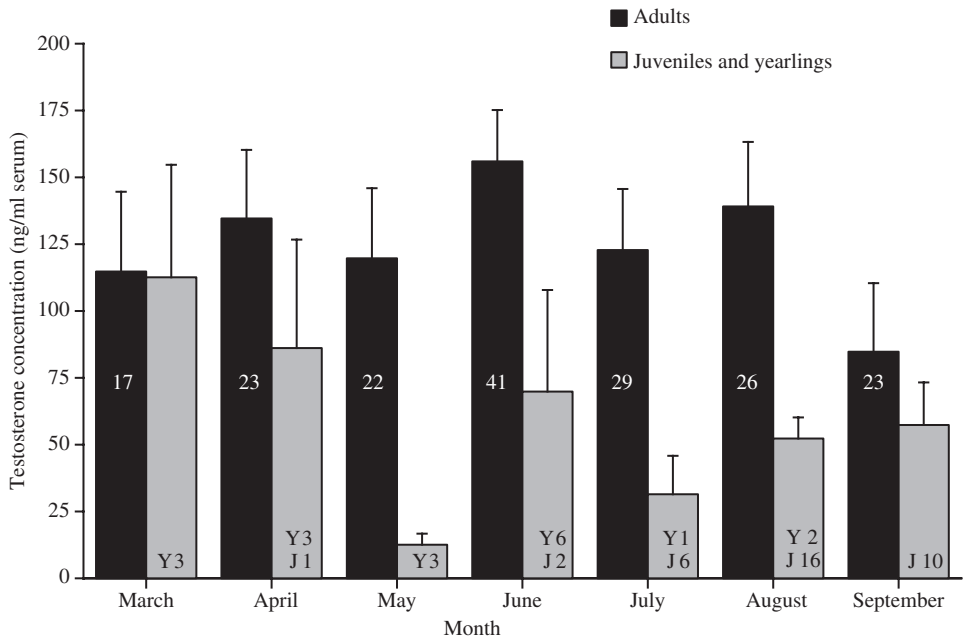

Figure 1 Monthly mean±SE serum concentrations of testosterone for adult and juvenile/yearling male nine-banded armadillos. Data were pooled across all sites and years of sampling. Sample sizes are provided within each bar. Abbreviations: J, juvenile male; Y, yearling male.

yearling/juvenile females (females of unknown age were excluded).

Among adult females, progesterone levels did not differ due to capture method, leprosy infection status, or between sites (Table 3). However, progesterone concentrations did vary across years at the Pinebloom West and Tall Timbers sites (Table 3), with levels higher in 2004 than in 2006 (Table 4), and across months at all sites (Table 3; see also Figure 3), with levels higher in August than in May, June or July (Tukey-Kramer HSD tests, all $p<0.006$), and higher in March than in June or July (Tukey-Kramer HSD tests, both $p<0.04$). Finally, circulating concentrations of progesterone were positively correlated with body weight ($r=0.33$, $F_{1,177}=21.85$,

