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Research paper

Pre-breeding androgen and glucocorticoid profiles in the eastern hellbender salamander (*Cryptobranchus alleganiensis alleganiensis*)

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ABSTRACT

Seasonally breeding species exhibit cyclical changes in circulating steroid hormone profiles that correspond with changes to their reproductive behavior and ecology. Such information is critical to the conservation of imperiled and data-deficient species, such as the eastern hellbender salamander (*Cryptobranchus alleganiensis alleganiensis*). We determined changes in plasma testosterone (T), dihydrotestosterone (DHT), 11-ketotestosterone (11-KT), 11-ketoandrostenedione (11-KA), dehydroepiandrosterone (DHEA), cortisol, corticosterone, and progesterone (P_4) during a four-month period preceding breeding in adult male and female eastern hellbenders. This pre-breeding period is characterized by increased diel movement and aggression by both sexes, follicular development and yolk production in females, and sperm production, territoriality, and nest site establishment in males. In both males and females, we observed a progressive increase in circulating T and DHT during the pre-reproductive season, both peaking in August (17 days before breeding), but concentrations of both hormones were higher in males. Conversely, 11-KT was higher in females, but did not vary significantly by date. These results suggest that T and DHT are the predominant androgens in eastern hellbenders and are likely important regulators of reproductive processes in both males and females. The detection of significant quantities of DHT and 11-KT in females is particularly interesting, considering that unlike T, neither of these androgens can be converted to estrogens. Therefore, it seems possible that aggression or some aspect of reproduction in the female eastern hellbender may be directly mediated by androgen signaling. Baseline cortisol did not vary throughout the pre-breeding period but was higher in females than males, and also became highly variable in females leading up to breeding. Progesterone, 11-KA, DHEA, and corticosterone were rarely or never detected, and thus, do not appear to be important during the pre-reproductive season. This study provides a physiological framework for future studies of hellbender reproductive biology, which could ultimately be important for their conservation.

1. Introduction

Characterizing and monitoring endocrine physiology in imperiled wildlife can provide information that is useful for effective conservation and management, such as identifying and mitigating causes of population decline or supporting successful reproduction in both wild and captive populations. Because anthropogenic disturbances (e.g., pollution, climate change, and habitat destruction) can impact wildlife populations through endocrine mechanisms (i.e., anthropogenic factors affect endocrine physiology that impairs individual fitness and ultimately cause shifts at the population level), hormone measurements can help investigators identify causes/mechanisms of declines and predict population responses to perturbation (reviewed: McCormick and Romero, 2017). For example, by measuring sex hormones in American alligators, Guillette et al. (1994) determined that reduced alligator recruitment in a polluted lake resulted from reproductive failure induced by endocrine disrupting chemicals. Furthermore, to overcome impediments to natural breeding and/or promote genetic diversity,

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captive wildlife breeding and reintroduction programs often rely on assisted reproduction technologies, the success of which fundamentally require a thorough understanding of the species' basic reproductive physiology and endocrinology (reviewed: Herrick 2019, Madliger et al., 2021). For these reasons, androgens and glucocorticoids (GCs), two classes of vertebrate steroid hormones, are commonly measured in wildlife. Androgens, such as testosterone (T), dihydrotestosterone (DHT), and 11-ketotestosterone (11-KT), are generally regarded as "male sex hormones" due to their role in regulating male reproductive processes, though androgens are also critical for female reproductive physiology (reviewed: Norris and Carr, 2013). Glucocorticoids, such as cortisol and corticosterone, stimulate catabolic processes to mobilize energy to support energetically demanding processes, like reproduction and responses to adverse stimuli (reviewed: Norris and Carr, 2013; Romero, 2002). In this study, we sought to assess androgen and GC physiology in a species of critical conservation concern, the eastern hellbender salamander (*Cryptobranchus alleganiensis alleganiensis*).

The eastern hellbender is a long-lived (25 + years), fully aquatic giant salamander (up to 74 cm total length) native to freshwater rivers and streams of the central and eastern United States (Nickerson and Mays, 1973; Taber et al., 1975). It is one of two subspecies of *C. alleganiensis*. Both subspecies have exhibited population declines throughout their ranges over the past few decades (Freake and DePerno, 2017; Jachowski and Hopkins, 2018; Wheeler et al., 2003). The Ozark subspecies (*C. alleganiensis bishopi*) and the evolutionarily distinct lineage of the eastern hellbender in Missouri are currently protected as endangered under the Endangered Species Act (USFWS, 2011a, USFWS, 2011b, USFWS, 2021). The causes of hellbender declines are poorly understood, but reproductive impairment is considered a possible contributing mechanism. Some populations of eastern hellbenders have exhibited a demographic shift towards a geriatric population age structure, suggesting a lack of reproduction and/or recruitment (Burgmeier et al., 2011; Jachowski and Hopkins, 2018; Wheeler et al., 2003). However, almost nothing is known about the reproductive physiology of wild hellbenders, which limits our ability to assess potential reproductive mechanisms underlying population declines and shifts in demographic structure. Thus, understanding hellbender reproductive physiology is a necessary step towards developing effective hellbender conservation and recovery strategies.

