Behavior of Male and Female *Rana Clamitans* Under Social Stress: Using phonotaxis to quantify response to territorial calling

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ABSTRACT:

Territorialism is an important behavior in resource competition that is widespread throughout the animal kingdom. Like other frogs of the Ranid family, male Northern Green Frogs (*Rana clamitans*) use vocal calling to defend spatial territories throughout the summer mating season. Previous work has suggested that males make behavioral decisions based on nearby calls, while females are generally non-responsive and instead localize based on environmental features. We completed phonotaxis experiments on 14 female and 15 male green frogs by playing high and low amplitudes of a territorial call recording from the edge of a cross-grid. Our purpose was to determine if the amplitude of the call impacted movement decisions such as latency, directionality or displacement. We also tested for relationships between the sex and the mass of frogs with these same response variables. We predicted that lower body condition males would exhibit more extreme movements of retreat behavior under high amplitudes while those with higher body condition would less readily move away from calls, and that females would show no significant responses. We found that amplitude had no effect on the end displacement from origin or speaker but that the latency to movement increased with increasing amplitude (p =0.007), suggesting that awareness of nearby competitors may delay movement decisions. We also found that mass correlated with latency and speed measurements differently in males and females.

INTRODUCTION:

In many populations that evolved under conditions of limited food, water, space, or access to mates, spatial territorialism is observed as a competitive strategy for allocation of resources (Wells 1978). Territorialism refers to the exclusion of conspecific members, usually of the same sex and most often male, from defined areas through the use of sensory signaling or aggressive behaviors (Giuggioli et al 2011).

Although such territorial behavior is widespread across the kingdom Animalia, from birds to mammals to fish (Potts and Lewis 2014), many studies on territorialism have centered on anurans due to the spatially intensive competition that exists during the brief mating season and their highly audible calls that serve as signals to breeding activity (Wells 1978). Worth noting is that these features have also made anuran reproduction a signal for climate change, as the time

and duration of the breeding season are highly sensitive to year-to-year changes in temperature and precipitation (Ospina et al 2013).

Vocal calling in anurans serves several purposes. Male ranids, known as true frogs, use calling to defend favorable areas throughout the summer mating season. In other species, calls also have the potential to attract nearby receptive females, making them an important metabolic investment for survival and reproductive fitness, despite the high amount of energy they require (Humfield 2013).

Competitive calling initiates behavioral and hormonal responses in other frogs inhabiting the surrounding vicinity. In addition to sex hormones that are stimulated in both nearby males and females, male green tree frogs also experience increased levels of the stress hormone corticosterone (Leary et al 2014), production of which has been previously linked to stressful scenarios such as that of an approaching predator (Narayan et al 2013).

Hormonal activity stimulates a "fight-or-flight" like behavioral decision in which a call-hearing male can respond with aggression toward the competitor, or otherwise resort to defensive retreat. Garcia et. al found that the response pathway carried out by a hearing male could be predicted by factors related to the male's competitive ability, such as body composition and/or frequency of competitor's call (Garcia et al 2019).

Male frogs with a higher body condition, as indicated by high snout-vent length (SVL) or larger mass, are more likely to engage competitively, while those having comparatively poorer body condition are more apt to retreat from confrontation. Likewise, a low-frequency call (indicating a higher fitness of competitor), will cause retreat in hearing males more often than will high-frequency calls. Males deciding not to competitively engage may sometimes resort to

non-traditional satellite tactics for acquiring a mate. The most common strategy for a satellite male is to remain near a calling male, and intercept only once a female approaches. Although generally less successful (Leary and Knapp 2005), this decision does allow for some metabolic conservation of energy.

Thus, current findings suggest that hearing males of several frog species may use fitness indicators to assess their competitive odds with a nearby calling male, and that this ultimately influences the behavioral response. However, the exact behavioral link associated with the known corticosterone neuroendocrinological stress pathway is still poorly understood for both tree frogs and for the more territorial Ranids.

