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Cortisol is the predominant glucocorticoid in the giant paedomorphic hellbender salamander (*Cryptobranchus alleganiensis*)

William A. Hopkins[a,*], Sarah E. DuRant[b], Michelle L. Beck[a,c], W. Keith Ray[d], Richard F. Helm[d], L. Michael Romero[e]

[a] Dept of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, VA 24061, USA
[b] Dept of Biological Sciences, University of Arkansas, Fayetteville, AR 72701, USA
[c] Dept. of Biology, Rivier University, Nashua, NH 03060, USA
[d] Dept of Biochemistry, Virginia Tech, Blacksburg, VA 24061, USA
[e] Dept of Biology, Tufts University, Medford, MA 02144, USA

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ABSTRACT

Corticosterone is widely regarded to be the predominant glucocorticoid produced in amphibians. However, we recently described unusually low baseline and stress-induced corticosterone profiles in eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*), a giant, fully aquatic salamander. Here, we hypothesized that hellbenders might also produce cortisol, the predominant glucocorticoid used by fishes and non-rodent mammals. To test our hypothesis, we collected plasma samples in two field experiments and analyzed them using multiple analytical techniques to determine how plasma concentrations of cortisol and corticosterone co-varied after 1) physical restraint and 2) injection with adrenocorticotropic hormone (ACTH), the pituitary hormone responsible for triggering the release of glucocorticoids from amphibian interrenal glands. Using liquid chromatography-mass spectrometry, we found that baseline and restraint-induced plasma concentrations of cortisol were more than five times those of corticosterone. We then demonstrated that plasma concentrations of both glucocorticoids increased in response to ACTH in a dose-dependent manner, but cortisol concentrations were consistently higher (up to 10-fold) than corticosterone. Cortisol and corticosterone concentrations were not correlated with one another at basal or induced conditions. The extremely low plasma concentrations of corticosterone in hellbenders suggests that corticosterone could simply be a byproduct of cortisol production, and raises questions as to whether corticosterone has any distinct physiological function in hellbenders. Our results indicate that hellbenders produce cortisol as their predominant glucocorticoid, supporting a small and inconclusive body of literature indicating that some other amphibians may produce appreciable quantities of cortisol. We hypothesize that the use of cortisol by hellbenders could be an adaptation to their fully aquatic life history due to cortisol's ability to fulfill both mineralocorticoid and glucocorticoid functions, similar to its functions in fishes. Given the large number of amphibian species that are fully aquatic or have aquatic life stages, we suggest that the broadly held assumption that corticosterone is the predominant glucocorticoid in *all* amphibians requires further scrutiny. Ultimately, multi-species tests of this assumption will reveal the ecological factors that influenced the evolution of endocrine adaptations among amphibian lineages, and may provide insight into convergent evolution of endocrine traits in paedomorphic species.

1. Introduction

Corticosterone is widely regarded as the predominant glucocorticoid produced in most amphibians in response to energetically demanding and "stressful" conditions (Greenburg and Wingfield, 1987; Herman, 1992; Norris and Carr, 2013; Wingfield and Romero, 2001; Moore and Jessop, 2003). Anurans (frogs and toads) release corticosterone when faced with a vast array of environmental stimuli including

Abbreviations: ACTH, adrenocorticotropic hormone; EIA, enzyme immunoassay; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography mass spectrometry; MDLC, multi-dimensional liquid chromatography; RIA, radioimmunoassay; P_4, Progesterone; $17OHP_4$, 17α-hydroxyprogesterone; DOC, 11-deoxycorticosterone; S, 11-deoxycortisol

* Corresponding author.
E-mail address: hopkinsw@vt.edu (W.A. Hopkins).

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exposure to pollutants (Hopkins et al., 1997), capture and handling (Belden et al., 2010), pathogens (Gabor et al., 2013), pond drying (Denver, 1998), predators (Maher et al., 2013), competition (Glennemeier and Denver, 2002a), and food deprivation (Glennemeier and Denver, 2002a; Reeve et al., 2013). In addition, corticosterone modulates energetically expensive anuran calling behaviors during the reproductive season (Hopkins et al., 1997; Emerson and Hess, 2001; Leary et al., 2006; Moore and Jessop, 2003; Eikenaar et al., 2012) and key developmental events associated with anuran metamorphosis (Hayes, 1997; Denver, 1998; Wright et al., 2003). Likewise, Urodeles (salamanders) release corticosterone in response to an equally diverse array of stimuli (e.g., Gendron et al., 1997; Homan et al., 2003; Cooperman et al., 2004; Schubert et al., 2009; Hopkins and DuRant, 2011; Ricciardella et al., 2010; Davis and Gabor, 2015; DuRant et al., 2015; but see Fonner and Woodley, 2015). Although generalizable patterns (e.g., corticosterone as the dominant glucocorticoid in amphibians) are widespread in endocrinology, exceptions to these generalizations can reveal important areas of ecological and evolutionary divergence among taxa.

