INDIVIDUAL AND ECOLOGICAL SOURCES OF VARIATION IN SEXUAL SIGNALS AND MATE CHOICE

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ABSTRACT

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Sexual signals are some of the most complex and variable communicative signals found in nature, making them ideal tools for studies of behavioral evolution. In this dissertation I examine the sources of individual level variation in sexual signaling and female mate choice in a gray treefrog (*Hyla versicolor*) study system. Like many other anurans, gray treefrog males form large choruses during the breeding season where males produce acoustic calls to attract females. These calls are extremely energetically costly to produce and females select mates based on specific properties of male calls. The high costs of calling in this species makes gray treefrogs an ideal system for studies of individual variation in sexual signals and mate choice. Using a series of experiments I identified several covariates of individual variation in male calling behavior and female mate choice. The variation observed in these studies may have consequences for the evolution of male signals, the maintenance of genetic diversity in male secondary sexual characteristics, and the evolution of female preferences. Accounting for the potential sources of individual variation is therefore necessary for building a more complete understanding of sexual selection.

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INTRODUCTION

Sexual signals are among the most complicated and variable communicative signals observed in nature, making them powerful tools for understanding behavioral evolution. In this thesis I examine the sources of individual level variation in sexual signaling and female mate choice using a gray treefrog (*Hyla versicolor*) study system. As with many other anurans, male gray treefrogs produce acoustic advertisement calls to attract females, and females base mate choice decisions on specific call properties. Calling is a very costly activity for males, with previous research demonstrating that calling in *H. versicolor* ranks as one of the most energetically expensive activities ever measured in an ectothermic vertebrate. The extreme costs of calling makes this species ideal for studies of individual variation in sexual signaling.

In Chapter 1 I used a broad, observational survey of male calling behavior in the field to quantify individual variation in signaling and to identify sources of this variation, focusing specifically on male age and physical condition as potential drivers of variation. In accordance with current theory, I hypothesized that older males or males in poor condition with fewer future reproductive opportunities (lower residual reproductive value) would increase their investment in current reproduction by calling with greater effort. Contrary to my predictions, I did not observe any significant relationship between age or physical condition and male calling behavior. This puzzling result led to a more thorough examination of variation in calling behavior to determine if the influence of these male characteristics are more prominent in different contexts.

In Chapter 2 I conducted a controlled calling experiment under laboratory conditions to examine the influence of male size, condition, and age on changes in calling behavior made in the presence of a sexually receptive female. Behavioral plasticity may allow males to limit the costs of calling by reserving intense signaling for contexts when it is likely to be most effective, such as in the presence of a female. I compared recordings of male calling behavior in the absence and presence of a receptive female to determine the influence of size, age, and condition on the magnitude of signaling plasticity. I found that female presence did significantly increase the intensity of male signaling, but the effect was not uniform across males. Specifically, I found that lighter males, and males in better physical condition exhibited significantly greater plasticity in call length. My results demonstrated the importance of context in size and condition driven variation in sexual signaling.

In Chapter 3 I further investigated variation in signaling in different contexts by examining signaling under high risk conditions. Current models predict that an individual's sensitivity to risk may be influenced by their residual reproductive value. Individuals with fewer future reproductive opportunities are expected to exhibit more risky behavior. I assessed differences in risk taking between males of different age and physical condition by timing how long it took males to resume calling following a simulated predator attack. I found evidence that males' physical condition was significantly predictive of risk taking, with males in low condition resuming calling more rapidly (i.e. more risk) following a simulated attack than their high condition counterparts. The variation in risky behavior observed in this experiment may have consequences for male mating success. These results highlight the importance of accounting for differences among males in risk aversion when studying how sexual selection acts on males and male traits.

The variation in courtship signals, both among males and across contexts, documented in chapters 1-3 may have different impacts on different females. In Chapter 4 I therefore switched my focus to female responses to male signals, in order to investigate the influences of female size, condition, and age on mate choice. Specifically, I used a simulated costly choice playback experiment to examine how these variables covary with female choosiness. I found that larger females maintained their preference for long (attractive) calls over greater simulated distances (i.e. were choosier than their smaller counterparts). This variation in mate choice can have important evolutionary consequences, as it can alter the strength and direction of sexual selection on male secondary sexual characteristics, as well as facilitate the evolution of female preferences themselves.

The research presented in this dissertation is part of and informs a growing trend in sexual selection towards studies of individual level variation. I have documented extensive variation in one of the most expensive sexual signals recorded in ectothermic vertebrates, and identified several specific covariates that predict individual variation for both male signaling and female choice. Many of the experiments in my research have relied on examining individual responses to pairwise manipulation of relevant variables (e.g. presence or absence of female, choosing between two call alternatives, etc.). Future research is needed to examine if similar trends in individual variation is observed in more natural conditions where male and female mating behavior may be influenced by multiple interacting factors.

CHAPTER 1

No evidence of terminal investment in the gray treefrog (*Hyla versicolor*): older males do not signal at greater effort

ABSTRACT

Current models indicate that life history trade-offs between current and future reproduction can have a major influence on sexual signaling. Individuals with fewer future reproductive opportunities--regardless of current effort--are expected to allocate greater resources to current reproductive effort (terminal investment) because of the low marginal survival cost to signaling. In this study we examined the effect of age and physical condition on the calling behavior of the Gray Treefrog (*Hyla versicolor*) to test the prediction that older males should exhibit greater signaling efforts compared to younger males of similar condition. Contrary to our predictions, calling males showed no significant effect of age or condition on any of the three call properties measured (call length, call rate, call effort). We offer possible explanations for the apparent discrepancy between theoretical predictions and our observations from the field.

INTRODUCTION

In nature, females of many species select mates based on the elaborate displays of males (Andersson and Iwasa 1996). Females typically prefer bigger, more intense displays because these observable signals are often correlated with unobservable qualities of value to females (Grafen 1990; Getty 1999, 2002; Dall et al. 2005). By mating with males producing these intense signals, females can gain direct benefits (e.g. access to high quality territories, nuptial gifts, or paternal care: Kirkpatrick and Ryan 1991; Forsgren et al. 1996) and/or indirect benefits (i.e. good genes: Welch et al. 1998; Kokko et al. 2003). Female preference for elaborate signals raises the question of why males do not simply “cheat” by signaling at high levels all of the time regardless of their quality. One of the ways signal honesty (the degree to which the signal accurately reflects the quality of the male) can be ensured is through condition-dependent marginal costs and benefits. If bigger signals are more costly or less beneficial for low quality males, the cost of cheating can outweigh the benefits (Otte 1974; Zahavi 1977; Grafen 1990). Consistent with this prediction, multiple studies on different species have confirmed condition dependent sexual signaling (Green 1991; Nicoletto 1993; Kotiaho 2000; Scheuber et al. 2003; Freeman-Gallant et al. 2009), with individuals in better condition producing more intense sexual signals preferred by choosy females.

Although differences in signal costs can promote honesty, situations involving unreliable signaling can still arise, particularly when signaling investment is influenced by life history trade-offs. Current models suggest that life history trade-offs can play a critical role in signaling and sexual selection (Enquist and Leimar 1990; Kokko 1997; Kemp 2002; Getty 2006). One such trade-off involves current versus future reproductive effort: investment in current sexual signaling can impose costs to longevity and future reproduction (Williams 1966; Stearns 1992). Under this scenario, the marginal costs of signaling decline as a male's future reproductive opportunities decline for reasons unrelated to signaling effort, e.g. male senescence or decreased mating opportunities near the end of the breeding season. Males with low residual reproductive value (few future reproductive opportunities) are expected to increase investment in current reproductive effort (terminal investment hypothesis; Clutton-Brock 1984). These may include males who are old, ill, or infested with parasites (Evans et al. 2011; Kuriwada and Kasuya 2011; Copeland and Fedorka 2012; Hayes et al. 2013; Gonzalez-Tokman et al. 2013).

A handful of empirical studies have supported the terminal investment hypothesis. For example, older males of the moth *Ostrinia scapulalis* practice higher courtship rates and produce larger nuptial gifts (spermatophores) than younger males (Win et al. 2013). In banded wrens

(*Thryophilus pleurosticus*), males vocalize at higher song rates during their last year of life (Hall et al. 2009). Sadd et al. (2006) found that when male mealworm beetles (*Tenebrio molitor*) were subjected to an immune challenge (insertion of a monofilament into the abdomen) they responded by increasing production of pheromones used in sexual signaling making them more attractive to females. In these studies, the influence of life history trade-offs obscures the relationship between observable signals and unobservable qualities of interest to females, resulting in unreliable signaling.

Typically, studies of sexual signals as they pertain to the terminal investment hypothesis are based in laboratory settings (but see Hall et al. 2009; Hayes et al. 2013). In this study we employ field observations to examine the role of terminal investment in sexual signaling in an acoustically communicating species. We investigated the effects of physical condition and age on calling behavior in Gray Treefrogs, *Hyla versicolor*. Like most other anurans, *Hyla versicolor* males produce vocal advertisement calls to attract gravid females (Gerhardt 1994). Studies have found these calls to be very energetically expensive to produce, ranking as one of the most energetically costly activities performed by an ectothermic vertebrate (Taigen and Wells 1985; Wells and Taigen 1986; Wells et al. 1995). Female Gray Treefrogs base mate choice decisions on the properties of male calls, showing preference for long calls, high call rates, and greater call effort, a composite of call length and rate (Gerhardt 1991; Gerhardt et al. 1996; Schwartz et al. 2001). It is often assumed that calling is a condition dependent behavior. In a study examining male swimming ability, Schwartz and Rahmeyer (2006) found that males producing calls at higher effort performed better in sustained swimming trials. However, recent work with the closely related sister species *Hyla chrysoscelis* found no relationship between physical condition and call effort (Ward et al. 2013). We propose that incorporating terminal investment could explain this lack of relationship. We tested the hypothesis that the life history trade-off between current and future reproductive effort of males would influence their calling behavior. Specifically, we examined the prediction that older males should invest more in signaling (resulting in greater call efforts) than younger males of similar physical condition due to reduced residual reproductive value.

METHODS

Study site

Research took place between May and July of 2010 and 2011 at the Lux Arbor Reserve (Kellogg Biological Station, Michigan State University) in Barry County, Michigan (42°29´N, 85°28´W). We sampled males and male calls in several large *Hyla versicolor* breeding choruses at four separate ponds within the reserve. Recordings of male calling behavior were made between 2200 and 0100 hours on every night during the breeding season when there was chorus activity.

Recordings

We recorded calls using a Sennheiser ME 66 microphone and K6 power supply connected to a Marantz PMD620 digital recorder. Focal males were recorded from a distance of approximately 1-2 meters (recording from this distance did not disturb male behavior). Each recording consisted of 20 consecutive calls. After recording, we captured focal males and measured individual snout-vent-length (SVL) to the nearest millimeter using a caliper and following a gentle compression to rid the body of excess water, weight was measured to the nearest 0.1 gram using a Pesola spring scale. Following previous methodology (Baker 1992;

Ward et. al. 2013), we calculated an index of physical condition as the residuals of a linear regression of the cube root of weight on SVL, divided by SVL. Air and water temperature were recorded at the calling site of each male using an alcohol thermometer to the nearest degree C. We toe clipped the fourth digit on the hind foot of each male (we clipped from the right foot in 2010 and the left foot in 2011) and stored the toe in 10% formalin for use in age determination via skeletochronology. We qualitatively scored the overall chorus intensity after each recording on a scale of 1-3 (3 = very dense chorus, calls heavily overlap, no silent gaps; 2 = moderate chorus, calls overlap, some silent gaps; 1 = weak chorus, calls do not overlap, large silent gaps). Males were released at their calling site after measurements and toe clipping were completed. Over the 2010 and 2011 breeding seasons combined, we recorded a total of 178 males.

Acoustical analysis

We analyzed field recordings using Audacity v 2.0.0, focusing on three call properties: call length, call rate and call effort. Using cursors in the waveform display we measured call length to the nearest millisecond for all twenty calls in each recording to calculate average call length for each male. Call rate was calculated in calls/min by measuring the total amount of time required to complete all twenty calls. We computed call effort as the product of call length and call rate (s/min). This measure can be interpreted as the amount of time in a given minute that a male is calling.

