# An acute increase in the stress hormone corticosterone is associated with mating behavior in both male and female red-spotted newts, *Notophthalmus viridescens*

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## ARTICLE INFO

## ABSTRACT

Hormones play key, functional roles in mediating the tradeoff between survival and reproduction. Glucocorticoid hormones can inhibit reproduction and improve chances of survival during periods of stress. However, glucocorticoid hormones are, at times, also associated with successfully engaging in energetically costly courtship and mating behaviors. Corticosterone (CORT), a primary glucocorticoid hormone in amphibians, reptiles and birds, may be important in activating or sustaining energetically costly mating behaviors. We used a non-invasive, water-borne hormone assay to measure CORT release rates of male and female red-spotted newts (*Notophthalmus viridescens*) collected when either engaged in amplexus or when not engaged in amplexus. Because amplexus is energetically costly for males, we predicted that males would have higher CORT release rates than females. We also predicted that females in amplexus would have elevated CORT release rates because the restraint of amplexus prevents foraging and breathing and may be costly. Here we show that an acute increase in CORT is associated with amplexus behavior in both male and female red-spotted newts. Additionally we demonstrate that males have higher overall CORT release rates both in and out of amplexus than do females. Our results support the hypothesis that glucocorticoid hormones are associated with energetically costly courtship and mating behaviors for both sexes.

## 1. Introduction

The tradeoff between reproduction and survival has long been the basis of models of life history theory (Reznick, 1985; Stearns, 1989; Roff, 1992). Building on the wealth of evidence for a negative correlation between survival and reproductive success, the physiological mechanisms behind this tradeoff are frequent avenues of investigation (Rose and Bradley, 1998; Ketterson and Nolan, 1999; Zera and Harshman, 2001). Hormones play important roles in functionally mediating the tradeoff (Harshman and Zera, 2007).

Steroid hormones of the hypothalamic–pituitary–gonadal (HPG) axis are linked to reproductive behaviors and commonly induce measurable costs via reduced immune function, increased parasitism, shortened life span and decreased short-term survival (Grossman, 1985; Folstad and Karter, 1992; Cox and John-Alder, 2007; Flatt and Kawecki, 2007). By comparison, the relationship among glucocorticoid steroid hormones (GCs), the hypothalamic–pituitary–adrenal (HPA) axis (or hypothalamic-pituitary-interrenal (HPI) in amphibians, reptiles and birds), and reproduction are less established (Moore and Jessop, 2003; Breuner et al., 2008; Bonier et al., 2009). Stressors or behavioral interactions may induce the release of GCs by HPA or HPI and these often impose costs that reduce or entirely inhibit reproduction in response to stress (Moore and Miller, 1984; Greenberg and Wingfield, 1987; Wingfield et al., 1998; Salvante and Williams, 2003). In cases of prolonged stress, GCs can inhibit reproduction by causing direct interference at every level of the HPG axis (Rivier and Rivest, 1991). Aside from inhibitory effects on reproduction, elevated

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levels of corticosterone (CORT), a primary GC in birds, amphibians and reptiles, can have a host of other well documented physiological costs (Sapolsky et al., 2000). Experimentally elevated CORT decreases growth efficiency and diminishes cognitive ability later in life (Kitaysky et al., 2003) and also increases metabolic energy demands (Wack et al., 2012).

The context in which an animal mounts a CORT response is crucial to the cost that will be realized (Moore and Jessop, 2003; Leary et al., 2008). In many animal systems, CORT and cortisol, another primary GC hormone in vertebrates, are associated with, and possibly required, to maintain courtship and reproductive activity (Orchinik et al., 1988; Borg et al., 1991; Schramm et al., 1999; Jessop et al., 2002; Leary et al., 2008). Elevated GC hormones facilitate the mobilization of stored energy resources for energetically expensive mating behaviors, such as calling and amplexus by male frogs (Harvey et al., 1997; Emerson and Hess, 2001). Narayan et al., 2013 and Narayan and Hero, 2013 also suggest a link between CORT and other reproductive hormones in both male and female Fijian ground frogs, *Platymantis vitiana*. Given that elevated glucocorticoid hormones have physiological costs, acutely elevated glucocorticoid levels associated with mating behaviors are likely functionally involved in the tradeoff between reproduction and survival. However, it remains largely unknown how common the association between mating behavior and acute elevations of glucocorticoid hormones are.