We recently described unusual corticosterone profiles in eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*), the only North American member of the ancient giant salamander Family Cryptobranchidae. Hellbenders are obligate paedomorphs that display a number of unusual physiological, genetic, and morphological traits compared to other amphibians (Nickerson and Mays, 1973; Jerrett and Mays, 1973; Sun and Mueller, 2014), but their endocrine physiology remains poorly studied. We demonstrated that this giant salamander produces atypically low concentrations of corticosterone in response to handling and restraint (Hopkins and DuRant, 2011; DuRant et al., 2015). Baseline concentrations of corticosterone in this species average ~0.25 ng/ml and increase to ~1.0 ng/ml after 1–3 h of restraint (Hopkins and DuRant, 2011; DuRant et al., 2015). Generally speaking, these concentrations are a fraction of those observed in other salamander species (Cooperman et al., 2004; Ricciardella et al., 2010; Schubert et al., 2009; Woodley and Lacy, 2010). Understanding whether hellbenders physiologically operate with unusually low concentrations of corticosterone, or if they use an alternative glucocorticoid, could further our understanding of ecological factors that influence the evolutionary divergence of endocrine adaptations among vertebrate lineages.

Because hellbenders belong to one of the earliest diverging lineages of modern day salamanders (Frost et al., 2006) and exhibit a fully aquatic lifecycle, we hypothesized that their glucocorticoid profiles might more closely resemble an ancestral state that is more similar to fishes than many other amphibians. Most teleosts produce cortisol which performs both mineralocorticoid and glucocorticoid functions (Mommsen et al., 1999; Bentley, 2002; McCormick and Bradshaw, 2006; Takahashi and Sakamoto, 2013). This possibility is supported by a small body of evidence suggesting that some fully aquatic amphibians produce appreciable quantities of cortisol (Phillips and Chester Jones, 1957; Chester Jones et al., 1959; Bern and Nandi, 1964; Nandi, 1967; Norris and Carr, 2013). As an alternative explanation to the ancestral osteichthyian condition, use of cortisol could be an adaptation to aquatic life in paedomorphic species, which has independently evolved in multiple salamander families including Cryptobranchidae, Proteidae, Sirenidae, Ambysotomatidae, Amphiumidae, and Salamandridae (Frost et al., 2006). Little is known about the glucocorticoid physiology of species in several of these families. To address our hypothesis that cortisol could be an important glucocorticoid in hellbenders, we conducted two field experiments and then used several analytical techniques to determine: 1) if cortisol and corticosterone were both produced by hellbenders in response to handling, and 2) how these two hormones varied in concentration after injection with multiple concentrations of adrenocorticotropic hormone (ACTH), the pituitary hormone responsible for triggering the release of glucocorticoids from vertebrate adrenal glands (a.k.a. interrenal glands in amphibians).

2. Methods

2.1. Study species

Hellbenders are giant salamanders in the Family Cryptobranchidae, which only contains two other species globally (*Andrias davidianus* and *A. japonicus* in China and Japan). All three species are considered imperiled and population trends suggest they could eventually face extinction. In North America, there are two recognized subspecies of hellbenders; the eastern subspecies (*Cryptobranchus alleganiensis alleganiensis*) and the Ozark subspecies (*C. a. bishopi*). Both subspecies have experienced precipitous population declines in the last few decades (Wheeler et al., 2003; Briggler et al., 2007; Foster et al., 2009), resulting in the Ozark subspecies receiving federal protection and one evolutionarily distinct lineage of the eastern subspecies being considered for similar status (USFWS 2011a,b, 2019). The last population stronghold for eastern hellbenders is believed to occur across portions of Appalachia. In the area of Virginia where our work was conducted, the major threats to hellbender populations are most likely habitat degradation, disease and parasitism, and direct human persecution (Hopkins and DuRant, 2011; Davis and Hopkins, 2013; DuRant et al., 2015; Hopkins et al., 2014; Hopkins et al., 2016; Jachowski and Hopkins, 2018).

Hellbenders are large (up to 70 cm total length), long-lived (> 30 years) fully aquatic salamanders that thrive in cool, well oxygenated lotic habitats (Nickerson and Mays, 1973; Taber et al., 1975; Petranka, 1998). Adult and subadult age classes require large cover objects such as boulders and bedrock crevices, and they feed predominantly on crayfish. In most populations, hellbenders are thought to be primarily nocturnal, but diurnal movement may become more frequent in mid- to late-summer when males become more aggressive and attempt to commandeer large nest rocks (Noeske and Nickerson 1979; Humphries and Pauley, 2005) and in the spring following prolonged fasting after overwintering (Hopkins, unpublished). Reproduction takes place in late August and early September under nest rocks where males externally fertilize eggs, and then remain to guard the eggs and larvae until the following April when larvae disperse from the nest (Hopkins, unpublished).

2.2. Hellbender sampling & treatment

We sampled hellbenders from a stream in the Tennessee River Basin in Southwest, VA that has been one of our focal study areas since 2007 (Hopkins and DuRant, 2011; Davis and Hopkins, 2013; DuRant et al., 2015; Hopkins et al., 2016; Jachowski and Hopkins, 2018). During diurnal skin-diving surveys (from 0942 to 1636 h), we collected hellbenders by turning cover objects and searching crevices with dive lights. We conducted all surveys in late summer (31 July – 21 Aug 2012; IACUC #11-140 FIW) prior to nesting because at this time adult male and female hellbenders can be distinguished by the swollen cloaca of males (Makowsky, et al., 2010). Once a hellbender was captured, we immediately (average time 130 ± 6 s) obtained an initial baseline blood sample from the caudal vein using a heparinized 26 gauge needle affixed to a 1 cc syringe (Hopkins and DuRant, 2011).

