The association between male-biased sex ratio and indicators of stress in red-spotted newts

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HIGHLIGHTS

- Female activity did not differ in response to chemical cues from one vs three males.
- Females increase activity with increasing stress hormone to chemical cues of three males.
- Females in male-biased enclosures had higher CORT and testosterone release rates.
- Sexual conflict in male-biased environments can reduce female fitness.

ARTICLE INFO

ABSTRACT

In populations with a male-biased operational sex ratio, coercive mating by males can have fitness consequences for females. One component of reduced fitness for females in populations with a male-biased OSR may be greater activation of the stress response, resulting in higher corticosterone release rates (CORT; a glucocorticoid stress hormone in amphibians). We test the hypothesis that a male-biased sex ratio affects female activity and release rates of CORT and testosterone (T) in male and female red-spotted newts (*Notophthalmus viridescens*). First, we evaluated if chemical cues from a male-biased sex ratio affect activity and CORT release rates in females. We predicted that females exposed to chemical cues of three males would be less active and have higher CORT release rates than those exposed to chemical cues of one male. Second, we measured CORT release rates of red-spotted newts in field enclosures with either a male-biased or a female-biased sex ratio. We predicted that females in the male-biased treatment would have higher CORT and T release rates than those in a female-biased treatment, owing to higher levels of male harassment. We also predicted that males would have higher CORT and T release rates in male-biased treatments due to higher levels of male-male competition. Females were not less active in response to chemical cues from more males over fewer males, but there was a positive relationship between female activity and CORT when they were exposed to the cues of three males. We also found that females, but not males, in the male-biased sex ratio treatment had higher CORT and T release rates than those in the female-biased treatment. Our results support the hypothesis that a male-biased sex ratio leads to a higher stress response, which may underlie the observed decrease in immune function and body condition in previous work exposing female red-spotted newts to a male-biased sex ratio. This study furthers our understanding of the mechanistic basis for costs associated with a male-biased sex ratio in a pond-breeding amphibian.

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1. Introduction

How males and females of the same species maximize fitness often differs, which sets the stage for conflicts of interest during male-female interactions. This conflict is referred to as sexual conflict when males and females interact in a mating context [1]. Sexual conflict occurs if the individual fitness of one sex is increased as a function of a trait that alters the behavior of the other sex, at an expense to the latter's own fitness. Sexual conflict can lead to a decrease in the fitness of one sex through a number of different actions, such as mating harassment, coercive behaviors, or injury during mating. Sexual selection favors traits that increase an individual's ability to differentially access mates

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and to be successful in syngamy. Sexual selection is stronger when the asymmetries in male and female mating success are larger, which is influenced by the degree of operational sex ratio bias (OSR; [2–4]). Although the processes of sexual selection and sexual conflict have traditionally been presented as opposing explanations for mating behaviors, Kokko and Jennions [1] argue that sexual selection and sexual conflict are conceptually linked in a number of ways, including the observation that there are likely no biologically plausible conditions under which either exists without the other.

The outcome of sexual conflict is important because in addition to effects on evolutionary trajectories, it can have significant effects on population-level processes [e.g., reviewed in 5,6]. For example, if females are on the winning side in sexual conflict, population fitness should be higher than if males are on the winning side because females are more likely to control demography; the more females in a population, the higher number of offspring that are produced [1,7]. Understanding the mechanisms by which sexual conflict plays out can help us understand which sex might be in the "winning role" in a population. For example, males of many species engage in coercive behaviors, including harassment of solo and mating females, and these behaviors could lead to a reduction in female fitness, and/or costly modifications in female behavior [8–17]. Coercive behaviors are expected to be higher in frequency in populations when the OSR is male-biased, as has been found in several taxa (*Aquarius*: [18,19]; *Thamnophis*: [19]; *Gambusia*: [20]).