The purpose of this study was to characterize fundamental aspects of steroid hormone physiology in a relatively healthy population of eastern hellbenders. Specifically, we aimed to: (1) identify the predominant androgens in male and female hellbenders, (2) characterize seasonal shifts in androgen and GC profiles during the pre-breeding period in adult males and females, and (3) develop a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method that would enable us to measure a suite of steroid hormones with an efficiency that cannot be achieved through traditional immunoassay methods. We hypothesized that plasma steroid dynamics in eastern hellbenders during the pre-breeding period would be comparable to that of other salamanders, with distinct differences between the sexes. Specifically, based on patterns observed in other amphibians, we predicted that T and/or DHT would be the predominant androgens, and that the secretion of androgens in male and female hellbenders would increase during the months preceding breeding to facilitate reproductive readiness (Bliley and Woodley, 2012; Bolaffi and Callard, 1979; Bolaffi et al., 1979; Cooperman et al., 2004; Garnier, 1985a, b; Hasumi et al., 1993; Houck et al., 1996; Moore and Jessop, 2003; Norris et al., 1985; Rivarola et al., 1968; Romero, 2002; Tanaka and Takikawa, 1983; Woodley and Lacy, 2010). However, we predicted that androgens would be higher in males than females. Likewise, we also expected that GCs would increase in a similar manner during the pre-breeding season in both male and female eastern hellbenders in preparatory anticipation of reproductive energy demands, as is observed in many vertebrates (Romero, 2002), including amphibian and reptilian taxa (Moore and Jessop, 2003). We also predicted one notable departure from the general patterns observed in other salamanders; based on the recent work by Hopkins et al., (2020), we predicted that the predominant GC in eastern hellbenders would be cortisol, rather than corticosterone which is more typically employed in amphibians (reviewed: Norris and Carr, 2013).

2. Materials and methods

2.1. Study species and sampling methods

Hellbenders are seasonal breeders and cavity nesters that perform solitary paternal care of eggs and larvae for at least 7–8 months (Hopkins, per obs). In our study system in southwestern Virginia, mating occurs during the late summer (early September). In early July, sexually mature males begin to exhibit cloacal swelling, which we suspect coincides with the onset of gonadal activity (i.e., spermatogenesis and steroidogenesis). Beginning in mid-August, milt (sperm and associated fluids) production is evident because it is sometimes emitted upon capture. During this time, both sexes exhibit increased diel movements and aggression. Adult males seem particularly aggressive and territorial as they begin to select nest sites during this time, sometimes escalating to direct combat and significant injuries (Hopkins, pers. obs). In female hellbenders sampled from the Niangua River in Missouri, USA, yolk was evident in ova throughout the year, but there is an apparent peak in yolk production and deposition between May and September, with spawning occurring between mid-September and early October (Ingersol et al., 1991). Therefore, we suspect that in our system vitellogenesis – an estrogen-dependent process in other vertebrates – begins in late spring/early summer and continues until oviposition. After mating/oviposition, females leave the nest cavity and males continue to guard their nests and care for offspring until larvae emerge from the nest the following spring (late April). Androgens and/or GCs likely regulate many of the physiological and behavioral shifts that occur during the pre-breeding, breeding, and parental care periods, but seasonal steroid hormone profiles have not been described in hellbenders.

In this study, we sampled a relatively stable and high-density population of eastern hellbenders inhabiting a stream in the Tennessee River basin of southwestern Virginia. This population is the subject of ongoing long-term study by our research group, where reproduction has been observed on an annual basis since monitoring began in 2007. The section of river used in this study encompasses 2.26 fluvial river kilometers, characterized by relatively high surrounding forest cover (average percent of riparian area in the upstream watershed characterized by forest = 65.6%). In this study system, we utilize artificial underwater shelters to augment habitat and facilitate detection and sampling of hellbenders (Jachowski et al., 2020; Button et al. 2020a; Button et al., 2020b). Artificial shelters have recently been adopted across the species' range, and are designed to emulate the natural cavities and crevices found under large boulders in the streambed that hellbenders use for shelter and nesting (Briggler and Ackerson, 2012). Thus, we located and sampled individuals both by manually checking artificial shelters and searching natural cavities. Due to the imperiled status of the eastern hellbender, we are prohibited from providing the specific geographic coordinates of our study population.

Most hellbender breeding and nest initiation in southwestern Virginia occurs within an approximately 14-day period between late August and mid-September. During the year of this study, we first detected oviposition in our study stream on September 3rd 2014 in our artificial shelters. We sampled adult male and female hellbenders (i.e., all exceeded 340 mm in total length; minimum size at male maturity in our system = 290 mm; Hopkins and DuRant, 2011) in May (118–119 days before oviposition), June (86–88 days preceding oviposition), July (47–60 days preceding oviposition), early August (17 days preceding oviposition). Although our focus in this study was on the pre-breeding period, we also analyzed plasma samples from individuals captured in December (90–91 days post oviposition), and February (155–158 days post oviposition) as seasonal outgroups for comparison (Table 1).

Table 1
Sampling distribution of adult hellbenders by sex and season.