To verify which steroid hormones increased in response to handling stress, we conducted an initial pilot experiment where 5 adult hellbenders (3 females, 2 males) were restrained in mesh bags submerged in the stream after the initial blood collection. These individuals were bled a second time after one hour of restraint. Blood from the initial and post-restraint bleeds was immediately placed on ice and returned to the laboratory where plasma was separated by centrifugation. Plasma from these individuals was stored frozen (-80° C) and later subjected to liquid chromatography-mass spectrometry (see methods below) to quantify changes in cortisol and corticosterone due to capture and restraint. This pilot experiment verified that both hormones were present in plasma (see results), and justified a more in depth exploration of their

dynamics.

For the comprehensive experiment, we transported each hellbender (after the initial blood sample) to the stream bank to be assigned to a treatment group and measured. Hellbenders were immediately weighed, sexed, and then assigned to one of four treatment groups: saline [control], 50, 100, or 200 IU kg^{-1} of ACTH (N = 11 per treatment group). In total, 44 hellbenders were included in the study. We stratified sexes (N = 6–7 females & 4–5 males per treatment) and body sizes (average 40.3–41.5 cm total length in each treatment) across all four treatment groups. We used intraperitoneal injections of porcine ACTH (Sigma Chemical A6303) because it provoked adrenal responses in previous studies with amphibians and reptiles (e.g. Glennemeier and Denver, 2002b; Romero and Wikelski, 2006), including hellbenders (DuRant et al., 2015). We transported aliquots of different pre-made stock solutions for each ACTH treatment group to the field on liquid nitrogen. We defrosted aliquots at the time of capture and adjusted volumes of injections according to the hellbender's mass, and used similar volumes of saline for the control group (total volume range across treatments = 120–160 μL). To monitor responses to injections, we bled hellbenders again at 1- and 3-h post capture by holding them in mesh dive bags submerged in shaded areas within the stream near the site of capture. Our prior work demonstrated that peak adrenal responses to restraint and ACTH injection occurred within 3 h of capture (DuRant et al., 2015). After completing the third blood draw at 3 hrs post-capture, we released each hellbender under the rock in which it was initially found.

Before placing a hellbender into a mesh bag for the three hour restraint period, we processed each individual according to our previously described methods. Briefly, we measured (total length and snout-vent length) each hellbender and classified them as juvenile or adult based on size (adult: ≥29 cm total length in our study system; Hopkins and DuRant, 2011). Only adults were used in this study. We documented the presence/absence of ectoparasites (leeches), because our previous work demonstrated that parasitism influences hellbender physiology (DuRant et al., 2015; Hopkins et al., 2016). We also injected a unique passive integrated transponder tag (PIT tag) into the tail of each hellbender as part of our long-term mark recapture efforts.

2.3. Hormone analyses

We used three techniques to measure glucocorticoid hormones. We first used mass spectrometry to measure both corticosterone and cortisol in individuals before and after being subjected to short term restraint. For the larger ACTH experiment, we used an enzyme immunoassay (EIA) to measure corticosterone that we had validated in previous studies (Hopkins and DuRant, 2011; DuRant et al., 2015), and a second EIA that we validated to measure cortisol herein. We also used mass spectrometry results as an external verification of our EIA results.

2.3.1. Liquid chromatography–mass spectrometry (LC–MS)

We transferred plasma (50 μL) to a 1.5 mL EtOH-washed tube followed by a standard solution (5 μL) containing 75 ng/mL each of d4-cortisol and d8-corticosterone. We mixed the sample with a precipitation solution (160 μL) that contained methanol, ethanol, acetone; (1:1:1; v/v/v) and vortexed for 1 min. We centrifuged the resulting sample (13,000 × *g*; 10 min) and the supernatant was transferred to a fresh tube and taken to dryness on a high-vacuum line. We then reconstituted the sample in water: methanol (50 μL; 80:20, v/v) to be used in LC–MS analysis.

We injected 10 μL of each sample onto a Jupiter 4 μ Proteo 90 Å 50 × 0.5 mm reversed phase column (Phenomenex) using a G1313A 1100 series autosampler (Agilent). The HPLC gradient was developed utilizing a Tempo nano MDLC (Eksigent) and column effluent was introduced into an 4000 QTrap mass spectrometer (Applied Biosystems) utilizing a TurboIonSpray electrospray source (Applied Biosystems). Solvents A and B were LC-MS grade water (Spectrum Chemical) supplemented with 0.1% (v/v) formic acid and LC–MS grade acetonitrile (Spectrum Chemical) supplemented with 0.1% (v/v) formic acid, respectively. Flow rate was 20 μL/min throughout the entire 30 min gradient and initial conditions were 80% solvent A and 20% solvent B. Initial conditions were maintained for 5 min following injection of the sample. A linear gradient from 80% A to 20% A over 10 min followed. The 20% A was maintained for 5 min followed by a linear gradient back to 80% A and a re-equilibration at 80% A for 5 min prior to injection of the next sample. We quantified cortisol and corticosterone utilizing the sum of the transition intensities described in Table 1 following normalization to the sum of the transition intensities for the internal standards d4-cortisol and d8-corticosterone, respectively, also listed in Table 1. Data acquisition conditions for the mass spectrometer were: ion spray 5200 V, source temperature 180 °C, interface heater on, CAD gas medium, entrance potential 10 V. Curtain gas, ion source gas 1, and ion source gas 2 were set to 12, 12 and 24, respectively.