The OSR may vary within a species temporally and at different scales. At a broader scale, a highly male-biased OSR in a population is expected to lead to greater variation in reproductive success, and therefore an increase in the opportunity for sexual selection [2–4, but see 21]. Male sexual harassment increases with a more male-biased OSR, which can detrimentally impact almost every aspect of female behavior and life history [reviewed in 8]. Females in male-biased OSR populations may respond both behaviorally and physiologically to male coercive behaviors, and these responses have implications for female health and therefore population fitness. Similar to the indirect effects of predator risk on prey ("ecology of fear"), which include morphological or behavioral responses that can be costly (e.g., reduced survival, growth or reproduction) [reviewed in 22], breeding females may also experience indirect effects from the risks of male harassment. For example, when female seed beetles (*Callosobruchus maculates*) are both harassed by or could mate with more than one male, they had decreased life spans and reduced reproductive rates relative to monandrous females [23]. In the Atlantic molly (*Poecilia mexicana*), male mating behavior often consists of constant female harassment by males and attempted forced copulations, and female feeding rates are lower when the female is accompanied by a male due to increased vigilance to potential harassment [24]). In a viviparous lizard (*Lacerta vivipara*), a male-biased OSR affects immediate female body condition and reduces lifetime reproductive rate [25].

Theory predicts that females can evolve counterstrategies to reduce the costs of male coercion, or to reduce the frequency of mating. These counterstrategies are in the form of morphology [reviewed in 8], as well as behaviors. For example, female guppies (*Poecilia reticulata*) that are not in mating condition associate with more attractive females in response to increases in male harassment [26]. Female red-spotted newts (*Notophthalmus viridescens*) avoid large groups of males [27], and spend more time in refuge habitat in male-biased sex ratio treatments [28]. Aside from changes in behavior, physiological changes can occur alongside reductions in fitness and observable countermeasures to an overabundance of a particular sex. The endocrine systems of animals are dynamic, such that rapid changes in circulating hormones arise from the various social and ecological environments that individuals may encounter, and the fitness correlates of these changes may vary across contexts [29–31].

The red-spotted newt is a pond-breeding amphibian that exhibits complex courtship and mating behaviors, including amplexus [32,33], and breeding populations are often male-biased [28,33–35]. Grayson et al. [28] showed that a male-biased OSR in the red-spotted newt

affects female body condition and decreases total lymphocyte and leukocyte abundance in blood, a potential indication of physiological stress. As the adaptive immune system is sensitive to elevated glucocorticoids [GC; 36], these effects on female condition and immunity may be proximally linked to an increase in corticosterone (CORT; a primary GC in birds, amphibians and reptiles) during amplexus, which may occur at greater rates in male-biased OSR populations. Further, high reproductive effort by males is expected to be associated with higher CORT levels in males [e.g., the CORT-Adaptation Hypothesis 37,38]. Higher rates of amplexus and aggressive male behaviors may also be costly to females in terms of loss of time foraging, increased risk of predation, and the potential for injuries, which could manifest as an increase in CORT levels in amplectant females relative to those not in amplexus. Indeed, amplexus has been associated with an increase in CORT release rates in both male and female red-spotted newts [39].

In addition to effects on CORT, population sex ratios during the mating season could also affect release of the steroid hormone, testosterone (T). For example, in the bank vole, *Clethrionomys glareolus*, (T) levels can influence both intra- and inter-sexual selection [40]. However, there is not a significant relationship between T and OSR [40]. Testosterone is the primary androgen hormone stimulating secondary sexual characteristics but at the same time can be immunosuppressive (and thus may be negatively associated with CORT) due to the negative feedback between the endocrine and immune systems [41]. The immunosuppressive effects of sexual hormones may be adaptive as resources can be redirected toward mating [42]. Yet, most studies of male amphibians have found a positive correlation for CORT and T [43–46], but in some species CORT and T are inversely correlated [47]. Little is known about the relationship between CORT and reproductive hormones such as T in female amphibians [but see 48,49]. Harvey et al. [48] found that in explosive breeding spadefoot toad, *Scaphiopus couchii*, T and estradiol increase in amplexed females but CORT did not change across the reproductive cycle of females.

In this experiment, we tested the hypothesis that a male-biased sex ratio will affect female activity and release rates of CORT and T in male and female red-spotted newts. We first conducted a lab experiment to evaluate how sex ratio affects activity and CORT levels in females. We exposed females to water-borne chemical cues of either one male or three males and we measured pre and post-experiment water-borne CORT release rates. We predicted that females exposed to cues of three males would have a higher CORT response and lower activity than those exposed to the cues of one male. In the second experiment, newts were placed in field enclosures in groups of four at either a male- or female-biased (3 males: 1 female or 3 females: 1 male) sex ratio for seven days and we measured post-experiment water-borne hormone release rates. We predicted that males, as well as females, exposed to a male-biased sex ratio will have higher CORT and T.