Skeletochronology

We determined the age of calling males using skeletochronolical techniques known to reliably age several anuran species (Hemelaar 1985; Acker et al. 1986; Lecair and Castanet 1987). The fourth digit of the hind foot from each male was clipped and fixed in 10% formalin solution. The second phalange was dissected and decalcified in a 14% EDTA/dH2O solution (pH: 7.59) for 5 days at room temperature on a rotator.

The phalange was paraffin embedded and 5 μm thick sections were made through the diaphyseal region of the phalange using a Reichert Jung 2030 rotary microtome. Sections were mounted to slides and stained with hematoxylin and eosin. Sections were examined under an Olympus AH2 light microscope using SPOT software 3.5.9 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and resting lines were counted. These lines correspond to periods of no growth for the males during hibernation over winter. In addition to counting resting lines, we also examined the thickness of bone layers and osteocytes in different bone layers to further distinguish between males of different age.

Statistical analyses

To examine which factors may have influenced male calling behavior, we fitted general linear models for the three call properties measured (call length, call rate, call effort) to several candidate predictor variables. We were primarily interested in age and condition as predictor variables, but also included temperature (air and water), chorus intensity, and time (i.e. number of days from the onset of the breeding season) in the models. These additional variables were chosen based on prior research indicating their ability to influence male calling behavior (Gayou 1984; Runkle et al. 1994; Schwartz et al. 2002). We used F-tests to compare models when selecting the minimal adequate model for each call property. We used $\alpha = 0.05$ as our significance criterion for all analysis. Data analyses were performed in R v 2.15.2 (R Core Team 2012).

RESULTS

Age

We were able to separate males into three different age categories, 1.5, 2 and 3 years post metamorphosis. These categories represent an ordinal ranking of age rather than absolute values. The distinction between 1.5 and 2 was made based on the thickness of the second bone layer and the presence of osteocytes. Of the 178 individuals analyzed, we placed 63 males in the 1.5 year category, 89 in the 2 year category, and 26 in the 3 year category.

Calling behavior

Mean call length ranged from 0.377 to 1.234 s (Mean ± SD = 0.682 ± 0.166 s; N = 178), call rate ranged from 6.2 to 26.0 calls/min (14.4 ± 3.9 calls/min; N = 178), and call effort ranged from 3.706 to 13.990 s/min (9.398 ± 2.041 s/min; N = 178).

We found no significant effect of age or condition on call effort (Fig. 1). The best fitting model for call effort ($F_{2,175} = 6.13$, $p = 0.003$) identified chorus intensity as the sole predictor; age, condition, time, air temperature and water temperature had no significant effect (Table 1).

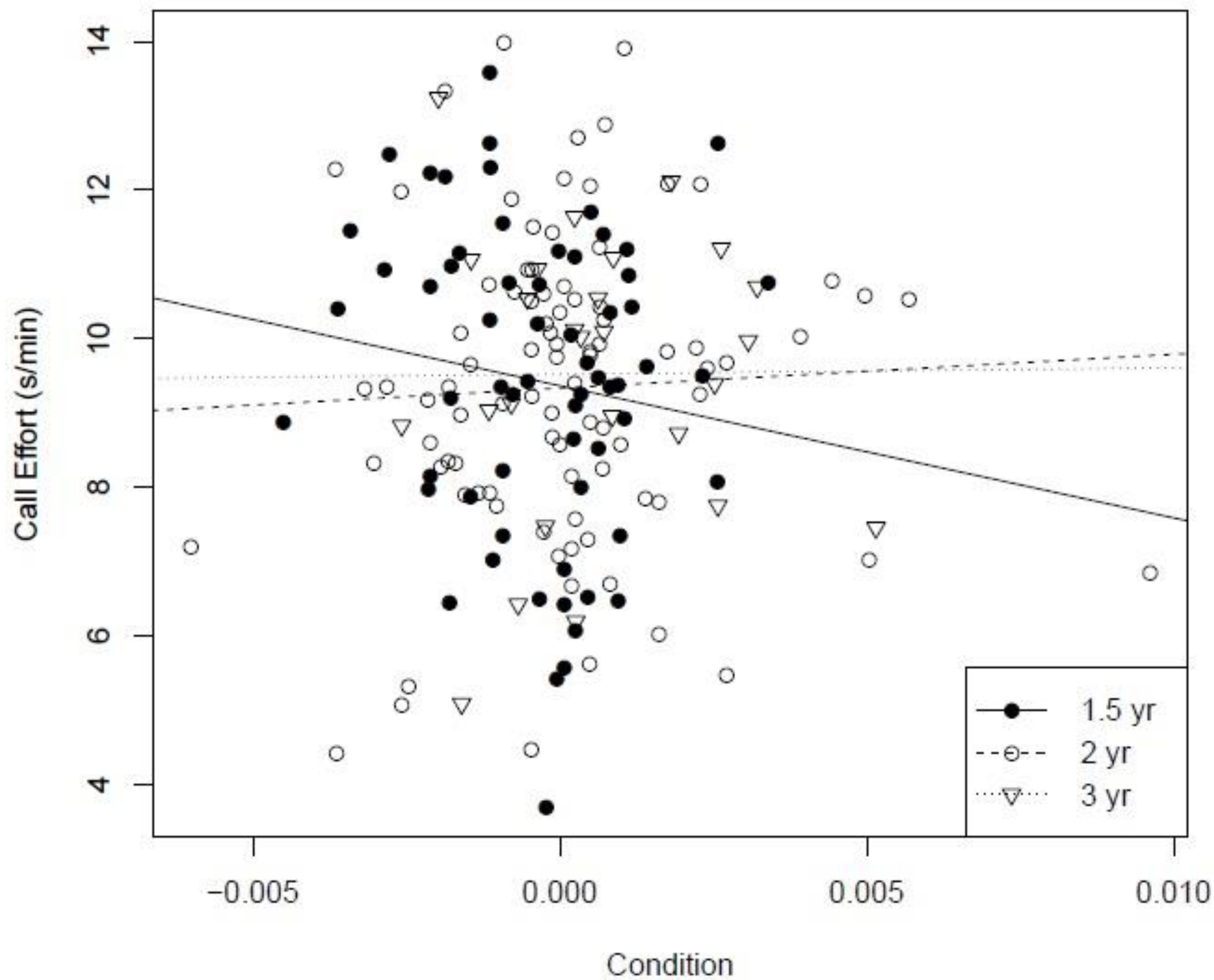

Figure 1. Relationship between physical condition and calling effort for three different age classes of Gray Treefrogs (*Hyla versicolor*) sampled at Lux Arbor, MI in 2010-2011. Neither age nor physical condition had a significant influence on calling effort.

Table 1. p-Values from general linear models for all candidate predictor variables for call length, call rate and call effort for gray treefrogs (*Hyla versicolor*) measured in the field. Age and physical condition were not significant predictors for any of the three measured call properties.

Call property	Age	Condition	Air temp.	Water temp.	Chorus	Time
Call effort	0.9	0.464	0.798	0.695	**0.003**	0.149
Call length	0.388	0.255	**< 0.001**	0.845	**< 0.001**	**0.003**
Call rate	0.51	0.338	**0.01**	0.341	0.203	0.348

Similarly, age and condition had no significant effect on call length or call rate. The best fitting model for call length ($F_{4,173} = 13.22$, $p < 0.001$) included chorus intensity ($p < 0.001$), air temperature ($p < 0.001$), and time ($p = 0.003$) as predictor variables; age, condition, and water temperature had no significant effect. The best fitting model for call rate ($F_{1,176} = 6.857$, $p = 0.010$) identified air temperature as the only predictor; chorus intensity, age, condition, water temperature and time had no significant effect (Table 1).

DISCUSSION

Our field recordings revealed extensive variation among male calling effort. Contrary to predictions of the terminal investment hypothesis, this variation was not explained by differences in male age or condition. Several explanations may account for this discrepancy. One possibility is that the recording methods used may not have been sensitive enough to detect differences between males of different ages amidst variable nightly environmental and acoustic conditions. We attempted to account for these conditions by including them as predictor variables in our models, however a more acoustically controlled environment such as a sound chamber may be a more sensitive method for detecting differences between males.

Another possibility is that increased investment in sexual signaling may be occurring on a broader temporal scale than what was measured in this study. The measures we used as indicators of investment in sexual signaling, call length, rate and effort, were all measured within a single calling bout for each focal male. Only males engaged in calling that night were sampled. A broader examination of signaling effort could involve measuring the number of nights during the breeding season a male attends the chorus and the amount of time spent at the chorus each night. Several previous studies have shown considerable variation between males in their chorus attendance and that males who attend the chorus more often have higher mating success than other males (Ritke and Semlitsch 1991; Sullivan and Hinshaw 1992; Bertram et al. 1996). Instead of altering call properties with age, older males with lower residual reproductive value may increase investment in reproduction by attending the chorus on a greater number of nights. At the beginning of this experiment, we attempted to record nightly chorus attendance in addition to male call properties, but unfortunately the ponds used in this study were vegetated in such a way that certain areas were inaccessible, making it impossible to gather complete data on nightly chorus attendance.

In addition to different temporal scales, the influence of life-history trade-offs may be more salient over a different spatial scale. Specifically, differences in call properties may be more prevalent when males are within visual range of a female. In fall field crickets (*Gryllus pennsylvanicus*), males with lower residual reproductive value increased their effort in producing short-range courtship calls while many properties of the long-distance mate attraction call remained a reliable indicator of condition (Harrison et al. 2013). Male *Hyla versicolor* will alter their calling behavior when in the visual or tactile range of females, producing very long calls at elevated rates (Fellers 1979; Schwartz et al. 2001; Reichert and Gerhardt 2012). The magnitude of this alteration may be influenced by life history trade-offs with males with low residual reproductive value increasing their calling effort in proximity to females to a greater extent than other males.

Another possible explanation could relate to the year to year survival of males. Given the relatively small number of older males (only 26 out of 178 males were classified as 3 year olds), over-winter mortality in this population may be high enough to preclude any expectation of surviving to additional breeding seasons. In this situation, there is no benefit to modulating signaling effort with respect to age and males may then signal at their maximum effort at all times.

Interestingly, in addition to finding no effect of age on call properties, we also failed to find any significant relationship between physical condition and signaling. Considering the very high energetic costs of calling (Taigen and Wells 1985; Wells and Taigen 1986; Wells et al. 1995), we predicted that current signaling effort would correlate positively with current condition, a pattern that has been reported from diverse taxa (Green 1991; Nicoletto 1993; Kotiaho 2000; Scheuber et al. 2003; Freeman-Gallant et al. 2009). It is possible that condition itself is an indicator of future reproductive opportunities where males below a certain condition can be considered to have reduced residual reproductive value. In this situation, poor condition males would be expected to increase signaling effort as a terminal investment. Consistent with this prediction, studies of other species have revealed several examples of poor condition males increasing signaling activity (Candolin 1999; Svensson et al. 2004; Harrison et al. 2013). In the present study however, our results do not corroborate these previous experiments as we do not find a curvilinear relationship between condition and signaling effort.

To conclude, our experiment failed to find any support for the hypothesis that trade-offs between current and future reproductive effort would influence male signaling in *Hyla versicolor*. Older males showed no difference in their call properties from younger males. This study serves as initial starting point in examining terminal investment theory in this species. Future experiments should focus on utilizing different recoding methodologies that may be more sensitive to detecting difference between males as well as examining signaling effort across different temporal and spatial scales to gain a more complete understanding of how males allocate resources to sexual signaling.

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CHAPTER 2

Context and condition dependent plasticity in sexual signaling in gray treefrogs

ABSTRACT

For many species sexual signaling is a very costly activity, both in terms of energetic expenditure and increased conspicuousness to predators. One potential strategy to limit the costs of signaling is to only signal at maximum effort in contexts when signaling is expected to be most effective. Multiple studies have documented extensive plasticity in sexual signaling within a variety of contexts, however fewer experiments have examined individual-level variation in the extent of signaling plasticity and the causes of this variation. In this study we examined the influence of size and physical condition on the magnitude of signaling plasticity using a gray treefrog (*Hyla versicolor*) study system. We quantified signaling plasticity by recording male calling behavior first in the absence and then in the presence of a sexually receptive female. For one call property, call length, we found that both weight and condition had a significant influence on the magnitude of plasticity. Smaller males, and males in higher condition exhibited the greatest degree of plasticity. We discuss several possible explanations for this pattern and provide suggestions for future work to examine the consequences of this plasticity and the potential interactive effects of multiple biotic and abiotic contexts on signaling plasticity.