Season	Male (n =)	Female (n =)
May	6	4
June	5	6
July	10	9
August	7	6
Winter (Dec and Feb)	4	6

Immediately upon capture of each individual, we collected a baseline blood sample from the caudal vein via heparinized needles and syringes. We obtained 89% of blood samples within 3 min of initiating handling (mean = 2.00 min, median = 1.87 min, range 1.02 to 3.93 min) to minimize the effect of capture-induced secretion of GCs (Romero et al., 2005). Samples were collected within an 8 hr period (mean = 12:35 hr ± 110 min [std dev]), median = 12:23 hr, range = 8:29–16:11 hr), with 68% of samples collected within one standard deviation of the mean (10:45 hr to 14:25 hr). We stored whole blood samples on ice until they were returned to the laboratory < 8 h later. We separated plasma from whole blood by centrifugation at 6000 rcf for 5 min. We then aliquoted plasma into 1.5 mL microcentrifuge tubes and stored them at −80 °C until analysis. All samples were from unique individuals, as determined by implanted tags (see below).

After blood collection, we followed standardized procedures for assessing health, sex, and morphometrics. We measured the mass and total length of each individual, which were used to calculate scaled mass index (SMI) as a measure of body condition (Peig and Green, 2009). We also visually quantified leech infection prevalence and assessed trypanosome infection by analyzing buffy coat smears under a microscope using methods described previously (Hopkins et al., 2016). Sex was determined by visual examination of the cloaca during the breeding season, at which time sexually mature males in this system exhibit pronounced cloacal swelling (Hopkins and DuRant, 2011). Finally, we implanted a passive integrated transponder (PIT) tag (HPT12; Biomark Inc., Boise ID, USA) in the lateral tail musculature. These tags can be read with a portable handheld reader, allowing for unique identification and long-term study of individuals (Connock et al., 2019; Unger et al., 2012). We have PIT tagged hellbenders in this stream each year since 2007. Recapture of PIT tagged individuals enabled us to confirm sex of individuals that were captured outside of the breeding season, when sexes cannot be distinguished based on external morphology.

2.2. Calibration standards and steroid extraction

To extract steroids, we transferred 100 µL of plasma (thawed at room temperature), calibration standard mixtures (prepared by serial dilution into HPLC-grade methanol), or methanol (blank) to 1.5 mL microcentrifuge tubes containing 300 µL of methanol and 100 µL of 50 ng mL ^{1} deuterated progesterone (P_4-d_9) in methanol. All solvents used throughout these extraction methods were HPLC-grade (Thermo Fisher; Waltham, MA, USA). We vortexed these tubes and incubated them at 20 °C for 16 h. We then centrifuged these mixtures at 13,000 rcf for 2 min to precipitate the plasma proteins. We transferred the supernatant to a new microcentrifuge tube. We washed the protein pellets twice with 100 µL of methanol by vortexing and centrifugation, as before, and each time we removed the supernatant and added it to the original supernatant. We reduced supernatant volume to approximately 100 µL by centrifugal evaporation at 30 °C and completed drying using a high vacuum line. We reconstituted dried extracts in 200 µL of methanol and diluted them with 800 µL of sodium acetate buffer (0.01 M, pH 5), which we further processed via reverse phase solid phase extraction (SPE) using Waters (Milford, MA, USA) Oasis PRiME HLN 3 cc (60 mg) extraction cartridges. Briefly, we placed the SPE cartridges onto a Supelco Visiprep vacuum manifold and sequentially conditioned each with 1 mL each of methanol, water, and sodium bicarbonate buffer (0.01 M, pH 5). We loaded samples into the cartridges and brought them to dryness with a low vacuum. We washed the cartridges with 1 mL of water and 0.42 mL of 80:20 water:acetonitrile (v:v), applying a vacuum as necessary. We eluted steroids with 1 mL methanol into ethanol-rinsed glass culture tubes and dried these extracts as above. We reconstituted dried extracts in 100 µL of 50:50 water:methanol. We confirmed method accuracy by performing a spike recovery analysis (Supplemental Experiment and Figure S1).

2.3. Steroid quantification

We quantified eight plasma steroids by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Using a Shimadzu (Columbia, MD) LCMS-8060 interfaced with a Nexera LC-20 UPLC (Shimadzu), we measured T, DHT, 11-KT, 11-ketoandrostenedione (11-KA), corticosterone, progesterone (P_4), and dehydroepiandrosterone (DHEA). We measured cortisol using a Waters ACQUITY UPLC® H-Class liquid chromatography system linked to a Waters Xevo TQS. All steroids were separated using a Waters (Milford, MA, USA) ACQUITY UPLC® BEH C18 1.7 µm column (2.1 mm × 100 mm) and the gradients detailed in Tables S1 and S2. Injection volume was 10 µL. Each individual sample was injected in duplicate (cortisol was analyzed in triplicate), while standards and blanks were injected in triplicate. Column effluent was introduced into the mass spectrometer using an ESI probe in positive ion mode. We used selected multiple reaction monitoring (sMRM) to monitor two transitions per analyte (Tables S3 and S4).

We quantified steroids by interpolation on extracted standard curves. Briefly, peak areas for each analyte were divided by the area of the internal standard (P_4-d_9) for each sample, standard, and blank. We produced standard curves by linear regression (Table 2), and sample and blank area ratios were interpolated onto the standard curves to calculate the concentrations. We report the average concentration from each technical replicate.