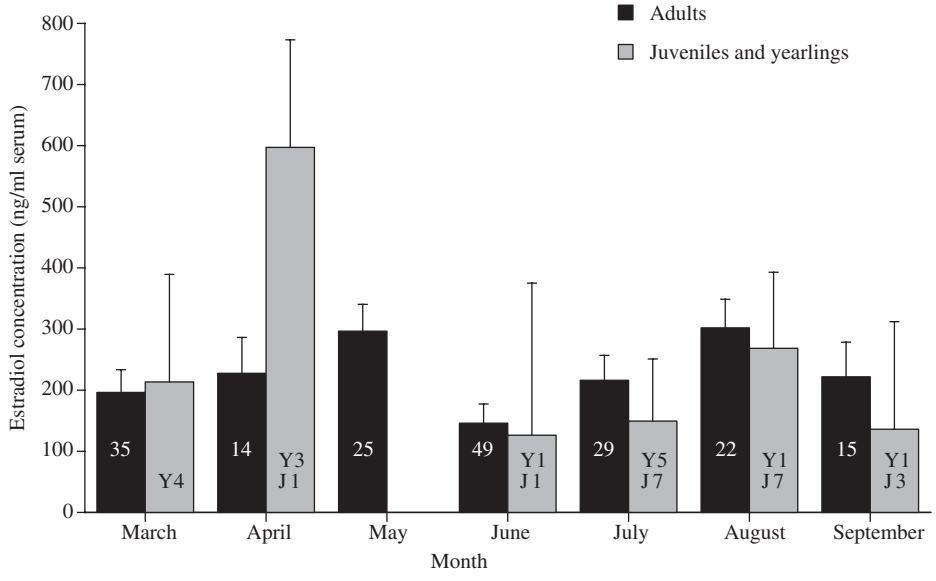

Figure 2 Monthly mean±SE serum concentrations of estradiol for adult and juvenile/yearling female nine-banded armadillos. Data were pooled across all sites and years of sampling. Sample sizes are provided within each bar. Abbreviations: J, juvenile female; Y, yearling female.

Table 4 Year-to-year variation in the mean±SE serum concentration (ng/ml) of progesterone for female nine-banded armadillos.

	2004	2005	2006
Progesterone (adult females)	96.12±7.87[a] (57)	73.02±8.40[a,b] (50)	70.19±7.21[b] (68)
Progesterone (juvenile and yearling females)	30.92±6.93[a] (11)	56.76±9.38[a] (6)	27.61±7.26[a] (10)

Data were pooled across all sites. Sample sizes are provided parenthetically. Shared superscripts indicate years that did not differ from one another (p>0.05) in Tukey-Kramer HSD pair-wise comparisons. See Table 3 for results of ANOVA comparisons.

Table 5 Mean±SE values (ng/ml) of circulating progesterone and cortisol in wild nine-banded armadillos that that were live caught *versus* those that were shot.

	Live-caught	Shot
Progesterone (juvenile and yearling females)	42.11±4.42 (29)	13.71±10.65 (5)
Cortisol (all males)	726.44±28.34 (228)	238.23±118.69 (13)
Cortisol (all females)	1004.63±44.81 (210)	230.21±138.43 (22)

Sample sizes are provided parenthetically. See Table 3 for results of ANOVA comparisons.

p<0.0001), but not with concentrations of estradiol (r=0.08, $F_{1,187}$=1.17, p=0.28).

For yearling and juvenile females, progesterone concentrations did not differ between sites, across months, or between seropositive and seronegative individuals (Table 3). There was marginally significant year-to-year variation at the Pinebloom West and Tall Timbers sites (Table 3), but there were no significant pair-wise comparisons among years using Tukey-Kramer HSD tests (Table 4). Progesterone concentrations were higher in animals that were live-caught instead of shot (Table 5), and were positively correlated with concentrations of estradiol (r=0.44, $F_{1,32}$=7.80, p=0.009), but not with body weight (r=0.09, $F_{1,32}$=0.27, p=0.61).

Cortisol

Cortisol concentrations differed dramatically between animals that were live-caught *versus* shot (Table 5). Consequently, we performed all remaining analyses separately for each group. There were no sex differences in cortisol concentrations of armadillos that were shot ($F_{1,33}$=0.02, p=0.89), but sample sizes were too small to allow analyses of any other sources of variation.

For live-caught animals, cortisol concentrations varied by sex ($F_{1,416}$=12.58, p=0.0004; see Figure 4 and Table 5) but not with age ($F_{2,416}$=2.34, p=0.16; there was also no significant age x sex interaction, $F_{2,416}$=0.23, p=0.79). Post-hoc Tukey-Kramer HSD tests showed that both yearling