Here we test for an association between courtship behavior and elevated CORT levels in male and female red-spotted newts (*Notophthalmus viridescens*). This species uses amplexus during courtship, so a CORT response associated with amplexus may activate stored energy to aid in successful courtship behavior. In general, energetically costly courtship behavior of males, such as male anurans calling and engaging in amplexus, generates a CORT response (Orchinik et al., 1988; Harvey et al., 1997; Leary et al., 2008). Although the female CORT response to mating has not been tested in many taxa, mating has been linked to an acute increase of CORT in female Japanese quail, *Coturnix japonica* (Rutkowska et al., 2011). Moreover, gravid female Fijian ground frogs have a higher baseline CORT as well as estrogen and progesterone than do non-gravid females (Narayan and Hero, 2013). In red-spotted newts, male biased sex ratios impose costs for females in terms of immunity and body condition and a HPI response was proposed as the proximate mechanism for this decrease in immunity and body condition (Grayson et al., 2012).

We performed three experiments during the late breeding season in a population of Eastern red-spotted newts. For all experiments we used a recently developed water-borne hormone collection method. The water-borne hormone assay was originally developed for fish (Scott and Ellis, 2007). This non-invasive method measures steroids that passively diffuse from the bloodstream into the water through urine and feces (Scott et al., 2008). Gabor et al. (2013) recently demonstrated a positive correlation between plasma CORT and water-borne CORT levels for multiple amphibian species. First, we performed an adrenocorticotropic hormone (ACTH) challenge on male newts to determine whether we could measure an HPI response using a non-invasive, water-borne hormone assay. Second, we examined how long it took newts to mount a CORT response after handling and captivity in the laboratory using the water-borne hormone assay. Finally, we evaluated water-borne CORT release rates associated with courtship activity in both sexes. We tested whether acutely elevated GC hormone levels are associated with courtship activity by measuring CORT release rates of male and female newts engaged in amplexus and compared them to release rates of non-amplectant (non-mating) individuals. We predicted that, given its role in mobilizing energy reserves, CORT release rates should be acutely elevated in both males and females during amplexus.

## 2. Materials and methods

### 2.1. Study system

We collected sexually mature red-spotted newts (*N. viridescens*) with dip nets at Riopel Pond (0.65 hectares) on the premises of Mountain Lake Biological Station in Giles County, VA (37.37 N, 80.52 W) in June 2013. Mating occurs at this location from March through June (Grayson et al., 2012). Males of this species engage in amplexus behavior with a female prior to any signs of female receptivity, typically grasping the female around the head and neck (Verrell, 1982, 1983). During amplexus, males exhibit tail fanning and chin rubbing behavior, which induces mating receptivity in the female via glandular secretions (Arnold and Houck, 1982; Verrell, 1982). Amplexus, in this population, lasts an average of 204 min before leading into the spermatophore transfer stage (Massey, 1988). During this time, the pair does not return to the surface to breathe (Verrell, 1982, 1983). At the completion of amplexus, males release the amplected female and then deposit a spermatophore that she may choose to pick up with her cloaca (Verrell, 1982, 1983). Animal welfare was approved by University of Virginia Animal Care and Use Committee (Protocol 3927-04-12).

