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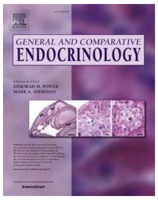

# Androgen and glucocorticoid profiles throughout extended uniparental paternal care in the eastern hellbender salamander (*Cryptobranchus a. alleganiensis*)

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A B S T R A C T

The behavioral endocrinology associated with reproduction and uniparental male care has been studied in teleosts, but little is known about hormonal correlates of uniparental male care in other ectotherms. To address this gap, we are the first to document the seasonal steroid endocrinology of uniparental male hellbender salamanders during the transition from pre-breeding to nest initiation, and through the subsequent eight months of paternal care. In doing so, we investigated the correlates of nest fate and clutch size, exploring hellbenders' alignment with several endocrinological patterns observed in uniparental male fish. Understanding the endocrinology of hellbender paternal care is also vital from a conservation perspective because high rates of nest failure were recently identified as a factor causing population declines in this imperiled species. We corroborated previous findings demonstrating testosterone and dihydrotestosterone (DHT) to be the primary androgens in hellbender reproduction, and that cortisol circulates as the most abundant glucocorticoid. However, we were unable to identify a prolactin or a "prolactin-like" peptide in circulation prior to or during parental care. We observed ~ 80 % declines in both primary androgens during the transition from pre-breeding to nest initiation, and again as paternal care progressed past its first month. In the days immediately following nest initiation, testosterone and DHT trended higher in successful individuals, but did not differ with males' clutch size. We did not observe meaningful seasonality in baseline glucocorticoids associated with breeding or nesting. In contrast, stress-induced glucocorticoids were highest at pre-breeding and through the first two months of care, before declining during the latter-most periods of care as larvae approach emergence from the nest. Neither baseline nor stress-induced glucocorticoids varied significantly with either nest fate or clutch size. Both stress-induced cortisol and corticosterone were positively correlated with total length, a proxy for age in adult hellbenders. This is consistent with age-related patterns in some vertebrates, but the first such pattern observed in a wild amphibian population. Generally, we found that nesting hellbenders adhere to some but not all of the endocrinological patterns observed in uniparental male teleosts prior to and during parental care.

## 1. Introduction

Obligate paternal care is common in vertebrates, especially within a biparental care structure (Royle et al., 2012). The case of solitary or uniparental male care occurs less often, though is frequently expressed in fish (Reynolds et al., 2002; Goldberg et al., 2020). In teleosts with

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uniparental male care, individuals allocate attention towards three temporally overlapping behavioral requirements during their seasonal reproductive cycles. This includes territoriality, breeding, and parental care (Gross and Sargent, 1985; Smith and Wootton, 1995; Reynolds et al., 2002; Goldberg et al., 2020). Individuals first experience a pre-breeding territorial acquisition period where males construct and compete for control of advantageous nesting sites (Rushbrook et al., 2010; Lehtonen et al., 2015). This is followed by a breeding period when males attract and often mate successively with more than one female (Coleman and Jones, 2011). In many cases, breeding occurs in the presence of smaller "sneaker" males, which threaten to compromise the attending male's paternity (Neff and Gross, 2001; Neff et al. 2003; Cogliati et al., 2014). Paternal care commences during a third stage (sometimes coinciding with secondary breeding), when males guard young from predators (Lissaker and Kvarnemo, 2006; Gravel and Cooke, 2013; Gravolin et al., 2021). During this time, they ensure proper oxygenation and embryonic development of eggs via tail fanning, agitation, and cleaning strategies (Knouft et al., 2003; Karino and Arai, 2006; Hopkins et al., 2011).

The endocrinology of uniparental male care is relatively well documented in teleosts. For example, changes in androgen profiles during nest establishment, reproduction, and subsequent paternal care are well studied (Kindler et al., 1989; Sikkel, 1993; Borg, 1994; Knapp et al., 1999; Pall et al., 2002; Sisneros et al., 2004). This includes a lack of support in fish for the Challenge Hypothesis (Wingfield et al., 1990)—a theoretical framework that describes the role of androgens in mediating behavioral tradeoffs between breeding, male-male competition, and parental care. Specifically, despite some evidence for androgens' role in teleost males' response to conspecific challenges (Dey et al., 2010; O'Connor et al. 2011a; Rodgers et al., 2012), an interference with parental care is almost never observed (reviewed in Moore et al., 2020), when examined across several uniparental species (Oliveira et al., 2001; Magee et al., 2006; Hanson et al., 2009; Neff and Knapp, 2009; Dey et al., 2010; O'Connor et al., 2012; Rodgers et al., 2012; but see Rodgers et al., 2013). Additionally, studies in these systems have explored the role of glucocorticoids in supporting the energetic demands of caring for developing eggs and offspring (Magee et al., 2006; O'Connor et al. 2011b). This includes the role of glucocorticoids in modulating tradeoffs between care and survival across individuals of differing nesting statuses (O'Connor et al. 2011b; Jeffrey et al., 2014), and in responding to unexpected challenges or life-threatening circumstances (O'Connor et al., 2009; Dey et al., 2010; Zolderdo et al., 2016).

Despite considerable knowledge of behavioral endocrinology of uniparental male care in fish, very little is known about the modulation of similar life histories in other ectotherms, where the expression of male-only care is less common than in fish (Reynolds et al., 2002). The hellbender giant salamander (*Cryptobranchus alleganiensis*) provides the opportunity to investigate the endocrinology of uniparental male care in an amphibian. Hellbenders are large (up to 74 cm in total length; 2.2 kg), long lived (25 + years), fully aquatic salamanders that inhabit rivers of the Appalachian and Ozark regions of the U.S. (Nickerson and Mays, 1972; Taber et al., 1975; Petranka, 1998). They are seasonal breeders with males providing solitary care to developing eggs and larvae for an extended eight-month period (Hopkins et al., 2023). Similar to uniparental teleosts' nesting biology, hellbender males exhibit territoriality for control of prospective nesting sites (Unger et al., 2020; Hopkins, pers obs), can obtain multiple clutches from different females during the relatively brief two-week nest initiation period (Crowhurst et al., 2009; Unger et al. 2015; O'Brien et al., 2024), and engage in tail fanning, egg agitation, and nest defense behaviors throughout parental care (Smith, 1907; Settle et al., 2018; Unger et al., 2020; O'Brien, 2023). However, unlike many teleosts examined to date, hellbenders lack sequential nesting opportunities over the course of a breeding season. Therefore, nest failure defers males' opportunity to re-nest until the following year.

The work reported herein had several related objectives. First, because the endocrinology of hellbender breeding and parental care remains unknown, we set out to describe the seasonal variation of eight plasma steroids in a healthy, actively reproducing hellbender population, during the transition from pre-breeding to nest initiation, and from nest initiation through the eight months of care. In doing so, we explored several lines of inquiry to ascertain whether hellbenders align with the endocrinological patterns observed in uniparental teleosts. Given that androgens do not typically interfere with parental care in male fish (reviewed in Moore et al., 2020), we sought to determine whether this observation would hold in an amphibian. We also investigated if hellbenders' androgens during the nest initiation period were inversely related to males' clutch sizes. We predicted this might be the case given the tendency for androgens to decline to non-breeding levels more quickly in teleost males with larger nests, and therefore a reduced need for these males to acquire additional clutches during that breeding cycle (Knapp et al., 1999; Pall et al., 2002). We also sought to explore whether glucocorticoids were related to nest success or clutch size. With regard to baseline concentrations, in avian species increased glucocorticoids can play a role in supporting the expected seasonal energetic demands of parental care (Bonier et al., 2009a; Bonier et al., 2011; Crossin et al., 2012; Ouyang et al., 2013), a phenomenon also observed in some (Magee et al., 2006), but not all uniparental male teleosts (O'Connor et al. 2011b). If seasonal elevations in baseline glucocorticoids help to mediate the seasonal energetic requirements of paternal care in hellbenders (e.g., nest cleaning, nest guarding, and egg fanning), we predicted to observe higher baseline glucocorticoids in successful males relative to those that fail, as well as in males attending to larger clutch sizes, which may be more behaviorally taxing to properly oxygenate (Hopkins et al., 2023; O'Brien, 2023). Separately, given that the glucocorticoid stress-response and entry into the "emergency life history stage" (Wingfield et al., 1998; Wingfield and Sapolsky, 2003) can disrupt parental care across many vertebrate taxa (Lendvai and Chastel, 2010; Miller et al., 2009; Ouyang et al., 2011; Ouyang et al., 2012), including fish with male care (O'Connor et al. 2011b; Jeffrey et al., 2014), we predicted successful nesting males and those with larger clutches (i.e., greater reproductive value) would attenuate their glucocorticoid stress response relative to males with smaller nests, or those who fail during care.