INTRODUCTION

Males from a wide variety of taxa produce elaborate displays to attract potential mates (Anderson and Iwasa 1996). When these signals differ between individuals, females often prefer to mate with males who produce larger, and/or more intense displays, which are often assumed to communicate some aspect(s) of male quality (Grafen 1990; Getty 2002; Dall et al. 2005). These preferred (intense) signals can incur multiple costs for male signalers including, for instance, energetic demands and conspicuousness to predators. While these costs can constrain male signals (Anderson 1984; Magnhagen 1991; Prestwich 1994; Zuk and Kolluru 1998), the costs can sometimes, in principle, be ameliorated by behavioral plasticity. In general, behaviorally plastic male signalers are predicted to reserve intense (and costly) signals for the contexts that maximize anticipated fitness returns (Patricelli et al. 2002). Despite extensive evidence that signal plasticity is common in nature (reviewed below), it is still common practice to ignore individual-level variation in signaling plasticity (but see Peretti et al. 2006; Bertram et al. 2013; Sullivan-Beckers and Hebets 2014).

Previous work shows that changes in sexual signaling can be induced by a variety of abiotic and biotic environmental conditions. Males in several species adjust their signaling behavior to overcome abiotic constraints on signal transmission and efficacy (McNett et al. 2010; Wilgers and Hebets 2011; Montague et al. 2013). For example, male blue-black grassquits (*Volatinia jacarina*) accelerate their display rates in direct sunlight, which maximizes the conspicuousness of their iridescent plumage to female receivers (Siscu et al. 2013). In the wolf spider (*Schizocosa ocreata*), where males use both visual and seismic signals to attract females, males accentuate visual signal components whenever substrate restricts vibratory signal propagation (Gordon and Uetz 2011).

There are also many examples of males adjusting signals in response to biotic variation, including the presence of conspecific rivals, the proximity and behavior of receptive females, and presence of potential predators (Kelso and Verrell 2002; Patricelli et al. 2006; Akre and Ryan 2011; Gavassa et al. 2013; Kim and Velando 2014). Male zebra finches (*Taeniopygia guttata*), for example, produce redder bills in the presence of female conspecifics (Gautier et al. 2008). In a study of the Australian toadlet (*Pseudophryne bibronii*), nesting males accelerated their calling rates in the presence of receptive females (Byrne 2008). And in the fiddler crab (*Uca perplexa*), males increase claw waving when females approach (How et al. 2008). In each of these examples, male signaling plasticity appears to minimize signal cost/benefit ratios, and thereby maximize expected payoffs to males. This, in turn, should promote or maintain plasticity in male signaling behavior (Patricelli et al. 2002; Sullivan-Beckers and Hebets 2014).

Although previous studies have measured individual variation in sexual signaling plasticity, the factors that drive this variation have received considerably less attention. A complete understanding of the drivers behind variation in the magnitude of plasticity is crucial to building a more comprehensive model of behavioral plasticity (Bretman et al. 2011). What research has been done indicates that multiple factors can influence the magnitude of plasticity (Jacot et al. 2008; Bertram et al. 2013; Fitzsimmons and Bertram 2013). In the special case of alternative mating tactics, previous research has found a variety of intrinsic characteristics such as body size, physical condition, and age can interact to catalyze a plastic switch between reproductive tactics (Howard 1984; Gross 1996; Leary et al. 2005; Wilgers et al. 2009; Humfeld 2013). For example, in Woodhouse's toad (*Bufo woodhousii*) and the Great Plains toad (*Bufo cognatus*) males adopting a "satellite" reproductive strategy, who remain silent and target females lured by their rivals' calls, were found to be significantly smaller than males producing mate attraction calls (Leary et al. 2005). Smaller males may deplete energy reserves required for sustained calling more rapidly than larger males, or smaller males may be less attractive to females making costs of calling outweigh the benefits, leading to the adoption of a "satellite" strategy. Similarly, in green treefrogs (*Hyla cinerea*), males in poor physical condition were more likely to switch mating tactics to a "satellite" strategy than high condition competitors (Humfeld 2013). These examples underscore how the adoption of different mating tactics may be influenced by individual variation in some aspect of status (size, condition, age, etc.) These same characteristics may influence less extreme forms of behavioral plasticity and may affect the extent to which males modulate their signaling in response to abiotic and biotic environmental variables.

In this study we investigate signal plasticity 1) in response to the presence or absence of females, and 2) as it related to male size (length and weight), and condition (after controlling for correlations with age; see methods section). Our model for this study, the gray treefrog (*Hyla versicolor*), exhibits several features that make it well-suited to studies of inter-individual variation in signaling plasticity. Like many other anurans, gray treefrogs produce acoustic advertisement calls to attract females (Gerhardt 1994), and these calls are very energetically costly to produce (Taigen and Wells 1985; Wells and Taigen 1986). Females base mate choice decisions on certain call properties, preferring long calls, rapid call rates, and high call efforts, a composite of call length and rate (Gerhardt 1991; Gerhardt et al. 1996; Schwartz et al. 2001). In this study we chose to focus on these temporal call properties because they are highly dynamic (Gerhardt 1991; Gerhardt et al. 1996), and as such these properties are more likely to exhibit signal plasticity. Previous research has shown that male gray treefrogs alter their calling behavior in response to varying levels of vocal competition, typically producing longer calls at shorter rates as competition increases (Wells and Taigen 1986; Schwartz et al. 2002). Additionally, males have been shown to increase their calling behavior in the presence of females, producing call lengths and call efforts approaching the upper limit of signal performance for this species (Fellers 1979; Reichert and Gerhardt 2012; Reichert 2013). Although these studies document extensive signaling plasticity, the extent and causes of individual-level variation in signal plasticity remain unexplored.

In this study we test whether male treefrogs that differ in length, weight and/or condition exhibit differences in behavioral response to female presence. Specifically, we predicted that smaller, poor condition males would show a greater escalation in calling behavior in the presence of females than larger, high condition males. Our rationale was that the extreme energetic demands of calling (Taigen and Wells 1985; Wells and Taigen 1986) may be more difficult to bear by smaller, poor condition males, and as a result, these males should restrict high signaling effort to contexts where mating is most likely to occur (such as proximity to receptive females). Additionally (or alternatively), small males may face tradeoffs between energetic investments in signaling and growth that are less limiting for larger males (Stearns 1992; Hoglund and Sheldon 1998; Heino and Kaitala 1999).

In contrast to small males, large and high condition males may exhibit less dramatic responses to female proximity because a) they can readily maintain high signal effort, at comparatively low cost, even when mating is unlikely, and/or b) females can identify and prefer these larger and/or higher condition males (Gatz 1981; Morris 1989), obviating any need to increase calling effort above a baseline level.

METHODS

Specimen collection and testing apparatus

Male and female gray treefrogs were collected between May and July 2013 from the Lux Arbor Reserve (Kellogg Biological Station, Michigan State University) in Barry County, Michigan (42°29´N, 85°28´W). Individuals were collected from several ponds within the reserve as amplected pairs (to ensure that females were sexually receptive), and brought back to the lab for testing. All recordings took place under dark lighting conditions within a simple sound chamber at the Kellogg Biological Station consisting of a 2x2 meter platform covered with a sound-dampening blanket and tarp, and encircled by additional draped sound-dampening blankets.

Recording signal plasticity

To examine the signaling plasticity of males in response to female proximity we recorded male calling behavior in the absence and presence of a nearby female. At the start of testing, isolated males were placed in a small (~10x10 cm) wire cage in the center of the sound chamber. We recoded 20 consecutive calls produced in the absence of a female as a baseline of comparison. Recording began after approximately 1 minute of calling to allow males to establish consistent calling behavior and minimize any warming up period. Following baseline recordings, we placed a single receptive female in a separate wire cage adjacent to the male and recoded an additional 20 consecutive calls. We followed other investigators in their decisions to perform all tests in the same order (recordings of baseline calling behavior followed by recordings in the presence of stimuli, e.g. Wells and Taigen 1986; Reichert and Gerhardt 2012, Reichert 2013). This approach is commonly used because male responses to manipulated stimuli can persist for unknown periods of time following stimulus removal, and thus complicate interpretation of results (Reichert and Gerhardt 2012). To confirm that the changes in male calling we observed were caused by the addition of female stimuli, we analyzed a random sample of 10 recordings of males calling in the field (recorded for a separate study in 2011) over comparable time spans as the trials in this study (~2-3 min). This showed no systemic changes in calling behavior over the course of the recording and a paired t-test revealed no significant difference in call length from calls sampled at the beginning of the recording versus calls sampled near the end ($t = -0.86$, df =

9, p = 0.41). All recordings were made using a Sennheiser ME 66 microphone and K6 power supply connected to a Marantz PMD620 digital recorder. Male snout-vent-length (SVL) was measured to the nearest millimeter using a caliper and following a gentle compression to rid the body of excess water, weight was measured to the nearest 0.1 gram using a digital scale. We calculated an index of physical condition as the residuals of a linear regression of the cube root of weight on SVL, divided by SVL (Baker 1992; Ward et. al. 2013). This index of condition provides a measure of weight controlled for body size such that for a given SVL a heavier male would be in higher condition than a lighter male. The use of residual mass as a proxy for physical condition is common in studies of amphibians, though its efficacy is debated (Kotiaho 1999; Green 2001; Tomkins et al. 2004). To ensure that any significant effects of body size could not simply be attributed to variation in age, we toe clipped the fourth digit on the left hind foot and stored the toe in 10% formalin for use in age determination from skeletochronology (described below). Males and females were returned to the pond from which they were collected following testing (typically within 24 hours). In the 2013 breeding season we recorded a total of 50 males.

Acoustical analysis

Recordings were analyzed using Audacity v 2.0.0 sound analysis software. For each male we separately analyzed recordings made in the absence and presence of a female, focusing on three commonly studied call properties: call length, call rate and call effort. We measured call length to the nearest millisecond using cursors in the waveform display for all 20 calls and calculated an average call length. Call rate was calculated in calls/min by determining the total amount of time required to produce all 20 calls. Call effort was calculated as the product of call length and call rate (s/min) and can be interpreted as the amount of time a male spent calling in a given minute. For each male we compared call length, rate, and effort in the absence and presence of a female to obtain a (separate) plasticity score for each call trait.

Skeletochronology

Following previous studies, we used skeletochronology as a reliable way to determine male age (Hemelaar 1985; Acker et al. 1986; Lecair and Castanet 1987). The fourth digit of the hind foot from each male was clipped and fixed in 10% formalin solution. The second phalange was dissected and decalcified in a 14% EDTA/dH2O solution (pH: 7.59) for 5 days at room temperature on a rotator.

Bone was paraffin embedded and 5 µm thick sections were made through the diaphyseal region of the phalange using a Reichert Jung 2030 rotary microtome. Sections were mounted to slides and stained with hematoxylin and eosin. Slides were analyzed with an Olympus AH2 light microscope using SPOT software 3.5.9 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and resting lines were counted. These lines correspond to periods of no growth for the males during hibernation over winter.

Statistical analysis

To examine the extent of signal plasticity across all males that was induced by the presence of females we used paired t-tests to compare the three call properties (length, rate and effort) in the absence and presence of females. We examined the influence of four intrinsic characteristics (length, weight, condition and age) on the magnitude of plasticity using multiple regression models. The magnitude of change in each call property in the presence of a female served as response variables (hereafter referred to as delta call length, delta call rate or delta call effort) and each was fitted to the candidate predictor variables of length, weight, condition and age. F tests were used to compare models when selecting the minimal adequate model. We used $\alpha = 0.05$ as our significance criterion for all analyses, we also used a Bonferroni corrected α (0.0125) for our multiple regression models to examine the robustness of outcomes to multiple hypothesis testing. Data analysis was performed in R v 2.15.2 (R Core Team 2012).

RESULTS

Males show signal plasticity in the presence of females

In the presence of a female males produced significantly longer calls (average call length: female absent = 0.530 s, female present = 0.708 s; paired t-test: $t = 14.33$, $df = 49$, $p < 0.001$), while call rate decreased slightly but was non-significant (average call rate: female absent = 21.08 calls/min, female present = 20.00 calls/min; paired t-test: $t = -1.97$, $df = 49$, $p = 0.054$). This increase in call length while call rate remained relatively unchanged resulted in a significant increase in call effort (average call effort: female absent = 10.99 s/min, female present = 13.85 s/min; paired t-test: $t = 8.67$, $df = 49$, $p < 0.001$) (Figure 2).