### 2.2. ACTH challenge

On 14-June-2013 we collected male newts ($n = 36$) from 0900 to 0930 h to test whether the water-borne hormone collection technique measured an increase in CORT release in response to an adrenocorticotropic hormone (ACTH) challenge. We placed all newts into individual containers (473 ml) filled with pond water immediately after capture. We then walked to the on-site laboratory (30 m away) and measured the mass (g) of each newt. We assigned newts to one of three treatment groups at random. One group ($n = 12$) received an injection of 0.03 ml of ACTH (Sigma–Aldrich #A6303 – porcine) dissolved in ringer solution to deliver a 50 IU/g dose (ACTH). Another group ($n = 12$) received an injection of 0.03 ml of ringer solution (saline) as a control for the injection. A second control group ($n = 12$) received no injection, but was momentarily inverted to replicate the physical handing during injection of the other two treatment groups. We administered injections with a 29 gauge needle, into the coelom, lateral of the midline and at a low angle relative to the body wall to avoid injury to organs. Following injection (saline or ACTH) or control treatment, we immediately placed each newt in a 150 ml glass beaker, filled with 80 ml of spring water and lined with a Nalgene HDPE plastic sleeve modified with drainage holes on the bottom, for 60 min. Beakers were always cleaned with 95% ethanol and rinsed with DI water before being filled with spring water. Due to a limitation in number of hormone collection cups, hormones were sampled over two consecutive time periods. At time 1 (45–55 min in captivity, hereafter ∼50 min), we performed the appropriate treatment on seven animals (one escaped from the saline treatment) at which point they were placed in the water-borne hormone collection beaker for 60 min. At time 2 (105–115 min in captivity, hereafter ∼110 min), after the first group was finished and we cleaned the beakers, we then performed appropriate treatments on 5 animals from each group and placed them in water-borne hormone collection beakers for 60 min. Following leaching of hormones into the water, we measured snout to anterior vent length (SVL; mm) of each individual.

### 2.3. CORT response to handling and captivity over time

To examine how long it took newts to mount a CORT response after handling and captivity in the laboratory, we conducted an

experiment to induce stress via handling. We collected male newts ($n = 29$) from 0900 to 0930 h on 27-June-2013. As in the previous experiment, newts were collected with dip nets, immediately transferred to individual containers, and then transported to the on-site lab, where we removed them from containers and obtained their mass (g). We randomly assigned these newts to one of three groups to measure CORT release over 60 min beginning at three time points in captivity: at 50 min ($n = 10$), 110 min ($n = 9$), or at 170 min ($n = 10$). All water-borne hormone collection and sample processing followed the same procedures used in the ACTH challenge experiment. Following leaching of hormones into the water, we measured the SVL (mm) of each individual.

### 2.4. CORT and other variables as related to amplexus

We observed and recorded behavioral data on amplectant pairs ($n = 20$), non-amplectant (hereafter solo) females ($n = 20$) and solo males ($n = 20$) on 19–21-June-2013 from 1030 to 1410 h. Individuals and pairs were haphazardly chosen from newts nearby the observer. We recorded the number of individuals within a 1-m radius of the focal individual or amplectant pair each minute for a 10-min observation period. We also scored the total number of chin rubs (applying cheek gland secretions to the female's nares) and total number of tail fanning behaviors of amplectant males at 1-min intervals during the 10 min observation period. We did not use data from amplectant pairs that were in the thrashing stage that immediately precedes dismounting (Massey, 1988) and selected pairs never reached this stage during our observations. This ensured that we did not observe newts that were within 10 min of dismount.

After the observation period, we quickly captured the observed pair or individual and immediately placed each individual into a separate clean 150 ml beaker filled with 80 ml of local spring water for 60 min to collect water-borne hormones. Newts were caught rapidly before they could flee, so there was little variation between individuals in the time it took to catch and place them in the beakers, which were placed on a table adjacent to the pond. Following hormone collection (see below), we brought the newts into the laboratory and measured the mass (g), SVL (mm), tail length (mm) and tail height (mm) of each individual to examine the relationship between behavior or CORT and morphological variables.