2.3.2. Corticosterone enzyme immunoassay

We determined plasma corticosterone levels using enzyme immunoassays (EIAs; Cat: No. 900-097, Assay Designs) that were previously validated and optimized for hellbenders (Hopkins and DuRant, 2011; DuRant et al. 2015). These EIAs have low cross-reactivity with other major steroids such as cortisol (0.046%) according to the manufacturer.

Based on our optimization, we analyzed samples at a 1:3 plasma dilution with 2% steroid displacement buffer. Each 96-well plate included a standard curve ranging from 15.63 to 2000 pg/ml with standards (non-specific binding and total binding) assayed in triplicate. Each plate also contained a 500 pg/ml standard assayed in quadruplicate to allow us to estimate interplate variability. We ran samples in duplicate. Assay detection limit ranged from 0.016 to 0.06 ng/ml. When plasma corticosterone concentrations fell below the detection limit, which occurred in 16 out of 132 samples, we used the plate's detection limit as the plasma corticosterone concentration for that individual sample. We calculated intra-assay variation as the average coefficient of variation across all samples within each plate and inter-

Table 1
Mass spectrometer selected reaction monitoring, transition, and instrumental parameters. Retention times: Cortisol = 6.5 min; corticosterone = 11.9 min.

Analyte	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	DP (V)	CE (V)	CXP (V)
Corticosterone	347.1	311.0	100	81	23	18
	347.1	121.0	100	81	35	20
Cortisol	363.1	121.0	100	81	33	20
	363.1	97.0	100	81	51	16
d4-Cortisol	367.1	121.0	100	86	33	20
	367.1	97.0	100	86	51	16
d8-Corticosterone	355.1	337.0	100	91	23	20
	355.1	125.2	100	93.5	40	14

DP = declustering potential; CE = collision energy; CXP = collision cell exit potential.

assay variation across plates as the coefficient of variation of the 500 pg/ml standards. Intra- and inter-assay variation was 3.74% and 3.23%, respectively.

2.3.3. Cortisol enzyme immunoassay

We used Enzo Life Sciences EIAs (Cat. 900-071) to quantify cortisol levels in eastern hellbenders. To validate the kits, we quantified cortisol concentrations in the same samples using side-by-side comparisons of mass spectrometry and the kit, and obtained highly concordant cortisol concentrations with the two methods ($r = 0.97$, $p < 0.001$; $n = 10$). In the course of our validation of the EIA, we determined that cortisol concentrations were not quantifiable unless we extracted the samples prior to running the assay. To extract samples, we placed 25 µL of plasma in glass tubes and diluted the sample with 400 µL of distilled water and then vortexed the mixture. We extracted the cortisol by adding 4 mL of dichloromethane to the samples and placed the samples on a shaker for 10 min after which samples sat for 1 h at room temperature without shaking. We removed the lower layer and dried it in a water bath of 38 °C under nitrogen gas until they were dry. Samples were reconstituted in 250 µL of assay buffer and vortexed for the assay. Average recovery of cortisol from extractions was 61% and we corrected our final cortisol concentrations based on this extraction efficiency.

We distributed samples across seven 96 well plates. EIA ingredients were allowed to warm to room temperature for at least 30 min prior to running the assay. Each plate contained a standard curve that ranged from 67.2 to 10,000 pg/ml and each standard curve sample was run in triplicate. We ran a single plasma sample in duplicate on multiple plates in order to assess inter-assay variation that was due to the extraction procedure and the variation among plates. All samples were run in duplicate and an additional standard of 3 ng/ml cortisol was run on each plate as well. The assay had a detection limit of 0.085 ng/ml. We calculated intra-assay variation as the average coefficient of variation between duplicate samples on each plate and inter-assay variation as the coefficient of variation among the standards on every plate. Intra-assay variation was 13.71% and inter-assay variation was 20.64%.

2.4. Statistical analyses

All statistical analyses were run in Microsoft Excel or SAS 9.1 (SAS Institute Inc., Cary, NC, USA) and statistical significance was recognized at $\alpha < 0.05$. Where appropriate, we tested for normal distribution of the data and homoscedasticity using Ryan-Joiners and Bartlett's tests, respectively. Corticosterone was $\log_{10}$–transformed to better meet assumptions of normality, but raw data were used in other statistical models. We obtained insufficient plasma from three individuals injected with saline to run both hormone analyses; only corticosterone is reported for these three individuals.

To test for the effects of ACTH or saline injection on $\log_{10}$–corticosterone and cortisol concentrations over time, we performed two repeated measures ANOVAs (SAS Proc mixed) that included treatment, leech, and time as main effects and all 2- and 3-way interactions. We included leech in our models to account for the variation that they can provoke in hellbender hormone concentrations (DuRant et al., 2015), but a thorough discussion of this effect is reserved for companion manuscripts (DuRant et al., 2015; DuRant et al., unpublished). We also tested for relationships between corticosterone and cortisol concentrations at each sampling time point using correlation analysis.