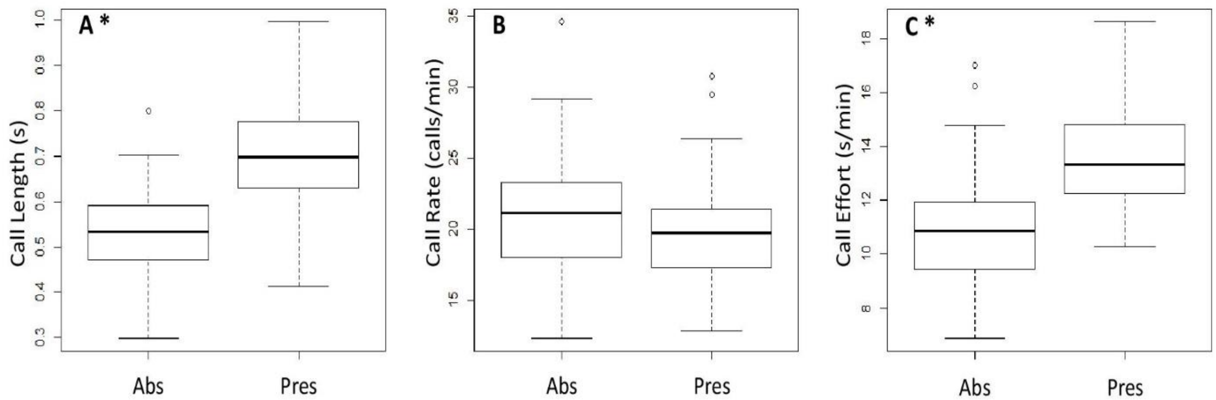

Figure 2. Call length (A), call rate (B) and call effort (C) in the absence (Abs) and presence (Pres) of a nearby female. Asterisks indicate significant differences. Call length and call effort significantly increased in the presence of a female, while call rate did not significantly change.

Lightweight, high condition males exhibit greatest signal plasticity

The best fitting model for delta call length ($F_{2,47} = 9.36$, $p < 0.001$) included weight ($p < 0.001$) and physical condition ($p = 0.003$) as significant predictor variables. There was an inverse relationship between weight and delta call length, with lightweight males showing the greatest plasticity, while the relationship between delta call length and condition was positive (Figure 3). To ensure there were no problems with multicollinearity in a model with both weight and condition we examined the variance inflation factor and found it to be sufficiently small (VIF = 1.12). Age and length were not significant predictors of delta call length (age: $p = 0.855$, length: $p = 0.070$).

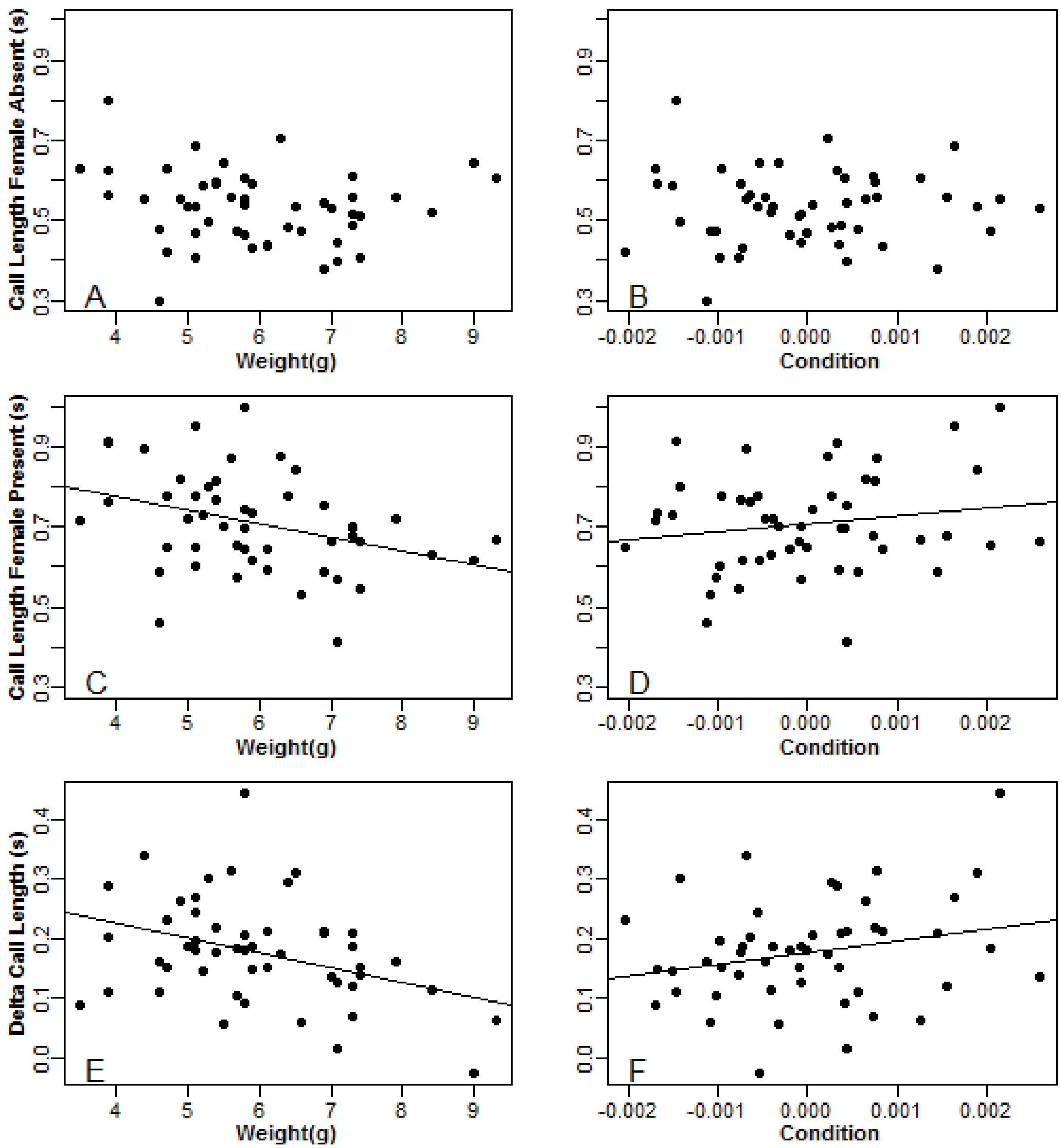

Figure 3. Relationship between male weight and physical condition and call length in the absence (A, B) and presence (C, D) of a female, and the magnitude of change in call length (E, F). Weight and condition are significantly predictive of call length in the presence of a female and on the magnitude of change in call length, but not predictive of call length in the absence of a female.

We could not fit any of our candidate predictor variables to delta call rate or delta call effort. Weight (delta call rate: $p = 0.494$; delta call effort: $p = 0.541$), length (delta call rate: $p = 0.463$; delta call effort: $p = 0.656$), physical condition (delta call rate: $p = 0.428$; delta call effort: $p = 0.389$) and age (delta call rate: $p = 0.326$; delta call effort: $p = 0.456$) all had no influence on the magnitude of plasticity for these call properties.

DISCUSSION

As with previous studies (Reichert and Gerhardt 2012; Reichert 2013) we found that female presence induced changes in male gray tree frog calls. In addition, we provide a clear demonstration of predictable differences among individual males in signaling plasticity induced by female presence. Specifically, the plasticity of call length was predicted by male weight and physical condition. We discuss this below, in light of both treefrog natural history and general life history theory.

Why are lightweight males more plastic than heavy counterparts?

Consistent with our predictions, lighter males exhibited a greater increase in call length in the presence of a female. There are several potential explanations for why lighter males are more plastic. As stated above, the high energetic demands of calling (Taigen and Wells 1985; Wells and Taigen 1986) may lead lightweight males to reserve high effort calls for situations where receptive females are unambiguously present. Additionally, faced with the life-history trade-off between growth and reproduction (Stearns 1992; Hoglund and Sheldon 1998; Heino and Kaitala 1999), lighter males may be reserving energetic resources for growth allocation. These explanations rely on the energetic costs of signaling to explain the difference in plasticity between heavy and lightweight males. Our results however showed that weight only significantly influenced the change in call length, not call effort which would have the greatest impact on energetic expenditure (Wells and Taigen 1986). Additionally, lightweight males in our study produced calls of comparable length to heavy males when a female was absent, and actually produced longer calls than heavy males in the presence of a female, making it unlikely that plasticity reflects greater energetic constraint on lightweight males.

Another possible explanation for the greater plasticity in call length among lightweight males could involve small males compensating for lower attractiveness to females. Previous research has shown that large males may have a mating advantage over smaller males (Gatz 1981). Additionally, in a study of *Hyla versicolor*'s sister species, *Hyla chrysoscelis* (Morris 1989), researchers found that females prefer to initiate amplexus with larger males among local groups likely to be assessed by females. If, all else being equal, small males are at a disadvantage in terms of attractiveness, then enhanced call lengths in the presence of females reduce or eliminate this disadvantage. Studies have shown that female gray treefrogs prefer longer calls over shorter call alternatives, even when the calls are broadcast at equal effort (Klump and Gerhardt 1987; Gerhardt et al. 1996; Schwartz et al. 2001). By producing a more attractive call, small males may experience a greater increase in attractiveness to females compared to large males. In natural chorus settings, however, additional complexities may arise as neighboring competitor males are free to react to these plastic changes in call length. Additional work is needed to fully understand how the presence of both females and rival males interact.

Large males on the other hand, may be less plastic in their call length because their size gives them an initial advantage in attractiveness over small males (Gatz 1981; Morris 1989). Additionally, some evidence suggests that large males may have a slight advantage over small males in male-male aggressive interactions (Gatz 1981; Reichert and Gerhardt 2011). This may give large males an advantage in controlling favored calling spaces, which may aid in attracting females.

Why are high condition males more plastic than low condition counterparts?

In addition to weight, physical condition also had a significant influence on the magnitude of plasticity in call length. Contrary to our predictions high condition males exhibited greater plasticity than low condition males. It is possible that being highly plastic carries its own costs (DeWitt 1998; Relyea 2002) and high condition males may be better able to bear these costs. Such costs could include, for example, physiological capacity for plasticity and/or costs of energetic expenditure to future growth and survival, which are likely greatest for low condition individuals (DeWitt et al. 1998). Additionally, perhaps low condition males are not as effective at assessing their local environment and responding strategically as are high condition males; an ability which can limit the extent of plasticity (Moran 1992; Getty 1996).

What does variation in signaling plasticity mean for sexual selection?

The signaling plasticity we observed in this study is likely to have substantial impacts on male fitness. In the presence of females males alter their calling behavior to exaggerate preferred call properties. Our research has also shown that the magnitude of this plasticity for at least one call property (call length) is significantly influenced by the intrinsic male characteristics of weight and physical condition. Lightweight, high condition males show the greatest increase in call length in the presence of females, and may therefore experience the greatest boost in attractiveness. These results highlight the importance of examining the extent and sources of individual variation in signaling plasticity to achieve a more complete understanding of sexual selection. For example, despite numerous laboratory experiments showing female preference for long calls (Gerhardt 1991; Gerhardt et al. 1996; Schwartz et al. 2001), field studies frequently find no relationship between call length and mating success (Ritke and Semlitsch 1991; Sullivan and Hinshaw 1992). We suggest that failure to account for context-dependent signal variation (e.g. individual-specific effects of female presence) may contribute to the discrepancies between lab- and field-based studies.

Future experiments should focus on quantifying how signal plasticity affects the mating success of males that differ in size and condition. Dynamic playback experiments, possibly manipulating visual as well as acoustic signals and cues, or phonotaxis experiments using live, caged males could ascertain whether the greater signaling plasticity exhibited by lightweight males is sufficient to make them more attractive to females than heavy counterparts.

Additionally, future research should also focus on examining any interactive effects between the presence of females and the presence of other male competitors. Numerous studies in *Hyla versicolor* and other anuran species have examined the effects of the presence of competitors on calling behavior (Schwartz et al. 2002; Marshall et al. 2003; Martinez-Rivera and Gerhardt 2008) but relatively little research has focused on the presence of receptive females (Byrne 2008; Akre and Ryan 2011; Reichert 2013). By examining signaling plasticity in response to variable combinations of competitors and receptive females we can gain a better understanding of how males are likely to alter their signaling behavior in natural choruses where females assess potential mates.

