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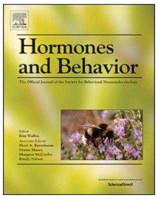

Multimodal stimuli regulate reproductive behavior and physiology in male túngara frogs

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ABSTRACT

Unlike in terrestrial animals, the boundary between internal (e.g., hormones) and external (e.g., social) stimulation can be blurred for aquatic and amphibious species. When chemicals such as hormones and glandular secretions leach into the water, they can further interact with other signaling systems, creating multimodal stimuli. It is unclear, however, whether water-borne chemical secretions from courting male frogs affect the physiology and behavior of their rivals. In order to address this question we first established non-invasive, continuous sampling methods for simultaneously measuring both hormones and behavior in amphibious species. Then, we examined whether interactions between water-borne chemical secretions and conspecific calls affect reproductive behavior and physiology (testosterone and corticosterone) of courting male túngara frogs. Our results demonstrate that conspecific acoustic stimulation alone increases locomotor activity, decreases latency to call, and increases calling behavior but does not alter the amount of hormones excreted. In response to water containing chemical secretions from rivals, but in the absence of calls from other males, males excrete more testosterone. Interestingly, the combined acoustic and chemical stimulus causes a multiplicative increase in both calling behavior and hormonal excretion. Taken together, our results suggest that a multimodal chemical-acoustic stimulus physiologically primes males for aggressive behavior.

1. Introduction

The timing and expression of reproductive behavior is regulated by contextual information provided by both external sensory stimulation and internal hormonal correlates (Adkins-Regan, 2005). Both cyclical changes in the abiotic environment and social stimulation are involved in physiologically priming individuals for reproductive behaviors such as attending mating aggregations, producing sexual advertisement displays, and engaging in male-male competition (Adkins-Regan, 2005; Allee et al., 2009; Chu and Wilczynski, 2001; Crews, 1987; Dzieweczynski et al., 2006; Lehrman, 1965; McGregor and Peake, 2000).

For aquatic and amphibious species, the boundary between internal and external stimulation becomes blurred when examining the influence of chemical communication and hormonal pathways on behavior. Most anurans (frogs and toads) are semi-aquatic as they commonly need water to call and to deposit eggs (Wells, 2010; Wells and Schwartz, 2006). Males of many species aggregate at aquatic sites and form lek-like choruses, producing species-specific advertisement calls to attract mates (Gerhardt and Huber, 2002; Kelley, 2004; Ryan, 2001). Both steroid hormones and glandular secreted proteins can be excreted or otherwise leached into the water by calling males at the breeding sites (Baugh et al., 2018; Gabor et al., 2013; Woodley, 2015). Due to the permeability of their skin, amphibians can be particularly sensitive to these chemicals (e.g., Hayes et al., 2010); this might in turn affect the steroid-dependent behavior of neighboring males directly, or may be indirectly integrated as social cues for neighboring males. Exogenous chemicals, including androgens and glucocorticoids, can be absorbed directly or perceived via the main or auxiliary olfactory pathways. Therefore, it is plausible that steroid-dependent behavioral feedback loops could emerge among calling males sharing an aquatic chorus site.

In frogs with prolonged breeding seasons, choruses occur as discrete daily events. Chorus participation is limited in part by energetic demands and thus varies among individuals both within and among nights (Emerson, 2001; Ryan, 1985). Calling behavior is typically associated with higher levels of androgens (testosterone and dihydrotestosterone)

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than those found in noncalling males (Burmeister and Wilczynski, 2000; Joshi et al., 2017; Leary and Harris, 2013; Marler and Ryan, 1996; Townsend and Moger, 1987). Conversely, exposure to acoustic stimuli from rival males is generally associated with changes in circulating androgens and glucocorticoids (corticosterone) (de Assis et al., 2012; Emerson and Hess, 1996). The relative roles of diurnal hormonal patterns and social stimulation in driving chorus participation are unclear (Leary and Knapp, 2014). Therefore the causal relationship between calling behavior, exposure to social stimulation, and directional changes in steroids over time must be addressed together to fully understand individual differences in reproductive behavior. Moreover, the role of exogenous chemical secretions from rival males in these hormone-behavior relationships has not previously been considered.

Although chemical communication is far less well studied than acoustics in frogs, the importance of multimodal communication is increasingly recognized in anuran systems (Starnberger et al., 2014). Males respond to seismic and visual cues while chorusing (e.g., Amézquita and Hödl, 2004; Gridi-Papp and Narins, 2010; Halfwerk et al., 2014) and females integrate visual cues of the vocal sac or aposematic coloration while making mating decisions (e.g., Crothers et al., 2011; Taylor et al., 2011). Sexual displays generally are quite complex, often involving multiple interacting components and spanning multiple sensory modalities (Callander et al., 2012; Candolin, 2003). Both the aspects of displays that evolved for the purpose of communication (signals) as well as the components that provide indirect information as a byproduct of signaling behavior (cues) affect behavior and physiology of receivers. More specifically, display components can be redundant, reducing uncertainty in noisy environments and having equivalent effects on the receiver, or nonredundant, providing different information about the signaler and resulting in unique receiver responses not predicted by the quantitative assessment of each stimulus in isolation. Our use of the term "redundancy" is in accordance with Hebets and Papaj (2005), Johnstone (1996), Moller and Pomiankowski (1993), Otovic and Partan (2009), Partan and Marler (1999, 2005), Partan (2017), Ronald et al. (2012), and Rowe (1999), which focuses on function irrespective of sensory structure. We should note that recent authors have advocated a systems approach which reserves "redundancy" for cases in which both function and structure are shared (Hebets et al., 2016). Given that acoustic and chemical sensory structures are clearly different, these patterns would be termed "degenerate" using that convention.

When display components are combined, they can elicit different behavioral and physiological responses than they do in isolation. For example, Narins et al. (2003) found that males of the poison frog, *Epipedobates femoralis*, infrequently approach a rival male's call in isolation. They will, however, consistently approach the combined stimulus when visual cues of the rival are also present. Similarly, male cichlid fish, *Astatotilapia burtoni*, respond aggressively to the visual stimulus of an intruding male but not to the chemical stimulus in isolation. Testosterone increases in response to the chemical cue of an intruder, however, and not to the visual stimulus alone (O'Connell et al., 2013). Therefore, in order to obtain a fuller understanding of the receiver's response, the effects of multimodal stimuli should be examined for both hormones and behavior.