2. Materials and methods

2.1. Study site

This research was conducted at Mountain Lake Biological Station (MLBS; Giles County, Virginia, USA) from June–July 2015. We conducted a behavioral experiment to test for effects of male chemical cues on female activity and CORT levels in a laboratory room at MLBS. We also performed an experiment to evaluate the effects of biased sex ratio on male and female CORT and T in a field experiment in Riopel Pond (0.65 ha). Riopel Pond contains a large population of red-spotted newts [estimated newt population = 7600–12,700 individuals; 28,50].

2.2. Effects of male chemical cues on female activity and CORT

To acquire male chemical cues, we collected male newts (N = 16 total males) from the adjacent pond and immediately placed either one male or three in a container (12 × 18 × 31 cm) with 1 L of spring

water for one hour on each of the four days we tested (following [27]). We started testing at the same time of day (0800 h) on 23 June–1 July 2015. We then collected female newts (N = 34 total) from the pond and measured their water-borne CORT release rates (pre-stimulus) following established methods [see below; 39]. Briefly, we placed individual female newts each into a 150 mL glass beaker with 80 mL of local spring water for 1 h. We then removed females from the beakers and placed individual females (N = 32, as two were excluded for not moving in the pre-treatment trial) in 37 L aquaria with 5 L of spring water. After a 10 min acclimation period we then recorded their activity (time (s) spent moving) for 10 min. After we measured this "pre-treatment activity", we slowly injected 60 mL (less than half the concentration per ml used by [27]) of the water samples containing the male chemical cues corresponding to one of the two treatments (chemical cues collected from 1 male or 3 males) into the middle of the tank. We then measured and recorded the "post-treatment activity" for 10 min. Immediately following this activity assay, we obtained a second sample of water-borne CORT (post-stimulus) for each female as above.

2.3. Short-term effects of biased sex ratio on CORT and T

This study was based on the experimental design of Grayson et al. [28], which was an experiment testing the effects of short-term exposure to a biased sex ratio on newt immune function. Here we use a similar design to test for the effects of short-term exposure to a biased sex ratio on newt CORT and T concentrations. We collected 96 red-spotted newts (48 of each sex) over the two testing periods and recorded data on each individual's mass (g) and snout-vent length (SVL; mm). We then randomly placed newts into a total of 24 pond enclosures. Each enclosure was a plastic bin (62 L) with mesh sides allowing for water flow-through, which contained a measured combination of mixed-species leaf litter, rocks and sand which served as substrate, microhabitat, and refugia (obtained from Riopel pond). The top was covered with fiberglass screening secured with clothespins to prevent newt escape and to allow us to check on the enclosures daily. We placed the enclosures in a shallow area along the southern shore of the pond such that each tank was about 3/4 submerged. We placed four newts in each enclosure such that each enclosure had either a male- or a female-biased sex ratio (3 males: 1 female or 3 females: 1 male, randomly assigned). As in Grayson et al. [28], the male-biased sex ratio treatment is based on the estimated sex ratio of the newts in Riopel Pond [2.4–2.8 males: 1 female, KL Grayson, unpublished data; comparable to 33]. While these enclosures confined the experimental individuals, we used within pond enclosures to mimic the microhabitat, weather, ambient water chemistry, and natural diet of newts in this pond.

The newts remained in the pond enclosures for seven days. On day 7 we collected all newts and recorded the mass (g) and SVL (mm) of one focal male and one focal female from each enclosure. Both were haphazardly chosen to minimize disturbance based on collection as we also collected water-borne hormone samples from these two individuals per enclosure.

Testing was performed over two 7-day blocks from June 18–July 2, 2015, due to a limited number of enclosures. Replicates were excluded from analysis when newt loss occurred due to failure of the mesh in some enclosures (which affects the sex ratio of the treatment). Our final sample size across the two blocks of data was: male-biased sex ratio (N = 14) and female-biased sex ratio (N = 11).