## 2. Methods

### 2.1. Study Species' seasonal behavior

Hellbender salamanders in southwest Virginia prepare for breeding in late July and early August, when adults exhibit numerous physiological and behavioral changes. Pre-breeding is characterized by cloacal swelling and milt discharge in males (Smith, 1907; Nickerson and Mays, 1972), yolking of follicles in females (Topping and Ingersol, 1981), as well as increased diel movement (Bishop, 1941; Nickerson and Mays, 1972), conspecific aggression, and territoriality (Smith, 1907; Alexander, 1927, Unger et al., 2020). Seasonal changes in behavior are especially evident in reproductive males because they compete with one another for control of prospective nesting sites (Unger et al., 2020; Hopkins, pers obs). In some cases, hostile interactions can rise to the level of direct physical combat (e.g., attacking behavior and prolonged "bite holds"; Unger et al., 2020), which can lead to lacerations and even the loss of limbs (Hopkins, pers obs). Following this pre-breeding period, mating commences (late August through early September in our system). During this time, males establish nesting sites beneath submerged boulders, mating with females by fertilizing eggs externally (Smith, 1907; Bishop, 1941; Nickerson and Mays, 1972; O'Brien et al., 2024). Following mating, nesting males remain to provide solitary care for eggs and subsequent larvae for at least eight months, until larval emergence in the following Spring (Hopkins et al., 2023).

The description of hellbender paternal care behavior is limited, but new evidence suggests that nesting males engage in numerous activities to ensure proper embryonic development. These behaviors include egg agitation and tail fanning to provide sufficient oxygenation for developing embryos (Settle et al., 2018; O'Brien, 2023). Nesting males also defend nesting cavities from conspecific and interspecific intruders (Smith, 1907; Settle et al., 2018; Unger et al., 2020; O'Brien, 2023). Clutch attendance and cavity defense requires consistent guarding from the nesting male, with minimal time spent away foraging or otherwise away from the nest (Settle et al., 2018; O'Brien, 2023). Our observations suggest that the first 60 days of parental care are the most crucial in determining eventual nest outcomes (Hopkins et al., 2023). This is the period when embryos develop (~first 6 wks post-oviposition; Smith, 1912), and when developing embryos hatch (~6–9 wks post-oviposition; Smith, 1912; Nickerson and Mays, 1972; Hopkins et al., 2023). In our system, 70 % of nest failures occur within the first 30 days of nest initiation. As a nest progresses past this early period without failure, probabilities of success greatly increase. In fact, males whose nests contain viable hatchlings after 60 days of care have an 83 % chance of yielding larvae emerging at the conclusion of the care period the following spring (based on 182 nests; Hopkins et al., 2023).

### 2.2. *Artificial shelters and study population*

For the purposes of this study, we deployed and utilized four artificial shelter arrays (n = 120 total shelters), across four stream reaches (30 shelters per reach), within a single river of the Tennessee River Basin in southwest Virginia, spanning 13 fluvial river kilometers. A more comprehensive description of our shelter construction and deployment methods can be found in Button et al., (2020a & 2020b). These four stream reaches are located in watersheds possessing between 64.3 and 67.9 % of upstream riparian area classified as forest (Hopkins et al., 2023). The hellbender populations in these study reaches are part of a long-term study by our research group. They are relatively stable, densely populated, actively reproducing populations (Jachowski and Hopkins, 2018; Hopkins et al., 2023), where breeding activity has been observed on an annual basis since monitoring began in 2007. Due to the imperiled status of the species, we are prohibited from providing the specific coordinates of our study populations.

### 2.3. *Nest sampling, hellbender processing, and nest fate categorization*

Beginning in the last week of August for each successive year between 2013 and 2021, we surveyed artificial shelters every 2–5 days for the presence of nesting activity within each study reach. Following the first detection of a nest during each breeding year, we increased shelter inspection frequency to every 2–3 days until the conclusion of breeding. We surveyed shelters when snorkeling by partially lifting shelter lids and visually inspecting the shelter chamber and tunnel with a dive light. Upon discovery, the date of nest detection was recorded. Given nest survey frequency, nest detection dates were within 72 h post-oviposition, but most often within 48 h.

We processed each nest and the attending male (e.g., enumerated clutch size, measured, weighed, and bled males) at four distinct periods during parental care following nest establishment, which occurred in a narrow 2 week period each year. These sampling periods are associated with life history milestones in embryonic and larval development, and encapsulate the period when nests of diverging fates become evident. The sampling intervals were: (1) nest initiation (~1 day (range 0 – 3) after nest detection [mean = 0.8 days ± 0.1 SE]), (2) mid-embryonic development (~30 days after detection [mean = 30.9 days ± 0.5 SE]), (3) larval hatching within the nest (hereafter "hatching"; ~60 days after detection [mean = 61.6 days ± 1.5 SE]), and (4) emergence of hatched larvae from the nest (hereafter "emergence"; ~200 days after detection [mean = 200.0 days ± 1.5 SE]). Our recent work demonstrated that male hellbenders are resilient to disturbance related to our sampling regime and that it does not affect nest fates (Hopkins et al., 2023).

We also sampled a subset of 22 adult males during the pre-breeding period before nest initiation (~20 days prior to first nest detection in a given year [mean = 21.8 ± 1.3 SE]). This served as a pre-breeding reference for all hormones analyzed, and demonstrated analytical consistency with previous investigations of pre-breeding hellbender endocrinology by our research group (Galligan et al., 2021).

To quantify the seasonal endocrine profiles of hellbender males caring for nests at each sampling interval, we captured and removed the attending male from his artificial shelter and immediately collected a baseline ("T0" avg. elapsed time since capture: 2.2 ± 0.1 SE minutes) blood sample from the caudal vein using a heparinized needle and syringe (Hopkins and DuRant, 2011). The elapsed time for baseline samples commenced from the moment each individual was captured from their shelter, and concluded at the completion of the blood draw. To describe GC changes in-response to a standardized stressor (i.e., capture and restraint), we collected a second ("T60") blood sample one hour following initial capture (avg. elapsed time from the initial capture: 61.6 ± 0.2 SE minutes). All blood samples were collected between the hours of 9:30 and 19:45 during each sampling day. In all cases, the volume of whole blood drawn (summed between baseline and stress-induced samples) was always well below the IACUC allowable limits of 1 mL per 100 g of body mass. Whole blood samples were stored on ice for less than 8 h before returning to the laboratory. Samples were centrifuged at 6000 rcf for 5 min to separate plasma from whole blood. Plasma was then aliquoted into microcentrifuge tubes (1.5 mL) and stored at −80 °C until analysis.

During each capture event, we identified each male's unique identification using passive integrated transponder (PIT) tags implanted into the hellbender's lateral tail musculature from previous captures. For those nesting males who had not previously been captured, a unique PIT tag was implanted prior to release (Unger et al., 2012, Connock et al., 2019).

Between the first and second blood samples at each sampling interval, each individual was processed for a suite of morphometric and health measurements. We measured the total length (head to end of tail), the snout-vent length (head to cloaca), and the mass of each individual using previously described methods (Hopkins and DuRant, 2011). Using the mass and total length of each individual, we computed a scaled body condition variable that enables comparison among individuals (scaled mass index (SMI); Peig and Green, 2009). Scaled mass, as estimated by the SMI, is interpreted as the expected mass of an individual if it were of a reference structural size. For all SMI calculations, we used the same scaling equation and reference length (41.0 cm) as in Hopkins et al., 2023. We used SMI as a body condition measure because it has been shown to be a more accurate indicator of relative energy reserves than commonly used indices of condition (e.g., residuals from an ordinary least squares regression; Peig and Green, 2009). It also allows for comparison of individuals' condition across time and between different studies of the same reference population (Peig and Green, 2009). Finally, as leech infection has been shown to attenuate the corticosterone response in hellbenders (DuRant et al., 2015), we visually quantified the number of leeches on each captured individual at the time of sampling.

Clutch size and the progress of each nest's embryonic or larval development were also assessed during each of the four sampling intervals. At nest initiation and mid-embryonic development, we removed each clutch from the shelter chamber and placed it in a sterilized plastic bin with 2 cm of water. Clutches were then gently compressed with a piece of plexiglass so that eggs formed a single or double layer of eggs, with all embryos visible when viewed from above. Overhead photographs were taken and later digitally analyzed using Image J software (NIH, Bethesda MD, USA) to obtain an exact count of eggs at these first two sampling periods. In a subset of cases (N = 18) at mid-embryonic development, weather and logistical constraints (e.g., high stream flows) made removing and quantifying the clutch unsafe. However, we were always able to confirm the presence or absence of viable eggs in the shelter to determine the nest's status (i.e., ongoing or failed) at mid-embryonic development. In subsequent periods (hatching and emergence), clutches were enumerated and monitored via a combination of underwater photography and videography to monitor nest progress and to confirm that eggs and/or larvae remained present and viable at each nest (Hopkins et al., 2023). If eggs and/or larvae did not appear to be present during any sampling interval following nest initiation, we exhaustively searched the internal chamber of the artificial shelter to verify their absence. Using the aforementioned combination of periodic parental monitoring and clutch enumeration observations at each sampling interval, we classified nest fates via a binary measure of success and failure using the criteria utilized by Hopkins et al., 2023.