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CHAPTER 3

Male treefrogs in low condition resume signaling faster following simulated predator attack

ABSTRACT

Current models indicate that an organism's sensitivity to risk may be heavily influenced by the trade-off between current and future reproduction. Individuals that have fewer future reproductive opportunities are expected to show more risky behavior as they have less to lose if captured by a predator (the asset protection principle). In this study we examined the effects of age and physical condition on risk taking behavior during sexual signaling in the gray treefrog (*Hyla versicolor*) to test the prediction that older and poor condition males will take greater risks than their younger or higher condition counterparts. In accordance with these predictions we found that males in low physical condition resumed signaling activity more rapidly following a simulated predator attack than their higher condition counterparts, although this effect was only apparent in one of two study years. Further, males that resumed calling early did not offset their risk of detection by predators via reduced calling effort. Contrary to our predictions, we did not find age to be a significant predictor of risk taking in male signaling behavior. We conclude with a discussion of possible explanations for the discrepancy observed between years and highlight the potential reproductive consequences of variation in risk taking behavior.

INTRODUCTION

Predation is a strong selective force that influences numerous aspects of prey behavior (Lima and Dill 1990). Mating behavior is often hazardous, potentially placing both sexes at risk of capture by predators (Magnhagen 1991; Zuk and Kolluru 1998). The act of mate-searching, the production of elaborate sexual signals, and the act of copulation can each be exploited by predators (Lloyd 1965; Tuttle and Ryan 1981; Pocklington and Dill 1995; Igaune et al. 2008). To cope with predation risk many species have evolved antipredator tactics that modify mating behavior when predation risk is high. These antipredator tactics can include reduced intensity of sexual signals, decreased courtship behavior, and altered preferences and choosiness in mate selection (Brenden and Stoner 1987; Forsgren and Magnhagen 1993; ter Hofstede et al. 2008; Bonachea and Ryan 2011a).

The amount of risk an organism is willing to accept during courtship and mating is likely to differ between individuals and should be heavily influenced by life history trade-offs, particularly the trade-off between current and future reproduction (Williams 1966; Stearns 1992; Clark 1994). Individuals with low residual reproductive value (few future reproductive opportunities) are expected to take greater risks in order to maximize current reproduction, while those with high residual reproductive value are expected to be more risk averse (the "asset protection principle"; Clark 1994). Individuals that differ in age, physical condition, health, or parasite load (factors that can affect residual reproductive value) are expected to show different reactions to predator cues (Magnhagen and Vestergaard 1991; Kotler et al. 2004; Kuriwada et al. 2011; Schwanz et al. 2012). Assuming these traits have little influence on individuals' abilities to escape predators, the asset protection principle predicts individuals with higher residual reproductive value to exhibit greater caution where predators are present.

Several empirical studies have demonstrated the importance of residual reproductive value in risk taking within the context of mating behavior. In a study of gobiid fish, Magnhagen (1990) found that older black gobies (*Gobius niger*), which have fewer future mating opportunities than their younger competitors, are more likely to mate in the presence of a predator than younger individuals. In a study of reproductive effort and nuptial coloration in the three spine stickleback (*Gasterosteus aculeatus*), Candolin (1998) found that males showed smaller reductions in red coloration when exposed to predators as the breeding season neared its end (i.e. as future reproductive opportunities declined). Lafaille et al. (2010) found that in an acoustic moth (*Achroia grisella*) older males were less likely to pause their calling behavior following playback of a predator cue, and males that did interrupt calling required the most intense predator cues to elicit silence.

As in the examples above, much of the work on mate signaling in the presence of predator cues has focused on age as a proxy for residual reproductive value, while the role of physical condition remains comparatively unexplored. We suggest that condition is likely to influence signal strategy in many systems, since it is potentially an important determinant of residual reproductive value (e.g. Svensson et al. 2004; Harrison et al. 2013). In one of the few studies that examined the role of physical condition in sexual signaling under predation risk, Candolin (1999) found that male three spine sticklebacks in poor physical condition maintained greater red coloration in the presence of a predator than high condition counterparts. In all of these examples, individuals with lower residual reproductive value (either due to age, the end of a breeding season, or poor condition) showed greater riskiness in their mating behavior.

In the present study we use simulated predator attacks to test whether the asset protection principle predicts signaling behaviors among males of an acoustically communicating anuran species. We expected simulated predation attempts would influence behavior based on previous studies documenting that predation risk can significantly influence both male and female mating behavior in chorusing frogs (Bonachea and Ryan 2011b; Dapper et al. 2011; Höbel and Barta 2014). Specifically, we investigated the effect of physical condition and age on risk aversiveness in chorusing male gray treefrogs (*Hyla versicolor*). Like many other anurans, *H. versicolor* males gather in large choruses during the breeding season where they produce vocal advertisement calls to attract gravid females (Gerhardt 1994). In addition to being very energetically expensive to produce (Taigen and Wells 1985; Wells et al. 1995), these calls also potentially increase male conspicuousness to predators. Previous research has shown that bullfrogs (*Rana catesbeiana*) and giant water bugs (*Lethocerus americanus*) will attack and consume calling male treefrogs (Hinshaw and Sullivan 1990).

Here we test a prediction of the asset protection principle in the context of sexual signaling under predation risk. Specifically, we examined the prediction that both older, and lower-condition males would accept greater signaling risks to offset their comparatively low residual reproductive value. Such males, we predicted, should resume calling activity more rapidly after a predator cue is detected.

In addition to measuring the latency to resume calling after a predator disturbance, we also investigated how specific temporal call properties changed after a simulated attack. Female gray treefrogs base mate choice decisions on male call properties, showing preference for calls broadcast at higher efforts (call length x call rate; Gerhardt 1991; Gerhardt et al. 1996; Schwartz et al. 2001), so any changes in these properties after a disturbance may alter the attractiveness of a male’s sexual signal. Additionally, low effort calling contains more and/or longer periods of silence, therefore males producing calls at a greater effort may be more conspicuous to predators. We investigated the relationship between the magnitude of change in signal properties and the latency to resume calling following a simulated predator attack. We wished to determine if males taking greater risks by resuming calling activity more rapidly were simply offsetting this increased risk by producing less conspicuous calls. Lastly, we also examined the relationships of age and physical condition to the magnitude of change in call properties in order to test the prediction that older and poor condition males would show less sensitivity to predation risk by maintaining risky (and attractive) calls after a simulated predator attack.

METHODS

Study site

All research took place between May and July of 2013 and 2014 within the Lux Arbor Reserve (Kellogg Biological Station, Michigan State University) in Barry county Michigan (42°29´N, 85°28´W). We sampled males and male calls from a large *H. versicolor* breeding population at a pond within the reserve. Blind data collection was not possible in this study because sampling involved focal animals in the field. The perimeter of the pond where sampling occurred was uniformly vegetated with woody shrubs, which maximized the similarity between calling environments. To minimize the effect of variable numbers of calling neighbors and overall background noise on antipredator behavior, data was only collected on nights with high levels of chorus activity between 2200 and 0100 hours during peak chorusing activity.

Simulated predator attack protocol

To investigate the effects of age and physical condition on risk taking behavior, we measured the duration of silence following a simulated predator attack before males resumed calling activity. We simulated a predator attack by grabbing males by hand to provide a tactile predator cue. We selected this form of stimulus after prior experimentation using auditory playbacks (of potentially threatening sounds: rustling bushes, bullfrog calls) failed to elicit any response in the behavior of calling males, and because a previous study in this species found no changes in male calling or female mate choice following playback of acoustic predator stimuli (bullfrog calls; Schwartz et al. 2000). We feel that a hand grab is an appropriate method for simulating a predator attack as we have witnessed bullfrogs attempt to grab, but ultimately fail to consume calling males within our focal populations.

Calling males were located in the field, typically perched in woody shrubs near the surface of the water, and approached to within one meter using a red light headlamp. After a brief waiting period (~1 min) to ensure male behavior was not disturbed by the approach, males were grabbed by hand and held firmly for 15 seconds before being placed in a floating calling platform approximately 1 meter away from the male’s calling site. The uniform woody shrub vegetation around the perimeter of the pond ensured that there were no systematic differences across individual tests in the amount of vegetation cover a male could potentially see while inside the calling platform. The calling platform consisted of a 30 x 20 cm clear plastic box with an elevated flat perch in its center. The platform was used as a means to further standardize the calling environment to minimize the potential confounding effects of different amounts of cover or perch position on risk taking. Upon being placed on top of the perch in the calling platform, we timed how long it took males to resume calling, up to a maximum of 300 seconds. In addition to recording the latency to resume calling, each male was weighed to the nearest 0.1 gram using a MS-600 Digital Pocket Scale following a gentle compression to rid the body of excess water. Snout-vent-length (SVL) was measured to the 0.1 millimeter using digital calipers. All measurement were made by a single researcher to eliminate any potential error from individual variations in measurement technique. Following the methods of previous studies (Baker 1992; Ward et. al. 2013), we calculated an index of physical condition as the residuals of a linear regression of the cube root of weight on SVL, divided by SVL. We toe clipped the fourth digit on the hind foot of each male (right foot was clipped in 2013, left foot was clipped in 2014) and stored the toe in formalin for use in age determination via skeletochronology (see below). Air and water temperature were recorded at each calling site. Males were released at their calling site after measurements and toe clipping were completed. Between 2013 and 2014 we tested a total of 123 males (2013: n = 50; 2014: n = 73).

Recordings

During the 2014 breeding season we made audio recordings of the calling behavior of a subset of tested males. These males were those that resumed calling within our 300 second observation period following simulated attack, allowing us to compare calls made before and after attack. We recorded calls using a Sennheiser ME 66 microphone and K6 power supply connected to a Marantz PMD620 digital recorder. Focal males were recorded from a distance of approximately 1-2 meters (recording from this distance did not disturb male behavior). Each recording consisted of 20 consecutive calls made before the simulated predator attack described above, followed by an additional 20 consecutive calls made once the male resumed calling after the predator disturbance. After recording, air and water temperature was measured and physical measurements were taken and condition calculated as described above. In total 46 males were recorded.

Acoustical analyses

We analyzed field recordings using Audacity v 2.0.0 focusing on three call properties: call length, call rate and call effort. For each male we separately analyzed properties of 20 calls made before and 20 calls made after the simulated predator attack. We used the number of pulses per call as a proxy for call length; previous studies have focused on this parameter, in part because field calling males add or subtract pulses in order to alter their call length. This metric is also less temperature sensitive than call length measured directly (in seconds), which is heavily influenced by individual body temperature (Gerhardt 1978; Gerhardt et al. 1996; Reichert 2013). We used the waveform display to count the number of pulses in each call and calculated an average number of pulses per call. Call rate was calculated in calls/min by measuring the total amount of time required to complete all twenty calls. We computed call effort as the product of call length and call rate (pulses/min). This measure can be interpreted as the amount of time during a given minute that a male is producing sound.

Estimating male age

Skeletochronology techniques have been used to reliably age a variety of frog and toad species (Hemelaar 1985; Acker et al. 1986; Lecair and Castanet 1987). Due to time and resource constraints we were able to obtain age estimates for the individuals in the 2013 field season only. The fourth digit of the hind foot from each male was clipped and fixed in 10% formalin solution. The second phalange was dissected and decalcified in a 14% EDTA/dH2O solution (pH: 7.59) for 5 days at room temperature on a rotator.

Bone was paraffin embedded and 5 µm thick sections were made through the diaphyseal region of the phalange. Sections were mounted to slides and stained with hematoxylin and eosin. Slides were analyzed with an Olympus AH2 light microscope using SPOT software 3.5.9 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and resting lines were counted. These lines correspond to periods of no growth for the males during hibernation over winter. In addition to counting resting lines, we also examined the thickness of bone layers and osteocytes in different bone layers to further distinguish between males of different age.

Statistical analysis

To examine how residual reproductive value influenced risk taking we fit censored regression (Tobit) models for the latency to resume calling after simulated predator attack to the primary candidate predictor variables of age, physical condition, body length, and weight. We also included air and water temperature in the models as these variables can influence calling behavior. We analyzed data from 2013 and 2014 separately due to incomplete sampling of skeletochronology across years (see above). Censored regression was used due to the 300-second upper limit on the latency to resume calling, and accounts for the fact that males that did not resume calling within 300 seconds may have resumed shortly after 300 seconds or may have taken much longer than 300 seconds to resume calling.