Female response to competitive calling in Ranids has also been left largely unexplored. It has been shown that unlike tree frog species, Ranid females select mates primarily through evaluation of the area that they occupy. Females are more likely to spend their time in spaces with certain physical features, such as vegetation density (Wells 1997). Males who occupy high quality territories have higher fitness and acquire more mates throughout the season. Thus, male-male competitive encounters for space are self-selecting for mates and calls themselves are not used directly to attract females. However, we wanted to know whether or not females may have developed the means to indirectly locate quality territories using the presence of a chorus or the fitness qualities of calls. If this were the case, both males and females would actively respond to call stimuli.

We sought to determine the response of both male and female green frogs to conspecific calling by analyzing their spatial movements following a nearby audible call in a laboratory setting. The call amplitude (used to simulate distance from calling frog), and the body condition

of the hearing frog will serve as potential covariates that may impact movement. The hypothesis being tested is that as amplitude of competitor call is increased, males with a lower mass will exhibit more extreme movements of retreat behavior, while those with a higher mass will less readily move away from calls. We also hypothesize that female response should be random if their spacing and mate selection is in fact independent of male calls.

MATERIALS AND METHODS:

We analyzed movement responses to calling in 29 green frogs on the University of Notre Dame Environmental Research Center (UNDERC) property in the Upper Peninsula of Michigan and Wisconsin. This included 14 females and 15 males ranging from 4 to 68 grams in body mass. This was done through the use of simple phonotaxis experiments in which a male *Rana clamitans* call recording was repeatedly played via a speaker near the frog placed on a cross-grid at different amplitudes.

The grid we used extended 1.7 meters on either side the central origin along the axis in the +/- y-direction, and 1.3 meters in the +/- x directions. The speaker was placed on the edge of the grid at y =+1.7m away from the frog's start position at the origin. We randomized the orientation of the entire set-up (grid and speaker) at the beginning of each session of trials in order to control for any asymmetries (window light, furniture, etc.) present in the room.

We ran trials using three different call amplitude levels. This included high amplitude (90 decibels), low amplitude (70 decibels) calling treatments, and silent (non-calling) treatment. Amplitude was measured and calibrated at a standard distance of 50 cm. Amplitude serves as an effective proxy for distance due to ease in adjusting volumes, as attempting to manually replace

speaker position may have interfered with natural movement responses. During calling treatments, we used a playback of a single-note territorial green frog call, repeated on an interval of 15 seconds.

Every frog underwent three total trials, one for each amplitude treatment. The order in which the trials were completed was randomized for each individual. Trials began with a one-minute acclimation period during which the appropriate stimulus was played, and the frog was kept in a nontransparent holding container at the origin that allowed sound to easily pass through. Following this period the container was lifted from a distance using a stick attached to a string, and the movements of the frog were recorded for five minutes, or until the frog left the boundaries of the grid.

Movement itself can be characterized through several different descriptors. We chose to measure the latency to first movement, rotation towards or away from the stimulus, estimated speed of travel throughout the trial, and final displacement from the speaker and from the origin. Latency (s) refers to the period of time before a first movement in any direction; rotation was determined by recording the frog's orientation to the nearest quadrant or axis; and speed (cm/s) was taken to be the total displacement from the origin divided by the trial time.

RESULTS:

In our survey of 14 female and 16 male green frogs (Avg.mass = 32.24 +/- 16.03 g), notable variation was observed between individual responses. We transformed all measurements on displacement and latency using a log (value +1) function to improve normality before statistical testing.

The mean distance traveled away from the origin across all trials was 0.783 m. We ran 2-way analyses of variance (ANOVAs) using amplitude and sex as factors and mass as a covariate on the log transformed displacements from the origin and speaker. We found that none of these significantly impacted the total distance a frog traveled from the origin (Fig. 1A; Table 1), but that males did on average travel closer to the speaker than did females (Fig. 1B; Table 2; df 1, p = 0.01556).

We also wanted to know which variables would affect the latency time for a frog to make a movement decision. In a 2-way ANOVA using mass and amplitude as factors, males and females together had a significantly longer delay to movement when the amplitude was highest, and were quickest to move during the silent treatment (Fig. 2; Table 3; df 2, p = 0.007224).