### 2.4. Calibration standards and steroid extraction

We used the same methodology for plasma steroid extraction as described in Galligan et al. (2021). This procedure utilizes several HPLC grade solvents (e.g., water, methanol, sodium acetate buffer, acetonitrile), numerous incubation, drying, reconstitution steps, and filtration techniques (e.g., reverse solid phase extraction; Galligan et al., 2021). In this study, we followed these prior methods with a few minor modifications. First, at the outset of extraction, we utilized 50 uL of aliquoted hellbender plasma, calibration standard mixtures, and methanol (for sample blanks), instead of 100 uL. In accordance with this change, we added 50 uL of internal standard deuterated progesterone ($P_4$-$d_9$; 50 ng $mL^{-1}$ dissolved in methanol) to each plasma sample, calibration, or blank (rather than 100 uL) at the beginning of extraction. When drying supernatants following protein pellet washing, and when drying extracts following the steps of solid phase filtration, we submerged the glass culture tubes in a water bath at 40 °C, while simultaneously applying a gentle stream of pressurized ultra-pure nitrogen gas through methanol-rinsed capillary alloy tubes from above. Extraction method accuracy and efficiency was confirmed previously in Galligan et al. (2021) via spike recovery analysis.

### 2.5. Steroid quantification

As in previous investigations (Galligan et al., 2021), we quantified eight plasma steroids using liquid chromatography-tandem mass spectrometry (LC-MS/MS). In this study, we utilized a Shimadzu (Columbia, MD) LCMS-8060 interfaced with a Nexera LC-20 UPLC (Shimadzu) to quantify all eight compounds, including testosterone, dihydrotestosterone (DHT), 11-ketotestosterone (11-KT), 11-ketoandrostenedione (11-KA), cortisol, corticosterone, progesterone, and dehydroepiandrosterone (DHEA). All steroids were separated with a Waters (Milford, MA, USA) Acquity UPLC BEH C18 1.7 um column (2.1 mm x 100 mm). Gradients are detailed in Table S1. Injection volume was 10 uL for each sample, calibration standard, and blank. Samples were injected once, while all standard curves and blanks were injected in duplicate. Column eluent was introduced directly into the mass spectrometer using an ESI probe in positive ion mode. Multiple reaction monitoring (MRM) was selected to monitor two transitions per analyte (Table S2).

Samples were analyzed across three separate LC-MS/MS runs. We quantified steroids by interpolation on extracted standard curves as in Galligan et al., 2021, and used equivalent quantification ranges across each run (Table S3). To measure extraction efficiency and monitor instrument variability, we extracted and analyzed two plasma pools (A: stress-induced samples, 6 pooled male samples, from July and August; and B: baseline samples, 3 pooled female samples, from July and August) for every 100 extracted samples (Table S5). We also re-injected calibration curves at the conclusion of each run to confirm homogeneity of the calibrations' slope through the run duration. To monitor instrument sensitivity to each steroid and the internal standard more frequently throughout each run, we also injected and visually monitored the intensity of a performance verification standard (20 ng $mL^{-1}$ of each quantified hormone, and internal standard dissolved in 50:50 water: methanol) after every 10 samples injected (Table S5).

### 2.6. Prolactin

We also attempted to quantify prolactin (or a "prolactin-like" peptide) within male hellbender plasma during parental care using both radioimmunoassay and LCMS. We hypothesized that prolactin may be important to hellbenders because of its role across numerous vertebrate systems in the transition from breeding to paternal care (Schradin and Anzenberger, 1999; Lynn, 2016), including uniparental male fish (Rall et al., 2004), and the upregulation of care behaviors in successful individuals across an array of environmental contexts (Kindler et al., 1991; Thierry et al., 2013; Angelier and Chastel 2016).

The heterologous radioimmunoassay methodology for prolactin (as described in Angelier et al., 2006) was performed at the Centre d'Etudes Biologiques de Chizé (Villiers-en-Bois, France). In this attempt, samples of nine nesting males of divergent nest fates were assayed. This included samples from five successful males and two failed males at nest initiation, as well as samples from two successful individuals at mid-embryonic development. In these assays, we were not able to detect the presence of prolactin in any of the hellbender plasma samples tested. Because tertiary structural differences between the antibody used (designed for avian species) and hellbender prolactin structure (if present) may have compromised the utility of this assay, we also attempted to identify prolactin via LCMS-based proteomic techniques. To do so, we utilized the prolactin primary amino acid sequences of the closely related *Andrias* sequence (Yang et al., 2010) as a guiding target. This scan included 10 samples at nest initiation (six successful males and four failures) and 11 additional samples at mid-embryonic development (six successful males and five failures). After finding no evidence of prolactin in protein extracts from the aforementioned samples, we also attempted to sequence three exons of the hellbender prolactin gene to dismiss the existence of major changes to the amino acid sequence between *Cryptobranchus* and *Andrias* that may have obscured our search. We successfully sequenced 320 bp of the hellbender prolactin gene (~30 % of the gene, half of the protein sequence) via Sanger techniques (Sanger et al., 1977). We found no nonsynonymous differences and only 4 synonymous differences with the closely related *Andrias* precursor. Using this partial hellbender prolactin sequence as a guide, we were still unable to find evidence for prolactin within the previously examined extracts via a second round of proteomic analyses.

### 2.7. Statistical analysis

#### 2.7.1. Non-detects and exclusion of data

For all hormones analyzed, the concentration of the lowest value in the standard curve was used as the reporting limit (RL). Reporting limits did not differ for any hormone between LCMS runs (Table S3). All values below the RL were assigned the value of the RL for both statistical analysis and graphical representations. Unless otherwise noted, in all cases where a proportion of values fell below the reporting limit, we utilized a variety of left-censored statistical techniques that are robust to the inclusion of these censored values, as described in Helsel (2011). All statistical analyses were performed in R (R Development Core Team, 2023).

Numerous steroids were detected at low frequencies across all sampling periods during nesting. As a result, we were limited in our ability to model these hormones seasonally across intervals, and between nest fates within sampling periods. Throughout sampling, baseline corticosterone was detected in 2 % samples (n = 4), DHEA was detected in 3 % of samples (n = 7), 11-KA in 1 % (n = 2), and 11-KT in 8 % (n = 17; Table 2). Consequently, baseline corticosterone, 11-KA, 11-KT, and DHEA were dropped from all further quantitative statistical analyses. As nesting progressed past nest initiation, DHT and progesterone were scarcely detected (0 % and 4 % of samples respectively), and were therefore only analyzed across nest outcomes at nest initiation (Table 2). Finally, as cortisol was not detected in baseline samples at nest emergence, we were unable to model nest fates in relation to baseline cortisol

**Table 1**
Blood plasma sample sizes of pre-breeding and nesting hellbender males by period, nest outcome, and sample type (baseline (T0) and following standardized stressor (T60)). The mid-embryonic development period is abbreviated to "mid-embryonic".

| Period | Success | | Failure | | Total | |
|---|---|---|---|---|---|---|
| | T0 | T60 | T0 | T60 | T0 | T60 |
| Pre-Breeding | – | – | – | – | 18 | 12 |
| Nest Initiation | 33 | 27 | 34 | 32 | 67 | 59 |
| Mid-Embryonic | 31 | 26 | 34 | 34 | 65 | 60 |
| Hatching | 16 | 12 | 12 | 11 | 28 | 23 |
| Emergence | 35 | 32 | 7 | 8 | 42 | 40 |

at this period.

### 2.7.2. Potential confounding covariates

Prior to modeling the relationships between quantified steroids, season, clutch size, and nest fates, we assessed relationships between steroids and additional covariates that are known to influence variance in their concentrations in other vertebrates.

First, androgens can fluctuate diurnally in vertebrates (Lorenzi et al., 2008; Laucht et al., 2011), including hellbenders (Galligan et al., 2021). Therefore, we tested for significant relationships between androgens (T and DHT) and sampling time. We modeled the relationship between androgens and sampling time (hour of day) using a nonparametric approach, given the heavily skewed distribution of T and DHT across periods (Kendall's tau and a significance test of the Akritas-Theil-Sen line (ATS regression; *ATS* function of the *NADA2* package; Akritas et al., 1995; Helsel, 2011; Julian and Helsel, 2021)). We found no evidence of a significant relationship between sampling time and T ($T = 0.04$, $p = 0.42$) or DHT ($T = 0.003$, $p = 0.97$) during the periods in which each steroid was detected (Table 2). As a result, we did not include sampling time as a covariate in any subsequent models of androgen concentrations.