3. Results

Our liquid chromatography-mass spectrometry analysis confirmed that cortisol is present in much higher concentrations in hellbender plasma than corticosterone (Fig. 1). Specifically, baseline and restraint stress-induced concentrations of cortisol were 5.5 and 5.7 times higher than corticosterone concentrations, respectively. In addition, the corticosterone values quantified from mass spectrometry were well aligned with concentrations of corticosterone that we previously reported using EIA and radioimmunoassay methods (Hopkins and DuRant, 2011; DuRant et al., 2015).

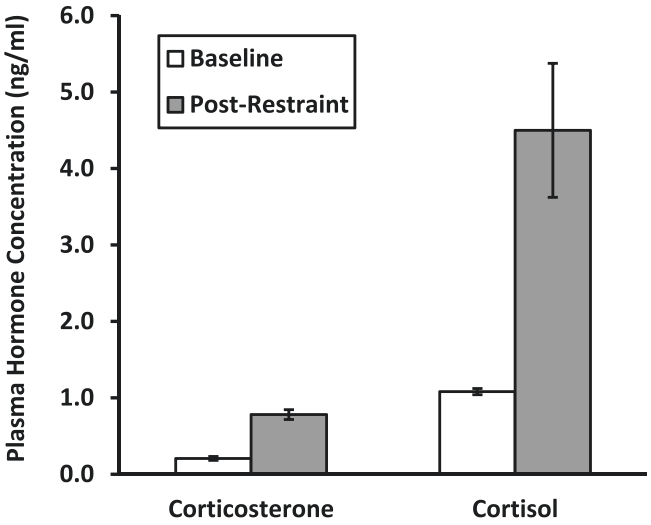

Fig. 1. Plasma concentrations of cortisol and corticosterone (ng/ml) in hellbenders at the time of capture and after one hour of restraint. Concentrations were measured from the same 5 individuals using liquid chromatography-mass spectrometry. Values are reported as mean ± 1 SE.

In our second field experiment, both plasma corticosterone and cortisol concentrations increased due to capture and injection with saline and ACTH, but cortisol concentrations were an order of magnitude higher than corticosterone at every time point (Fig. 2). In general, injection of 50 IU/kg ACTH provoked similar increases in plasma glucocorticoids as injection with saline alone (Fig. 2). However, injection with 100 and 200 IU/kg ACTH induced the release of higher concentrations of both glucocorticoids over time (cortisol: Time: $F_{2,64} = 58.06$; $p < 0.0001$ Treatment × Time: $F_{6,64} = 2.42$; $p = 0.036$; corticosterone: Time $F_{2,61} = 27.54$; $p < 0.0001$ Treatment × Time × Leech $F_{5,61} = 3.29$; $p = 0.011$). The ACTH dose-dependent temporal dynamics of these changes differed between the two glucocorticoids. Whereas the highest plasma concentrations of corticosterone were induced by 200 IU/kg ACTH after one hour, cortisol concentrations continued to rise in plasma and were highest at 3 h in response to the same dose of ACTH. Interestingly, plasma corticosterone and cortisol concentrations did not correlate with each other at any time point (in all cases $r \leq 0.10$; $p \geq 0.52$).

4. Discussion

We demonstrated that plasma concentrations of both corticosterone and cortisol increase in hellbenders responding to capture and restraint, but that cortisol is the predominant glucocorticoid in hellbenders. Baseline and restraint-induced plasma cortisol concentrations were > 5 times higher than corticosterone concentrations. Furthermore, injection with ACTH resulted in dose-dependent increases in both plasma glucocorticoids, but the induced concentrations of cortisol were again much higher than corticosterone. In fact, ACTH-injection produced up to 10-fold higher plasma cortisol concentrations compared to plasma corticosterone concentrations. Such large differences in circulating levels of the two glucocorticoids suggest that cortisol is likely the predominant glucocorticoid produced by hellbenders, and that corticosterone's physiological significance is uncertain. While it is possible that the two hormones have different physiological roles (e.g., tissue-specific importance in developing songbirds; Schmidt and Soma, 2008), it is also possible that corticosterone has negligible importance.

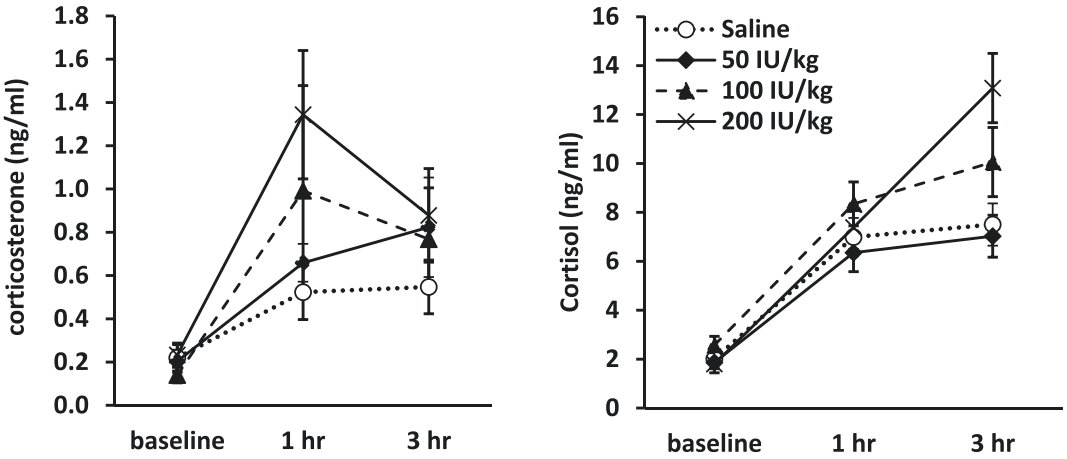

Fig. 2. Plasma concentrations of corticosterone and cortisol (ng/ml) in hellbenders at the time of capture and after injections with saline or one of three doses of ACTH (N = 11 per treatment group). Concentrations were measured using EIAs that were verified against results from liquid chromatography-mass spectrometry. Values are reported as mean ± 1 SE. Note difference in scale on y-axes.