2.4. Hormone assays

We measured the release rates of corticosterone (CORT) and testosterone (T) using a recently developed, non-invasive process for measuring water-borne hormone levels in amphibians [51]. This method relies on steroids that are released from the bloodstream into the water

through the skin, urine, mucus and bile and is proportional to their concentration in plasma (reviewed by Scott et al. 2008). Water samples were maintained at −20 °C until hormone assays were performed. We primed C18 solid phase extraction columns (Sep-Pak, Waters Inc.) with 4 mL of HPLC-grade methanol and 4 mL of distilled water. We extracted the hormones from the water samples by passing the water samples through the primed columns using a vacuum manifold. Hormones were then eluted from the columns into borosilicate test tubes using 4 mL of HPLC-grade methanol, placed in a 37 °C waterbath, and dried under a stream of low flow nitrogen gas. Prior to assaying samples, we resuspended hormones in a solution of 5% ethanol and 95% EIA buffer for a final resuspension volume of 260 μL. We used commercially available EIA plates (Cayman Chemicals Inc.) to measure CORT and T concentrations. We ran samples in duplicate on 96 well plates that were read by a fluorescent plate reader set at 405 nm (BioTek ELX800).

Reedy et al. [39] validated the use of the water-borne hormone assays in red-spotted newts by showing that CORT increased after an adrenocorticotropic hormone challenge (ACTH). Reedy et al. [39] also validated the use of water-borne CORT on Cayman Chemical CORT plates. Here we also validated the use of water-borne T on Cayman Chemical T plates. We assessed parallelism of the serial dilution curve, using a pooled control from 10 non-experimental red-spotted newts diluted 1:2. The dilution curve (from 1:2 to 1:16 dilution) was parallel to the standard curve for T (comparison of slopes, t = 0.91, df = 9, p = 0.38).

We determined the quantitative recovery of the water-extracted T by spiking the pooled control samples for red-spotted newts with each of the eight standards and running an unmanipulated 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 levels of T ($F_{1,7}$ = 216.89, r^2 = 0.97, p < 0.0001).

To measure CORT, we used five plates with two pooled samples on each. The inter-plate assay variation for CORT was 5.73%. The intra-plate variation for CORT ranged from 1.68–7.62%. To measure T for the sex ratio field data we used three plates. The inter-plate assay variation was 3.76% and intra-plate variation for T ranged from 0.64–4.97%.

2.5. Statistical analyses

All CORT and T release rate values were normalized to the SVL of individuals, to account for the effect of body size (pg/svl/h). We used JMP Pro (v12) to perform all analyses.

2.5.1. Effects of male chemical cues on female activity and CORT

We next used repeated measures ANOVA to examine if activity increased or decreased over time (the two testing periods) as a function of the CORT release rate response (ln post-cue CORT–ln pre-cue CORT) and treatment, as well as with any interaction between the CORT release rate response and treatment.

2.5.2. Short-term effects of biased sex ratio on CORT and T

We used a linear mixed effects model to analyze the relationship between sex ratio and hormone release rates (T and CORT). As fixed effects, we used sex and treatment (male or female-biased sex ratio), as well as the sex × treatment interaction in the model. The enclosure number was modeled as a random effect. Visual inspection of residual plots did not reveal any heteroscedasticity or deviations from normality. p-Values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. Trial number had no effect on CORT or T release rates for both males and females in both treatments, and was thus not entered in the model to conserve DF.

3. Results

3.1. Effects of male chemical cues on female activity and CORT

Activity level overall decreased from pre- to post-treatment (significant Time effect; Table 1). There was also a significant CORT release rate response × treatment interaction on the change in activity; CORT release rate response and the change in activity are positively correlated, but only in the three male treatment (Table 1; Fig. 1).

3.2. Short-term effects of biased sex ratio on CORT and T

For CORT, there were no overall significant effects of the fixed effects Sex or Treatment, but there was a significant interaction between these terms (Table 2). Females in the male-biased treatment had the highest CORT release rates (Fig. 2). Similarly, for T, there were no overall significant effects of the fixed effects Sex or Treatment, but there was a significant interaction between these terms (Table 3). Females in the male-biased treatment had the highest T release rates (Fig. 3).