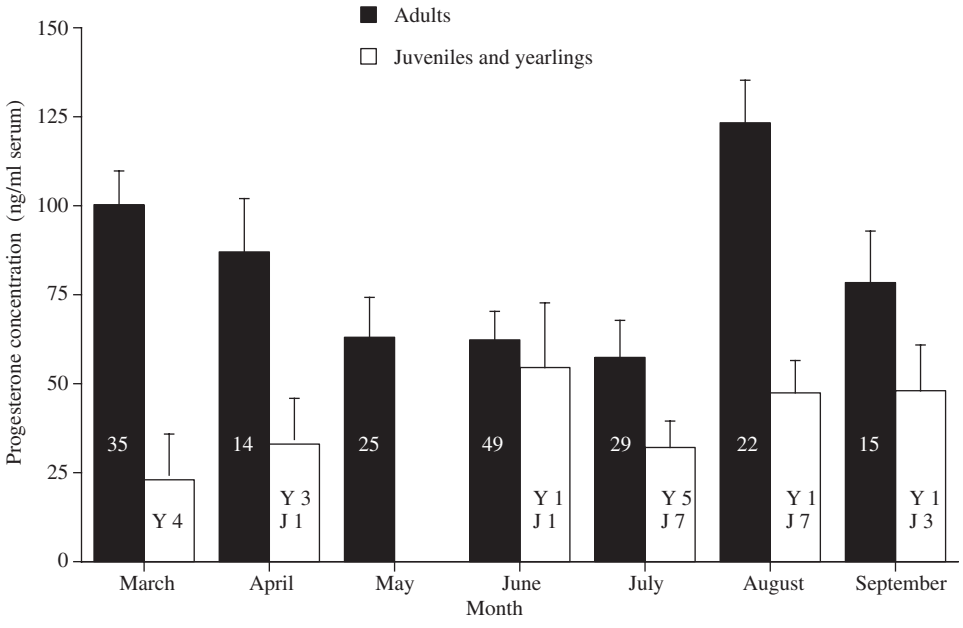

Figure 3 Monthly mean±SE serum concentrations of progesterone for adult and juvenile/yearling female nine-banded armadillos. Data were pooled across all sites and years of sampling. Sample sizes are provided within each bar. Abbreviations: J, juvenile female; Y, yearling female.

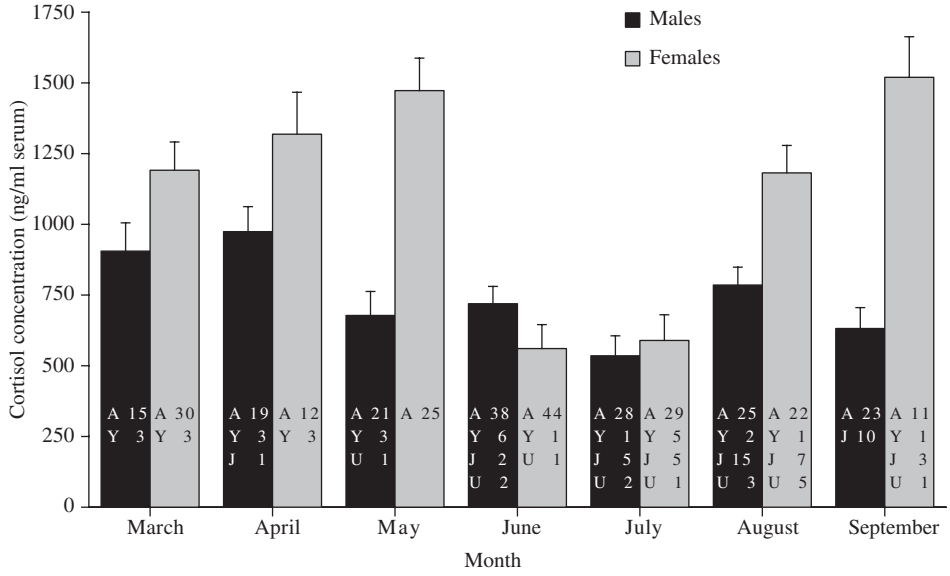

Figure 4 Monthly mean±SE serum concentrations of cortisol for male and female nine-banded armadillos. Data are from animals that were live-caught, and were pooled across age groups, all sites and years of sampling. Sample sizes are provided within each bar. Abbreviations: A, adult; Y, yearling; J, juvenile; U, age unknown.

and adult females had higher circulating levels of cortisol than did adult males (p=0.013 and 0.0004, respectively); levels in yearling females were also higher than in juvenile males (p=0.03; all other pair-wise comparisons were non-significant). Because of these findings, we further analyzed variation in cortisol concentrations separately for each sex, but because there were no age differences, males and females of unknown age were included where possible.

For live-caught males, there was significant variation across months (Figure 4), but no other comparisons were significant (Table 3). There were also no significant correlations between cortisol concentrations and body weight (r=-0.04, $F_{1,216}$=0.35, p=0.55), or with concentrations of testosterone in either adults (r=0.12, $F_{1,167}$=2.51, p=0.11) or juveniles and yearlings (r=0.24, $F_{1,49}$=3.08, p=0.09). Regarding month-to-month variation, concentrations of cortisol were significantly higher in March and April than in July (Tukey-Kramer HSD tests, p=0.04 and 0.003, respectively), and in April *versus* September (p=0.05; all other pair-wise comparisons between months were non-significant).