Here we investigated the effects of multimodal (chemical and acoustic) social stimuli on the competitive interactions and hormonal response of male túngara frogs (*Physalaemus* = *Engystomops pustulosus*). Túngara frogs are small, nocturnal anurans commonly found throughout much of Mesoamerica and northern South America. During the breeding season (May – December), males aggregate in shallow ponds most evenings around dusk and produce species-specific advertisement calls to attract mates. Competitive interactions at the chorus include acoustic responses such as call rate changes and escalation of call type as well as locomotor responses such as phonotaxis, adjustments in spatial proximity, and physical combat (Baugh and Ryan, 2010; Bernal et al., 2009, 2007; Burmeister et al., 1999; Ryan, 1985). No previous studies have examined the potential detection of chemical stimuli in male or female túngara frogs, but chemical stimuli could be relevant in this species in particular because of the close proximity with which males are often found calling (Lea, 2016; Ryan, 1985) and because call sites generally consist of pools and pond peripheries where water currents are low. Moreover, túngara frog choruses are variable and noisy environments. These conditions favor the evolution of more complex, multimodal displays that reduce the uncertainty in signal reception (Ay et al., 2007; Dall and Johnstone, 2002). Thus our primary goal was to determine whether males exhibit behavioral and physiological responses to the experimental addition of conspecific chemical cues in a chorus. Our secondary goal was to classify the chemical component as redundant or nonredundant to the primary mode of communication, the acoustic signal.

We presented male frogs with a conspecific acoustic stimulus (the sound of a chorus), a conspecific chemical stimulus (water containing excretions from calling males), or a combination of both social stimuli on consecutive days. We measured each male's locomotor activity, latency to call, and calling effort in addition to water-borne testosterone and corticosterone levels prior to, during, and following social stimulation. In order to acquire multiple hormone samples from the males while minimizing the known confounds of handling stress (Narayan, 2013), we developed a non-invasive methodology in which the holding water for frogs was remotely added and removed during behavioral experiments. In this way, our experimental design permitted repeated, continuous, and simultaneous behavioral and hormonal assays.

2. Methods

2.1. Animal collection

Experiments were performed using the laboratory facilities of the Smithsonian Tropical Research Institute (STRI) in Gamboa, Panama from June–August 2014. Adult, male *Physalaemus pustulosus* were collected at night from nearby calling sites and tested over the subsequent four days. During this time, frogs were maintained on a 12:12 h light cycle in individual critter cages within the laboratory. After behavioral testing, frogs were measured (SVL), weighed (g), marked with a unique toe-clip number to prevent recapture, and released at the original capture location (n = 8 frogs, average ± SD SVL = 26.38 mm ± 0.81, average ± SD mass = 1.72 g ± 0.19). Marking procedures were approved by the Institutional Animal Care and Use Committees at the University of Texas at Austin (UT Austin) and at STRI.

2.2. Experimental methodology

Traditional collection procedures require blood collection from individual frogs. Not only is this approach highly invasive, especially for smaller animals, but it also limits the frequency at which hormones can be sampled due to extraction and handling-induced stress (Greenberg and Wingfield, 1987). Narayan et al. (2012) demonstrated in cane toads that CORT increases and T decreases with increased intervals of handling, limiting hormone collection to either a single time point or multiple points over extended periods of time. Urine sampling and water-borne hormone collection are less invasive than blood collection and still an accurate and biologically informative means of sampling hormones in aquatic and semi-aquatic species (Gabor et al., 2013; Kidd et al., 2010). For example, it has been previously demonstrated in túngara frogs that the concentration of water-borne testosterone (W. Wilczynski, personal communication) and corticosterone (Baugh et al., 2018) is directly correlated with the plasma concentration. Yet, many experimental designs still require the animal to be handled and thus impose similar practical limitations to blood collection.

We developed a novel, non-invasive method that allowed for simultaneous, repeated, and continuous measurements of hormones and behavior. Individual frogs were placed in testing arenas at least one