Second, because leech infection prevalence has previously been linked to the inhibition of the hellbender's corticosterone stress response (DuRant et al., 2015), we also tested for relationships between leech infection and stress-induced concentrations of cortisol and corticosterone. When doing so, we included the number of days between the day that the sample was taken, and the nest's detection date as a covariate in models with each glucocorticoid. Pre-breeding samples were assigned negative days relative to their site's first detected nest of that year. This approach was appropriate to account for the seasonal increase in leech prevalence and infection magnitude during the late summer in our system (Jachowski et al., 2024). For both glucocorticoids, we utilized parametric survival model regressions to account for censored values below the RL (*survreg* function from the *Survival* package), and assigned the PIT identifier of the sampled adult as a cluster variable (Therneau and Grambsch, 2000; Therneau, 2023). We log-transformed cortisol and corticosterone to better align with the model's normality assumptions. In both instances, leech infection magnitude was unrelated to stress-induced cortisol ($\beta = -0.00 \pm 0.01$ SE, $p = 0.91$) and corticosterone ($\beta = 0.00 \pm 0.02$ SE, $p = 0.81$). Likewise, we observed no significant interaction between leech infection magnitude and elapsed days from nest detection for either glucocorticoid (cortisol: $\beta = 0.00 \pm 0.00$, $p = 0.14$; corticosterone: $\beta = 0.00 \pm 0.00$, $p = 0.37$). This result contrasted with our previous study (DuRant et al., 2015), likely because we only measured the stress response at 1-hour post-capture, and the inhibition of the corticosterone stress response in the previous study occurred in samples taken 3-hours post-capture. In addition, the previous study examined blood samples from the summer months only, and never during the immediate pre-breeding period or during parental care. Because there are seasonal differences in leech prevalence and infection intensity in this system, as well as clear seasonal differences in endocrinology of the host, differences in sampling season may also contribute to discrepancies between the two studies. Nevertheless, we did not include leech infection as a covariate in models of stress-induced glucocorticoids.

Finally, because body condition can affect circulating hormone concentrations (Galeotti et al., 1997; Romero and Wikelski, 2001; Narayan et al., 2013), we sought to confirm whether morphometric characteristics of hellbenders affected any of the steroids that we quantified. We performed ATS regressions between hormones and total length and SMI to ascertain the influence of body size or condition on hormone concentrations within each period retained for statistical analysis. However, we only explored relationships of hormones with SMI at nest initiation because some males will cannibalize their nests, leading to greatly inflated SMIs when captured at latter sampling periods due to excess stomach contents (Hopkins et al., 2023). Overall, body condition never varied with hormones in baseline or stress-induced samples (in all cases $T \leq 0.03$, $p \geq 0.66$). Likewise, total length was unrelated to all baseline hormone concentrations across all periods (in all cases, $T \leq 0.12$, $p \geq 0.07$). However, both stress-induced cortisol and corticosterone were positively correlated with total length throughout sampling (cortisol: $T = 0.17$, $p < 0.001$; corticosterone: $T = 0.19$, $p = p < 0.001$). As a result, we retained total length as a covariate in all subsequent statistical models of stress-induced glucocorticoids when used as a predictor of nest fate or clutch size.

### 2.7.3. Seasonal fluctuations, clutch size, and nest fates

To describe the seasonal changes in steroid concentrations irrespective of nesting fates from pre-breeding through parental care, we analyzed data using left-censored statistical methods as described in Helsel (2011). Given the high frequency of non-detects and the right skewed distributions of testosterone, DHT, and baseline and stress-induced glucocorticoids across season, we employed a Peto-Peto nonparametric approach. This was followed by pairwise multiple comparisons with the BH method for p-value adjustment (*cen1way* function of the *NADA2* package) to ascertain seasonal differences in steroid concentrations when each were detected (Peto and Peto, 1972; Benjamini and Hochberg, 1995; Helsel, 2011; Julian and Helsel, 2021).

We employed the same Peto-Peto approach to describe statistical

**Table 2**
Hormone detection frequency in hellbender males across sampling period (pre-breeding (pre), nest initiation (0d), mid-embryonic development (30d), hatching (60d), and emergence (200d)). For cortisol and corticosterone, both baseline (T0) and standardized stressor (T60) detection frequencies are reported, with T60 detection frequency in parentheses.

| Hormone | Sampling Period Detection Frequency (%) | | | | | |
|---|---|---|---|---|---|---|
| | Pre | 0d | 30d | 60d | 200d | Overall |
| Testosterone (T) | 100 | 97 | 37 | 57 | 48 | 65 |
| Dihydrotestosterone (DHT) | 100 | 90 | 0 | 0 | 0 | 35 |
| Dehydroepiandrosterone (DHEA) | 0 | 4 | 3 | 7 | 0 | 3 |
| 11-ketotestosterone (11-KT) | 0 | 6 | 11 | 18 | 2 | 8 |
| 11-ketoandrostenedione (11-KA) | 0 | 1 | 0 | 4 | 0 | 1 |
| Progesterone ($P_4$) | 0 | 22 | 3 | 7 | 5 | 10 |
| Cortisol (F) | 0 (100) | 19 (98) | 22 (95) | 26 (96) | 0 (75) | 16 (92) |
| Corticosterone (B) | 11 (58) | 3 (60) | 0 (65) | 0 (65) | 0 (23) | 2 (54) |

differences between baseline and stress-induced cortisol irrespective of sampling period or nesting outcome. In all cases, we report and plot the Kaplan-Meier mean estimate when discussing global differences in steroids between season or sample type (baseline vs. stress-induced).

To determine whether total length or SMI affected nest fates, we performed two generalized linear mixed models (GLMM; *glmer* function of *lme4* package) with nest fate (success vs. failure) as the response, either total length or initiation SMI as the dependent variable, and the male's PIT identifier as a random effect (Bates et al., 2015). In both instances, we specified a binomial distribution of the response variable.

Depending on the steroid and proportion of values below detection (Table 2), we utilized several different statistical approaches to examine differences in steroid concentrations between males of differing nest fates and clutch sizes. For baseline cortisol (within initiation, mid-embryonic development, and hatching), and progesterone (nest fate within initiation only), we employed non-parametric approaches to explore differences in steroid concentrations between nest fate (Peto-Peto) and clutch size (ATS regression of baseline glucocorticoids at initiation and mid-embryonic development). To test for differences between successful and failed males' T and DHT at nest initiation, we substituted the value of the RL for censored data (3 % for T, 10 % for DHT; Table 2), and fitted two linear mixed models (*lmer* function of *lme4* package) with each androgen as the response, nest outcome as a fixed effect, and each individual's PIT identifier as a random effect (Bates et al., 2015). Likewise, we fitted linear mixed models with clutch size as the fixed predictor to examine differences in androgens across nest sizes at nest initiation. We focused on the nest initiation period during these analyses because androgen levels dropped below or near our RL in the weeks following nest detection. We used the same approach to test for differences in stress-induced cortisol between both nest fate (nest initiation through emergence) and clutch size (within initiation and mid-embryonic development only). In both instances, we included the male's total length as a fixed covariate. In the case of nest fate, we also included nest period with models of stress-induced cortisol (and their interaction). We again substituted the value of the RL for the small number of non-detects during analysis (8 % for stress-induced cortisol; Table 2). Finally, to examine the same predictive variables (nest fate and clutch size) with stress-induced corticosterone, we employed survival model regressions (to account for greater number of non-detects; Table 2) assuming a log-normal response distribution (*survreg* function of the *Survival* package) with nest fate or clutch size as fixed effects, along with sampling period (and its interaction with nest fate) in nest fate models only. In all cases, the hellbender's PIT identifier was used as a clustering variable (Therneau and Grambsch, 2000; Therneau, 2023), and total length was included as a fixed covariate. For both stress-induced cortisol and corticosterone, we utilized package *emmeans* to conduct post-hoc pairwise comparisons on nest fate model results to deduce differences between fates' stress-induced glucocorticoids within sampling periods. We report results from the corticosterone comparisons on a log scale.

## 3. Results

From the 2013 to 2021 breeding seasons, we surveyed 90 nests from 54 unique males, collecting 202 baseline and 182 stress-induced plasma samples across the four nesting periods (Table 1). Among surveyed nests, 47 were successful (32 unique males) and 43 failed (29 unique males). During the pre-breeding period, we collected 30 blood samples (18 baseline, 12 stress-induced) from 22 unique males, approximately 20 days prior to nesting [mean = 21.8 days ± 1.3 SE] (Table 1). Though all 90 nests were monitored for nest fate at all periods for as long as they remained viable, due to logistical challenges (e.g., strong stream flows), we did not obtain blood samples from every nesting male at each period (Table 1). Clutch sizes at nest initiation ranged from 51 to 1152 eggs (mean = 333 ± 25 SE) and were obtained at initiation for all nests where blood samples were taken (Table 1). At mid-embryonic development, we were able to quantify clutch sizes in 47 of the 65 nests where we obtained blood samples (Table 1; range = 0 – 801 eggs; mean = 146 ± 21 SE inclusive of failed nests; mean = 167 ± 23 SE for viable nests only).