We used paired t-tests to examine how each of the measured call properties changed after a simulated predator attack. We fit general linear models to examine the relationship between the magnitude of change for each of the call properties (e.g. call effort before disturbance – call effort after disturbance), physical condition, length, weight, air and water temperature, and the latency to resume calling. We used $\alpha = 0.05$ as our significance criterion for all analyses. Data analysis was performed in R v 2.15.2 (R Core Team 2012).

RESULTS

High condition males delay calling longer following a simulated predator attack

Between our two study years we observed a substantial range of latency to resume calling, from 11 seconds to the 300 second time limit. In total only 33 out of 123 males (approximately 27%) did not resume calling within the 300 second limit. In 2013 (n = 50 males), physical condition, but not length or weight, was found to have a significant effect on the latency to resume calling (condition: $z = 3.19$, $p = 0.002$; length: $z = 0.61$, $p = 0.55$; weight: $z = 1.87$, $p = 0.06$). Specifically, low condition males resumed calling more quickly than high-condition counterparts (Figure 4a). We did not find a significant effect of age or temperature on the latency to resume calling (age: $z = 0.48$, $p = 0.6$; air temperature: $z = 1.25$, $p = 0.21$; water temperature: $z = -0.23$, $p = 0.78$). All males were identified as being either 2 years old ($n = 30$), suggesting that 2013 was their first breeding season, or 3 years old ($n = 17$), suggesting 2013 was their second breeding season. A post-hoc power analysis for age revealed low statistical power (power = 0.127).

In 2014 ($n = 73$ males), we found no significant effect of physical condition, length, weight, or temperature on latency to resume calling (condition: $z = -1.07$, $p = 0.3$; length: $z = 0.46$, $p = 0.64$; weight: $z = 0.17$, $p = 0.87$; air temperature: $z = -0.04$, $p = 0.97$; water temperature: $z = -1.17$, $p = 0.25$; Figure 4b), perhaps indicating effects of differences in the sampling environment or population structure between years (see discussion).

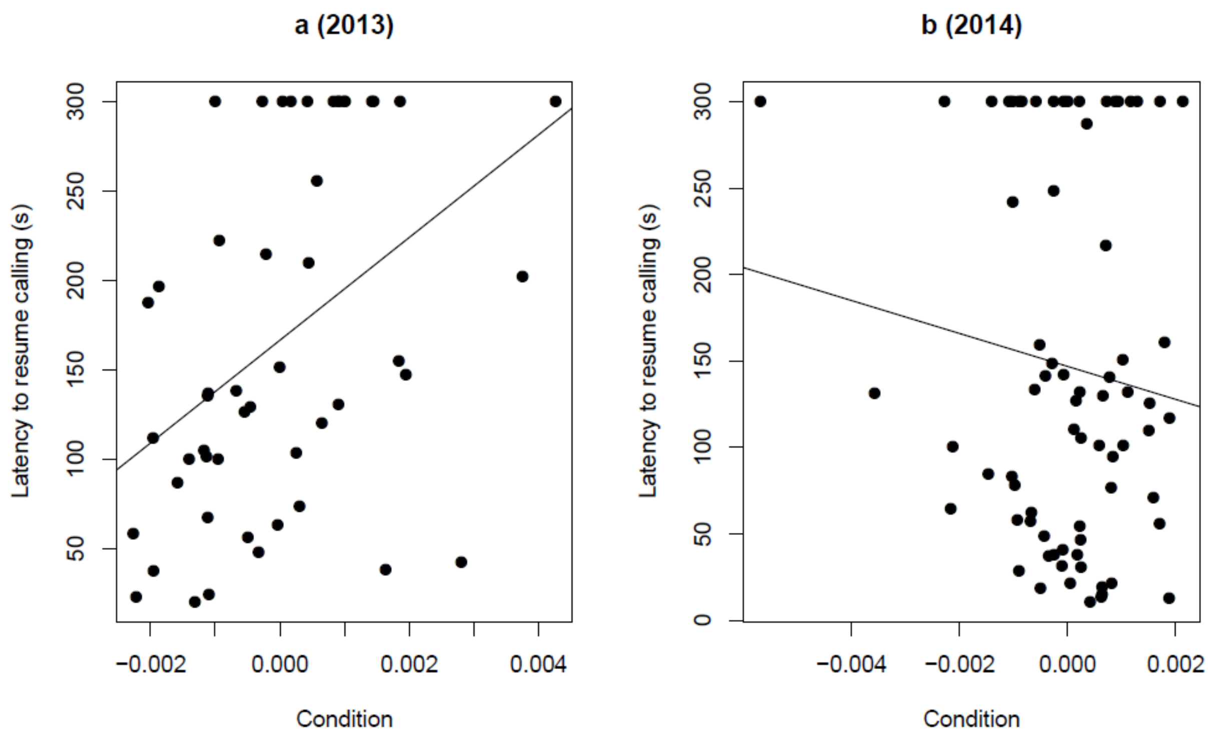

Figure 4. The relationship between physical condition and risk taking as measured by the latency to resume calling after a simulated predator attack. (a) depicts data from the 2013 breeding season showing males in higher condition taking significantly longer to resume calling, while (b) depicts data from the 2014 breeding season showing no significant relationship between condition and latency to resume calling.

Males reduce call effort after simulated predator attacks

Following a simulated predator attack, all males showed a significant decrease in call effort ($n = 46$, $t = 3.93$, $df = 45$, $p < 0.001$; Fig. 5). This relationship was driven by a significant decrease in call length, whereas call rate remained unchanged (call length: $n = 46$, $t = 8.14$, $df = 45$, $p < 0.001$; call rate: $n = 46$, $t = -0.13$, $df = 45$, $p = 0.90$).

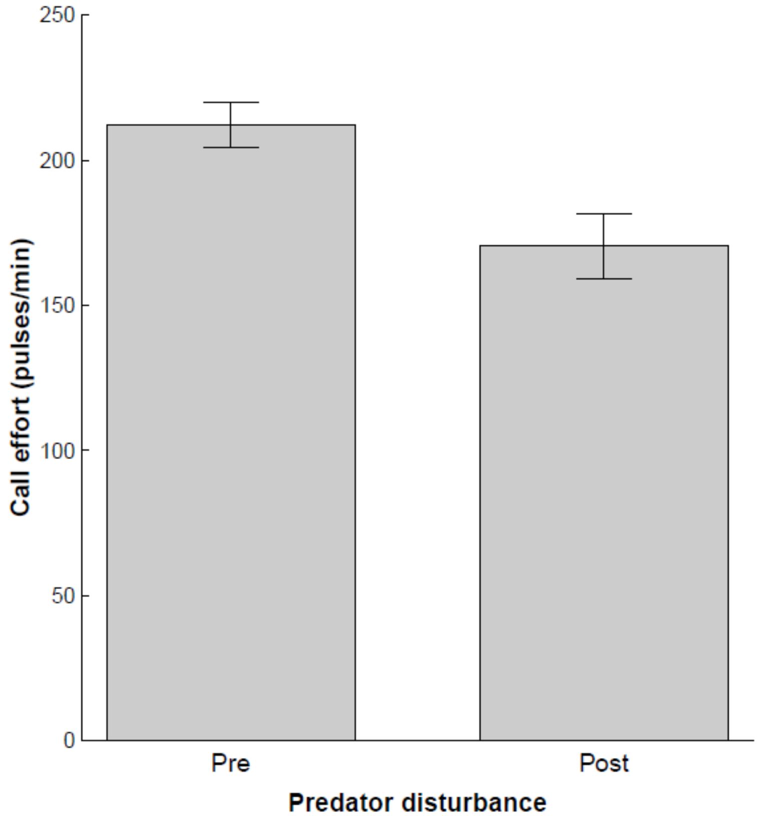

Figure 5. Mean calling effort before and after a simulated predator attack. Following a disturbance males significantly decrease call effort. Error bars represent standard error of the mean.

Neither physical condition, length, nor weight predicted call properties measured before or after simulated predator disturbance (Table 2). These measures also failed to predict the magnitude of change in the call properties following the disturbance (Table 3).

Air and water temperature also failed to predict call properties with the exception of water temperature significantly influencing call rate prior to simulated attack (Table 2). Neither air nor water temperature had any significant influence on the magnitude of change in call properties following the disturbance (Table 3).

Table 2. Results from general linear models examining the effect of physical condition, length, weight, air and water temperature on three call properties before and after a simulated predator attack. All relationships are not significant except for water temperature and call rate before simulated attack.

	Call Length Pre-attack	Call Rate Pre-attack	Call Effort Pre-attack	Call Length Post-attack	Call Rate Post-attack	Call Effort Post-attack
Condition	$F_{1,44} = 0.11$ $p = 0.75$	$F_{1,44} = 0.10$ $p = 0.75$	$F_{1,44} = 0.30$ $p = 0.59$	$F_{1,44} = 0.36$ $p = 0.55$	$F_{1,44} = 0.48$ $p = 0.49$	$F_{1,44} = 1.06$ $p = 0.31$
Length	$F_{1,44} = 0.05$ $p = 0.82$	$F_{1,44} = 0.53$ $p = 0.47$	$F_{1,44} = 0.41$ $p = 0.53$	$F_{1,44} = 0.26$ $p = 0.61$	$F_{1,44} = 0.07$ $p = 0.80$	$F_{1,44} = 0.37$ $p = 0.54$
Weight	$F_{1,44} = 0.08$ $p = 0.77$	$F_{1,44} = 0.27$ $p = 0.60$	$F_{1,44} = 0.13$ $p = 0.72$	$F_{1,44} = 0.55$ $p = 0.46$	$F_{1,44} = 0.33$ $p = 0.57$	$F_{1,44} = 1.18$ $p = 0.28$
Air Temp	$F_{1,44} = 2.18$ $p = 0.15$	$F_{1,44} = 3.86$ $p = 0.06$	$F_{1,44} = 0.25$ $p = 0.62$	$F_{1,44} = 0.23$ $p = 0.64$	$F_{1,44} = 2.34$ $p = 0.13$	$F_{1,44} = 1.51$ $p = 0.22$
Water Temp	$F_{1,44} = 2.74$ $p = 0.11$	**$F_{1,44} = 6.37$** **$p = 0.02$**	$F_{1,44} = 0.99$ $p = 0.32$	$F_{1,44} = 0.42$ $p = 0.52$	$F_{1,44} = 3.39$ $p = 0.07$	$F_{1,44} = 3.04$ $p = 0.09$

Table 3. Results from general linear models examining the effect of physical condition, length, weight, air and water temperature on the magnitude of change in the three call properties measured following a simulated predator attack.

	Δ Call Length	Δ Call Rate	Δ Call Effort
Condition	$F_{1,44}$ = 0.31, p = 0.58	$F_{1,44}$ = 0.42, p = 0.52	$F_{1,44}$ = 0.44, p = 0.51
Length	$F_{1,44}$ = 0.30, p = 0.59	$F_{1,44}$ = 0.61, p = 0.44	$F_{1,44}$ = 1.25, p = 0.27
Weight	$F_{1,44}$ = 0.72, p = 0.40	$F_{1,44}$ = 1.09, p = 0.30	$F_{1,44}$ = 2.00, p = 0.16
Air Temp	$F_{1,44}$ = 1.45, p = 0.23	$F_{1,44}$ = 0.41, p = 0.52	$F_{1,44}$ = 0.81, p = 0.37
Water Temp	$F_{1,44}$ = 1.31, p = 0.26	$F_{1,44}$ = 0.48, p = 0.49	$F_{1,44}$ = 1.10, p = 0.30

Males who are slow to call after simulated predator attacks also call with lower effort

We found that latency to resume calling was significantly predictive of the magnitude of change for all three call properties (call length: n = 46, $F_{1,44} = 5.25$, p = 0.03, $R^2 = 0.11$; call rate: n = 46, $F_{1,44} = 5.32$, p = 0.03, $R^2 = 0.11$; call effort: n = 46, $F_{1,44} = 6.77$, p = 0.01, $R^2 = 0.13$). Males who resumed calling more rapidly after a simulated predator attack exhibited a smaller decrease in call effort than males who took a longer time to resume calling activity (Figure 6).