### 2.5. Water-Borne Hormone collection, extraction, validation, and analyses

We extracted hormones immediately after collection or stored samples at $-4\,°C$ for less than 48 h following Gabor et al. (2013). To obtain total CORT release rates, we primed C18 solid phase extraction columns (SepPak, Waters, Inc., Milford, MA, USA) with 4 ml of distilled water and high-performance liquid chromatography (HPLC) grade methanol. We then passed the entire water sample through Tygon tubing (Saint Gobain #2475) into the columns under vacuum pressure. After extraction, we used 4 ml methanol to elute the columns into borosilicate test tubes. We evaporated the methanol using nitrogen gas using an Evap-O-Rac (Cole-Parmer) placed in a $37\,°C$ water bath. We re-suspended the hormone residue to a volume of 250 μl in 5% EtOH, vortexed for 1 min, and then added 95% enzyme-immunoassay (EIA) buffer (Cayman Chemicals Inc., Ann Arbor, MI, USA). We diluted all samples to 1:2. Because all of our water-borne hormone samples used the same spring water from the on-site well, we did not subtract the CORT measured in the spring water. We did however measure the CORT in this water and as predicted the CORT level was very low (69.7 pg/ml or 17.423 pg/sample based on the resuspension volume of 0.25 ml). We measured CORT hormone release rates in

duplicate for all samples with an EIA kit (Cayman Chemicals Inc.) on a fluorescent plate reader set to 405 nm (BioTek ELX800).

To validate the EIA kits for water-borne hormones, we used samples collected from 10 adult red-spotted newts that were each placed in 150 ml beaker with 80 ml of water for 60 min. We resuspended evaporated samples in a final volume of 250 μl EIA buffer (following above) and combined them all into one pooled sample. We diluted the pooled sample to 1:2 for the serial dilutions and 1:4 for the quantitative recovery.

We assessed parallelism of the serial dilution curve, using the pooled control for red-spotted newts run in duplicate. We constructed the log–logit transformed dilution curve using average% maximum binding and pg/ml concentrations for six dilution samples (from 1:2 to 1:64 dilution). The dilution curve was parallel to the standard curve for CORT (comparison of slopes, $t = 1.390$, $df = 10$, $P = 0.195$).

We determined the quantitative recovery of the water-extracted CORT by spiking the pooled control samples for red-spotted newts with each of the eight standards and running an unmanipulated pooled control sample. We determined expected recovery concentrations based on the known amount of CORT in the standards and the pooled control sample. The minimum observed recovery was 79%. The slope of the observed vs. expected curves for red-spotted newts was 0.95 indicating a linear relationship between observed and expected ($F_{1,7} = 17236.8$, $r^2 = 0.999$, $P < 0.0001$).

We used five plates with two pooled samples on each: The intra-plate assay variation was 9.80%, 5.99%, 7.99%, 5.89%, and 12.06%. The inter-plate assay variation was 13.05%.

### 2.6. Statistical analyses

All CORT release rate values were normalized to account for the effect of body size (pg/svl/h). In cases where the paired samples had a coefficient of variation > 33%, data for the individual was not included in the analyses ($n = 3$). For the ACTH challenge, we used a two-way ANOVA to test for effects of treatment (ACTH, saline, control), time in captivity before the treatment ($\sim$50 min in captivity vs. $\sim$110 min in captivity), as well as the interaction between time in captivity and treatment on CORT release rates. Time in captivity and its interaction with treatment were included in our model to account for the different length of time that individuals were held in captivity ($\sim$50 min vs. $\sim$110 min) before injections (or control handling treatment) and hormone collection. We analyzed significant effects using post hoc paired comparisons (Tukey's HSD). For the capture and handling experiment, we used a single factor ANOVA to test for the effect of time in captivity on the CORT release rate (pg/svl/h) followed by post hoc paired comparisons (Tukey's HSD). We used a pairwise comparison between CORT and a principal component (PC) correlate of all measurements of the morphological variables (tail height, tail length, mass, SVL). We used Spearman rank correlations to examine the relationship between the number of individuals nearby and CORT release rates and male behavior. For the CORT response to amplexus experiment, we examined the effects of sex, amplexus and their interaction on CORT (pg/svl/h) using a two-way ANOVA, with sex and treatment (amplexus or solo) as main fixed effects. We performed all analyses in JMP 10 (SAS Institute, Cary, NC, USA).