### 3.1. Morphometrics of nesting males

At nest initiation, the size of nesting males was widely variable (TL: mean = 43.9, range = 33.0 – 50.5 cm; mass: mean = 513.5, range = 212 – 960 g). Across all nest sampling periods, mean SMI was 426.9 ± 3.8 SE (median SMI = 418.4; range: 322.5 – 597.1) and we rarely encountered a nesting male that was visibly underweight; throughout nest sampling, only 6 % of captured males' SMI fell below the population's historical 10th percentile (i.e., SMI = 357; Hopkins et al., 2023). Aligning with results reported previously by our research group (Hopkins et al., 2023), nesting success was not predicted by the total length nor the SMI of parental males at nest initiation (TL: $\beta$ = -0.13, SE = 0.10, $p = 0.19$; SMI: $\beta$ = 0.02, SE = 0.35, $p = 0.95$).

### 3.2. Androgens

We found testosterone and DHT to be the most abundant androgens associated with the onset of reproduction and subsequent parental care in male hellbenders. Testosterone and DHT were detected in all pre-breeding samples, while 11-KT, DHEA, and 11-KA were never detected during the pre-breeding period (Table 2). Concentrations of testosterone and DHT were dramatically elevated prior to breeding (T: KMmean = 95.0 ng $\mathrm{mL}^{-1}$; DHT: KMmean = 34.7 ng $\mathrm{mL}^{-1}$), but both decreased substantially as sampling progressed seasonally from pre-breeding and throughout parental care (T: $\chi 2$ = 494.2, DF = 4, $p <$ 0.001; DHT: $\chi 2$ = 115.3, DF = 1, $p <$ 0.001). Following the onset of parental care at nest initiation, average T and DHT across all nesting males declined to approximately 20 % of pre-breeding concentrations (T: KMmean = 18.7 ng $\mathrm{mL}^{-1}$; DHT: KMmean = 8.4 ng $\mathrm{mL}^{-1}$; Fig. 1; Table S4).

Within nesting males at nest initiation, there was appreciable variation in testosterone (range = 0.2(RL) – 91.8 ng $\mathrm{mL}^{-1}$) and DHT (range = 1(RL) – 26.4 ng $\mathrm{mL}^{-1}$; Fig. 2). An ATS regression of all androgen values (including those below the RL) confirmed that this wide variance in testosterone and DHT among males at nest initiation was unrelated to the number of elapsed days (range = 0 – 3d) between nest detection and blood sampling (testosterone: T = 0.11, $p = 0.17$, DHT: T = 0.08, $p =$ 0.29). On average, successful nesting males' testosterone at nest initiation (22.3 ng $\mathrm{mL}^{-1}$ ± 3.5 SE) trended 47 % higher than that of unsuccessful males (15.2 ± 3.4 SE), but this difference was not statistically significant ($\beta$ = 7.1, SE = 4.7, $p = 0.13$; Fig. 2). Similarly, successful males' DHT (9.8 ng $\mathrm{mL}^{-1}$ ± 1.2 SE) trended 38 % higher than that of unsuccessful males (7.1 ng $\mathrm{mL}^{-1}$ ± 1.1 SE), but not significantly so ($\beta$ = 2.6, SE = 1.5, $p = 0.09$; Fig. 2). Irrespective of nest fate, males' androgens did not vary significantly with clutch size at nest initiation (testosterone: $\beta$ = 0.02, SE = 0.01, $p = 0.16$; DHT: 0.00, SE = 0.00, $p =$ 0.52).

As parental care progressed to the end of its first month, testosterone significantly decreased (Table S4) by two orders of magnitude by mid-embryonic development (T: KMmean = 0.23 ng $\mathrm{mL}^{-1}$), and was never greater than 0.74 ng $\mathrm{mL}^{-1}$ in any sample (Fig. 1). From thereon through hatching and emergence, testosterone remained at a low concentration, near our RL (0.2 ng $\mathrm{mL}^{-1}$), and only exceeded 0.5 ng $\mathrm{mL}^{-1}$ in one sample (Fig. 1). Following nest initiation, DHT was not detected at mid-embryonic development, hatching, nor the emergence sampling intervals (Table 2).

Among samples where both testosterone and DHT were detected (n = 77), they were highly positively correlated (adjusted $R^2 = 0.89$), with testosterone consistently circulating at nearly three times that of DHT (T:DHT ratio = 2.76 ± 0.11 SE).

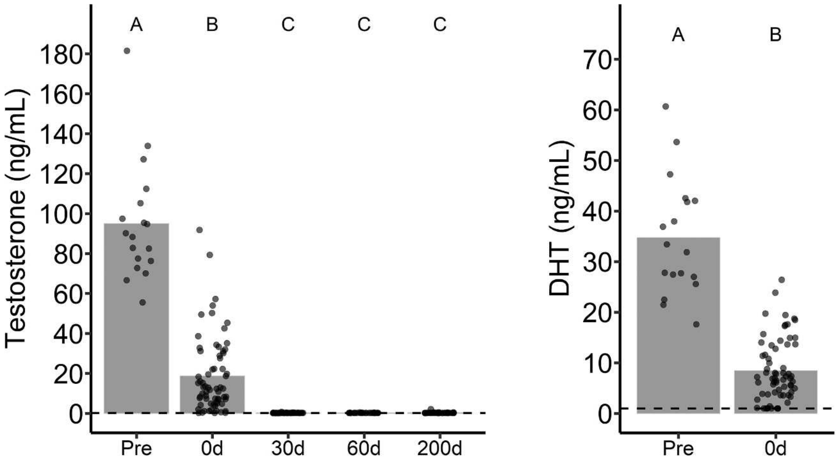

**Fig. 1.** Testosterone (left) and DHT (right) across the five sampling seasons (pre-breeding (pre), initiation (0d), mid-embryonic development (30d), hatching (60d), emergence (200d)), inclusive of all samples within parental care regardless of nest outcome (DHT not detected past nest initiation; Table 2). Points are individual samples of hellbender males. Bar magnitude represents Kaplan-Meier mean of a given sampling period. Letters for each period (top) signify statistical differences between period means (within each compound) via a Peto-Peto nonparametric multiple comparison test. Dashed line is the reporting limit (RL) for testosterone (0.2 ng mL$^{-1}$) and DHT (1.0 ng mL$^{-1}$; Table S3). Values below these thresholds censored to the RL for both statistical and visualization purposes. Note that the scale of the y-axes of both plots are not identical.

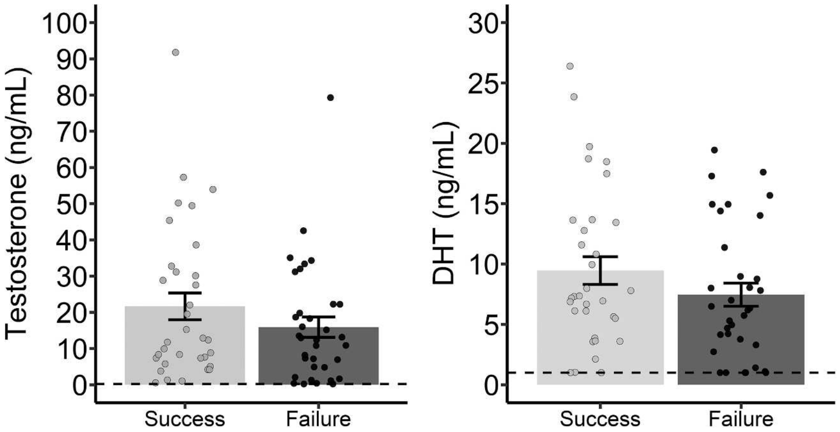

**Fig. 2.** Testosterone (left) and DHT (right) at nest initiation, by fate (success = light grey, failed = dark grey) for paternal hellbender males. The horizontal dashed line represents the reporting limit (RL) for T (0.2 ng mL$^{-1}$) and DHT (1.0 ng mL$^{-1}$) respectively (Table S3). Values below the RLs were censored to the RL for statistical and visualization purposes. Bars represent the group mean, error bars signify one standard error from the mean. Individual points represent the hormone concentrations of individual male samples. Note that the scale of the y-axes of both plots are not identical.