Mass also had a significant effect (Table 3; df 1, p = 0.087408). Independent regressions showed that higher mass male frogs were quicker to initiate movement (Fig. 3A; Table 4; R^2 =0.02617, p = 0.0682) and traveled faster than their lower mass counterparts (Fig. 3B; Table 4; R^2 = 0.1202, p < 0.001). In females, mass did not impact latency (Fig.3C; Table 4) and it decreased speed (Fig. 3D; Table 4; R^2 = 0.0504, p = 0.0225).

DISCUSSION:

Our preliminary finding that none of the response variables we used (sex, amplitude, or mass) affected the overall distance a frog moved from the origin was informative but not altogether surprising. In both males and females, the capacity for locomotion is determined by a number of physiological and metabolic factors unrelated to call response such as temperature,

aerobic capacity, foraging behavior and body composition (Taigen 1985). Thus a frog's motion may simply have more to do with overall energetics than to the particular stress stimulus used.

Despite this we did expect amplitude to have significant effects on certain aspects of movement response, such as orientation and total proximity to the speaker, that were not observed. We found that males on average indicated higher interest in the speaker by traveling closer than females; however we gained no evidence to support our hypotheses about the relation between fitness and competitive aggression. We expected that higher mass males would more readily approach the a louder call, while lower mass males would show signs of retreat as Garcia et al found green tree frogs, but neither mass nor amplitude were good predictors of movement toward or away from the speaker in our study (Garcia et al 2019).

One possible explanation for this discrepancy is that we were simply unable to fully replicate how male-male encounters would occur in natural settings. Because all phonotaxis experiments took place in a laboratory away from the actual territories of the males, this set-up is likely to simulate a scenario in which the experimental male has intruded into the foreign territory of a competitor. Thus responses may be overall more cautious and less competitive than might be seen if the male instead were in a position of actively defending his own territory (Wells 1997). Additionally,

Our expectation that females would not be affected significantly by calling was relatively well-supported; their movement was more likely to be in a direction away from the stimulus than males, and their proximity to the speaker did not differ substantially between the calling and non-calling treatment amplitudes. This serves as further evidence to the null that female green frogs choose location independently of male calls (Wells 1997).

A variable of interest considering the temporal span of this study, which took place during late June and early July, is the reproductive status of females. Abdominal observations led us to believe that several larger females were gravid during trials, while others --including most smaller juveniles-- were not. This factor could be important to female response as receptive and non-receptive females presumably have differing hormonal and behavioral effects to male calling (Leary 2014).

Gravidity itself was not considered however and so the best proxy for whether or not a female was gravid in our study was mass. Female body mass was not heavily correlated with any of the metrics used to quantify response, except for speed; unlike in males, heavier females actually traveled slower. This trend could highlight a reproductive cost associated with carrying eggs that was not observed in male counterparts.

Perhaps the most notable finding was that while amplitude didn't affect several variables as we anticipated, it did significantly influence the latency to movement for both female and male frogs. Individuals waited longer to move when the when a call was playing at a high amplitude, and traveled more readily away from the origin at low and silent treatments, suggesting greater caution and delay in decision-making when a frog was thought to be nearby.

This may simply indicate that the corticosterone stress pathway known to initiate movement choices is not immediate, but rather includes a period of hormonal acclimation before an individual is able to respond behaviorally. Thus, in closer proximity encounters causing greater stress (simulated by higher amplitude trials in our study), a frog would express longer delay before action, as we observed.

The finding that with increased mass, males were less latent and moved faster may indicate that these individuals were under less stress and practiced less cautious behavior as a result of their comparatively better fitness and improved competitive odds. Alternatively, larger and more developed males may have acquired learned behaviors that enable them to discriminate between stimuli and respond more efficiently to competitive encounters than juveniles with less experience.

Overall our results suggest that the eventual outcome of a stimulus response may be less relevant than the decision-making process itself that occurs understanding how males regulate their behavioral responses to competitor calling. This concept is particularly relevant for agent-based modeling, in which behavior of any number of individuals is simulated using a number of determinate variables that might impact a choice. In addition, because many other species utilize competitive calling --whether to attract mates, secure resources, or display dominance to competitors-- information on spatial behaviors may be valuable for creating models to simulate population interactions and reproduction among these species, which can be utilized larger-scale ecological monitoring and conservation efforts.