Our findings suggest that corticosterone could simply be a metabolic byproduct of cortisol production in hellbenders (Fig. 3). In other vertebrates, the biosynthetic pathways for both glucocorticoids involve several identical precursors (i.e., cholesterol, pregnenolone, and progesterone) and enzymes (e.g., 21-hydroxylase and 11β-hydroxylase), meaning that production of cortisol inherently requires production of corticosterone precursors and activation of corticosterone-producing enzymes (reviewed in: Payne and Hales, 2004; Norris and Carr, 2013). Considering that hellbenders exhibit robust production of glucocorticoids following treatment with porcine ACTH, it is reasonable to assume that hellbender interrenal physiology follows the general vertebrate paradigm (Fig. 3). Progesterone (P_4) is the immediate upstream shared precursor for both cortisol and corticosterone. To produce cortisol, P_4 is converted into 17α-hydroxyprogesterone ($17OHP_4$) by the enzyme 17α-hydroxylase. Thereafter, the pathways for cortisol and corticosterone production involve identical enzymes; 21-hydroxylase converts P_4 and $17OHP_4$ into 11-deoxycorticosterone (DOC) and 11-deoxycortisol (S), respectively. Similarly, 11β-hydroxylase then converts DOC and S into corticosterone and cortisol, respectively. Thus, when adrenal stimulation activates the cortisol biosynthesis pathway, the subsequent increase in production of shared precursors such as P_4 and activation of 21-hydroxylase and 11β-hydroxylase could logically lead to production of corticosterone as a byproduct (i.e., small amounts of P_4 are inadvertently converted to corticosterone while the majority of P_4 is processed into $17OP_4$ for cortisol production).

Interestingly, plasma concentrations of cortisol and corticosterone were not correlated with one another within individuals at any time point, and show different temporal profiles after hellbenders were

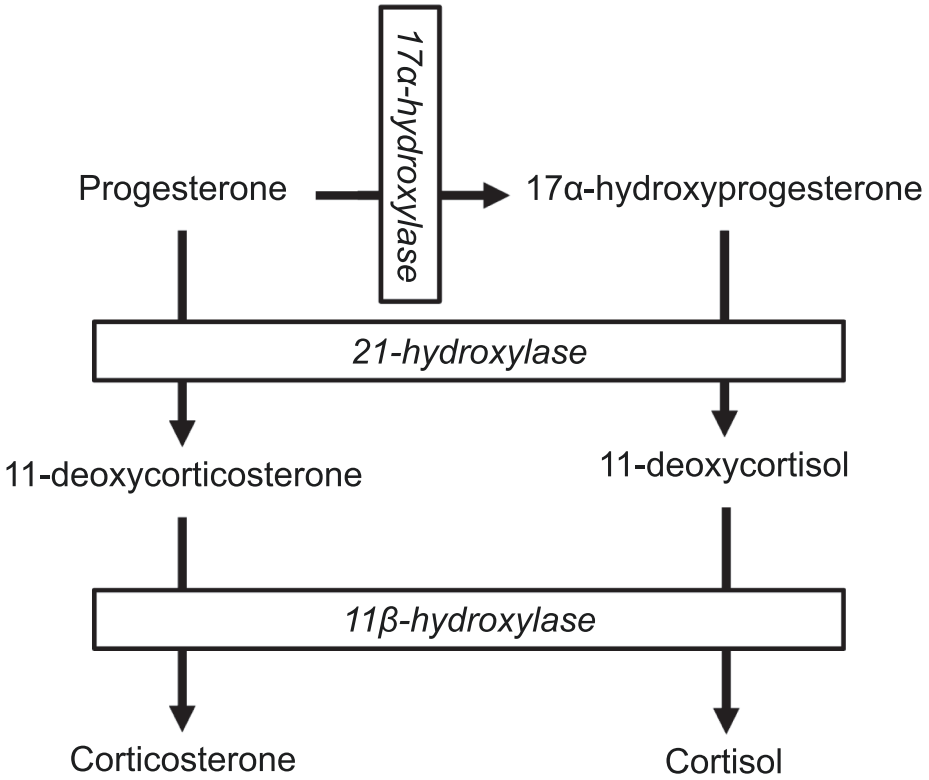

Fig. 3. Biosynthetic pathways of cortisol and corticosterone. Note the shared precursors and enzymes of the two pathways, suggesting the possibility that corticosterone could be the byproduct of cortisol production in hellbenders. Figure Credit: Thomas Galligan, based on Payne and Hales, 2004.

Table 2
Summary of studies that have measured both cortisol and corticosterone in amphibians. Detection of each steroid is presented as Detected (Yes) or Not Detected (No).