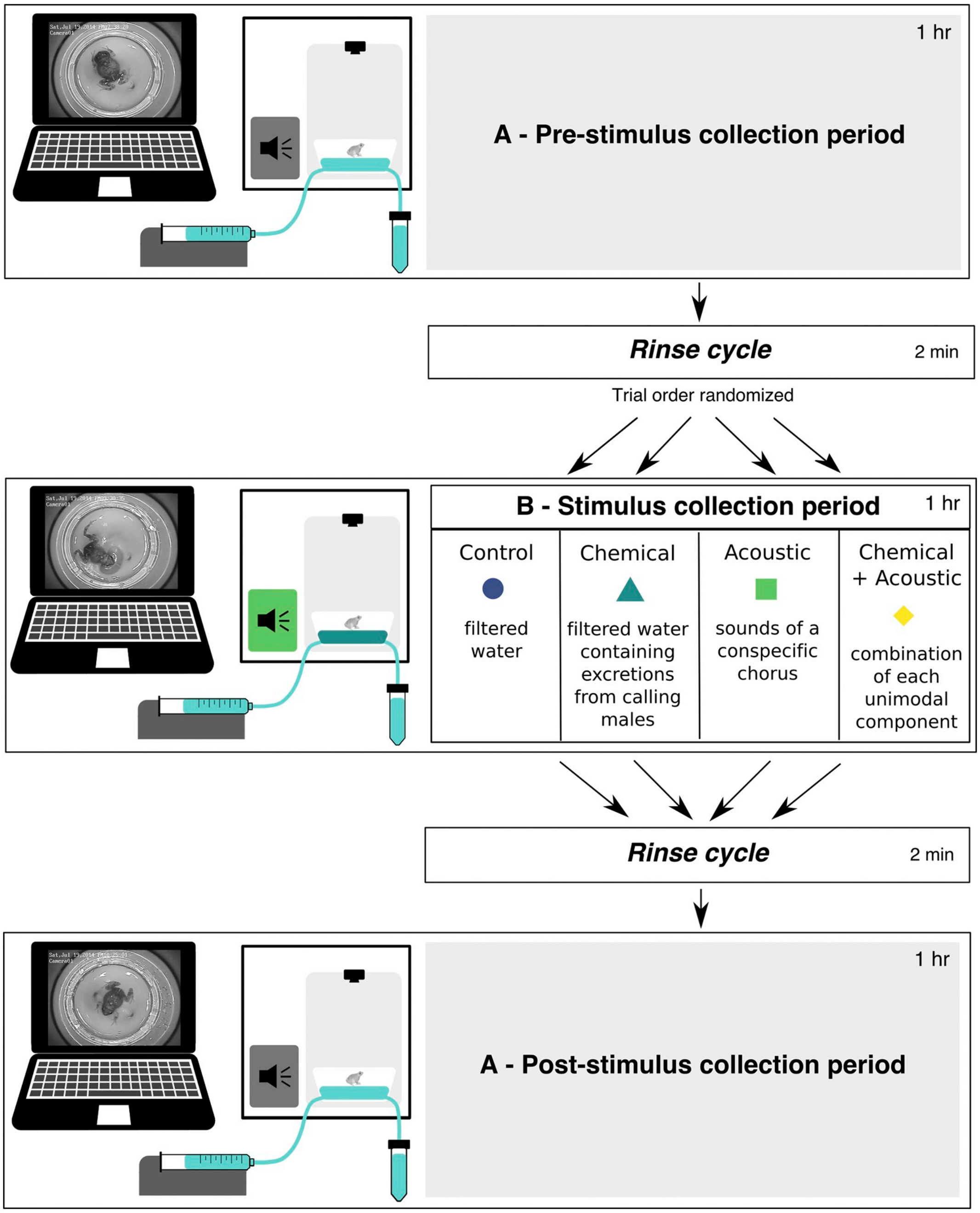

Fig. 1. Illustration of the non-invasive behavioral assay and water-borne hormone collection in male túngara frogs. One stimulus treatment was presented each night: chemical only (triangle), acoustic only (square), chemical and acoustic combined (diamond), or control (circle). Green box (left): speaker from which acoustic stimuli are played. Teal box (middle): water laden with chemical stimuli. Experimental setup not to scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hour before experimentation to minimize the capture of a handling stress response during experimentation. The testing arena consisted of a small glass bowl (dimensions: 9 cm dia. x 3.25 cm deep) with inflow and outflow tubes on either side that allowed us to collect water-borne hormone samples without directly handling the frog (Fig. 1). We used an infusion/withdrawal dual syringe pump (GenieTouch™, Kent Scientific, Torrington, CT, USA) to standardize the rate at which transfusion occurs when adding and withdrawing water in the chamber (60 ml/min). Withdrawn water samples were immediately placed in a − 20 °C freezer for subsequent hormone extraction and analysis and the chamber was rinsed with fresh water between collection periods. Hormone samples were exported from Gamboa, Panama to Austin, Texas, USA for analysis. An enzyme-linked immunosorbent assay was used to determine water-borne levels of corticosterone (CORT) and testosterone (T) (Cayman Chemical, Ann Arbor, MI, USA) as described by Baugh et al. (2018) and in accordance with the manufacturer's instructions. In order to assess the hormonal excretion of the test subject in response to the chemical stimulus, the amount of T and CORT measured in the pooled chemical stimulus was subtracted from the amount of T and CORT measured for each test subject. Four assay plates were analyzed for each hormone. Samples from a given trial (i.e., pre-, during, and post-stimulation) were assayed on the same plate, although individual frogs were distributed randomly across the four assay plates analyzed for each hormone. The average intra- and inter-plate variation was 11.2% and 17.5% for testosterone and 11.1% and 18.7% for corticosterone, respectively.

Males were tested using a repeated-measures, A-B-A experimental design such that every male is assayed for one-hour before (A-pre-stimulus collection period), one-hour during (B-stimulus collection period), and one-hour after (A-post-stimulus collection period) social stimulation (Fig. 1). Each collection period was measured by the total amount of time the frog spent in contact with the water (instead of a one-hour continuous period) as frogs had the ability to sit on the edge of the bowl just outside the water's edge. Each male was tested in one of the following four treatment groups each night until they completed all four trials: acoustic only, chemical only, combined chemical and acoustic, and control (filtered water). Frogs were tested in one treatment each day for four consecutive days. The order of treatments was randomized across frogs. Acoustic stimuli consisted of a naturally recorded chorus of túngara frog calls recorded in 2009 (Lea, 2016). Chemical cue stimuli were water samples collected previously from males stimulated with natural chorus sounds that were pooled together (and frozen at −20 °C until use) to ensure each test subject was exposed to the same chemical stimulus throughout the experiment.

Males were tested in sound-attenuated boxes (dimensions: $28 \times 28 \times 40\ \text{cm}^3$) between 1900 and 0230 h (Fig. 1). Acoustic stimuli were presented using Audacity (version 2.1.2) and broadcast at a peak level of 90 dB SPL (re. 20 μPa) at 10 cm measured by an Extech Instruments sound pressure level meter (model 407,764). Trials were video recorded using miniature infrared security cameras and later scored for behavioral analysis by naïve scorers; inter-rater reliability (i.e., agreement of repeated ratings from multiple raters that observed the same videos) was assessed for 5% of the trials (intraclass correlation coefficient > 0.9 for all measures). We measured three behaviors. First, we recorded locomotor activity as the number of times the frogs moved in and out of the water bowl during each stimulation period. Second, we measured latency to the male's first call. Finally, we recorded call number, defined as the total number of calls during each stimulation period.

2.3. Statistical analyses

Statistical analyses were conducted in R version 3.4.1 (R Core Team, 2017) using a significance threshold of $\alpha = 0.05$. To account for repeated measures of the same frog across treatments, mixed models were used for all analyses using an ID unique to each frog as a random effect. For testosterone and corticosterone, data were log transformed to meet the assumption of normality. A linear mixed model (LMM) was then fitted for each hormonal response and a generalized linear mixed model (GLMM) with negative binomial error distributions and log link function (glmer.nb) was used to account for zero-inflation and over-dispersion in behavioral counts (lme4 package; Bates et al., 2015). Latency to the first call was modeled using a mixed model Cox proportional hazard regression (MMCoxPH function in coxme package; Therneau, 2015). All models testing the effect of treatment on the response variable were initially modeled with treatment order, collection period (pre, during, and post-stimulation), social stimulus combination (chemical and acoustic), and all logical combinations as fixed effects and individual identification of frog as the random subject effect. The model was then reduced using an iterative model selection procedure. Hormone-behavior relationships were modeled using the same model type as noted above but only included the hormone type and the collection period as the predictor variables. Multimodal stimuli components were classified using pairwise post-hoc comparisons of treatment and control groups. We used the Tukey's procedure to conduct pairwise post hoc tests (with a significance threshold of $\alpha < 0.05$) (lsmeans package; Lenth, 2016). Effect sizes (Cohen's d, Cohen, 1988) were calculated from the estimated marginal means (lme.dscore function in EMAtools package; Kleiman, 2017). Intra-class correlation coefficients (ICC, Shrout and Fleiss, 1979) were used to calculate an index of inter-rater reliability. We used two-way models to treat both raters and subjects as each being randomly drawn from a population (icc function in irr package; Gamer et al., 2012). Descriptive statistics are presented as the mean ± standard error or 95% confidential interval as specified.