## 3. Results

### 3.1. ACTH challenge

CORT release rates were significantly higher in the ACTH injected newts (Two-way ANOVA: $F_{2,29} = 4.42$; $P = 0.021$), and we also found a significant effect of time in captivity ($F_{1,29} = 13.75$;

$P < 0.001$), but this effect of time in captivity was different across treatments (Fig. 1; $F_{2,29} = 12.29$; $P < 0.001$). Both the treatment effect and the interaction were driven by the large effect of the ACTH treatment on CORT levels in the group that spent an additional hour in captivity prior to hormone collection (Fig. 1). This effect of time spent in captivity was unique to the ACTH injection treatment as the saline injection treatment and the control treatment did not differ with time in captivity (Fig. 1).

### 3.2. CORT response to handling and captivity over time

We measured CORT release rates at three-time points in captivity. The elapsed time in captivity had an effect on the CORT release rates (ANOVA: $F_{2,26} = 5.44$; $P = 0.011$). CORT peaked at 110 min in captivity and there was no difference in CORT between 50 min and 170 min in captivity (Fig. 2).

### 3.3. CORT and other variables as related to amplexus

Amplexus had a significant effect on CORT values, with amplectant individuals of both sexes showing higher CORT release rates than solo individuals of both sexes (Two-way ANOVA: $F_{1,71} = 4.65$; $P = 0.035$; Fig. 3). This treatment effect was consistent across both sexes. ($F_{1,71} = 0.10$; $P = 0.751$). Additionally, there was an effect of sex on CORT, where males had higher CORT levels overall, and this was consistent in and out of amplexus ($F_{1,71} = 4.60$; $P = 0.034$; Fig. 3). We found no relationship between the CORT levels of the male and female for each amplexed pair (ANOVA: $F_{1,16} = 0.09$, $r^2 = 0.006$, $P = 0.76$).

Amplexed males and females did not differ from solo males and females in tail height, tail length, SVL, mass or a PC correlate of all measurements (Table 1). There was no relationship between CORT
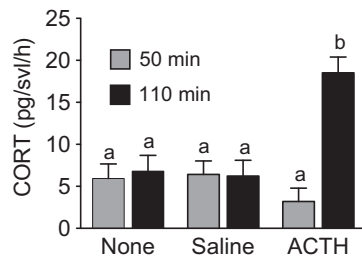


**Fig. 1.** Mean (±1 SE) CORT values (pg/svl/h) in male *Notophthalmus viridescens* treated with ACTH, saline injection, or nothing (none). CORT values for treatment at 50 min (±5 min) in captivity are shown with light bars ($n = 7$ for each) and with dark bars for treatment at 110 min (±5 min) in captivity ($n = 5$ for each). The same letters above the bars indicate no significant difference between the groups (Tukey's HSD, $p < 0.05$).
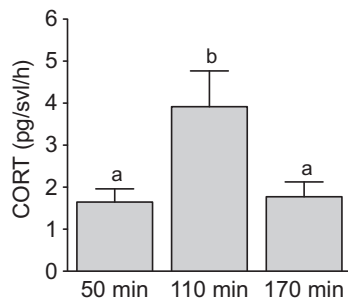