4. Discussion

Male coercive behavior can be costly to females as it may modify their behavior and physiology. In this experiment, we tested the hypothesis that a male-biased sex ratio is associated with lower levels of female activity and higher release rates of CORT and T in male and female red-spotted newts. First, in a lab study examining the effects of male chemical cues on female activity and CORT release rates, we found that for female red-spotted newts there is a positive relationship between the CORT release rate response and the change in activity, but only in the 3 male cue treatment; in that treatment group, females that had greater CORT release rate responses also were more active after the cue was introduced. Second, in our short-term field enclosure study we found that females in a male-biased sex ratio had higher levels of CORT and T than those in a female-biased sex ratio. Together these results indicate that male coercive behavior does come at a cost in terms of female behavior and physiology for red-spotted newts.

In the population we studied there is a strong male OSR and field studies at this site have shown that males use coercive behavior during courtship [33,52]. The increase in CORT release rates as a function of a male-biased sex ratio, combined with the decrease in female immune function [28] supports the hypothesis that sexual conflict arising from increases in male coercive behavior in male-biased sex ratio environments can ultimately influence female fitness [8–17]. Our results from a short-term experimental study that manipulates the sex ratio are relevant to the natural dynamics between males and females in newt populations. Male-male competition in newt mating systems is intense and very few females at our site are observed in open water in the spring without a male attached in amplexus [52]. The degree of male-bias in
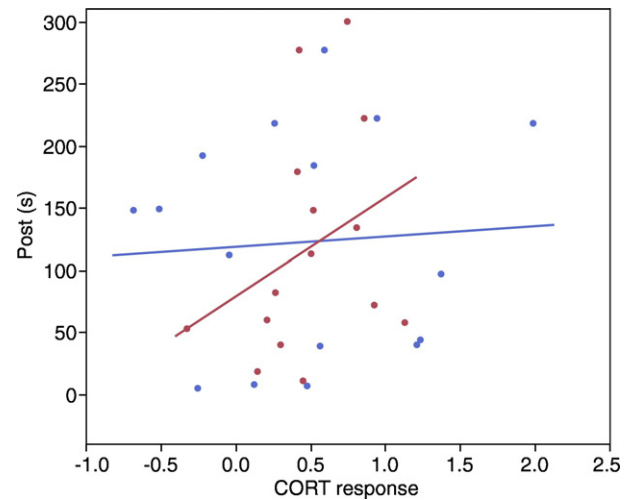

Fig. 1. Linear fit of the relationship between the CORT response (Ln post-CORT pg/svl/h) − (Ln pre-CORT pg/svl/h) and the amount of time female red-spotted newts spent in activity (s) after the introduction of chemical cues from one male (blue) and three males (red). The positive slope of the linear fit was greater for the three male chemical cue (red line) than for the one male cue (blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

newt populations may be a combination of several factors, such as collecting or disturbance (e.g. [53]) or the dynamics of the population (the degree of skipping breeding opportunities in females or the degree of residency in the population; [54]). Given that males are the winner in the sexual conflict in this system, the ramifications of this study along with the natural variation in OSR are that population viability might suffer from intense male harassment under highly biased OSRs. Rankin et al. [55] show that when there is a positive feedback between male harassment and a male-biased OSR, females in populations could theoretically go extinct; thereby causing an extinction of the population. Quantifying the feedback between male coercive behavior and the OSR of newt populations could help shed light on the effects on population fitness in red-spotted newts. In addition, a quantification of the effects of the increase in CORT and T on female fitness as a function of male-biased OSR could help us determine the true cost to females of the male-biased OSR in this newt system. Indeed, Hews and Baniki [56] found that lizards (*Sceloporus virgatus*) that were not investing heavily in reproduction had higher acute CORT following stressors.

While CORT and T were higher in females exposed to male-biased sex ratios than those exposed to female-biased sex ratios, it was not higher for males in male-biased sex ratios. The CORT- Adaptation Hypothesis [37,38] predicts that high reproductive effort by males would be associated with higher CORT levels in males. Our results do not support this prediction, assuming that the male-biased sex ratios did lead to higher reproductive effort by males. If males in the male-biased sex ratio treatment did indeed have higher reproductive effort than those males in the female-biased treatment, then some other factors that modulate

Table 1
Repeated measures ANOVA on Activity (time (s) moving) pre-cue exposure and post-cue exposure. Between subjects' factors include Treatment (1 male vs. 3 male cues), CORT release rate response (Ln (post-CORT pg/svl/h) − (Ln (pre-CORT pg/svl/h)), and their interaction.