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Table 1. The Effects of Amplitude and Sex on Displacement from Origin with Mass as a covariate. A 2-way ANOVA was run on log-transformed displacement from origin (in cm) using treatment amplitude (high, low and silent) and sex as factors and mass (g) as a covariate. None of the variables had a significant effect on displacement.

Variable	F-value	df	p-value
Treatment	0.1931	2	0.8248
Sex	1.3566	1	0.2476
Treatment*Sex	0.6922	2	0.2476
Mass	0.3316	1	0.5035

Table 2. The Effects of Amplitude and Sex on Displacment from Origin with Mass as a covariate. A 2-way ANOVA was run on log-transformed displacement from origin (in cm) using treatment amplitude (high, low and silent) and sex as factors and mass (g) as a covariate. Sex had a signficant effect on displacment with $p < 0.05$.

Variable	F-value	df	p-value
Treatment	1.1187	2	0.33177
Sex	6.1103	1	0.01556*
Treatment*Sex	0.9437	2	0.39348
Mass	0.0300	1	0.86283

Table 3. The Effects of Sex and Mass on Latency (s) with Amplitude as a covariate. A 2-way ANOVA was run on log-transformed latency (s) using mass (g) and sex as factors and teatment amplitude (high, low and silent) as a covariate. Both mass ($p < 0.1$) and treatment amplitude ($p < 0.001$) were shown to have significant effects on latency. A Tukey's HSD test confirmed that high and silent treatments were significantly different.

Variable	F-value	df	p-value
Sex	0.23611	1	0.477268
Mass	2.9559	1	0.087408*
Sex*Mass	5.0779	1	0.132798
Treatment	0.5074	2	0.007224*

Table 4. Regressions on Mass for male and female Latency (s) and Speed (cm/s). A 2-way ANOVA was run on log-transformed latency (s) using mass (g) and sex as factors and teatment amplitude (high, low and silent) as a covariate. Both mass ($p < 0.1$) and sex ($p < 0.001$) were shown to have signficant effects on latency.