We attempted to quantify estradiol, estrone, and aldosterone via LC-MS/MS, but we were unable to do so due to poor ionization, which could not be overcome through dansyl chloride derivatization or use of negative ion mode. Poor ionization of estrogens is a well-known problem in the analysis of steroid hormones by mass spectrometry (Blair, 2010). Future studies could potentially overcome this issue through use of alternative ionization/derivatization methods or instrumentation (Blair 2010; Wooding et al., 2015).

2.4. Statistical analyses

Because we did not detect all steroids in all samples, we employed the *Nondetects and Data Analysis for Environmental Data (NADA)* package (Lee, 2017) in R version 3.5.1 using RStudio (R Core Team, 2018; RStudio Team, 2016) to analyze our data with censored statistical methods, as described by Helsel (2011). The concentration of the lowest standard in each standard curve was utilized as the reporting limit (RL) for each hormone in all statistical analyses. Values below the RL were censored to the RL (i.e., assigned the value of the RL) for statistical testing and graphical representation; detection frequencies (i.e., number of samples where value exceeded RL) are reported in Table 2. We used backwards stepwise maximum likelihood estimation (MLE) lognormal regression (*cenreg* function in the *NADA* package) to examine the effects of sampling day (relative to oviposition), sex, time of day at sample collection, and the two-way interactions between these terms on hormone concentrations (Helsel, 2011). We included all potential explanatory variables and two-way interactions in the initial models. Time of day at sample collection was included to account for possible diurnal shifts in hormone secretion, but it should be noted that sample collection time was distributed similarly among factors of interest in our models sex ($p = 0.769$), month ($p = 0.150$), or their interaction ($p = 0.307$), per two-way analysis of variance (ANOVA). SMI was included as a potential explanatory variable for GCs because of the known relationship between

Table 2
Standard curve parameters and detection frequency for eight steroid hormones measured in hellbender plasma.

Hormone	Standard Range (ng mL ^{1})		R^2	Detection Freq. (%)		
	Min. (reporting limit)	Max.		Male	Female	Overall
Testosterone (T)	0.200	200	0.999	91	87	89
Dihydrotestosterone (DHT)	0.800	100	0.995	88	58	73
Dehydroepiandrosterone (DHEA)	2.00	200	0.999	0	0	0
11-ketotestosterone (11-KT)	0.500	100	0.998	47	58	52
11-ketoandrostenedione (11-KA)	0.500	100	0.999	0	0	0
Progesterone (P_4)	0.200	200	0.999	0	3.2	1.6
Cortisol	0.800	80.0	0.996	13	39	25
Corticosterone	0.800	100	0.997	0	0	0

body condition and these energy-mobilizing hormones (Romero, 2002). We eliminated variables based on p-value, with interaction terms eliminated first, until all remaining explanatory variables were significant ($p < 0.05$). Ectoparasites (leeches) may effect GC secretion (DuRant et al., 2015), but we lacked sufficient sample sizes of infected and uninfected individuals within each season to assess effects of parasites on cortisol, given that season and infection were partially confounded, particularly in males (Table S5). However, it has been shown that these ectoparasites specifically affect stress-induced GC secretion, not baseline concentrations (DuRant et al., 2015) which were the focus of this study, so ectoparasite infection should have minimal effect on our results.

3. Results

We detected five steroid hormones in hellbender plasma, including T, DHT, 11-KT, cortisol, and P_4 (Table 2). DHEA, 11-KA, and corticosterone were not detected in any samples (Table 2). P_4 was excluded from further analysis because it was only detected in one sample (Table 2).

We predicted that androgen levels would increase in both sexes prior to breeding, though males should have higher overall androgen levels compared to females. We found that both sex and sampling day had a significant effect on T and DHT, consistent with our predictions, with a significant interaction between sex and sampling day (Table S6, Fig. 1).