### 3.3. *Glucocorticoids*

We found no seasonal variation in baseline cortisol, but cortisol was consistently elevated in stress-induced samples relative to baseline samples. Cortisol was detected in 16 % of baseline, and 92 % of stress-induced samples—circulating five times higher after an hour of capture and restraint (KMmean = 5.05 ng mL$^{-1}$) compared to baseline (KMmean = 0.94 ng mL$^{-1}$; $\chi2 = 330.6$, DF = 1, $p < 0.001$; Fig. 3; Table 2). Cortisol was only detected in baseline samples at nest initiation, mid-embryonic development, and hatching (Table 2). Between these periods, we observed no seasonal variation in baseline cortisol (initiation KMmean = 0.97 ng mL$^{-1}$, mid-embryonic development KMmean = 1.01 ng mL$^{-1}$, hatching KMmean = 1.00 ng mL$^{-1}$; $\chi2 = 0.328$, DF = 2, $p = 0.85$; Fig. 3). Across seasons, baseline cortisol concentrations above the reporting limit did not vary with the elapsed time from capture to the conclusion of the blood draw (adjusted $R^2 = 0.01$, $\beta = 0.25$, SE = 0.21, $p = 0.26$).

Baseline cortisol did not vary between successful and failed nesting males within any of the parental care periods when it was detected. This included the initiation period (successful KMmean = 0.99 ng mL$^{-1}$; failed KMmean = 0.95 ng mL$^{-1}$; $\chi2 = 0.07$, DF = 1, $p = 0.79$), at mid-embryonic development (successful KMmean = 0.90 ng mL$^{-1}$, failed KMmean = 1.12 ng mL$^{-1}$; $\chi2 = 1.63$, DF = 1, $p = 0.20$), and hatching (successful KMmean = 0.88 ng mL$^{-1}$, failed KMmean = 1.15 ng mL$^{-1}$; $\chi2 = 0.48$, DF = 1, $p = 0.49$). Likewise, regardless of eventual nest fate, at both nest initiation and mid-embryonic development, males' baseline cortisol was unrelated to the number of eggs that they were attending at that time (in both cases $T \leq 0.04$, $p \geq 0.18$).

In contrast to baseline samples, we did observe seasonal variation in stress-induced cortisol ($\chi2 = 72.9$, DF = 4, $p < 0.001$). Stress-induced cortisol was highest during pre-breeding (KMmean = 6.45 ng mL$^{-1}$) and mid-embryonic development (KMmean = 6.90 ng mL$^{-1}$), with the latter being significantly elevated relative to nest initiation (KMmean = 5.09 ng mL$^{-1}$; Fig. 3; Table S4). Stress-induced cortisol at hatching (KMmean = 4.58 ng mL$^{-1}$) was significantly lower than pre-breeding and mid-embryonic development. Stress-induced cortisol at emergence (KMmean = 2.07 ng mL$^{-1}$) was lower than all other periods (Fig. 3; Table S4).

Despite seasonal differences in stress-induced cortisol, within parental care periods, we observed no statistical differences in stress-

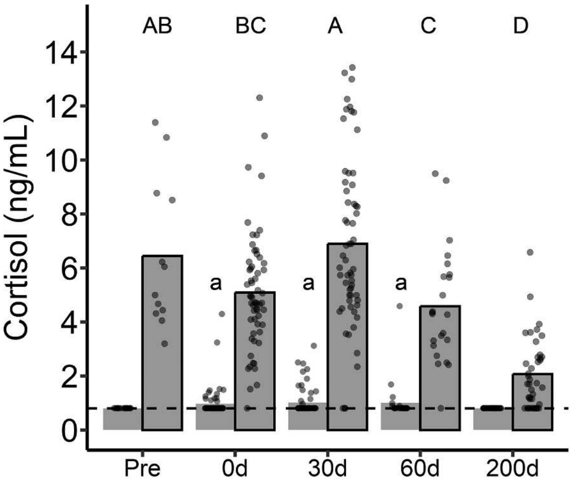

**Fig. 3.** Baseline (bars with no outline) and stress-induced (bars with black outline) cortisol across the five sampling seasons, inclusive of all samples within parental care regardless of nest fate. Points are individual sampling events of hellbender males. Bar magnitude represents Kaplan-Meier mean of a sample type (baseline or stress-induced) within a sampling period. Letter case above each bar (baseline = lowercase; stress-induced = uppercase) indicates global statistical difference between baseline and stress-induced cortisol inclusive of all periods via a Peto-Peto nonparametric comparison test. Letter identities (stress-induced: A – D; baseline: a) within each case signify statistical differences between period means amongst stress-induced and baseline categories via separate Peto-Peto comparisons (cortisol was never detected in baseline samples during pre-breeding, nor emergence). Dashed line is the reporting limit for cortisol (0.8 ng mL$^{-1}$) (Table S3). Values below this threshold are censored to the reporting limit for both statistical and visualization purposes.

induced cortisol between nest fates. This included initiation ($\beta = 0.34$, SE $= 0.61$, $p = 0.58$), mid-embryonic development ($\beta = -0.66$, SE $= 0.61$, $p = 0.28$), hatching ($\beta = 0.20$, SE $= 0.93$, $p = 0.83$), and emergence ($\beta = -0.40$, SE $= 0.89$, $p = 0.65$; Fig. 4). As with baseline cortisol concentrations, stress-induced cortisol was unrelated to males' clutch size at both nest initiation and mid-embryonic development (in both cases $\beta \leq 0.001$, $p \geq 0.55$). Consistent with our preliminary analysis, in our model of stress-induced cortisol between nest fates (inclusive of all samples), males' total length was a significant predictor of stress-induced cortisol ($\beta = 0.13$, SE $= 0.06$, $p = 0.04$). However, in models within sampling periods (either nest initiation or mid-embryonic development) exploring relationships between clutch size and stress-induced cortisol, total length was not significant (in both cases $\beta \leq 0.25$, $p \geq 0.06$).

Corticosterone was detected in only 2 % of baseline samples (KMmean = 0.82 ng mL$^{-1}$) and 54 % of the time following capture and restraint (KMmean = 1.63 ng mL$^{-1}$). Within stress-induced samples, we did observe seasonal variation in corticosterone ($\chi2 = 19.51$, DF = 4, $p < 0.001$). While there was no significant difference in stress-induced corticosterone between the pre-breeding (KMmean = 1.41 ng mL$^{-1}$), initiation (KMmean = 1.78 ng mL$^{-1}$), mid-embryonic development (KMmean = 2.01 ng mL$^{-1}$), and hatching periods (KMmean = 1.49 ng mL$^{-1}$)—all four of these intervals had higher stress-induced corticosterone than at nest emergence (KMmean = 0.97 ng mL$^{-1}$; Table S4). Across seasons, baseline corticosterone concentrations above the reporting limit did not vary with the elapsed time from capture to the conclusion of the blood draw (adjusted $R^2 = -0.19$, $\beta = -0.31$, SE $= 0.43$, $p = 0.55$). Successful males' average stress-induced corticosterone concentrations trended 19 % lower at nest initiation than that of failed males, but this difference was not significant ($\beta = 0.45$, SE $= 0.25$, $p = 0.07$). Across the latter three care periods, there were no differences in stress-induced corticosterone between nest outcomes at mid-embryonic development ($\beta = -0.28$, SE $= 0.25$, $p = 0.27$), hatching ($\beta = 0.31$, SE $= 0.34$, $p = 0.36$), or emergence ($\beta = 0.32$, SE $= 0.42$, $p = 0.44$). Stress-induced corticosterone did not vary with males' clutch sizes at either initiation or mid-embryonic development (in both cases $\beta = 0.001$, $p \geq 0.17$). Consistent with our preliminary analysis, total length was a significant predictor of stress-induced corticosterone in our model with nest fate inclusive of all samples ($\beta = 0.06$, SE $= 0.02$, $p = 0.005$), as well as models of clutch size within sampling periods at both initiation ($\beta = 0.08$, SE $= 0.04$, $p = 0.04$) and mid embryonic development ($\beta = 0.10$, SE $= 0.04$, $p = 0.03$).

Overall, there was a significant correlation between stress-induced cortisol and stress-induced corticosterone when regressed on a log–log scale via an ATS regression ($T = 0.37$, $p < 0.001$). The ATS estimator slope between corticosterone and cortisol was 1.38, intercept = 0.58 (Akritas et al., 1995; Helsel, 2011; Julian and Helsel, 2021).

### 3.4. *Progesterone*

Between successful and failed males at nest initiation (when the compound was detected in 22 % of samples), there was no difference in circulating progesterone ($\chi2 = 0.19$, DF = 1, $p = 0.66$; success KMmean = 1.07 ng mL$^{-1}$, failed KMmean = 0.85 ng mL$^{-1}$). Progesterone was detected in less than 7 % of samples in all other periods, and never at pre-breeding.