	F	p
Between subjects		
All between	3.59	*0.026*
Treatment	0.75	0.394
CORT release rate response	6.02	*0.021*
CORT release rate response × Treatment	7.30	*0.012*
Within subjects		
Time	5.85	*0.02*
Time × Treatment	1.77	0.194
Time × CORT release rate response	2.67	0.114
Time × Treatment × CORT release rate response	0.16	0.696

Italics indicate significant effects (p < 0.05).

Table 2
Parameter estimates for linear mixed model on CORT levels in male and female red-spotted newts exposed to a male-biased or a female-biased sex ratio.

	β	SE$_β$	z (β/SE$_β$)	p
Fixed effects				
Intercept	0.117	0.054	2.170	*0.039*
Sex	0.055	0.057	0.965	0.172
Treatment	−0.028	0.054	0.519	0.083
Treatment × Sex	−0.129	0.057	2.263	*0.032*
Random effects				
Var$_{(enclosure)}$	−0.010	0.034		
Var$_{(residual)}$	0.189	0.051		

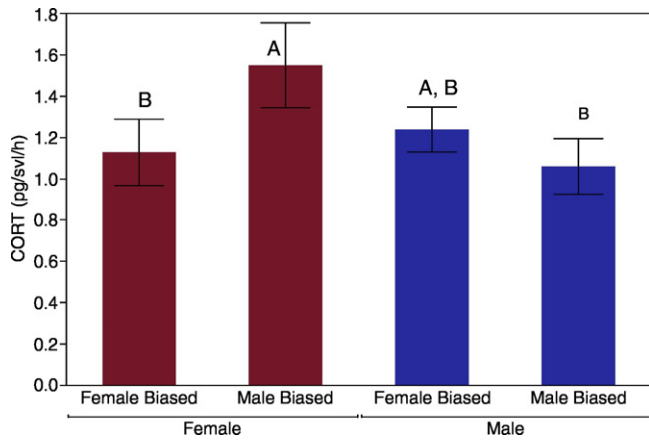Italics indicate significant effects (p < 0.05).

Fig. 2. Mean Ln CORT release rates (± S.E.M.) for male (blue) and female (red) red-spotted newts after seven days in an enclosure with 3 males and 1 female (male-biased sex ratio) or with 1 male and 3 females (female-biased sex ratio). Females in the male-biased sex ratio treatment had significantly greater CORT values than females in the female-biased sex ratio treatment, and then males in the male-biased sex ratio treatment (Tukey's HSD p < 0.05; letters above bars represent significant differences between groups). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
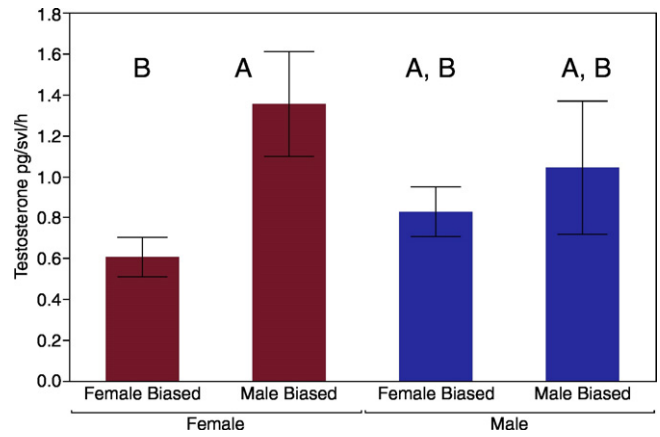

Fig. 3. Mean Ln T release rates (± S.E.M.) for male (blue) and female (red) red-spotted newts after seven days in an enclosure with 3 males and 1 female (male-biased sex ratio) or with 1 male and 3 females (female-biased sex ratio). Females in the male-biased sex ratio treatment had significantly greater T values than females in the female-biased sex ratio treatment (Tukey's HSD p < 0.05; letters above bars represent significant differences between groups). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CORT may mask the predicted relationship between male reproductive effort and CORT levels [reviewed in 57]. First, the male newts may not perceive that higher male numbers as stressful. Second, they may not increase secretion of CORT (even though they perceive the presence of more males as stressful) or may always have high CORT in the breeding season instead of CORT being responsive to the sex ratio. Third, the relationship between CORT and mating behavior may be more permissive than regulatory [46,58]. Finally, the males in the male-biased treatments may counteract the actions of CORT via compensatory stimulation of the gonadal axis. Alternatively, male reproductive effort may have been lower in general because the mating season was waning, and thus the male-biased sex ratio males did not have a high enough reproductive effort to have an effect on CORT release rates.