With regard to live-caught females, once again the only significant variation in cortisol concentrations was due to month (Table 3; see also Figure 4). Specifically, concentrations were lower in June and July than in other months (Tukey-Kramer HSD tests, all p<0.0008; levels in June and July did not differ from one another, nor did the other months differ among one another). There was no significant correlation between cortisol concentrations and body weight (r=-0.095, $F_{1,193}$=1.74, p=0.19), but there were positive correlations with estradiol (all females, r=0.44, $F_{1,208}$=50.65, p<0.0001) and progesterone (adult females, r=0.23, $F_{1,144}$=8.02, p=0.005; juvenile and yearling females, r=0.40, $F_{1,22}$=4.10, p=0.06).

Discussion

This study greatly expands our knowledge of hormone concentrations in wild nine-banded armadillos. We were able to identify several important sources of variation for each hormone, with most differences fitting predicted expectations. In what follows, we discuss our findings for each hormone in more detail.

Testosterone

The only significant variation found for testosterone was the predicted higher levels in adult males compared to younger males. This result provides biological validation of our assay, and is consistent with studies of captive males that found testosterone secretion did not reach adult levels until about 300 days of age, at which time sperm production began (Peppler 2008, see also McCusker 1977). Our data also support findings from captive animals that there is little seasonal variation in testosterone secretion (Peppler and Stone 1981). However, we did not sample year round so we cannot definitively rule out the possibility of

seasonal fluctuations. Other species of armadillos exhibit seasonal patterns of spermatogenesis (Superina and Jahn 2009, Luaces et al. 2012), and some field data from nine-banded armadillos suggest that sperm production (and, presumably, associated testosterone secretion) is reduced or even shut down during cold winter months (Czekala et al. 1980, Gause 1980, Torres et al. 1983, Schmidt 1990; see also Cardoso et al. 1985 for related data on seasonal changes in secretions from the accessory glands of males). Samples from the four months we did not collect (November to February) will be needed to clarify whether any seasonal changes in testosterone secretion occur in the wild.

One other issue regarding testosterone concerns the substantial differences in mean concentrations found in captive *versus* wild individuals. Peppler and Stone (1981) reported values of reproductively mature males in captivity that ranged from 9 to 14 ng/ml, whereas Czekala et al. (1980) reported values for wild individuals that were well over 100 ng/ml (see also Schmidt 1990). The assay we used for testosterone (and other gonadal hormones) differed from those employed in earlier field and lab studies. Thus, there is a strong possibility that the differences between our results and those of previous studies with captive animals are due to technical differences. Nonetheless, our data are consistent with findings from other field studies, which suggest there may be a fundamental difference between values obtained from wild-caught *versus* captive animals. One possible explanation, proposed by Schmidt (1990), is that captive animals were maintained on a controlled, winter daylight schedule year-round, whereas wild-caught animals were exposed to a natural daylight cycle. Testing this hypothesis will require sampling captive animals kept such that they are exposed to natural daylight conditions.

Estradiol

There were few differences in circulating concentrations of estradiol. Providing biological validation of our assay, we found evidence of seasonal variation, as predicted from results with captive animals (Peppler et al. 1986, Peppler 2008). However, there were no instances of specific pair-wise comparisons between months that were significant. On the basis of the captive data, we expected July and August to exhibit the highest estradiol concentrations because that is when most females ovulate (Peppler 2008). In contrast, our data indicated a trend towards higher concentrations in April, May, and August (for adult females only, see Figure 2). Collectively, these data point toward an earlier onset of reproductive activity by at least some females in the wild, although the underlying reasons for this phenomenon remain unknown.

Progesterone

Just as with testosterone, the higher concentrations of progesterone found in adult females compared to younger females matched predicted expectations and provide biological validation of the assay. This age difference was undoubtedly linked with reaching reproductive maturity. This interpretation is supported by data from captive animals that showed females did not secrete adult levels of progesterone until at least 15 months of age (Peppler et al. 1986). However, the actual values we obtained were much higher than those reported by Peppler (2008) for captive armadillos, and Truman et al. (1991) for wild females (in both studies values ranged from ~0 to 23 ng/ml over the course of a year). One other study of wild females (Nakakura et al. 1982) reported values similar to ours. Even so, comparing values between our study and any of these others is problematic because each used a different assay protocol. The fact that studies of wild and captive armadillos found levels of progesterone much lower than we did would imply the difference is not solely due to artificial conditions in the laboratory. Instead, it seems the most likely explanation for the difference between our study and those that reported much lower values probably has to do with assay protocols, e.g., perhaps the much lower extraction efficiencies of the earlier analyses, as well as the higher sensitivity of our assay.