**Fig. 2.** Mean (±1 SE) CORT values (pg/svl/h) in male *Notophthalmus viridescens* at 50 min ($n = 10$), 110 min ($n = 9$) and 170 min ($n = 10$) in captivity. The same letters above the bars indicate no significant difference between the groups (Tukey's HSD, $p < 0.05$).
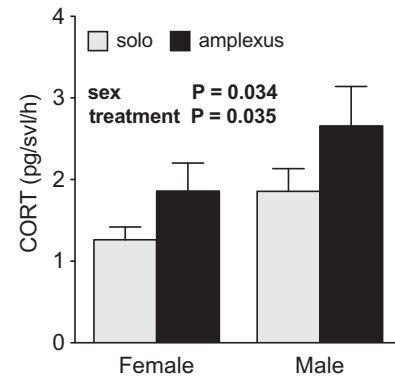


**Fig. 3.** Mean (±1 SE) CORT values (pg/svl/h) in females (left) and males (right). Data for females ($n = 19$) and males ($n = 17$) in amplexus are shown in black, while data for males ($n = 19$) and females ($n = 20$) caught solo are in grey.

and PC of the morphological variables (tail height, tail length, mass, SVL) for any treatment or sex (amplectant F: $r = 0.01$, $P = 0.97$; amplectant M: $r = -0.266$, $P = 0.32$; solo F: $r = -0.24$, $P = 0.32$; solo M: $r = -0.11$, $P = 0.66$). There was no relationship between CORT of the amplected individuals and the average number of individuals nearby (amplectant F: Spearman $\rho = -0.138$, $P = 0.57$; amplectant M: Spearman $\rho = 0.14$, $P = 0.60$). Males of amplectant pairs with higher average number of individuals within the 1 m radius of the pair, performed more chin rubs during amplexus (Spearman $\rho = 0.525$, $P = 0.02$). Males of amplectant pairs with higher average number of individuals within the 1 m radius also had shallower tail height (Spearman $\rho = -0.451$, $P = 0.046$).

## 4. Discussion

We used a newly developed, non-invasive method for examining water-borne hormones in amphibians. We demonstrated that ACTH injected newts had elevated CORT release rates. This demonstrates that the ACTH challenge initiates activation of the HPI axis and the downstream secretion of CORT can be detected with the water-borne CORT method. We also found that it takes close to 110 min in captivity to obtain peak CORT release rates in red-spotted newts. Finally, we found that the amplexus stage of courtship is associated with an increased level of CORT in both male and female red-spotted newts.

The results from our ACTH challenge indicate that the downstream secretion of CORT can be detected with the water-borne CORT method within an hour of administration. In addition, the ACTH challenge demonstrated that our study population is not likely chronically stressed, as they are capable of mounting a CORT response as demonstrated by the responsiveness of the HPI axis to ACTH (following Cyr and Romero, 2009). However, in the first ACTH treatment group of animals (injection after ~50 min in captivity) an increase in CORT release rate was not apparent, in contrast to the second ACTH treatment group of animals (injection after ~110 min in captivity) that show a significantly higher CORT release rate. The lack of a CORT response to ACTH injection in the first group may be due to the amount of time it takes for CORT to be released into the blood and then excreted into the water. In prior studies with amphibians, a plasma CORT response to ACTH challenge takes about 30 min (Belden et al., 2010; Chambers et al., 2011) and from 30–60 min due to the stress of confinement (Glennemeier and Denver, 2002; Belden et al., 2005, 2007, 2010). From our study on stress from handling and captivity we found that it takes CORT over 110 min to begin to peak. This follows with our peak in CORT release rates at about 110 min in the second ACTH treatment group. Given these results, we interpret the

**Table 1**
Comparison of morphological variables for male and female *Notophthalmus viridescens* that were captured either in amplexus or solo. Student's *t*-test values are presented for all comparisons.