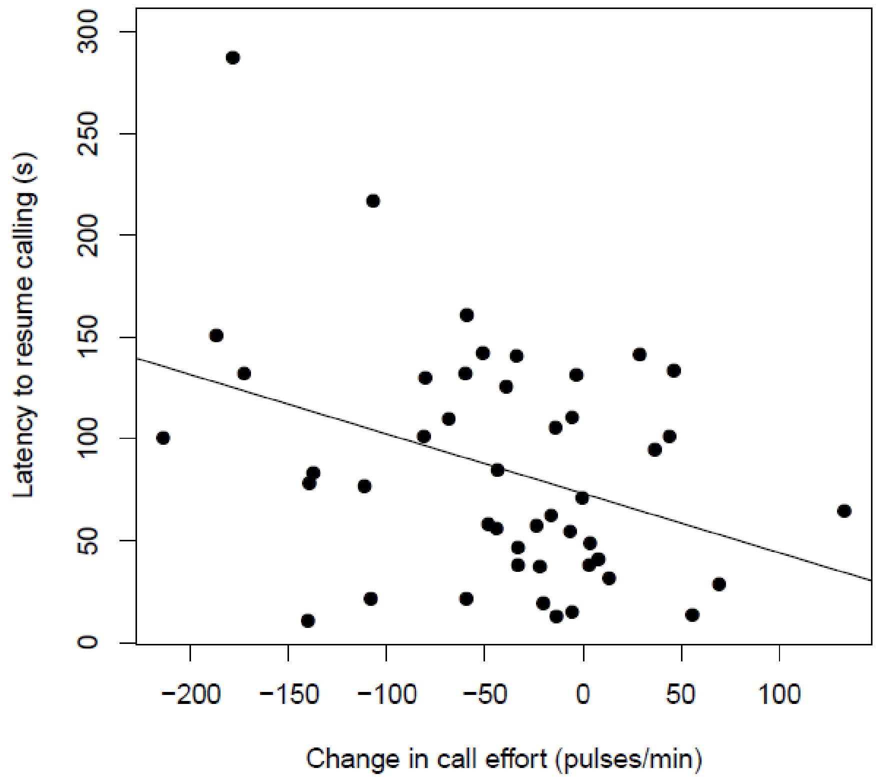

Figure 6. Relationship between the magnitude of change in call effort and the latency to resume calling after a simulated predator attack. Males that took longer to resume calling had a significantly greater decrease in call effort.

DISCUSSION

Low condition males take greater risk

Consistent with the predictions of the asset protection principle, we found that low condition males were quicker to resume calling following a simulated predator attack during the 2013 breeding season. Interestingly, our direct measures of body size (length and weight) were not significant predictors of a male's latency to resume calling. Our 2014 data confirmed that the risk of calling earlier following a simulated attack is not offset by the production of more cautious calls. Below, we discuss the implications of these findings for the asset protection principle, as well as potential sources and implications of differences in data between 2013 and 2014.

Our results revealed substantial variation in risk taking between individuals and between years. 2013 data supported the predictions of the asset protection principle, which proposes that males with fewer future mating opportunities should take greater risks during a given mating attempt. A relationship between the condition of males in our study and their residual reproductive value may result from multiple (non-exclusive) mechanisms. First, low-condition males might have lower survival, and thus, fewer future mating opportunities. The energetic costs of signaling may also limit low condition males' future mating opportunities in this system. For *H. versicolor* calling is an extremely energetically costly activity (Taigen and Wells 1985; Wells et al. 1995). As a result, males in poor physical condition may be more limited in the number of nights and number of hours per night they can spend calling at a chorus (factors known to predict male mating success; Ritke and Semlitsch 1991; Sullivan and Hinshaw 1992; Bertram et al. 1996). Regardless of the mechanism by which condition influences residual reproductive value (e.g. survival, signaling capacity), our 2013 results demonstrated that condition predicts risk taking behavior, with low condition males (i.e. those with lower predicted residual reproductive value) taking greater risks.

Interestingly, males' ages did not predict their responses to simulated predation contrary to the predictions of the asset protection principle, however the statistical power for age was very low (power = 0.127). The discrepancy between our results for age and condition may indicate that condition is more important in determining variation in male residual reproductive value. Males in our population never exceeded three years old, and overwinter mortality, if high enough, may make future breeding seasons unlikely for males of any condition. Therefore, male age might not be expected to play a role in risk assessment. Alternatively, age-related variation in behavioral strategies may be subject to context dependencies (e.g. environmental factors) that we were unable to detect using our sampling design. However, due to low statistical power we cannot rule out the possibility that age has an effect on risk taking behavior.

Variation in call properties following simulated predator attacks

As with other studies showing reduced intensity of sexual signals in high risk situations (e.g. Lloyd 1983; Candolin 1998), we found that males reduced call length and call effort following a simulated predator attack. Contrary to our predictions, however, the magnitude of this decrease did not depend on males' physical condition or any of our measures of size. Although this result was unexpected, it is consistent with the 2014 dataset, in which condition did not predict latency to resume calling. It would be interesting to know whether this pattern differed during 2013, when there was a significant relationship between condition and latency to resume calling, unfortunately in 2013 we did not record focal male calls after the predator simulations.

Although the magnitude of decrease in call properties was not predicted by physical condition, we did find a significant relationship between change in call properties and latency to resume calling. Males that resumed calling more rapidly after a predator disturbance also exhibited a smaller decrease in call length, rate, and effort. Considering that producing calls with greater acoustic energy is likely more conspicuous to predators (Akre et al. 2011), our results suggest that males taking greater risk by calling earlier following a simulated attack do not offset this risk by producing more cautious signals. In fact, males with shorter latency to resume calling produce more conspicuous signals than males who are slow to resume calling following a simulated attack.

Yearly variation in latency to resume calling

While our 2013 data corroborated the prediction that high condition males have more risk-averse courtship strategies (vs. low condition counterparts), this relationship was not apparent in 2014. Below, we suggest two explanations for this discrepancy, each of which merits future investigation.

First, differences in the distributions of male condition in 2013 and 2014 samples may explain observed differences in male behavior. In 2014 males were significantly longer, heavier, and in better physical condition than males in 2013 (Welch two sample t-test: length: $t = -3.6$, $df = 90.5$, $p < 0.001$; weight: $t = -5.0$, $df = 101.9$, $p < 0.001$; condition: $t = -3.2$, $df = 93.3$, $p = 0.002$). The influence of condition on risk taking may only become apparent in males of lower condition, so that having a population of high condition males in 2014 may mask any effect we may otherwise expect to observe.

Another, non-mutually exclusive explanation for the difference between years is the presence of an environmental/year effect on risk taking. Previous research has shown that a variety of environmental factors (both biotic and abiotic) may influence risk assessment and antipredator behavior (Killen et al. 2013; Dosmann and Mateom 2014; Leinart et al. 2014). It is possible that a difference in the environmental conditions during 2014 is playing a dominant role in determining risk sensitivity and limiting our ability to detect the influence of physical condition. Additionally, it is also possible that the role of physical condition in risk taking is context dependent and may change under different environmental conditions.

Consequences of variation in risk taking behavior

The differences between males in the latency to resume calling after a simulated attack have potential consequences for both male and female treefrog reproduction. In a field study of mate assessment time, Schwartz et al. (2004) found that female gray treefrogs likely spend approximately 2 minutes assessing the calls of potential mates before making a decision. In our study, males showed substantial variation in the latency to resume calling in both years, ranging from just over 10 seconds to beyond the 300 second upper limit of our measurements. Males that are more sensitive to risk, and take longer to resume calling activity, may therefore end up missing the entire assessment windows for nearby females. If such is the case, our data indicate that poor condition males might have experienced mating advantages in the presence of predators in 2013, while in 2014 they did not. These interacting effects of condition and extrinsic factors that influence mating behaviors may act to maintain population variation in response to predators and may limit any potential reproductive benefit of high physical condition.

Additionally, the variation in call properties after a simulated predator attack has implications for mate attraction. Males that engage in riskier behavior by resuming calling more rapidly after a disturbance also maintain greater call efforts. Previous research has shown that female gray treefrogs prefer calls broadcast at higher efforts (Gerhardt 1991; Gerhardt et al. 1996; Schwartz et al. 2001). Our results thus indicate that riskier males are producing more attractive calls, which may grant them a reproductive advantage in addition to the advantage gained by resuming calling activity more rapidly (as discussed above).

General conclusions and future directions

Our results provide partial support to the hypothesis that antipredator behavior is influenced by residual reproductive value as stated in the asset protection principle, and illustrate the importance of considering condition, as well as age, when assessing male residual reproductive value. In our study, age had no significant effect on risk taking, but physical condition was a significant predictor of riskiness in one of the two breeding seasons studied. Additionally, although physical condition did not affect the magnitude of change in call properties after a disturbance, males who exhibited greater risk in time to resume calling also showed the smallest decreases in call properties. Future research should focus on examining any extrinsic variables such as the social or physical environment that could potentially influence risk taking and may interact with the effects of physical condition, possibly contributing to the discrepancy between years observed in this study. Additionally, future work could examine how females respond behaviorally to predator cues. Although a previous study found that female mate choice was not influenced by auditory playback of bullfrog calls (Schwartz et al. 2000), future research could examine female responses to other predator cues (e.g. visual stimuli). Our results demonstrate the importance of individual variation in physical characteristics in antipredator behavior and highlight the potential for a complex interplay between environmental, temporal, and individual variables in sexual signaling in risky conditions.

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CHAPTER 4

Larger females are choosier in the gray treefrog (*Hyla versicolor*)

ABSTRACT

Individual variation in female mate choice has important implications for sexual trait evolution and the maintenance of phenotypic diversity. In this study we examined several potential drivers of individual variation in female choosiness for the well-studied, energetically expensive courtship signal of male gray treefrogs, *Hyla versicolor*. Specifically, we investigated the relationship between female choosiness and other female traits (female body size, physical condition, and age) using a costly choice playback experiment where females traveled different simulated distances to reach attractive mates. We found that larger females maintained their preferences for attractive male calls over greater simulated distances (i.e. were choosier) than smaller females. We discuss possible explanations for why larger females may be choosier and suggest several potential avenues of future research.

INTRODUCTION

Females in many species choose mates based on elaborate sexual displays or ornaments (Andersson 1994). Historically, these choices have chiefly been studied using pooled observations of many individual females, and were therefore unable to explore whether and how individual females varied in their mate choice behaviors (Jennions and Petrie 1997; Widemo and Saether 1999). More recent work suggests that such individual differences in female preference and/or choosiness are potentially common and evolutionarily significant (Jennions and Petrie 1997; Cotton et al. 2006; Chaine and Lyon 2008). As a result, the proximate and ultimate causes of individual variation in female choice are now a major area of research among biologists seeking to explain how nonrandom mating and sexual trait variation evolve.

Individual females can differ from one another in several components of the mate selection process that have important evolutionary consequences (for comprehensive reviews see: Jennions and Petrie 1997; Widemo and Saether 1999). In order to understand how these aspects of mate choice behavior evolve it is important to consider the multiplicative costs and benefits of mate choice (Cotton et al. 2006). In the past there has been extensive research on how trade-offs in males between the benefits of elaborate signals and the costs of producing and maintaining those signals can generate variation in sexual displays and ornaments (Andersson 1994; Moller and de Lope 1995; Getty 2006). Females are likely to face similar trade-offs during mate choice, balancing the benefits and costs of exhibiting mating preferences (Pomiankowski 1987; Reynolds and Cote 1995; Cotton et al. 2006). Benefits of mate choice can be direct (access to high quality territories, nuptial gifts, paternal care: Kirkpatrick and Ryan 1991; Forsgren et al. 1996) and indirect (good genes: Petrie 1994; Welch et al. 1998), while costs of choice may include predation and harassment risk, loss of time, energetic costs, and risk of disease transmission (Pomiankowski 1987; Pocklington and Dill 1995; Backwell and Passmore 1996; Grafe 1997). In general females are expected to show weaker preferences and less choosiness when the costs of choice are high (Milinski and Bakker 1992; Hedrick and Dill 1993; Slagsvold and Dale 1994). For example, when forced to swim against a current, female sticklebacks became less selective and were more likely to mate with dull, unattractive males (Milinski and Bakker 1992).