Grayson et al. [28] found that latency to initiate activity was lower, and overall movement time was greater, for females exposed to the chemical cues of four males vs. those exposed to pond water. They did not find this effect when comparing female responses to chemical cues from two males vs. pond water. Their results are congruent with those of Rohr et al. [27], in which female red-spotted newts avoided groups of four males. In our study, we did not detect effects of more male chemical cues on female activity or CORT release rates even though this research was also conducted at about the same time of year. However, unlike most years, we did not see many newts mating at this time and some newts were already migrating from the pond to their terrestrial habitat. The lack of a behavioral response to more or less males could have arisen from some males and/or females not being in reproductive condition. We also used a smaller volume of chemical cue than was used in the previous study. Regardless, we

found that female CORT response was positively correlated with their change in activity in the three male treatment. Females that were more active after the cue introduction also had higher CORT release rates, but only in the three male cue treatment. These results suggest that female behavior and physiology are linked, and that females show a physiological response to the chemical cues of more males than to less. Although we did not quantify the chemicals that were in the water used as cues, Rohr et al. 2005, found that there is a 33-kD protein secreted from male cloacal glands that is used in assessment of sex ratio. We do not know if female responses were the result of that same chemical cue, or a result of response to other cues, including, potentially, alarm cues from the three males. We do not suspect that females are responding to alarm cues given that these newts are poisonous and do not show a stress response to capture alone. Nevertheless, the association between female behavioral and physiological responses may reflect female counter-adaptations to male coercion.

The body condition of female red-spotted newts is negatively impacted by long-term exposure to male-biased sex ratios and female immune function (decreased leukocytes) is negatively impacted by short-term exposure to male-biased sex ratios [28]. Similarly, we show that male-biased sex ratio is associated with higher CORT and T release rates in females. Harvey et al. [48] also found that amplexed female spadefoot toads had higher T but not CORT. Increases in CORT and/or T during the breeding season occur in males in other species [48,58, reviewed by 59,60], but to the best of our knowledge, both CORT and T have not been examined often in females [but see 48]. Androgens in females may be associated with ovulation but the specific function is not clear [see references within 48]. In *Pleurodeles waltl* androgens (including T) increase until just after the end of oviposition [61]. In female red-spotted newts, the high T levels observed may not indicate that females are still maintaining reproduction, but may suggest that higher CORT is associated with increasing T levels which is associated with the end of oviposition (and hence migration) and may explain why so many females escaped the enclosures. Because CORT plays a role in regulating energy metabolism and is expected to be elevated during periods of negative energy balance [62,63], we hypothesize that the loss of female body condition over longer periods of time may be due to the costs associated with chronically higher CORT levels in females exposed to male-biased OSR. Further, chronic elevation of CORT levels can be immunosuppressive and thus might explain the decreased leukocytes in females exposed to male-biased sex ratios found by Grayson et al. [28].

Table 3
Parameter estimates for linear mixed model on T levels in male and female red-spotted newts exposed to a male-biased or a female-biased sex ratio.

	β	SE$_β$	z (β/SE$_β$)	p
Fixed effects				
Intercept	−0.324	0.112	2.890	0.007
Sex	0.007	0.067	0.104	0.915
Treatment	−0.160	0.112	1.429	0.164
Treatment × Sex	−0.170	0.067	2.537	*0.018*
Random effects				
Var$_{(enclosure)}$	0.233	0.101		
Var$_{(residual)}$	0.224	0.065		

Italics indicate significant effects (p < 0.05).

In summary, we found that increased CORT release rate may be the mechanism by which females suffer a cost to male-biased sex ratios. Physiological measures provide a means for quantifying these dynamics in the short-term and understanding the potential costs to individuals, which can translate into important fitness consequences. As populations of amphibians decline, or any species for that matter, the OSR can be skewed by a variety of mechanisms such as stochasticity, drift, or positive feedbacks [64,65]. Quantifying the degree of male-bias in populations of conservation concern may be an overlooked factor and important for assessing the current health and long-term viability of a breeding site.

Acknowledgements

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