Response variable	Sex	R^2	df	p-value
Latency (s)	Male	0.02617	43	0.0682*
Speed (cm/s)	Male	0.1202	43	<0.0001*
Latency (s)	Female	---	40	>0.01
Speed (cm/s)	Female	0.0504	40	0.0504*
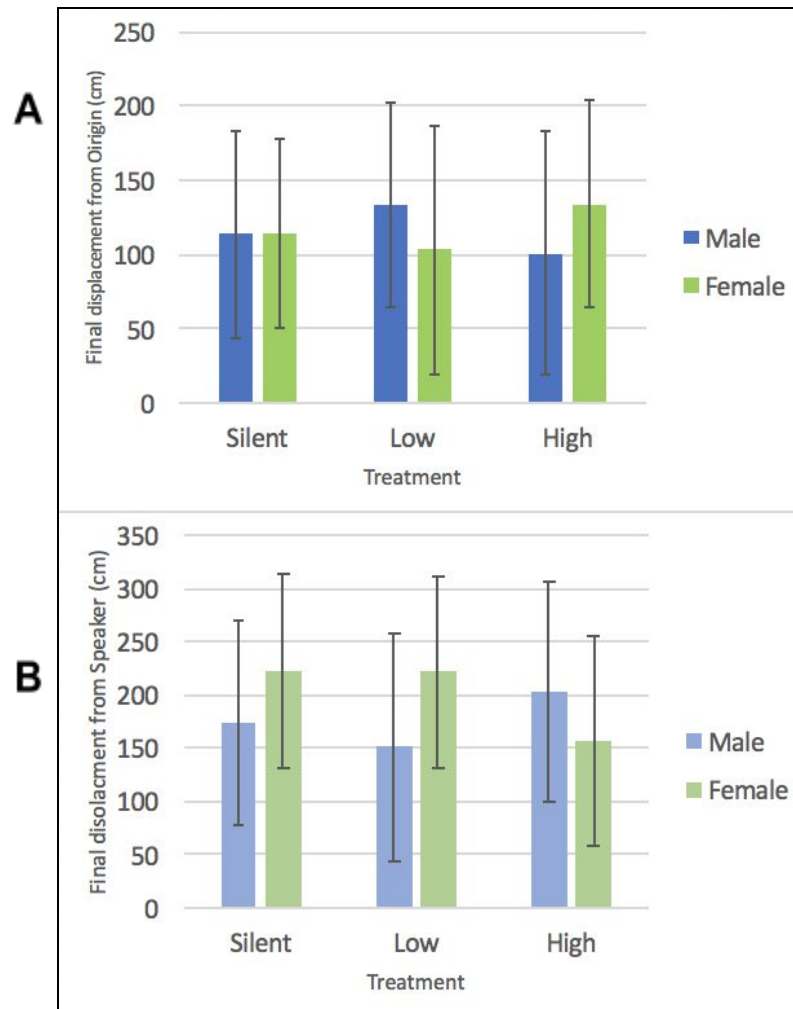
Figure 1: Final Displacements from the Origin (A) and Speaker (B). Displacement measures are averages taken from each treatment (Silent, Low, and High) and sex. Neither sex nor treatment significantly impacted displacement from the origin, but males had a lower average displacement from the speaker (p = 0.01556). Standard deviations are shown.
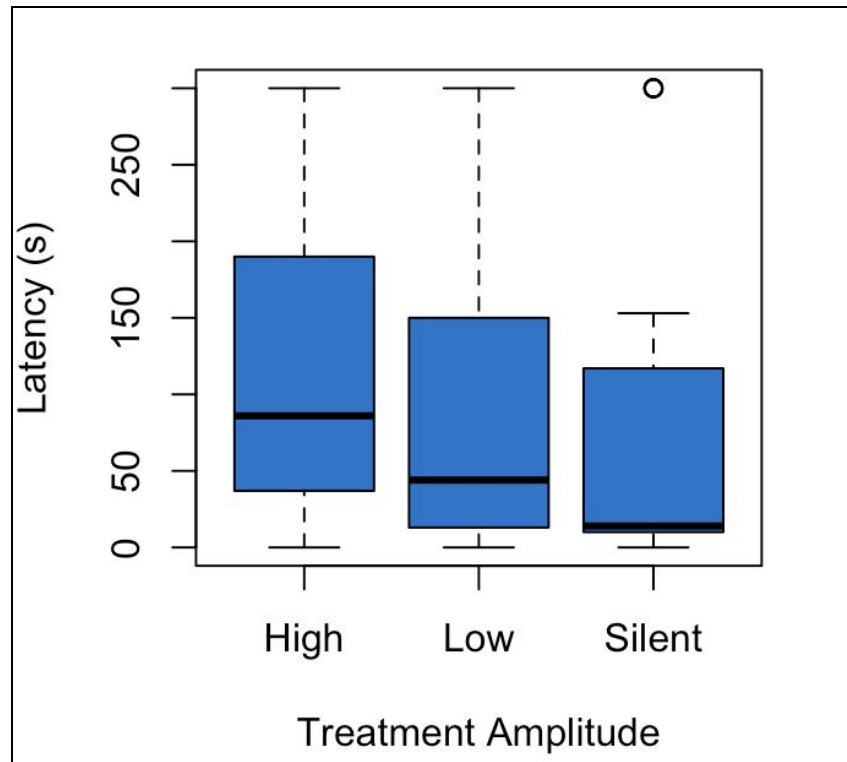