Some of the patterns in progesterone concentrations that we found were consistent with expectations, but others remain more difficult to explain. For example, among adult females the concentration of progesterone was expected to exhibit month-to-month variation (Peppler 2008), with lower levels during the period (June and July) when weaning and breeding occur, and to be positively correlated with body weight (Truman et al. 1991). To the extent that progesterone can be secreted by the adrenal glands and, thus, be associated with corticosteroid synthesis (Rideout et al. 1985, Superina et al. 2009), one also might expect the higher levels of progesterone found in yearling and juvenile females that were live caught as opposed to shot, and, for all live-caught females, the strong positive correlation with concentrations of cortisol. However, we can offer no convincing explanation for why progesterone concentrations were higher for all females in 2004 (see Table 4), nor why there was a positive association between circulating levels of progesterone

and estradiol for juvenile and yearling females, although the latter may be an artifact of small sample sizes.

Cortisol

As expected, we found that all armadillos were stressed by live capture, which provides biological validation of the cortisol assay. It seems obvious that initial capture is an acute stressor that triggers a high adrenocortical response. Yet, analyses of cortisol concentrations in the feces of captive three-banded armadillos (Howell-Stephens et al. 2012) suggest that long-term captive housing may be stressful as well. If so, this may have important ramifications regarding welfare concerns for armadillos housed in captivity (Howell-Stephens 2012), and may be one reason reproductive failure is so common in captive armadillos (Rideout et al. 1985, Loughry and McDonough 2013). Suggestions have been made about ways to ameliorate such stresses (Superina et al. 2008), but we are unaware of any tests of their efficacy.

We have mentioned previously the difficulties inherent in interpreting the results for cortisol from live-caught animals. This problem is highlighted by the fact that we found significant sex differences in cortisol concentrations among animals that were live-caught, but not among those that were shot immediately. These findings suggest that baseline levels of cortisol did not differ between males and females, but females responded more strongly to the stresses of capture.

Bearing in mind the important caveats about cortisol values obtained from live-caught animals, if we assume that significant differences represent real underlying biological patterns as opposed to differential responses to capture, certain inferences can be made. For example, the higher concentrations found in females during March to May may reflect the need to secrete cortisol to support lactation (Tucker 1988, Neville et al. 2002). Likewise, the much lower concentrations of cortisol in females during June and July are probably attributable to the termination of lactation by adult females (see also Superina et al. 2009). However, this supposition does not explain why values for males were also the lowest at this time. The reason cortisol in males was so low during the breeding season, as well as why the concentration of cortisol increased again for all animals beginning in August, is not clear.

We found a strong positive correlation between circulating levels of estradiol and cortisol in live-caught females. This finding may reflect the fact that estradiol can stimulate the hypothalamic-pituitary-adrenal axis (Kime et al. 1980). The cortisol values we found tended to be the highest (albeit not significantly) in May, which was similar to estradiol concentrations (see Figures 2 and 4). This result potentially may be related to estrus and the onset of the breeding season. Estrus can cause stress as observed in the cow (Lyimo et al. 2000). In fact, acute stressors may stimulate reproduction (reviewed in von Borell et al. 2007).

Conclusions

An important weakness of this study stems from the fact that it was a retrospective analysis, exploiting samples collected for other purposes. Consequently, much ancillary information about the animals sampled was lacking (e.g., reproductive condition of adults), which limited our ability to fully determine the functional significance of our findings. Nonetheless, our data generally corroborated previous work in that patterns of hormone secretion were similar to what was reported from earlier studies of captive armadillos, whereas the actual values we obtained were more consistent with those obtained in previous field studies.

Additionally, we have validated the use of our hormone assays for *Dasypus novemcinctus*. Our study is among the first to report field data on circulating levels of cortisol and estradiol in nine-banded armadillos, and to examine a large number of potential sources of variation in hormone secretion. Ideally, this study will set the stage for future field investigations of eco-physiology in *D. novemcinctus*, as well as spur similar analyses in related species.

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