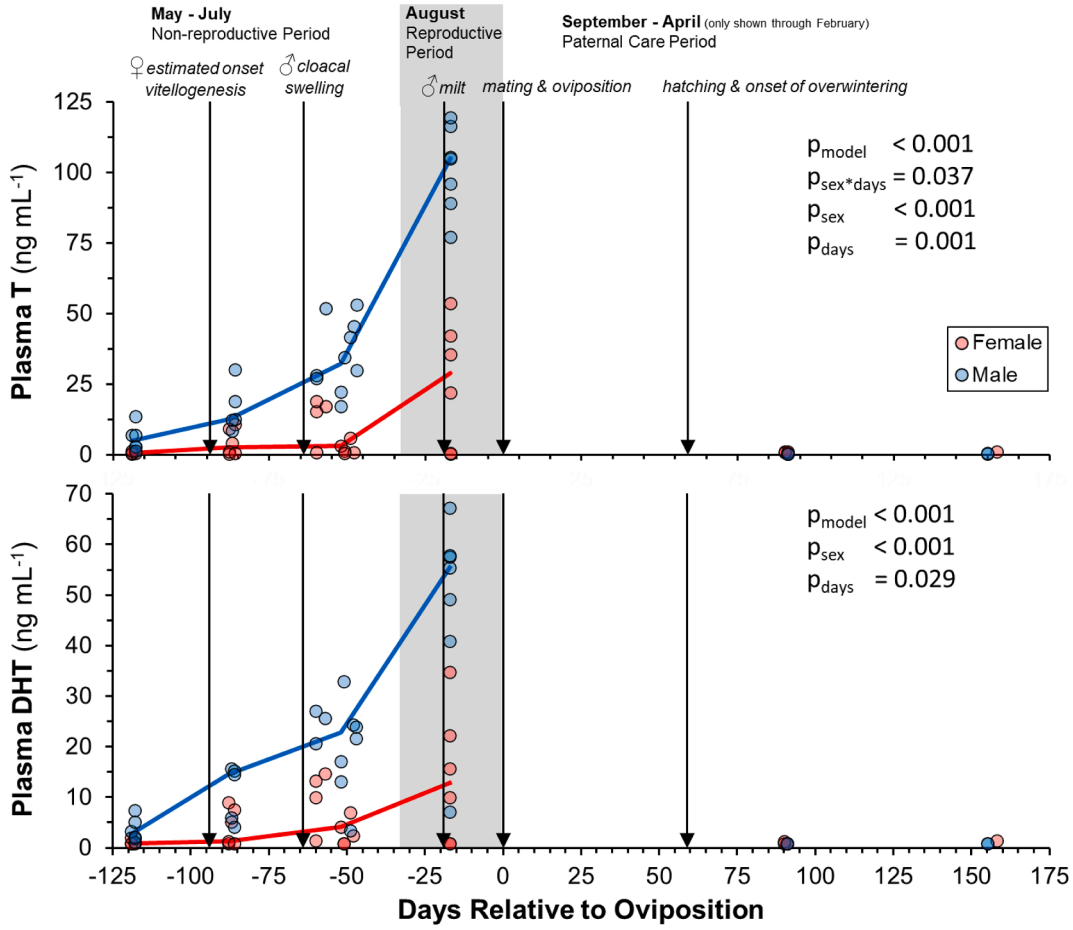

Fig. 1. Seasonal variation in circulating testosterone (T) and dihydrotestosterone (DHT) concentrations in adult male and female eastern hellbenders, relative to oviposition (day 0). Cortisol and 11-KT are not plotted seasonally because there was no relationship with sampling date relative to oviposition (see Fig. 2 and Figure S3). Scales of y-axes vary for each steroid. Blue lines indicate seasonal shifts in median hormone concentration in males, and red lines similarly indicate seasonal shifts in females. Differentially shaded regions along the x-axis represent categorical subdivisions of the year, and vertical black arrows denote key reproductive life events that occur throughout the annual cycle. Values below the reporting limits (RL) are censored to the RL for visualization purposes; RL for T = 0.200 ng mL ^{1}, RL for DHT = 0.800 ng mL ^{1}. See Table 1 for sample sizes and Table S6 for model outputs.

In both sexes, T and DHT concentrations exhibited a progressive increase throughout the pre-breeding period, peaking in August when milt production is evident in males, though the rate of increase was higher in males (Fig. 1). Concentrations of T and DHT were approximately two times higher in males compared to females (Fig. 1; Table S6; T: β_{sex} = -2.93; DHT: β_{sex} = -1.62). On average, T was higher than DHT, and the ratio between these two hormones was two times higher in males than females (when excluding individuals in which one or both hormones were not detected, male T:DHT ratio = 2.38 ± 0.49 [standard error], female T:DHT ratio = 1.20 ± 0.20, both sexes T:DHT ratio = 1.92 ± 0.32; or when including all individuals and censoring non-detects to RL, male T:DHT ratio = 2.13 ± 0.45, female T:DHT ratio = 1.00 ± 0.13, both sexes T:DHT = 1.59 ± 0.24). The main effect of time was significant for T (Table S6), suggesting a negative relationship between circulating T and time of day at which sampling occurred, although visual inspection of the data reveals no strong relationship (Figure S2). Sampling day did not have a significant effect on 11-KT, but this androgen was approximately 60% higher in females compared to males (Fig. 2; β_{sex} = 0.606). T and DHT concentrations overall were an order of magnitude higher than 11-KT (when excluding individuals in which one or both hormones were not detected T:11-KT ratio = 27.7 ± 6.4 and DHT:11-KT ratio = 15.7 ± 3.38; or when including all individuals and censoring non-detects to RL T:11-KT ratio = 27.7 ± 6.2 and DHT:11-KT ratio = 15.4 ± 3.08). The time of day at which sampling occurred was not significantly related to DHT or 11-KT (Table S6).

We predicted that baseline cortisol levels would increase during pre-breeding and that cortisol would be negatively related to SMI. However, neither sampling day nor SMI, nor their interaction, were significant predictors of baseline cortisol (Figure S3). We found that baseline cortisol was roughly 70% higher in females than males overall (Table S6, Fig. 2; B_{sex} = 0.691). Corticosterone was below our detection limit (<0.800 ng/ml) in all samples (Table 2), which was expected based on our prior work reporting very low circulating concentrations of corticosterone in eastern hellbenders (DuRant et al., 2015; Hopkins and DuRant, 2011; Hopkins et al., 2020). The time of day at which sampling occurred was not a significant predictor of cortisol (Table S6).