3. Results

We first examined the effect of chemical and acoustic stimuli combinations on hormone measures (T and CORT) prior to, during, and following social stimulation. We subsequently examined the effect of those stimuli on behavioral measures (locomotor activity, latency to call, and call number) over each collection period. Lastly, we evaluated the relationship between hormones and behavior over each collection period independent of the particular combination of social stimulation.

3.1. Stimulus – behavior relationships

3.1.1. Locomotor activity

Locomotor activity was not affected by chemical stimulation alone (GLMM: chemical, $p = 0.096$), but increased significantly in response to acoustic stimulation across the collection period (GLMM: acoustic * collection period, $p = 0.005$; Fig. 2a). Locomotor activity did not vary significantly across treatments prior to or following the stimulation period (pairwise p-values, range: 0.46–1.0). Within the stimulation period, locomotor activity significantly increased in response to acoustic stimulation by a factor of 5.2 ($p < 0.001$, $d = 0.71$). The effects of chemical and acoustic stimuli in isolation were additive and thus accurately predicted the response to the combined stimulus (i.e., chemical only + acoustic only = combined chemical and acoustic stimulus; $p = 0.85$). Therefore, locomotor activity significantly increased in response to acoustic treatments and declined once the stimulus was removed.

3.1.2. Latency to call

There was a trend for shorter latency to call in chemical treatment groups by a factor of 1.6 (MMCoxPH: chemical, $\chi^2_1 = 3.38$, $p = 0.066$). The latency to call varied significantly across collection period in response to acoustic stimulation (MMCoxPH: acoustic * collection period, $\chi^2_2 = 6.85$, $p = 0.033$; Fig. 2b). Latency to call did not vary significantly across treatments prior to or following social stimulation (pairwise p-values, range: 0.2–1.0). During the stimulation period, however, the latency to call significantly decreased in response to acoustic stimulation by a factor of 3.4 ($p = 0.046$). The effects of chemical and acoustic stimuli in isolation were additive ($p = 0.1$). Thus, similar to locomotor activity, the latency to call significantly decreased in response to acoustic treatments and declined once the stimulus was removed.

3.1.3. Call number

When all males are included in the model, the number of calls produced, referred to from here on as “calling”, is significantly greater in chemical treatment groups by a factor of 1.5 (GLMM: chemical, $p = 0.019$). When the male with the greatest influence was excluded from the model, however, the significance of this effect decreases to a non-significant trend (GLMM: chemical, $p = 0.089$). Calling varied significantly across the collection period in response to acoustic stimulation (GLMM: acoustic * collection period, $p < 0.001$; Fig. 2c) when modeled with and without the male of greatest influence. Calling did not vary significantly across treatments prior to the stimulation period (pairwise p-values, range: 0.44–1.0). During and following stimulation, calling significantly increased in response to acoustic stimulation by a factor of 12.2 when presented in isolation ($p < 0.001$, $d = -2.50$) and 18.9 when presented in combination with the chemical stimulus ($p < 0.001$, $d = -2.94$). The chemical stimulus in isolation elicited a slight increase in calling (1.5 times greater than the control respectively), but these levels were not statistically different from the control ($p = 0.44$). The calling response to the combined stimulus was multiplicative (i.e., combined chemical and acoustic stimulus > chemical stimulus + acoustic stimulus; $p < 0.001$). Therefore, calling

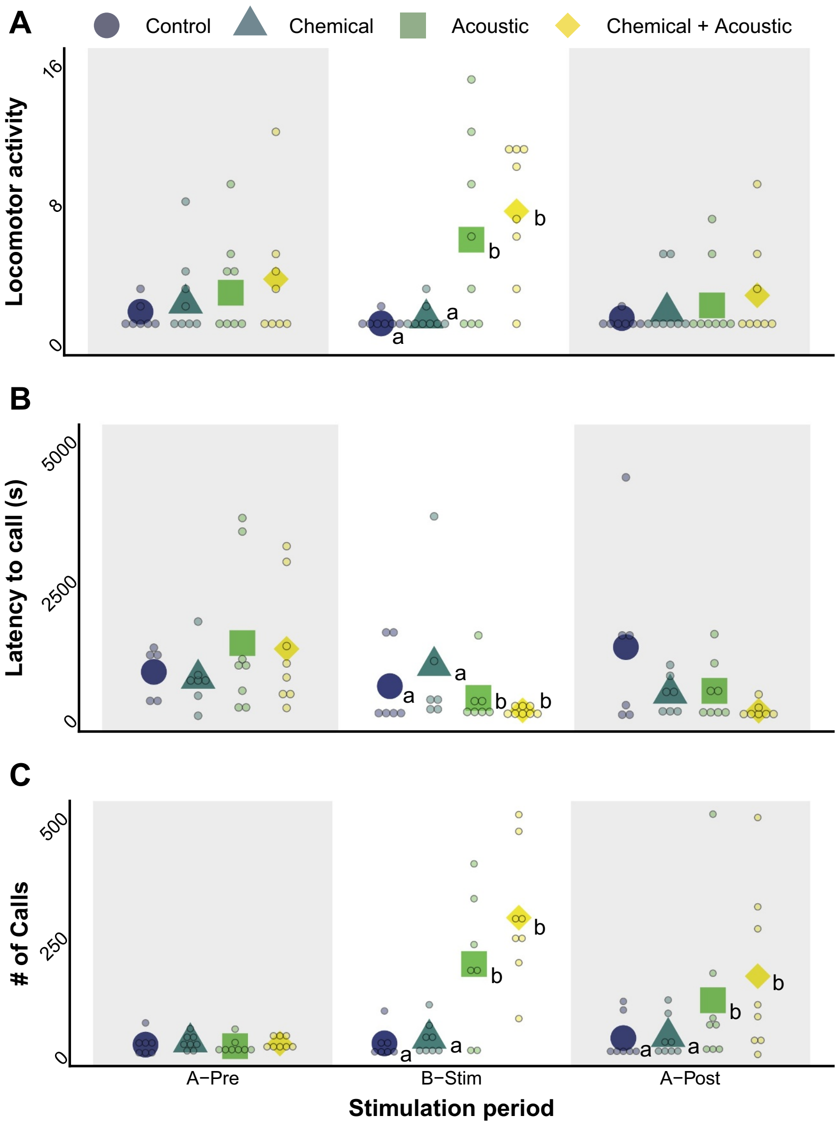

Fig. 2. In panels (A–C), each point represents an individual's hormone measurement grouped by treatment: chemical only (triangle), acoustic only (square), chemical and acoustic combined (diamond), and control (circle); n = 8. The colored shapes are the predicted means for each treatment group from the appropriate model for (A) locomotor activity and (C) numbers of calls. The colored shapes in panel (B) are the raw means for each treatment group for latency to call [note: raw values are depicted because a survival analysis was used to analyze the data instead of a regression model]. Letters (a-b) denote significant differences after Tukey's correction. Points are offset slightly on the x-axis for data visualization purposes only.