Species	Lifestage	Corticosterone	Cortisol	Predominant GC	Method or Source	Citation
Xenopus laevis	Adult[1]	No	Yes	Cortisol	Plasma, chromatography	Phillips and Chester Jones, 1957
Aphiuma sp.	Adult	Yes	Yes	Corticosterone	Whole Blood, chromatography	Chester Jones et al., 1959
Rana catesbeiana	Adult	Yes	No	Corticosterone	*In vitro* interrenal incubation, chromatography	Carstensen et al., 1961
Rana sylvatica	Larvae	No[2]	Yes	Cortisol	Urine, chromatography	Dale, 1962
Rana pipiens	Larvae	No[2]	Yes	Cortisol	Urine, chromatography	Dale, 1962
Bufo marinus	Adult	No[2]	Yes	Cortisol	*In vitro* interrenal incubation, chromatography	Crabbe, 1963
Pleurodeles waltlii	Adult	Yes	No	Corticosterone	*In vitro* interrenal incubation, chromatography	Ozon and Dupuis-Certain, 1967
Rana catesbeiana	Adult	Yes	No	Corticosterone	Plasma, chromatography	Johnston et al., 1967
Rana rugulosa	Adult	Yes	No	Corticosterone	*In vitro* interrenal incubation, chromatography	Chan et al., 1969
Xenopus laevis	Adult	Yes	No	Corticosterone	*In vitro* interrenal incubation, chromatography	Chan and Edwards, 1970
Rana pipiens	Adult	Yes	No	Corticosterone	*In vitro* interrenal incubation, chromatography	Jungreis et al., 1970
Andrias davidianus	Adult	Yes	No	Corticosterone	*In vitro* interrenal incubation, chromatography	Chan et al., 1975
Rana catesbeiana	Adult	Yes	No	Corticosterone	*In vitro* interrenal incubation, chromatography	Mehdi and Carballeira, 1971
Rana catesbeiana	Larvae	Yes	Yes	Corticosterone	Plasma, radioimmunoassay	Krug et al., 1983
Rana catesbeiana	Juvenile	Yes	Yes	Cortisol	Plasma, radioimmunoassay	Krug et al., 1983
Rana catesbeiana	Adult	Yes	Yes	Cortisol	Plasma, radioimmunoassay	Krug et al., 1983
Xenopus laevis	Larvae	Yes	No	Corticosterone	*In vitro* interrenal incubation, chromatography	Jolivet-Jaudet and Lelop-Hatey, 1984
Xenopus laevis	Juvenile	Yes	No	Corticosterone	*In vitro* interrenal incubation, chromatography	Jolivet-Jaudet and Lelop-Hatey, 1984
Ambystoma tigrinum	Larvae	Yes	Yes	Corticosterone	Plasma, radioimmunoassay	Stiffler et al., 1986
Ambystoma tigrinum	Adult	Yes	Yes	Corticosterone	Plasma, radioimmunoassay	Stiffler et al., 1986
Rana pipiens	Adult	Yes	No	Corticosterone	Plasma, radioimmunoassay	Stiffler et al., 1986
Triturus carnifex	Adult	Yes	Yes	~Equivalent	Plasma, radioimmunoassay	Zerani and Gobbetti, 1991
Triturus carnifex	Adult	Yes	Yes	~Equivalent	*In vitro* interrenal incubation, radioimmunoassay	Zerani and Gobbetti, 1991
Rana esculenta	Adult	Yes	Yes	~Equivalent	Plasma, radioimmunoassay	Gobbetti and Zerani, 1993
Rana esculenta	Adult	Yes	Yes	~Equivalent	*In vitro* interrenal incubation, radioimmunoassay	Gobbetti and Zerani, 1993
Rana catebeiana	Larvae	Yes	Yes	Corticosterone	Plasma, radioimmunoassay	Wright et al., 2003
Rana catebeiana	Adult	Yes	Yes	Corticosterone	Plasma, radioimmunoassay	Wright et al., 2003
Cryptobranchus alleganiensis	Adult	Yes	Yes	Cortisol	Plasma, LC–MS and enzyme immunoassay	Current Study

[1] Study does not provide lifestage, but we assume animals were adult because plasma was analyzed.
[2] Study states they measured corticosterone, but no concentration was reported. Thus, we assume it was not detected.

injected with ACTH. Whereas corticosterone decreased in plasma 3 h post-injection with 100 and 200 IU/kg ACTH, cortisol concentrations were still rising three hours after injection with the same two ACTH doses. These observations suggest that while more cortisol was being secreted into circulation, corticosterone production was either decreasing or it was being cleared from circulation. Similar lack of covariation between these two glucocorticoids has been described in several species of mammals that also primarily rely on cortisol (Koren et al., 2012). There are several possible mechanisms through which this could arise. For example, 17α-hydroxylase activity could steadily increase over the treatment period (3 hrs), leading to a progressive increase in the rate at which P_4 is metabolized into $17OHP_4$, effectively reducing the quantity of P_4 available for corticosterone production concomitantly with increased cortisol production (Fig. 3). Alternatively, cortisol might exhibit a greater affinity for hellbender corticosteroid binding globulin (CBG) compared to corticosterone, allowing cortisol to displace bound corticosterone in circulation, which progressively accelerates corticosterone clearance. Enzyme activity and CBG affinity studies would be required to test these hypotheses and fully understand these observed temporal dynamics.