4. Discussion

This is the first study to characterize several fundamental aspects of androgen and GC physiology in eastern hellbenders, with particular focus on hormone dynamics during the pre-breeding period (May through mid-August). We demonstrated that T and DHT are the predominant androgens in eastern hellbenders, with 11-KT occurring at much lower levels. Generally, androgens appeared at similar concentrations, with comparable seasonal patterns, in hellbenders as in other salamanders, especially mudpuppies (*Necturus maculosus*), another fully aquatic species (Table 3) (Bolaffi et al., 1979). We also corroborated recent work that demonstrated that cortisol is the predominant GC in hellbenders (Hopkins et al., 2020), but contrary to our predictions we did not observe seasonality in either cortisol or corticosterone concentrations. Our results provide a foundation for understanding the basic reproductive physiology of this imperiled amphibian species.

Seasonal shifts in androgens have been observed in other male salamanders (Japanese black salamander (Hasumi et al., 1993), spotted salamander (Cooperman et al., 2004), tiger salamander (Norris et al., 1985), marbled salamander (Houck et al., 1996), Iberian ribbed newt (Garnier, 1985b), and Japanese fire belly newt (Tanaka and Takikawa, 1983)). The magnitude of the shift in T concentration seen in male hellbenders (from < 0.200 ng mL ^{1} in December and February to 119 ng mL ^{1} in mid-August) was very similar to that observed in male Japanese black salamanders (approximately 0 ng mL ^{1} to 281 ng mL ^{1}, assuming a 2:1 ratio between DHT and T) (Hasumi et al., 1993). In most of the other species studied to date (excluding spotted and tiger [Ambystomatid] salamanders), peak circulating androgen concentrations coincided with expression of male physiological and behavioral reproductive traits. Consistent with these patterns, we report a progressive increase in androgen concentrations during pre-breeding in male eastern hellbenders that seasonally coincides with the expression of numerous physiological (e.g., cloacal swelling and milt production) and behavioral changes (e.g., increased diel movements, conspecific territoriality, and aggression) in the weeks prior to reproduction. Furthermore, circulating androgen concentrations were low during the non-breeding period (December and February), when expression of these physiological and behavioral traits has waned or ceased.

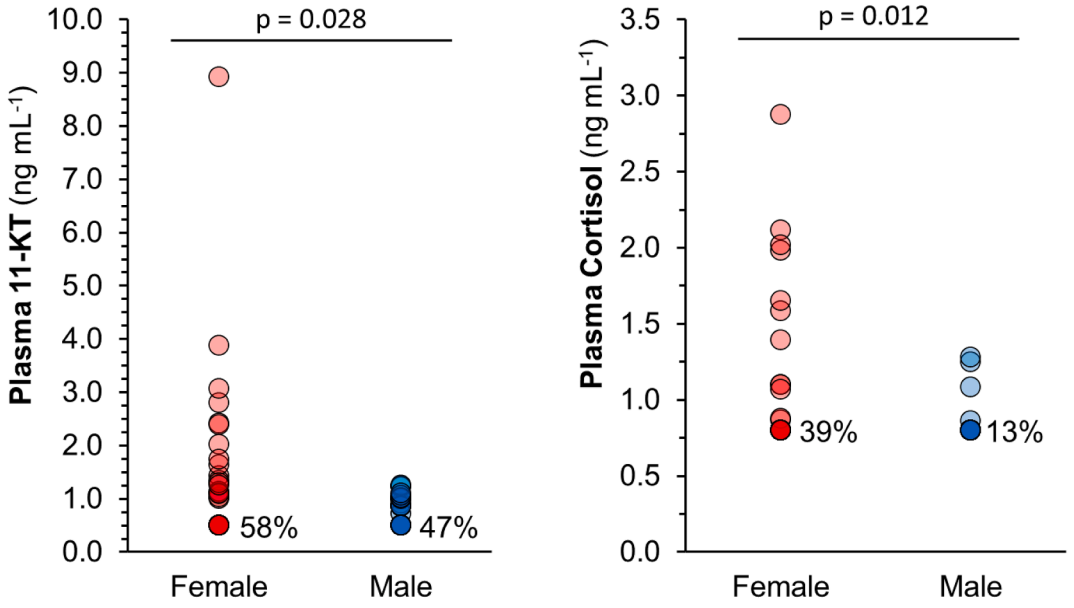

Fig. 2. Circulating 11-ketotestosterone (11-KT) and cortisol concentrations in male and female eastern hellbenders, regardless of sample day because there was no seasonal fluctuation detected in either hormone (see Figure S3). Percentages adjacent to points indicate detection frequency of each hormone within each sex. Values below the reporting limits (RL) are censored to the RL for visualization purposes; RL for 11-KT = 0.500 ng mL ^{1}, RL for cortisol = 0.800 ng mL ^{1}. See Table 1 for sample sizes and Table S6 for model outputs.

Table 3
Comparison of circulating androgen concentrations in males of select species of salamanders. When study design allowed, we report the average value during a season/period of maximal circulating androgen concentrations ("Seasonal Max. Average") and/or a global maximum (i.e., the maximum value measured in a single individual). All values are ng mL ^{1}.