| Sex | Treatment | n | Mean SVL | Mean mass | Mean TL | Mean TH | Mean PC |
|---|---|---|---|---|---|---|---|
| F | Amplex | 20 | 45.85 ± 0.75 | 2.74 ± 0.19 | 49.69 ± 0.67 | 6.64 ± 0.20 | −0.92 ± 0.20 |
| F | Solo | 20 | 46.40 ± 0.39 | 2.67 ± 0.09 | 49.06 ± 0.71 | 6.54 ± 0.20 | −0.98 ± 0.23 |
| | | | $t = 0.64$, $P = 0.52$ | $t = -0.52$, $P = 0.60$ | $t = -0.64$, $P = 0.53$ | $t = -0.34$, $P = 0.73$ | $t = -0.18$, $P = 0.85$ |
| M | Amplex | 19 | 46.86 ± 0.59 | 3.28 ± 0.12 | 51.15 ± 105 | 9.82 ± 0.31 | 0.68 ± 0.32 |
| M | Solo | 20 | 48.36 ± 0.60 | 3.55 ± 0.14 | 51.36 ± 1.14 | 9.99 ± 0.34 | 1.25 ± 0.37 |
| | | | $t = 1.77$, $P = 0.08$ | $t = 1.43$, $P = 0.16$ | $t = 0.14$, $P = 0.89$ | $t = 0.35$, $P = 0.72$ | $t = 1.15$, $P = 0.26$ |

elevated CORT release in the second group as evidence that our water-borne hormone collection and EIA method is capable of detecting elevated CORT levels.

The highest CORT levels observed in the ACTH challenge group were nearly triple the levels seen in the saline control or handling control groups and nearly six times greater than the CORT levels found in males during amplexus (Fig. 1 vs. Fig. 3). We interpret this as evidence that the CORT response mounted by red-spotted newts can vary from subtle to severe. Because high levels of CORT have many documented negative effects, it is possible that it is adaptive for animals to vary the magnitude of the CORT response to balance the net physiological costs with the potential net fitness benefits of the response (Busch and Hayward, 2009). This variation in the magnitude of the response may happen via habituation to amplexus over the mating season. Cyr and Romero (2009) suggest that it is possible for animals to desensitize their physiological response to a stressor whereby GCs are attenuated because of the HPI axis being altered due to repeated challenges, yet the challenge is not completely innocuous, so the animal still shows a reduced stress response.

In the handling and captivity experiment, we found that red-spotted newts require more than 110 min in captivity before CORT release rates reach peak levels and then began to fall after 170 min in captivity. These results are in contrast to Berner et al. (2013), who found a CORT response after 30 min in captive Eastern red-spotted newts, *N. viridescens viridescens*, by measuring plasma CORT levels. Given that amplexus lasts up to 204 min on average (Massey, 1988), our data suggests that an acute CORT response that takes 110 min to reach a peak could aid in amplexus even if not initiated until the onset of the behavior.

Both males and females showed elevated CORT release rates if they were collected while in amplexus, with males showing higher overall CORT release rates than females collected both in and out of amplexus. This may indicate sex differences in the role of CORT in courtship and mating. The higher CORT release rates in males may ultimately be tied to the highly male biased operational sex ratio (OSR) in this population. Not only may the intense male-male competition found in populations of red-spotted newts (Verrell, 1982, 1983; Able, 1999; Rohr et al., 2005; Grayson et al., 2012) drive up CORT release rates in males, but it may also increase the energetics required to maintain amplexus. Sustained muscle contraction involved in clasping may be energetically costly for newts (Coddington and Moore, 2003), and both courtship and male-male agonistic interactions have been shown to increase oxygen consumption in other salamanders (Bennett and Houck, 1983). It is worth noting that, in male marbled salamanders (*Ambystoma opacum*) that lack physical male-male competition and do not engage in amplexus behavior, no increase in plasma CORT is associated with courtship behavior (Houck et al., 1996). Thus, the physiological costs and behavioral changes associated with inter and intrasexual selection on males (Rowe et al., 1994) may be driving higher CORT release rates in males. For males, the higher CORT release