## 4. Discussion

This study is the first to describe the seasonal steroid endocrinology of an amphibian engaged in extended uniparental male care in the wild. We found that hellbender salamanders have both endocrinological similarities and differences compared to fish with comparable life histories. Because hellbender populations are rapidly declining, and the cessation of care leading to nest failure is hypothesized as a mechanism underlying declines (Hopkins et al., 2023), we also examined paternal endocrine correlates of nest fate and clutch size in hellbenders. By focusing our work on a relatively robust hellbender population with high annual rates of successful reproduction, our findings provide foundational information about the hormonal correlates of nesting and

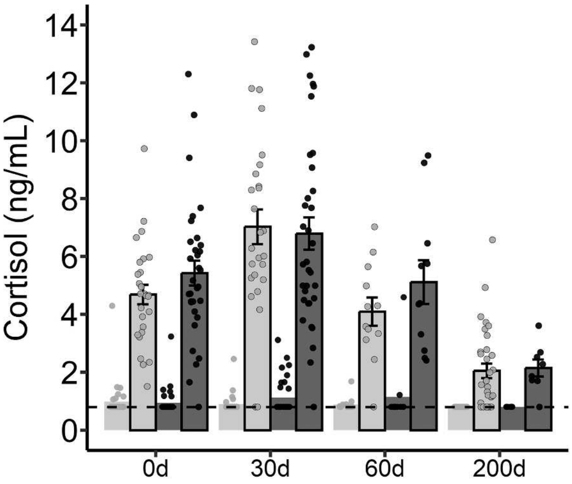

**Fig. 4.** Baseline (bars with no outline) and stress-induced cortisol (bars with black outline) across parental care sampling periods, separated by nest outcome (success = light grey, failed = dark grey). Points are individual sampling events of hellbender males. Bar magnitude of baseline samples represents Kaplan-Meier mean within a sampling period for a given nest outcome (baseline cortisol not detected at emergence). Baseline data displayed for visualization purposes only (see results for Peto-Peto comparisons between baseline cortisol concentrations across nesting outcomes). For stress-induced cortisol across season and between nesting outcomes, bars represent the group mean, while error bars signify one standard error from the mean. Dashed line is the reporting limit for cortisol (0.8 ng mL$^{-1}$) (Table S3). Values below this threshold are censored to the reporting limit for both statistical and visualization purposes.

paternal care in healthy hellbender populations.

We corroborated previous findings that demonstrated testosterone and DHT to be the primary androgens in hellbenders (Galligan et al., 2021), and that hellbenders' seasonal androgen profiles are consistent with patterns observed in male fish providing uniparental care. Namely, the steady decline in androgen concentrations we describe in hellbender males from pre-breeding to nest initiation, and from nest initiation through progressive periods of care, mirrors that of others with uniparental male care. This includes bluegill sunfish (*Lepomis macrochirus*; Kindler et al., 1989; Magee et al., 2006), three-spined stickleback (*Gasterosteus aculeatus*; Pall et al., 2002), plainfin midshipman (*Porichthys notatus*; Sisneros et al., 2004), and garibaldi (*Hypsypops rubicundus*; Sikkel, 1993). While we observed declines in hellbender males' androgens from pre-breeding to nest initiation, testosterone and DHT still circulated at ~ 20 % of pre-breeding levels in the days following hellbenders' initial nest establishment (Fig. 1). This observation suggests that circulating androgens are not incompatible with the initiation of care behaviors in hellbenders, especially given that both testosterone and DHT at nest initiation trended higher (47 and 38 % respectively) in successful males compared to those who failed (Fig. 2). These observations also align with multiple studies in bluegill sunfish, which demonstrate that while elevated androgens can lead to increased predatorial aggression (O'Connor et al. 2011a; Rodgers et al., 2012), and may rise during the midpoint of care prior to re-nesting (Magee et al., 2006)—they often have no influence on nest cleaning nor fanning behavior (Neff and Knapp, 2009; Rodgers et al., 2012; but see Rodgers et al., 2013). Likewise, in uniparental smallmouth bass (*Micropterus dolomieu*), while androgens have been experimentally connected to nest defense (Dey et al., 2010), they are unassociated with care behaviors (Dey et al., 2010), the likelihood of nest abandonment (Zuckerman and Suski, 2013), and overall nest fate (O'Connor et al., 2012). In other ectotherms, support for androgens' role in conspecific territoriality has recently been supported in a uniparental male anuran when exposed to simulated auditory intrusions (Rodriguez et al., 2022), though performance during parental care was not an objective of that study. Together, our findings and those in other species support revised theory that androgens may not mediate a behavioral tradeoff between territoriality, breeding, and care in uniparental males, because in many cases, these behaviors are not mutually exclusive (reviewed in Moore et al., 2020). In species that provide uniparental male care, while seasonal declines in androgens correspond with the onset of parental care, the literature on male teleosts, and the results reported herein, suggest that this decline is not necessarily related to the onset of parental care itself. Instead, androgens in uniparental male species may primarily mediate a response to social interactions related to breeding with both male and female conspecifics for as long as they are ongoing, and return to non-breeding baseline concentrations once the seasonality of these breeding interactions begin to slow towards a conclusion (reviewed in Goymann et al., 2019; Moore et al., 2020). In accordance with this hypothesis, circulating androgens remained at 20 % of pre-breeding levels as nests were initiated (when secondary mating events are still occurring; Hopkins et al., 2023; O'Brien et al., 2024), and these concentrations were compatible with the onset of care in successful males. However, as care progressed past the first month of care (when all mating and conspecific breeding interaction for that season has ceased in the weeks prior), we observed testosterone and DHT to be very low in all individuals. To further investigate the relationship between androgens, territoriality, breeding, and parental care behavior in hellbenders, future investigations could manipulate males' testosterone and DHT in-conjunction with in-situ video monitoring of breeding events, parental behavior, and via simulated predator intrusion.

Though androgens exhibited wide variance at nest initiation among individuals regardless of nest outcome (Fig. 1), we found no support for our hypothesis that testosterone might be inversely related to clutch size as nests were initiated. Presuming testosterone is a primary modulator of breeding behavior in hellbenders, we predicted males with smaller nests would exhibit higher testosterone to support further courting of females in the days immediately following our initiation sampling. While hellbenders only nest once per breeding season, there is evidence that alike teleosts (Coleman and Jones, 2011), hellbenders may obtain clutches from multiple females during their brief ~ 2 week annual breeding period (Crowhurst et al., 2009; Unger et al. 2015; Hopkins et al., 2023; O'Brien et al., 2024). Indeed, historically at the sites included in this study, 17 % of nests with quantified clutch sizes at both initiation and mid-embryonic development exhibit increases in clutch size after their initial establishment (based on 116 nests; Hopkins et al. unpub data). In this context, the absence of an inverse relationship between androgens and clutch size is notable given the sizable variation we observed in initiation clutch sizes (51 – 1152 eggs). Our findings are at odds with evidence in other species with uniparental male care that demonstrate a link between androgens and clutch size. For example, androgens decline faster as nests are established in males with multiple clutches in both plainfin midshipman and bluegill sunfish, compared to those who have only mated once (Knapp et al., 1999; Pall et al., 2002). Likewise, in species with re-nesting cycles, males with reduced clutch sizes show faster re-elevations in androgens as the initial nesting period concludes, bolstering their chances of obtaining a larger nest in subsequent bouts (Magee et al., 2006). Given the relative infancy of our understanding regarding multiple mating in hellbenders compared to other systems with uniparental male care, future investigation of the relationship between androgens and clutch size could consider more frequent blood draws and clutch enumeration as nests are established, clutch size manipulations, or video monitoring of shelters entrances to directly quantify mating events.

Our study also confirmed that cortisol functions as the most abundant glucocorticoid in hellbenders (Hopkins et al. 2020), but that baseline concentrations of cortisol do not exhibit seasonal elevations prior to breeding. In fact, consistent with a previous study (Galligan et al., 2021), during the period of activity immediately preceding nest establishment when male-male territorial conflict is common (Unger et al., 2020) and when prospective nesting males spend 75 % of their time guarding their nesting cavity and engaging in cavity cleaning behavior via the movement of sediment with their face, feet, and tail (O'Brien, 2023), we never detected cortisol in baseline samples of reproductive males (Fig. 3). This pattern is consistent with previous research on bluegill sunfish, which lack a strong seasonal pattern in cortisol in the days preceding breeding (Magee et al., 2006). The findings in hellbenders and bluegill sunfish are in stark contrast with the pronounced elevations in glucocorticoids observed prior to reproduction in most seasonally breeding birds, mammals, and reptiles (Romero et al 2002; Moore and Jessop, 2003), in trout and uniparental plainfish midshipman during sexual maturation and prior to breeding (Audet and Claireaux, 1992; Sisneros et al., 2004; Jamalzadeh et al., 2013), and in salmonids during their migration and osmoregulatory transitions (Fuller et al., 1976; Maule et al., 1993; Shrimpton and McCormick, 1998; Culbert et al., 2022).