Although the overwhelming majority of previous research shows that corticosterone is the predominant glucocorticoid in amphibians, our work supports a small and inconclusive body of literature that identifies cortisol production in some amphibian species (Table 2). It is has been postulated that the use of cortisol rather than corticosterone might be associated with the fully aquatic lifestyle of a small number of species and life stages (Norris and Carr, 2013). For example, there is some evidence that fully aquatic paedomorphic species such as *Amphiuma* produce appreciable quantities of cortisol (Chester Jones et al., 1959; Bern and Nandi, 1964; Nandi, 1967). Likewise, aquatic adult stages of the Italian crested newt (*Triturus carnifex*) produce nearly equivalent concentrations of both cortisol and corticosterone (Zerani and Gobbetti, 1991). However, a review of available evidence from studies that have measured both cortisol and corticosterone in amphibians yields equally contradictory evidence, and thus inconclusive patterns among species with different life histories (Table 2). Not only are results of these studies mixed, but many of them rely on *in vitro* incubation of interrenal tissue with exogenous precursors to yield glucocorticoids. Although these *in vitro* techniques confirm the glandular presence of enzymes important for steroidogenesis, they do not necessarily reflect the relative hormone concentrations that ultimately circulate in plasma. Additional comparative studies that measure plasma concentrations of both cortisol and corticosterone in amphibians are needed, ideally using LC–MS to eliminate cross-reactivity issues associated with antibody-based assays (Koren et al., 2012).

It is possible that the use of cortisol by hellbenders is an adaptation associated with paedomorphosis, and that other paedomorphic species exhibit similar endocrine characteristics. The switch in salamanders to fully-aquatic life stages or obligate paedomorphism has occurred independently multiple times (Bonett and Blair, 2017). Interestingly, recent research indicates paedomorphic amphibians share several convergent traits, including body morphology and expression of and insensitivity to thyroid hormones (Bonett and Blair, 2017; Aran et al., 2014). The use of cortisol as the primary glucocorticoid could also be the result of convergent evolution in paedomorphic species because it could provide advantages to an aquatic life style, but support for this hypothesis based on a small number of obligate and facultative paedomorphic species is also mixed (Table 2), including conflicting evidence between paedomorphic species within Cryptobranchidae (current study and Chan et al., 1975). Our findings suggest that additional work is required to verify the importance of cortisol across aquatic amphibian species and life stages, especially in paedomorphic species and in early divergent amphibian lineages.

The adaptive advantage of cortisol over corticosterone in hellbenders remains unknown, but it may be attributable to its ability to regulate plasma ion concentrations in aquatic species. Cortisol binds to both the mineralocorticoid and glucocorticoid receptors in other vertebrates (mammals: Pippal and Fuller, 2008; fishes: Takahashi and Sakamoto, 2013) and is known to have important ionoregulatory roles in some vertebrates (Mommsen et al., 1999; Bentley, 2002; McCormick

and Bradshaw, 2006; Takahashi and Sakamoto, 2013). For example, cortisol appears to be the predominant hormone regulating both mineralocorticoid and glucocorticoid functions in fishes, and they tend to lack appreciable quantities of aldosterone, the principle mineralocorticoid in terrestrial tetrapods (Mommsen et al., 1999; Bentley, 2002; McCormick and Bradshaw, 2006; Takahashi and Sakamoto, 2013). Thus, it seems possible that cortisol plays dual mineralocorticoid and glucocorticoid functions in hellbenders and some other fully aquatic amphibians (Norris and Carr, 2013). Future comparative work of cortisol, corticosterone, and aldosterone dynamics among terrestrial and aquatic lineages of amphibians could help elucidate whether reliance on cortisol is in fact an ionoregulatory adaptation to aquatic life.

4.1. Conclusions

Given the plight of hellbender populations in recent decades, understanding their glucocorticoid physiology will be important to their conservation. Glucocorticoid responses to environmental conditions are a key tool used by conservation physiologists in efforts to monitor individual health, especially with species of conservation concern like the hellbender (Wasser et al., 1997, 2000; Wikelski and Cooke 2006; Cook, 2012; Cooke et al., 2013; Narayan, 2013; McCormick and Romero, 2017). Our use of LC–MS provides the most conclusive evidence to date of an amphibian species relying on cortisol as their predominant glucocorticoid. Our work demonstrated that cortisol should be measured in future efforts to understand how hellbenders, and perhaps some other early diverging and/or paedomorphic amphibians, respond to environmental changes such as compromised water quality. Our LC–MS work confirmed our cortisol results from the EIA reported herein, as well as our corticosterone results from our previous studies that used EIA (Hopkins and DuRant, 2011; DuRant et al., 2015), and thus verified the utility of these two EIAs for future studies of hellbenders. Given the extremely low plasma concentrations of corticosterone in hellbenders, however, it is possible that corticosterone is simply a byproduct of cortisol production. Future work should determine whether corticosterone has any physiological importance in hellbenders, and whether cortisol influences multiple physiological processes such as iono-, osmo-, and blood glucose regulation. Moreover, additional work is needed to determine whether the patterns we observed in hellbenders occur in other early amphibian lineages and/or species that have evolved a paedomorphic lifecyle.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113267.

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