significantly increased in response to acoustic treatments and remained elevated once the stimulus was removed.

3.2. *Stimulus – hormone relationships*

3.2.1. *Testosterone*

There was a non-significant trend for T response to chemical and acoustic stimuli to vary across collection periods (GLMM: chemical * acoustic * collection period, $p = 0.064$; Table 1; Fig. 3a). As expected, T levels did not vary significantly across treatments prior to or following the stimulation period (pairwise p-values, range: 0.43–1.0). Within the stimulation period, however, chemical stimulation increased T levels by a factor of 2.6 and 5.1 when presented in isolation ($p = 0.07$, $d = -0.54$) and in combination with acoustic stimulation ($p < 0.001$, $d = -2.72$) respectively. T levels in response to acoustic stimulation alone (1.3 times greater than the control) were not statistically different from the control ($p = 0.56$). Moreover, the T response to the combined stimulus appears to be multiplicative ($p < 0.001$). Thus, T significantly increased in response to treatments with chemical stimulation and declined once the stimulus was removed.

3.2.2. *Corticosterone*

A similar pattern was observed for CORT. In response to chemical and acoustic stimulation, CORT levels varied across collection period (GLMM: chemical * acoustic * collection period, $p = 0.026$; Table 1; Fig. 3b). Similarly, CORT levels did not vary significantly across treatments prior to or following the stimulation period (pairwise p-values, range: 0.68–1.0). In contrast to T, however, CORT levels significantly increased in response to the combined chemical and acoustic stimulus (6.4 times greater than the control, $p < 0.001$, $d = -2.90$) within the stimulation period only. While there were slight CORT increases in response to chemical and acoustic stimulation in isolation (1.8 and 1.5 times greater than the control respectively), these levels were not statistically different from the control ($p > 0.1$). The CORT response to the combined stimulus was also multiplicative ($p < 0.001$). Therefore, CORT significantly increased only in response to the multimodal stimulus and declined once the stimulus was removed.

3.3. *Hormone-behavior relationships*

3.3.1. *Locomotor activity*

Across the collection period, locomotor activity varied significantly in its relationship with T but not with CORT (GLMM: T * collection period, $p = 0.045$; CORT * collection period, $p = 0.22$; Fig. 4a,d). T was positively correlated with locomotor activity prior to and during

Table 1
Mixed models investigating the influence of the collection period and social stimuli type on hormones (LMM).

Fixed effect		df	χ^2	p	Effect size (d)
Stimulus	Estimate ± SE				
Testosterone (pg/mL/SVL)					
(Intercept)	1.81 ± 0.33				
Collection period		2	2.12	0.347	
Pre-stim	0.00 ± 0.00				
Stim	0.43 ± 0.30				
Post-stim	0.25 ± 0.30				
Acoustic		1	**4.49**	**0.034***	
Absent	0.00 ± 0.00				
Present	0.63 ± 0.30				
Chemical		1	**4.68**	**0.030***	
Absent	0.00 ± 0.00				
Present	0.65 ± 0.30				
Collection period * Acoustic		2	1.07	0.586	
Pre-stim * Absent	0.00 ± 0.00				
Stim * Present	−0.31 ± 0.42				−0.169
Post-stim * Present	−0.42 ± 0.42				-0.227
Collection period * Chemical		2	1.15	0.562	
Pre-stim * Absent	0.00 ± 0.00				
Stim * Present	0.32 ± 0.42				0.174
Post-stim * Present	−0.11 ± 0.42				-0.062
Acoustic * Chemical		1	**5.81**	**0.016***	
Absent	0.00 ± 0.00				
Present	−1.02 ± 0.42				−0.549
Collection period * Acoustic * Chemical		2	5.49	0.064	
Pre-stim * Absent	0.00 ± 0.00				
Stim * Present	1.36 ± 0.60				0.517
Post-stim * Present	0.99 ± 0.60				0.375
Individual ID (Random Effect)	0.51 ± 0.25				
Corticosterone (pg/mL/SVL)					
(Intercept)	1.83 ± 0.29				
Collection period		2	3.40	0.183	
Pre-stim	0.00 ± 0.00				
Stim	0.58 ± 0.32				
Post-stim	0.21 ± 0.32				
Acoustic		1	**4.05**	**0.044***	
Absent	0.00 ± 0.00				
Present	0.64 ± 0.32				
Chemical		1	3.77	0.052	
Absent	0.00 ± 0.00				
Present	0.62 ± 0.32				
Collection period * Acoustic		2	1.78	0.411	
Pre-stim * Absent	0.00 ± 0.00				
Stim * Present	−0.23 ± 0.45				-0.114
Post-stim * Present	−0.60 ± 0.45				-0.301
Collection period * Chemical		2	0.57	0.754	
Pre-stim * Absent	0.00 ± 0.00				
Stim * Present	−0.05 ± 0.45				-0.026
Post-stim * Present	−0.32 ± 0.45				-0.160
Acoustic * Chemical		1	3.50	0.061	
Absent	0.00 ± 0.00				
Present	−0.84 ± 0.45				-0.427
Collection period * Acoustic * Chemical		2	**7.28**	**0.026***	
Pre-stim * Absent	0.00 ± 0.00				
Stim * Present	1.71 ± 0.64				0.610
Post-stim * Present	1.04 ± 0.64				0.370
Individual ID (Random effect)	0.27 ± 0.19				

Collection period refers to the three consecutive temporal intervals: before, during or after the experimental manipulation; *present* (experimental) vs. *absent* (control) refers to whether or not the social stimuli (chemical and/or acoustic cues) were presented during the stimulus interval. Regression estimates are presented in addition to results from ANOVA. A negative effect size (Cohen's d) indicates a negative effect. *Statistically significant effects ($p < 0.05$) are boldface.

stimulation but not after the stimulation ceased (pre-stim: p = 0.002; stim: p = 0.06; post-stim: p = 0.70). In contrast, CORT was positively correlated with locomotor activity regardless of the collection period (p = 0.004).