During parental care, the frequency of cortisol's detection in baseline samples remained low, and did not vary between nest fate nor clutch size. Overall, baseline cortisol was only detected between 19 – 26 % of time during the first three periods of parental care (Table 2), when oxygenation and egg cleaning behaviors such as tail fanning and clutch agitation are commonly employed by hellbenders (Settle et al., 2018; O'Brien, 2023). Further, considering cortisol's role in supporting the energetic demands of care in some vertebrates (Bonier et al., 2009a; Bonier et al., 2011), it was particularly surprising that we did not observe greater baseline cortisol in males with larger clutch sizes during the first month of care. We predicted this relationship because larger nests could reduce available dissolved oxygen, and in nest cavities with less oxygen, hellbender males are more active providing care (O'Brien, 2023). In fact, males with nests of low dissolved oxygen can spend up to 40 % of their time fanning their clutch (O'Brien, 2023). Even so, evidence for an adaptive role of elevated glucocorticoids in support of paternal care is understudied in systems with uniparental male care. In one case, Magee et al., 2006 observed an elevation in baseline glucocorticoids in-accordance with the most intense period of fanning behavior in bluegill sunfish. Though alike our findings, O'Connor et al. 2011b found no relationship between males' clutch sizes and baseline glucocorticoids in smallmouth bass. This is despite evidence in the avian literature for the "cort-adaptation hypothesis", which explains the role of moderately elevated glucocorticoids in parents with increased brood sizes and more robust nestling growth (Bonier et al., 2009b; Bonier et al., 2011), incubation behavior (Ouyang et al., 2013), and provisioning rates (Crossin et al., 2012; Ouyang et al., 2013). As in birds, parental behaviors in teleosts such as tail fanning and guarding are energetically costly (Cooke et al., 2002; Cooke et al., 2006). However, it may be the case that the relative costs bore by ectothermic males are less than those of birds engaged in incubation, nest defense, foraging, and offspring provisioning. To this end, additional research is needed to ascertain the applicability of the cort-adaptation hypothesis and the role of baseline glucocorticoids in supporting the energetic costs of uniparental male care in ectotherms.

While we did not observe any seasonality or pronounced elevations in male hellbenders' baseline glucocorticoids throughout pre-breeding and parental care, we did observe seasonal changes in their glucocorticoid stress response. Namely, we observed a robust stress-induced cortisol response during the pre-breeding period, which was maintained through the nest initiation and mid-embryonic development sampling periods (Fig. 3), when behavioral territoriality, breeding interactions, and care behaviors are at their peak in nesting male hellbenders (Bishop, 1941; Nickerson and Mays, 1972; Unger et al.; O'Brien, 2023). As eggs began to hatch, we observed an attenuation in nesting males' cortisol response, which ultimately reached its lowest level at spring emergence, when the primary parental care activity is likely limited to guarding the nest cavity entrance. The seasonal elevation of the glucocorticoid stress response in hellbenders during the pre-breeding and early reproductive periods is consistent with syntheses across birds, reptiles, amphibians, and mammals. Namely, the observed response is often highest during the breeding season (reviewed in Romero et al. 2002), when the frequency of challenges from unpredictable stimuli are more frequent (e.g., predation and hostile territorial interactions with conspecifics). However, the seasonal changes of glucocorticoid responsiveness in-association with pre-breeding, reproduction, and care have not been investigated in uniparental teleosts. Amongst fish without care, there are inconsistent seasonal patterns overall (e.g., Pottinger and Carrick, 2000; Belanger et al., 2016). In addition, a sizeable portion of the seasonal literature in teleosts is limited to captive individuals, or to species where it can be difficult to establish baseline concentrations due to a short latency in the glucocorticoid response (reviewed in Parkhurst, 2011).

The reduced cortisol and corticosterone stress responses we observed at nest emergence relative to earlier in parental care (Fig. 3) could also be influenced by water temperature, a known covariate of the glucocorticoid response in aquatic ectotherms (Lankford et al., 2003; Nazarudin et al., 2016; Samaras et al., 2018; Cockrem et al., 2019). However, if driven primarily by reductions in stream temperature (and associated changes in metabolism as the parental period progressed), the glucocorticoid stress response would have declined into early winter following the pre-breeding and nest initiation periods of August and September. This was not the case. In fact, despite a ~ 43 % decline in average stream temperature (>7 °C) between nest initiation and hatching (N = 85 temperature measurements; nest initiation mean = 17.1 ± 2.1 °C; hatching mean = 9.8 ± 2.9 °C; Hopkins et al. unpub data)—there was no statistical difference in the glucocorticoid stress response between these two care periods (Fig. 3). While the confounding influence of water temperature should not be dismissed, our findings suggest that the stage of parental care was a more influential factor than stream temperature in the seasonal variability of the glucocorticoid stress response.

Despite the seasonality of hellbenders' glucocorticoid stress response, we did not observe the predicted attenuation of the glucocorticoid stress response in males with larger clutches (e.g., greater reproductive investment or value within a given breeding season), nor in individuals who were ultimately successful in care (Wingfield and Sapolsky, 2003; Viblanc et al., 2016; Lynn, 2016). In uniparental male teleosts, the attenuation of the glucocorticoid stress response has been observed in caring versus non-reproductive individuals (O'Connor et al. 2011b; Jeffrey et al., 2014). However, in alignment with the results we observed in hellbenders, prolonged experimentally induced elevations in glucocorticoids are unrelated to care behaviors in smallmouth bass (O'Connor et al., 2009; Dey et al., 2010; Zolderdo et al., 2016), but are linked to an increased frequency of nest abandonment (O'Connor et al., 2009; Dey et al., 2010), as commonly observed in other vertebrates (e.g., Ouyang et al., 2011). In light of hellbenders' declining status in areas of impaired habitat, future study could integrate investigation of the parental glucocorticoid stress response in hellbender males and nest fates across a gradient of habitat quality. Given the hypothesized influence of increased environmental change upon allostatic demands of individuals (Angelier and Wingfield 2013), hellbenders offer a study system to further test hypotheses involving changes in the vertebrate glucocorticoid stress response during care and reproduction of both viable and imperiled populations.

The positive relationship we observed between hellbender males' total length and both stress-induced cortisol and corticosterone is consistent with studies linking age to glucocorticoid hypersecretion or a reduction in HPA/HPI-axis sensitivity (i.e., decrease in negative feedback) in older vertebrates (Sapolsky et al., 1986; Stamou et al., 2023). This phenomenon is most often observed experimentally, as in laboratory rats (e.g., Sapolsky et al.,1986), enclosed fish (e.g., Barcellos et al., 2012), and is well-studied in elder humans (e.g., Traustadóttir et al., 2005; Hatzinger et al., 2011, Stamou et al., 2023). In free living animals, the relationship is far less established, though has been documented previously in garter snakes (Moore et al., 2000). In the avian literature, studies have demonstrated downregulation of the glucocorticoid response with increasing age and decreasing reproductive opportunities (e.g., Angelier et al., 2020), but in other cases found no relationship (e.g., Angelier et al., 2007). To our knowledge, this is the first study to document a positive relationship between total length (correlated with age in hellbenders; Taber et al., 1975) and the glucocorticoid response in a free-living amphibian.

In conclusion, we provide one of the first descriptions of the steroid endocrinology of a non-teleost vertebrate that exhibits solitary paternal care. We demonstrate strong similarities as well as some notable differences between endocrinological patterns of hellbenders and uniparental male teleosts. Given the focal species' imperiled conservation status, and the recent discovery of filial cannibalism as a driver of reproductive failure in hellbenders (Hopkins et al., 2023)—this study also serves to contextualize the seasonal endocrinology of a successfully reproducing population. This latter contribution is critical for future investigations across the species' range exploring variance in nesting success, demography, and population viability.

## 5. Ethical considerations

All protocols were approved under a Virginia Department of Game and Inland Fisheries handling permit, and the Virginia Tech Institutional Animal Care and Use Committee (VT IACUC Numbers 08-085, 11-140, 13-128, 16-162, 18-186 and 19-147).

## CRediT authorship contribution statement

**Brian F. Case:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Jordy Groffen:** Writing – review & editing, Validation, Methodology, Investigation, Data curation.

**Thomas M. Galligan:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Catherine M. Bodinof Jachowski:** Writing – review & editing, Validation, Methodology, Investigation. **John J. Hallagan:** Writing – review & editing, Validation, Methodology, Investigation. **Sherry B. Hildreth:** Writing – review & editing, Validation, Supervision, Methodology. **Valentina Alaasam:** Writing – review & editing, Investigation. **W. Keith Ray:** Investigation, Methodology, Writing – review & editing. **Richard F. Helm:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition. **William A. Hopkins:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

## Declaration of competing interest

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

## Data availability

Data will be made available on request.

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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.2024.114547.

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