Species	Testosterone		Dihydrotestosterone		Citation
	Min. [a]	Seasonal Max. Average (Global Max.)	Min. [a]	Seasonal Max. Average (Global Max.)	
Eastern hellbender	< 0.200	101 (119)	< 0.800	47.9 (67.2)	this study
Mudpuppy	4.00	– (202)	2.00	– (133)	Bolaffi and Callard (1979)
	–	– (129)	–	–	Bolaffi et al. (1979)
	47.0	– (261)	–	–	Rivarola et al. (1968)
Japanese black salamander	–	104 # (281 #)		208 # (562 #)	Hasumi et al. (1993)
Spotted salamander	2.00[b]	17.0 [d] (–)	–	–	Cooperman et al. (2004)
Iberian ribbed newt	3.40	19.0 (–)	0.500	1.97 (–)	Garnier (1985b)
Marbled salamander	2.90	50.0[b] (78.3)	1.70	16.0[b] (38.3)	Houck et al. (1996)
Tiger salamander	0.350	32.0 (–)	0.290	7.00 (–)	Norris et al. (1985)
Ocoee salamander	–	322 (–)	–	–	Bliley and Woodley (2012)
	–	350[b] (–)	–	–	Woodley and Lacy (2010)
Japanese fire belly newt	0.72	21.3 (–)	–	20.9 (–)	Tanaka and Takikawa (1983)
Plethodon jordani	25	– (626)	2	– (41)	Woodley (1994)
Desmognathus ochropheus	11	– (823)	0.3	– (182)	Woodley (1994)
Axolotl	0.167 #	– (2.4 #)	0.333 #	1.68 # (4.8 #)	Eisthen and Krause (2012)
	–	– (0.363)	–	–	Jacobs and Kuhn (1988)

a. Value is either the global minimum or the average value from the time point at which the yearly minimum occurred.
b. Value visually estimated from figure.
#. Only report "androgens", which is assumed to be a summation of T and DHT; thus, the values reported here are calculated assuming a 2:1 ratio between DHT and T.

Therefore, we conclude that eastern hellbenders are associated breeders (i.e., maximum sex steroid secretion and, presumably, gamete maturation, coincide with mating behavior; Crews 1984), reflecting the general pattern observed in most other salamanders. The three androgens identified at detectable plasma concentrations in this study could potentially function independently or interactively in eastern hellbender physiology, as has been seen in the rough-skinned newt (Moore, 1978; Deviche and Moore, 1988) and other amphibians (reviewed: Moore 1983). Experimental manipulations would be required to characterize such relationships among androgens in the eastern hellbender.

Our results suggest that androgens play a role in female reproductive physiology and behavior in the eastern hellbender. Testosterone and DHT concentrations were lower in females than males, and the rate of increase in T during pre-breeding was higher in males, but both T and DHT progressively increased during the pre-breeding season in females as well. Moreover, 11-KT was higher in females than males throughout the pre-breeding period. Increases in circulating androgen concentrations have been observed in association with female reproductive processes (e.g., ovarian growth and breeding) in the Iberian ribbed newt, bullfrog, and many fish species (Borg, 1994; Garnier, 1985a; Licht et al., 1983). Notably, peak T and DHT concentrations in female hellbenders were higher than those observed in other female salamanders but were somewhat comparable to female bullfrogs (Table 4). Less is known about 11-KT concentrations and function in salamanders, but 11-KT was not detected in female mudpuppies (Bolaffi et al., 1979). From these patterns, we suspect that androgens could play both direct and indirect roles in regulating female hellbender reproduction. For example, we have observed aggressive/defensive behavior in females during the breeding season, which could be a reflection of their elevated androgen levels.

Based on Ingersol et al. (1991), it is likely that vitellogenesis, oocyte maturation, and ovulation all peak during the pre-breeding period in female eastern hellbenders. Vitellogenesis is generally considered an estrogen-mediated process (Norris and Jones, 1987), but in some fish, DHT and 11-KT have been shown to regulate vitellogenesis and ovarian physiology (Kim et al., 2003; Le Menn et al., 1980; Lokman et al., 2007; Riley et al., 2004; Thomson-Laing et al., 2019; Shilling and Williams, 2000). This could potentially occur through androgen signaling (i.e., activation of the androgen receptor by T, DHT, and/or 11-KT) or estrogen signaling, as DHT has been shown to activate the estrogen receptor in *Gobius niger* (Le Menn et al., 1980). Further, in mammals, androgen signaling is critical for normal ovarian function (Shiina et al., 2006; Weil et al. 1998). Thus, it is possible that androgen secretion increases during pre-breeding in female hellbenders to directly support ovarian function/vitellogenesis. Further, some androgens, such as T, but not DHT or 11-KT, are precursors for estrogen production (e.g., testosterone is the direct precursor to 17β-estradiol, the predominant estrogen in many species), and peripheral tissues produce estrogens from circulating T in mammals (Barakat et al., 2016), meaning the high concentration of T in circulation could serve as a pool of precursor to support peripheral estrogen production in female hellbenders. Because we were unable to measure circulating estrogen concentrations by LC-MS/MS due to technical limitations associated with estrogen ionization (Blair, 2010), we are limited in our ability to interpret the physiological functions of androgens in females.