3.3.2. *Latency to call*

Latency to begin calling and hormone levels did not vary across collection periods, and thus the interaction term was removed from the model. The latency to call significantly decreased as T and CORT increased (GLMM: T, $p < 0.001$; CORT, p = 0.001; Fig. 4b,e).

3.3.3. *Call number*

The calling and T relationship statistically varied across the collection period (GLMM: T * collection period, p = 0.001; Fig. 4c). Prior to stimulation, T was not significantly correlated with calling (p = 0.48). During and following stimulation, T was significantly

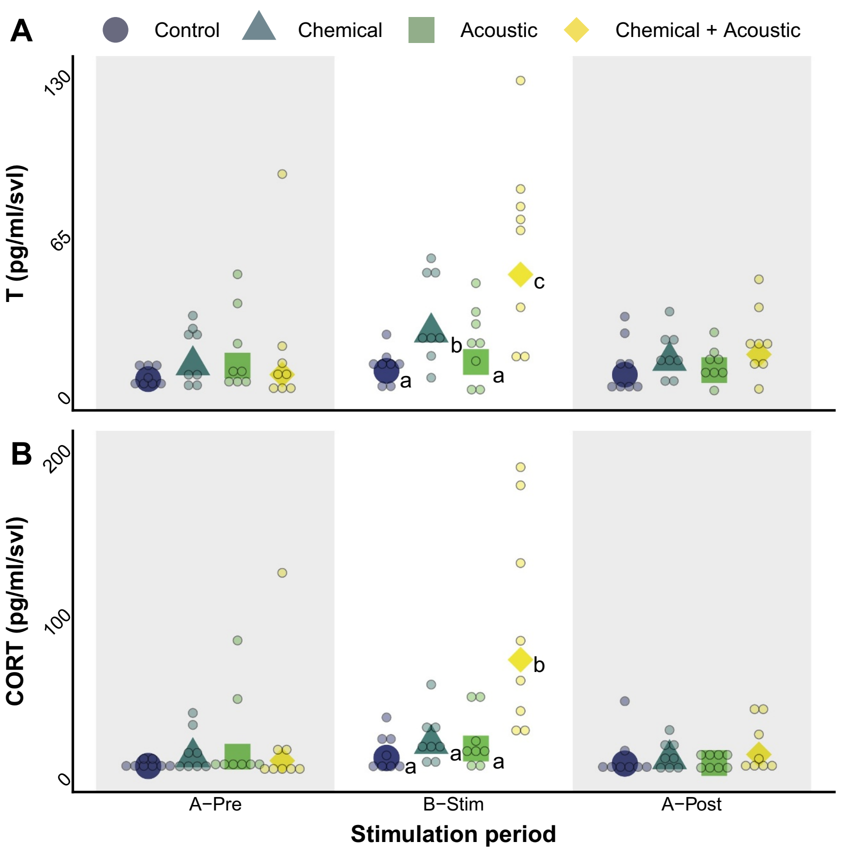

Fig. 3. In panels (A) and (B), each point represents an individual's hormone measurement grouped by treatment: chemical only (triangle), acoustic only (square), chemical and acoustic combined (diamond), and control (circle); n = 8. The colored shapes are the predicted means for each treatment group from the appropriate model for (A) T and (B) CORT. Letters (a–c) denote significant differences after Tukey's correction. Points are offset slightly on the x-axis for data visualization purposes only.

positively correlated with calling (during stimulation: $p = 0.007$; post stimulation: $p < 0.001$). The calling and CORT relationship also varied across the collection period (GLMM: CORT * collection period, $p = 0.015$; Fig. 4f). Similarly to T, CORT was significantly correlated with calling during ($p = 0.004$) and following the stimulation period ($p = 0.002$) but not prior to stimulation ($p = 0.92$).

4. Discussion

The importance of multimodal communication in reproductive behavior has been well studied with respect to female choice but its importance in inter-male competition has received significantly less attention. The role of chemical signals in mediating male competition is particularly under-studied. Unlike in terrestrial animals, where the border between internal (e.g., hormones) and external (e.g., social) stimulation can be concrete, the boundary is blurred for aquatic and semi-aquatic species. As a result of passive excretion of hormones and glandular secretions, amphibians might experience a quite different chemical milieu than do terrestrial animals. For this reason, the role of chemical cues in anuran communication systems may be particularly important. Additionally, while the same signals that are used to stimulate mating behaviors, such as increased calling, also stimulate endogenous signaling pathways, such as increases in testosterone, hormonal responses to multimodal stimulation are often not investigated. In this study we addressed the potential of internal and external chemical secretions to supplement and further interact with other signaling systems, creating multimodal signals. We first established non-invasive, continuous sampling methods for simultaneously measuring both hormones and behavior in semi-aquatic anurans. Next, we used a series of cue isolation tests to examine whether rival frogs secrete chemicals into the water that subsequently interact with acoustic calls to affect the physiology and behavior of competing male túngara frogs. Our results demonstrate that male túngara frogs perceive and respond to conspecific chemical secretions in the water, which interact with chorus sounds to alter physiological and behavioral responses in unexpected ways.

Our results are consistent with the anuran chemical communication literature, which have implicated predominantly non-volatile compounds in sexual communication (for a review, see Woodley, 2015). For example, hormone metabolites excreted in the water could have evolved to function like pheromones, a phenomenon referred to as hormonal-pheromones (Cardwell et al., 1995; Doving, 1976; Sorensen, 1992; Sorensen and Stacey, 2004; Stacey et al., 2003; Stacey and Sorensen, 2009; Stacey, 1991). In fish, hormonal pheromones can induce reproductive responses not only between, but also within the sexes (Kidd et al., 2013b, 2013a; O'Connell et al., 2013). Sansone et al. (2015) recently determined that the African clawed frog, *Xenopus laevis*, excrete compounds consistent with sulfated steroids and that those compounds activate a significant subset of steroid-sensitive neurons in the main and accessory olfactory systems, suggesting their involvement in pheromonal communication. In general, studies of chemical communication emphasize intersexual communication, while studies of intrasexual communication are rare. King et al. (2005) demonstrate that male mountain chicken frogs, *Leptodactylus fallax*, excrete peptides from their skin, which act as a pheromone that stimulates attraction and aggression (jumping and rearing behavior) towards rival males. Our study similarly suggests inter-male communication although it is not clear whether the chemical secretions are excreted actively or passively into the water. Chemicals contained in our water samples include, but are not limited to exogenous steroid hormones, secretions from the glandular dermis, and urine from chorusing male túngara frogs.