Figure 2. Latency to First Movement Across Treatments. Average and interquartile latencies are shown for each treatment (high, low and silent). As speaker amplitude increased, the time period before the first movement decreased (p = 0.007224). A Tukey's HSD test confirmed that high and silent treatments were significantly different.
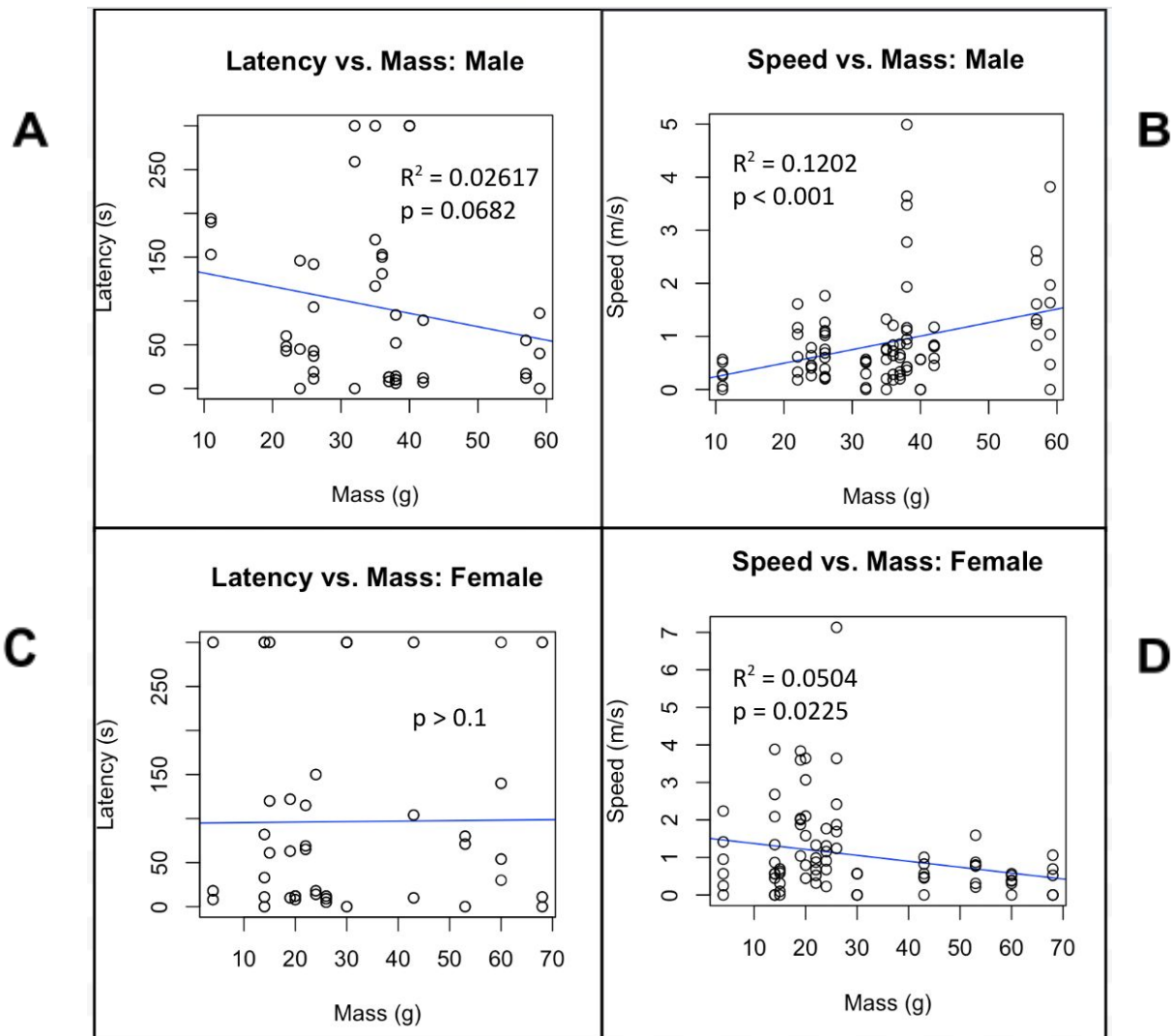
Figure 3. A-D: Regressions on Mass for Latency (s) and Speed (cm/s) in males and females.
Linear regressions were run for latency to movement (s) and estimated speed (cm/s) using mass as
a predictor variable. Latency decreased for with mass for males (A: $p < 0.1$) and did not change for
females ($p > 0.1$). Speed increased with mass for males ($p < 0.001$) and decreased with mass for
females ($p < 0.05$).