Glucocorticoids play critical roles in regulating the energetically demanding process of reproduction. Across vertebrate taxa, baseline concentrations of GCs tend to increase during the breeding season (Romero, 2002). Contrary to this, we found that baseline GC values did not significantly change during the months leading to the immediate pre-breeding period in either sex. However, because our sampling efforts ceased a few weeks prior to breeding, it remains unknown whether GCs increase at the peak of reproductive activity. Although seasonal changes in GCs were not detected, cortisol was significantly higher in females than in males. This sexual difference in baseline cortisol may suggest that females face different metabolic (e.g., yolking follicles, eating less, etc.), immunological, and/or behavioral demands during the pre-breeding season than males. Because we do not know if female hellbenders produce eggs every year, nor whether individuals in our study eventually laid eggs that season, it is unknown whether all of these females were actively experiencing the energetic demands associated with yolking follicles at the time of sampling. Ingersol et al. (1991) reported significant variation in ovarian/follicular characteristics among adult-sized female hellbenders. In fact, they classified three ostensibly adult individuals (total lengths 383 mm, 385 mm, and 415 mm) as sexually immature, despite their size, based on ovarian/follicular characteristics. Considering that it was not a longitudinal study, it is unclear whether those individuals were in fact sexually immature or simply did not breed that season. Given this variance in reproductive activity/status among female hellbenders in the adult-size range, it is interesting to note that we observed considerable variance in cortisol concentrations among adult females during the 90 days prior to breeding (Figure S3). Future studies with expanded sample sizes of females of known reproductive

Table 4
Comparison of circulating androgen concentrations in females of select species of amphibians. When study design allowed, we report the average value during a season/period of maximal circulating androgen concentrations ("Seasonal Max. Average") and/or a global maximum (i.e., the maximum value measured in a single individual). All values are ng mL ^{1}.

Species	Testosterone		Dihydrotestosterone		Citation
	Min. [a]	Seasonal Max. Average (Global Max.)	Min. [a]	Seasonal Max. Average (Global Max.)	
Eastern hellbender	< 0.200	– (53.5)	< 0.800	– (34.7)	this study
Mudpuppy	1.53	– (8.32)	2.99	– (11.4)	Bolaffi and Callard (1979)
	–	– (14.6)	–	–	Bolaffi et al. (1979)
Iberian ribbed newt	2.90	9.00 (–)	0.500	1.10 (–)	Garnier (1985a)
Bullfrog	–	140[b] (–)	–	–	Licht et al. (1983)

a. Value is either the global minimum or the average value from the time point at which the yearly minimum occurred.
b. Value visually estimated from figure.

status would allow us to better assess whether variance in cortisol is related to female reproductive status. Alternatively, Hopkins et al. (2020) recently proposed that cortisol could also be important for both osmo- and iono- regulation in eastern hellbenders, much like it is in fishes, raising the additional possibility of sexual dimorphisms in osmoregulatory demands during pre-breeding. Additional studies correlating GCs to measures of energetics, reproductive status, feeding ecology, immune function, and osmoregulation are necessary to test these hypotheses.

Our study significantly improves our understanding of the basic physiology of wild hellbenders by identifying four steroid hormones important for reproduction, energetics, and stress physiology, and thus may be useful for monitoring wild populations and for informing captive rearing programs. For example, we showed that eastern hellbenders are associated breeders, suggesting that the pre-breeding season is a period during which hellbenders may be particularly sensitive to environmental disturbances and endocrinological dysfunction (e.g., xenobiotic-induced endocrine disruption). Moreover, captive propagation of hellbenders has historically been challenging (Ettling et al., 2013). Recent success at zoos has been partly attributable to strategic trial-and-error modifications to husbandry conditions (e.g., water chemistry and temperature) that ultimately led to hellbenders breeding (Ettling et al., 2013). Our findings indicate that hormone concentrations in hellbenders could provide a tool for mechanistically linking husbandry conditions to reproductive readiness as well as identifying potential stressors associated with captivity. Likewise, hormone profiles will likely be valuable for informing reintroduction or translocation of hellbenders, just as they have been in diverse wildlife species (Madliger et al., 2021). Because our study was designed to assess hormone fluctuations leading up to breeding, future studies should similarly characterize hormone concentrations during breeding and the extended paternal care period of hellbenders. Such work will help further refine our understanding of the basic biology and potential periods sensitive to endocrinological disruption in this declining species.

Ethical considerations

This study and associated sampling methods were approved by the Virginia Tech Institutional Animal Care and Use Committee (IACUC).

CRediT authorship contribution statement

Thomas M. Galligan: Conceptualization, Formal analysis, Investigation, Methodology, Data curation, Project administration, Supervision, Validation, Visualization, Writing - original draft. **Richard F. Helm:** Conceptualization, Investigation, Methodology, Resources, Supervision, Validation, Writing - original draft, Funding acquisition. **Brian F. Case:** Conceptualization, Investigation, Methodology, Data curation, Validation, Visualization, Writing - original draft. **Catherine M. Bodinof Jachowski:** Data curation, Investigation, Writing - review & editing. **Clara L. Frazier:** Methodology, Validation, Writing - review & editing. **Valentina Alaasam:** Data curation, Investigation, Visualization, Writing - review & editing. **William A. Hopkins:** Conceptualization, Funding acquisition, Investigation, Methodology, Data curation, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft.

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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.2021.113899.

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