rate during amplexus, although it likely imposes physiological costs, may also facilitate the lengthy and energetically costly behavior of amplexus and contribute to reproductive success. Previous work in other amphibian systems further supports this hypothesis (Orchinik et al., 1988; Harvey et al., 1997; Emerson and Hess, 2001; Leary et al., 2008). Conversely, for females the benefit of increased reproductive success from more frequent and longer lasting amplexus would not be expected and could lead to sexual conflict over the optimum number of matings (Jones et al., 2002). The resulting difference between the sexes in CORT release rates during amplexus could be especially pronounced in a population with a highly male-biased OSR, such as the one we studied, and would further increase sexual conflict over optimum frequency of mating. Previous work with this population of newts found that male biased sex ratios lead to decreased body condition in females (Grayson et al., 2012). Our observed increase in CORT release rates for females in amplexus may explain this decrease in body condition as females likely experience a high frequency of amplexus during the mating season when subjected to a strongly male-biased sex ratio.

We found a difference in CORT release rates between solo individuals and individuals engaged in amplexus. We argue that the higher CORT release rates for individuals captured during amplexus, reflects an acute increase in CORT during amplexus. Because CORT levels can be negatively correlated with body condition (reviewed by Moore and Jessop, 2003), alternative hypotheses for explaining the higher CORT levels in amplexus are that individuals could be attracted to members of the opposite sex with similar CORT levels or that only higher quality pairs were in amplexus. Previous studies have found that CORT levels can be a factor in mate choice: female Great Plains toads (*Bufo cognatus*) (Leary et al., 2006) and female zebra finches, *Taeniopygia guttata,* ((Roberts et al., 2007) prefer to associate with males with lower CORT levels. Because high CORT levels may be associated with immune-incompetence or lower parental care, these females may be selecting for higher quality males. However, we did not find any relationship between CORT levels of males and females paired in amplexus. Furthermore there were no significant morphological differences (tail height, tail length, mass, SVL) detected between amplectant and solo individuals of either sex. Nor was there a relationship between CORT and the PC of the morphological variables for any treatment or sex.

In addition to the hormone data, our study also examined differences in behaviors of individuals in amplexus versus those that were not. During the 10 min observation periods, there were fewer newts around amplectant pairs when the male of the pair had a deeper tail. This result supports prior studies showing that males use their tailfin during male-male competition (Halliday and Tejedo, 1995) and that male newts with deeper tails have a greater ability to achieve amplexus (Able, 1999). Males with deeper tailfins are more successful at catching females and maintaining control of amplexus. In addition, males performed more chin rubs (genial

gland area) when more individuals were within 1 m of the amplectant pair, which may function to increase female receptivity when potential competitors are in the area. However, we found no relationship between CORT and any of the variables recorded: behavioral or morphological. This suggests that the elevated CORT release rates result from being engaged in amplexus rather than from any differences between amplectant and solo newts. However, our results showing an association between elevated CORT and amplexus should be considered with the caveat that our data cannot distinguish between CORT release rates due to engaging in amplexus and CORT release rates due to abrupt separation during amplexus. Though, solo individuals experienced the same capture protocol but do not show the same increase in CORT and previous work with male amphibians has shown an increase in CORT associated with amplexus. We argue that the observed increase in CORT in our experiment is associated with amplexus rather than any effect of capture during amplexus.

Amplexus is associated with an acute, albeit moderate, increase in CORT in both male and female red-spotted newts. A novel contribution of this work is the examination of a CORT response associated with mating behavior, simultaneously in both sexes. Additionally, CORT release rates in and out of amplexus were found to be higher in males than in females. Our findings highlight that the acute CORT response associated with amplexus may activate stored energy to aid in successful courtship behavior, but given the known costs of elevated CORT levels, it may simultaneously act as a constraint that mediates the cost of reproduction. Furthermore, our data add support to the hypothesis that CORT is not always inhibitory to reproduction and mating behavior (Woodley and Lacy, 2010; Narayan et al., 2013) in contrast to other studies (Moore and Miller, 1984; Wingfield et al., 1998; Salvante and Williams, 2003; Woodley and Lacy, 2010). We argue that an acute CORT response to mating may allow individuals to access the required energy needed during courtship encounters.

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