To classify the observed responses to the chemical stimulus as redundant or nonredundant to the acoustic stimulus, we compared the effect of each unimodal display component to the combined multimodal stimulus for both hormonal and behavioral responses for the stimulus

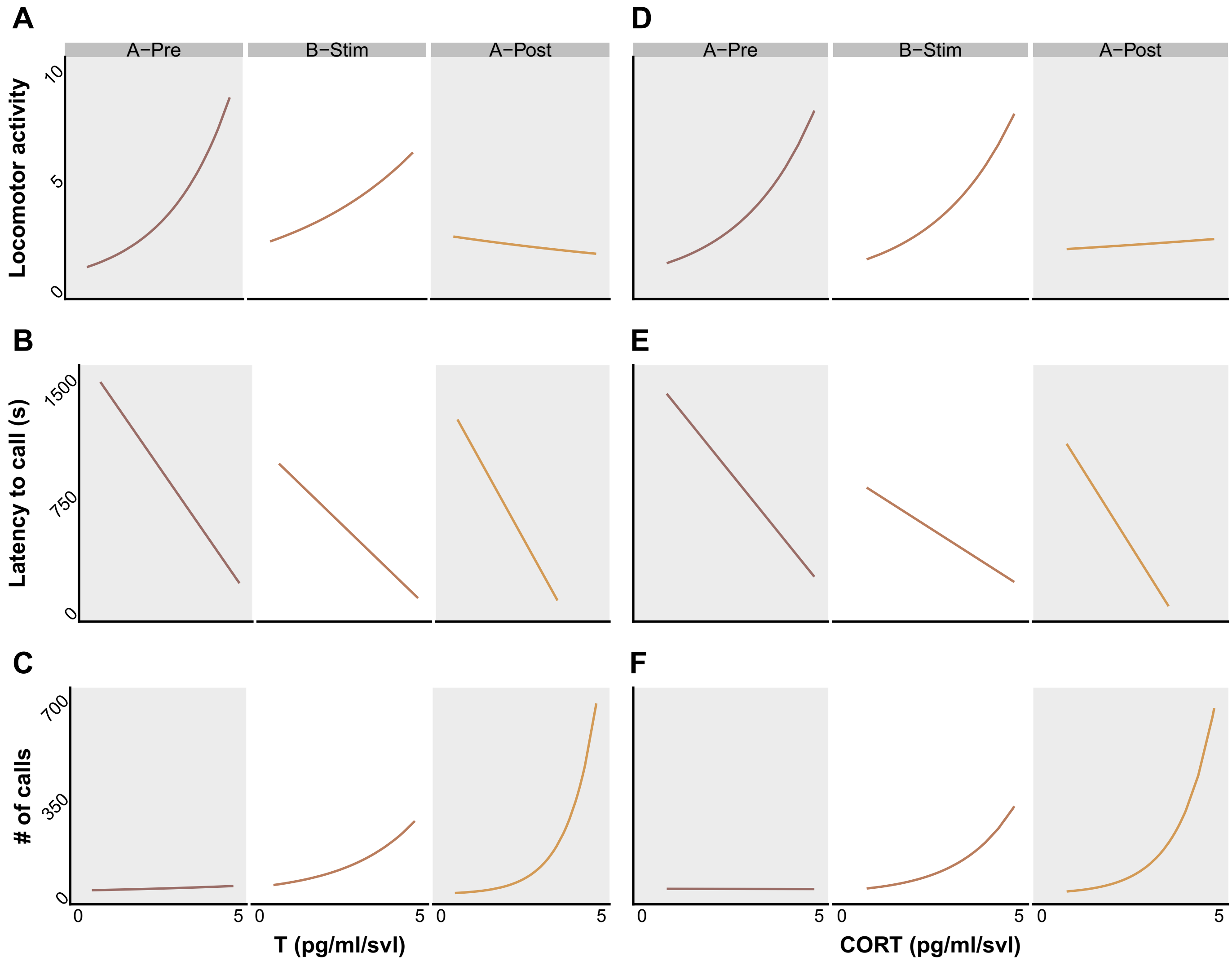

Fig. 4. In panels (A–F), each line depicts the relationship between hormones, T (A–C) and CORT (D–F) respectively, and behavior grouped by collection period, before (A-Pre), during (B-Stim), and after (A-Post) social stimulation; n = 8. The colored lines are the predicted regression output for each collection period from the appropriate model for (A, D) locomotor activity and (C, F) numbers of calls. The colored lines in panel (B, E) are for visualization of latency to call only and are depicted using the predicted model output from a GLMM (instead of the survival analysis).

period. Component redundancy (Rowe, 1999), and specifically enhancement (Partan, 2004), is common across animal communication systems. Interestingly, we see evidence for redundancy in behavioral responses but nonredundancy in physiological responses (Table 2). Redundant stimuli elicit the same qualitative responses to both unimodal and multimodal stimuli and can supplement the acoustic signal and ensure that the signal is detected in a noisy environment and increase the overall intensity (Hebets and Papaj, 2005; Partan, 2004; Partan and Marler, 2005; Scheffer et al., 1996). In our study, the acoustic component dramatically evoked behavioral changes whereas the chemical component did not. When presented in combination, however, the chemical component enhanced the behavioral responses to the acoustic stimulus. While chemical cues can be difficult to localize, in water they form a concentration gradient that rival males cannot assess unless they are in close proximity. Thus, the addition of a chemical component could be particularly useful to receivers in providing information about rival proximity during courtship displays. For example, as inter-male distances become smaller, and the risk of acoustic interference or masking becomes greater, chemical cues may serve as a redundant indicator of proximity.

Nonredundant stimuli, on the other hand, elicit distinct responses when combined from those of unimodal and multimodal stimuli; because each component serves a different function in the dyadic interaction between signaler and receiver, the amount of information communicated per unit time is increased. For example, as inter-male distances become larger, the chemical component could draw attention to the quieter, and thus farther, calls. In this sense, chemical cues may emphasize that the threat of either direct acoustic competition or physical contest is imminent. The chemical component can also inadvertently convey private information, such as the level of testosterone of the caller (e.g., Baeckens et al., 2017; Longpre and Katz, 2011; Stacey et al., 2003). Therefore, the use of multimodal information can provide a more accurate depiction about the quality or condition of the competitor and thus further lead to quicker recognition and assessment of threats.

When chemical and acoustic components are presented in combination, both androgens and glucocorticoids significantly increase. This hormonal peak, along with an increase in locomotor activity, a decrease in latency to call, and an increase in calling behavior in response to the multimodal stimulus suggests that the combination of the chemical and acoustic components sufficiently elevates the perceived level of competition to physiologically prepare the individual to fight (challenge

Table 2
Multimodal stimulus categorization.

		Chemical (response)	Acoustic (response)	Chemical + Acoustic (response/intensity)	Proposed stimulus category
Nonredundancy					
Hormones	*Testosterone*	Weak +	ND	Strong + / Multiplicative	Modulation
	Corticosterone	ND	ND	Strong + / Multiplicative	Emergence
Redundancy					
Behaviors	*Locomotor activity*	ND	Strong +	Strong + / Minor	Enhancement
	Latency to call	ND	Weak -	Weak - / Summation	Enhancement
	Call number	Weak +	Strong +	Strong + / Multiplicative	Enhancement

Note: The multimodal stimulus is made up of two components, the chemical and acoustic stimuli. Using results from Tukey's corrected pairwise post-hoc tests, the magnitude of the response was classified as not detectable (ND), trend, weak, moderate, or strong when p-value equaled 1–0.1, 0.1–0.05, 0.05–0.001, 0.001–0.0001, and < 0.0001 respectively. Hormonal and behavioral responses were classified as redundant and nonredundant, and into further sub-categories, according to the following classification system. Redundant stimuli be placed into three categories based on the intensity of the response to the multimodal stimulus relative to its unimodal components: antagonism, lower intensity: equivalence, equal intensity; and enhancement, greater intensity (Hebets and Papaj, 2005; Munoz and Blumstein, 2012; Otovic and Partan, 2009; Partan and Marler, 2002, 1999, 2005; Partan, 2004). Behavioral responses were further classified into three sub-categories of enhancement based on the intensity of stimulus enhancement: minor enhancement, when the response to the multimodal stimulus is greater than the response to the unimodal stimuli but less than the sum; summation, when the level of enhancement mathematically sums to the total of the unimodal components; and multiplicative increase, when the response to the multimodal stimulus is greater than the sum of its components (Partan and Marler, 2002; Partan, 2004). Non-redundant stimuli can be grouped into four categories based on the response to the multimodal stimulus relative to each of the components: dominance, one component can mask the effect of another; modulation, one component can alter the magnitude of the effect of another; independence, each component continues to have distinct effects, or emergence, when the multimodal response is novel altogether (Partan and Marler, 2002, 1999, 2005; Partan, 2004). The proposed stimulus category is included in this table to facilitate comparisons with the published literature.

hypothesis, Wingfield et al., 1990). While hormone-behavior relationships are rarely investigated in response to multimodal stimuli, similar results were found in two studies of intrasexual communication in cichlid fish and song sparrows (O'Connell et al., 2013; Wingfield and Wada, 1989).

A more detailed analysis of the unimodal responses might give us some clues about the salience of chemical stimuli in a competitive context. We observe a T increase in response to social stimulation, but specifically in response to the chemical stimulus and not to the acoustic stimulus as might be expected (Burmeister and Wilczynski, 2000). In contrast to the acoustic signal, however, the chemical component does not significantly impact the behavior of the males when presented in isolation. We do observe an increase in locomotor activity, a decrease in the latency to call, and an increase in calling in response to the acoustic stimulus, however, and males continue to call even after the stimulus is removed. This evidence suggests that competitive social stimulation not only differentially influences the time course over which physiological and behavioral states vary, but it also affects hormone excretion and behavior independently of one another. A similar result was found in the green treefrog (*Hyla cinerea*) where exposure to a conspecific chorus resulted in a simultaneous but independent increase in glucocorticoids, androgens, and calling behavior (Burmeister and Wilczynski, 2000).

Our results demonstrate that across treatments, androgens and corticosteroids are positively correlated with one another, and together they correlate with behavior. A survey of the literature suggests that the hormone-vocal relationship in frogs varies both across contexts within a species (e.g., in contrast to this study, CORT is not positively correlated with T and calling in túngara frogs in Marler and Ryan, 1996) as well as among different species (e.g., CORT higher in callers, Leary, 2004; CORT and T inversely correlated in callers, Leary and Harris, 2013; CORT high and T low in callers, Mendonça et al., 1985). The positive relationship between androgens and reproductive behavior—locomotor activity, motivation to call (i.e., inverse of latency to call), and calling—however, is unsurprising as it has been well documented across vertebrate groups (e.g., Klukowski et al., 2004; Perrot-Sinal et al., 1998; Ros et al., 2006; Solís and Penna, 1997; Wada, 1982).

The present study is one of only a few to demonstrate that waterborne chemical cues are perceived by and alter the reproductive behavior of rival males. When combined with the acoustic stimulus, the multimodal stimulus enhanced behavioral responses and produced unique hormonal responses not predicted by the responses to unimodal stimuli. Additional research is required to identify the mechanism of perception as well as the component of conspecific secretions that elicit physiological and behavioral response in male túngara frogs. Future studies could test the hypothesis that chemical cues provide information about rival proximity by titrating the typical proxy for proximity, call amplitude, with the chemical cues.

5. Conclusions

It is becoming apparent that multimodal signaling is widespread. Multisensory cues are important for a variety of behaviors including habitat selection, prey recognition, predator avoidance, and mate selection. The classification of multimodal stimuli is one means of deciphering the influence of each component on the receiver and plays an important role in the evolution of communication systems (Hebets and Papaj, 2005; Johnstone, 1997, 1996; Partan and Marler, 1999). More complex studies that integrate multimodal stimuli, receiver behavior, and physiology are sorely needed, as is a greater understanding of how animals utilize multiple sensory channels when communicating in changing environments. The experimental methodology introduced in this study can be particularly useful for a broad range of studies from additional fields such as conservation biology, where non-invasive methodologies can be essential to improve the success of captive breeding and reintroduction programs, as well as to monitor environmentally induced stress responses that contribute to population declines.

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