The kinds of costs and benefits enumerated above are likely to differ among individual females in accordance with multiple extrinsic and intrinsic factors, and thus promote variation in mate choice. These factors include female size (Hingle et al. 2001), condition (Bakker et al. 1999; Hunt et al. 2005; Burley and Foster 2006; Baugh and Ryan 2009), age (Kodric-Brown and

Nicoletto 2001; Moore and Moore 2001), parasite load (Poulin 1994; López 1999), reproductive state (Lynch et al. 2005, 2006), and prior experience (Dukas 2005; Hebets and Vink 2007; Bailey and Zuk 2008). For example, in a study of wolf spiders where females and males were raised on either a high or low quality/quantity diet, high-diet females were in better physical condition and were more likely to mate with good condition males raised on high quality diets compared to low-diet females who showed no mate selectivity (Hebets et al. 2008). In house crickets (*Acheta domesticus*), female age was found to influence mate choice with younger females exhibiting a preference for longer male songs with more pulses per chirp, while older females showed no preference (Gray 1999). In a study of the effects of prior experience, female Polynesian field crickets (*Teleogryllus oceanicus*) raised under silent acoustic conditions were found to be less discriminating among male songs compared to females that were exposed to conspecific song during development (Bailey and Zuk 2008).

Additionally, certain ecological and environmental factors such as predator abundance (Hedrick and Dill 1993; Johnson and Basolo 2003; Bonachea and Ryan 2011), the physical environment, and timing of the breeding season (Qvarnstrom et al 2000; Borg et al. 2006; Clark and Backwell 2015) can influence female mate choice. For example, female green swordtails (*Xiphophorus helleri*) typically prefer to mate with males with long, conspicuous swords; however following exposure to video playbacks of predation events they prefer to associate with cryptic males, presumably to minimize predation risk (Johnson and Basolo 2003). Female banana fiddler crabs (*Uca mjoebergi*), change their mate selection depending on their location within the inter-tidal zone, and across the breeding season to maximize successful larval emergence (Clark and Backwell 2015).

We suggest that one of the best ways to understand what drives or limits individual variation in mate choice is to focus on experimentally tractable systems with well characterized mating signals. Here we investigated the influence of body size, condition, and age on female choosiness in the gray treefrog (*Hyla versicolor*). As with many other anuran species, female gray treefrogs assess and select mates based upon specific properties of highly energetically demanding male acoustic advertisement signals (Taigen and Wells 1985; Gerhardt 1994). Females prefer long calls, fast call rates, and high call efforts--a composite of call length and rate (Gerhardt 1991; Gerhardt et al. 1996; Schwartz et al. 2001), and may receive indirect benefits in the form of improved larval fitness by mating with males producing long calls (Welch et al. 1998). Previous research has documented variation among *H. versicolor* females in the strength of their preference for long calls (Gerhardt et al. 2000), however this is the first study to examine what predicts this variation. In this study we utilized a costly choice playback design where we examined the simulated distances females were willing to travel to reach a more attractive mate to investigate how variation in the intrinsic factors of size, condition and age influence female mate choice. We define females that maintain their preference over greater simulated distances as being choosier. Specifically we tested the prediction that larger and higher condition females would be choosier and willing to travel greater simulated distances to reach a more attractive mate. Larger, higher condition females may be choosier because they are better equipped to handle the costs associated with mate choice than smaller, low condition females. This trend has been observed in empirical studies from multiple taxa (Bakker et al. 1999; Hunt et al. 2005; Hebets et al. 2008). Additionally, we predicted that older females would be less choosy because they are closer to the end of their lives when selection for costly mate searching is expected to be weaker (Stearns 1992). In support of this prediction, reduced preference strength and choosiness in older females has been observed in several species (Gray 1999; Kodric-Brown and Nicoletto 2001; Moore and Moore 2001).

METHODS

Experimental overview

We investigated the influence of female size (length and weight), condition, and age on choosiness using a simulated costly choice playback experiment (e.g. Gerhardt et al. 2000). In this experiment females were presented with a choice between a priori attractive and unattractive male calls that differed in playback amplitude. Because sound pressure level (SPL) is negatively correlated with distance from a sound source (6 dB SPL decrease per doubling of distance), altering playback amplitude simulated males calling from different distances. While maintaining the unattractive call at a constant "nearby" SPL, we sequentially decreased the SPL of the attractive call simulating a male that was farther and farther away. We then examined how "far" a female was willing to travel to reach an attractive mate, and whether the simulated distance a female was willing to travel depended upon female size, condition, or age.

Specimen collection and general testing procedure

Females were collected between May and July 2015 from several breeding aggregations within the Lux Arbor Reserve (Kellogg Biological Station, Michigan State University) in Barry County, Michigan (42°29´N, 85°28´W). Females were collected from amplected pairs to ensure that they were sexually receptive. Mated pairs were brought to the lab and stored in small plastic containers, and kept on ice to delay oviposition until testing. All playback trials took place in a simple sound chamber at the Kellogg Biological Station. The chamber consisted of a 2x2 meter platform covered with a sound dampening blanket and tarp and encircled by draped sound dampening blankets. Speakers (Bose Companion 2 Series II) were placed on opposite ends of the chamber and a small, ~ 8 cm diameter, hardware cloth cage was placed in the center between the two speakers. All testing took place under dark conditions, and a night-vision capable camera (VideoSecu Bullet Security Camera) was mounted above the chamber so that female responses could be observed remotely. Before testing, containers with the mated pairs were removed from ice and females were given ~1 hour to warm up. Trials began when a female was separated from her mate and placed within the hardware cloth cage in the center of the sound chamber. Females were given a one minute period of silence before the stimuli began. Females were allowed to hear 4 calls of each alternative before the top of the cage was remotely removed via a string pulled from outside the chamber, and female response to playback was observed. Females were given 5 minutes to make a choice (see below for a description of the choice criteria). A few females did not respond within 5 minutes; these individuals were considered to be sexually unresponsive and were dropped from the experiment.

At the completion of testing we measured each female's snout-vent-length (SVL) to the nearest 0.1 millimeter using a digital caliper. To avoid any confounding effects with egg mass, we waited until females had completed depositing eggs to measure female weight. Following a gentle compression to rid the body of excess water, we weighed females to the nearest 0.1 grams using a digital scale. As in previous studies (Baker 1992; Ward et al. 2013; Kuczynski et al. 2015) we computed an index of physical condition as the residuals of a linear regression of the cube root of weight on SVL, divided by SVL. To determine female age, we toe clipped the fourth digit on the right hind foot of each female for use in skeletochronology. Skeletochronological techniques have been reliably used to estimate anuran age in previous studies (Hemelaar 1985; Acker et al. 1986; Lecair and Castanet 1987; Kuczynski et al. 2015). For a detailed description of the methods used in this study see Kuczynski et al. (2015), briefly: toe bones were decalcified, cut into 5 μm sections, stained with hematoxylin and eosin, and resting lines corresponding to periods of no growth during hibernation were counted. Mated pairs were returned to the pond where they were collected, typically within less than 48 hours. In total 50 females completed this experiment.

Acoustic stimuli

We created playback stimuli using natural recordings of male calls made during summer 2014. We selected a call from a recording made at 20^{o}C (this temperature approximately matched the temperature of the sound chamber) and added and subtracted pulses using Audacity v 2.0.0 sound analysis software to create the attractive and unattractive alternatives. The attractiveness of longer calls has been well established in this species (Gerhardt 1991; Gerhardt et al. 1996; Schwartz et al. 2001). The long, attractive call contained 20 pulses/call which is approximately 1 standard deviation above the mean of calls from 50 males recorded in 2014 (mean = 16.67 pulses/call, SD = 3.2 pulses/call), while the short, unattractive call contained 10 pulses making it ~2 SD below the mean. Calls were broadcast on a loop at a call rate of 10 calls/minute which was slightly below the average rate (mean = 13 calls/min, SD = 6.4 calls/min). Because call length differed while call rate remained the same, the call effort of the attractive call was necessarily higher, but all values (call length, rate, and effort) used in this experiment fell within the range of natural variation. Four sets of stimuli were created using recordings from four different males, and during testing each female was randomly assigned to one of these stimuli for all trials.

During trials, calls were broadcast antiphonally with the attractive and unattractive calls playing on opposite ends of the sound chamber. In all trials the SPL of the unattractive call was fixed at 85 dB at a position 1 meter away from the speaker while the SPL of the attractive call varied between sets of trials. The SPL of both calls was calibrated at the beginning of testing and in between trial sets using an Extech Instruments model 407732 digital sound level meter.

Costly choice design

Each female was tested in a series of trials where the SPL of the attractive call was sequentially decreased. In the first set of trials the unattractive call was broadcast at 85 dB SPL, while the attractive call was 3 dB lower at 82 dB SPL. We did not include trials with equal playback SPL because the extent of the difference in attractiveness between our alternatives was such that in a previous unpublished experiment 100% of females (n = 55) chose the attractive call when playback SPL was equal. Furthermore, excluding trials with equal playback reduced the total number of trials each female needed to complete, which reduced the risk that a female would lose sexual responsiveness before testing was complete. Using the methodology from a previous study (Gerhardt et al. 2000) we determined that a female preferred the attractive call if she moved to within 50 cm of the speaker broadcasting the attractive call in a minimum of 3 out 4 trials. We justify using the 50 cm response criteria based on previous unpublished observations (n = 55) where we did not observe any females reversing direction after approaching a speaker to within 50 cm. This response criteria is further justified in previous literature (Gerhardt et al. 2000). Females were given a minimum of a one minute break in between trials before being tested again. If a female exhibited a preference for the attractive call according to our criteria at the end of a set of trials, the SPL of the attractive call was decreased an additional 3 dB (simulating increased distance from the center of the test chamber) and a new set of trials began.

Testing continued in this manner with the SPL of the attractive call decreasing in 3 dB steps until the female failed to show a preference for the attractive call. To ensure that females were able to hear and evaluate both test stimuli, our lowest stimulus level in all trials (73 dB) was well above the hearing threshold required to elicit a behavioral response in this species (37-43 dB; Beckers and Schul 2004).

Our measure of female choosiness was then quantified as the maximum simulated distance over which females still preferred the longer (more attractive) call. Females that did not exhibit a preference for the attractive call in the first set of trials when there was only a 3 dB difference between calls were considered to have a choosiness score of 0 dB. This measure of choosiness can be interpreted as the simulated distance a female is willing to travel. For example, a female with a choosiness score of 9 dB is willing to travel as much as 1.82 meters extra distance to reach the longer (more attractive) call, whereas a female with a choosiness score of 3 dB will travel no further than 0.41 meters extra distance to reach the longer call.

Statistical analysis

Due to the ordered nature of our response variable (e.g. near—far simulated distance), we examined the intrinsic factors influencing female choosiness using ordinal logistic regression models. Female choosiness score served as the response variable and was fit to the candidate predictor variables of length, weight, and age. Due to issues with multicollinearity when including condition in the same models with body size, we fit an additional ordinal logistic regression model to analyze condition. We also used a second approach in which we used binary logistic regressions to compare choosier vs. less choosy females, and repeated this analysis for all possible break points (i.e. 3 dB, 6 dB, 9 dB). Regardless of the selected threshold, the binary logistic regression identified the same predictor variable (female body length; see results) as the ordinal models. For simplicity, we report only the results from the ordinal logistic regression and provide proportional odds ratios as effect sizes for significant predictors. We used likelihood ratio tests to compare nested models, removing the term that explained the least amount of variance in a given model to arrive at a simpler (nested) model (model 1: choosiness score ~ length + weight + age; model 2: choosiness score ~ length + weight; model 3: choosiness score ~ length), and $\alpha = 0.05$ as our significance criterion for all analysis. All data analysis was performed in R v 2.15.2 (R Core Team 2012).

RESULTS

Female characteristics

Female length ranged from 40.7-53.7 mm (Mean ± SD = 47.3 ± 2.9 mm), and weight ranged from 5.4-11.7 g (8.3±1.6 g). Due to small numbers of very old females, age was collapsed into two categories, young and old, with old females including all individuals that were 3 years or older. This categorization of age yielded 30 young females and 20 old females.

Female choosiness

The best fitting model for female choosiness contained only length as a significant predictor variable ($t = 3.89$, $p < 0.001$, odds ratio: 1.54). Larger females were significantly choosier, i.e. retained their preference for the longer (more attractive call) over greater simulated distances (Figure 7). After controlling for length, female weight and age were not significantly predictive of choosiness (weight: $t = 0.95$, $p = 0.34$; age: $t = 0.74$, $p = 0.46$). Although older females do appear to have greater choosiness scores than young females (Figure 8), this is likely the result of a significant relationship between age and length (mean length young = 46.2 mm, mean length old = 48.9 mm; Welch's t-test: $t = -3.45$, $p = 0.001$). We found no significant influence of the computed condition index on female choosiness ($t = 0.63$, $p = 0.53$) (Figure 9).

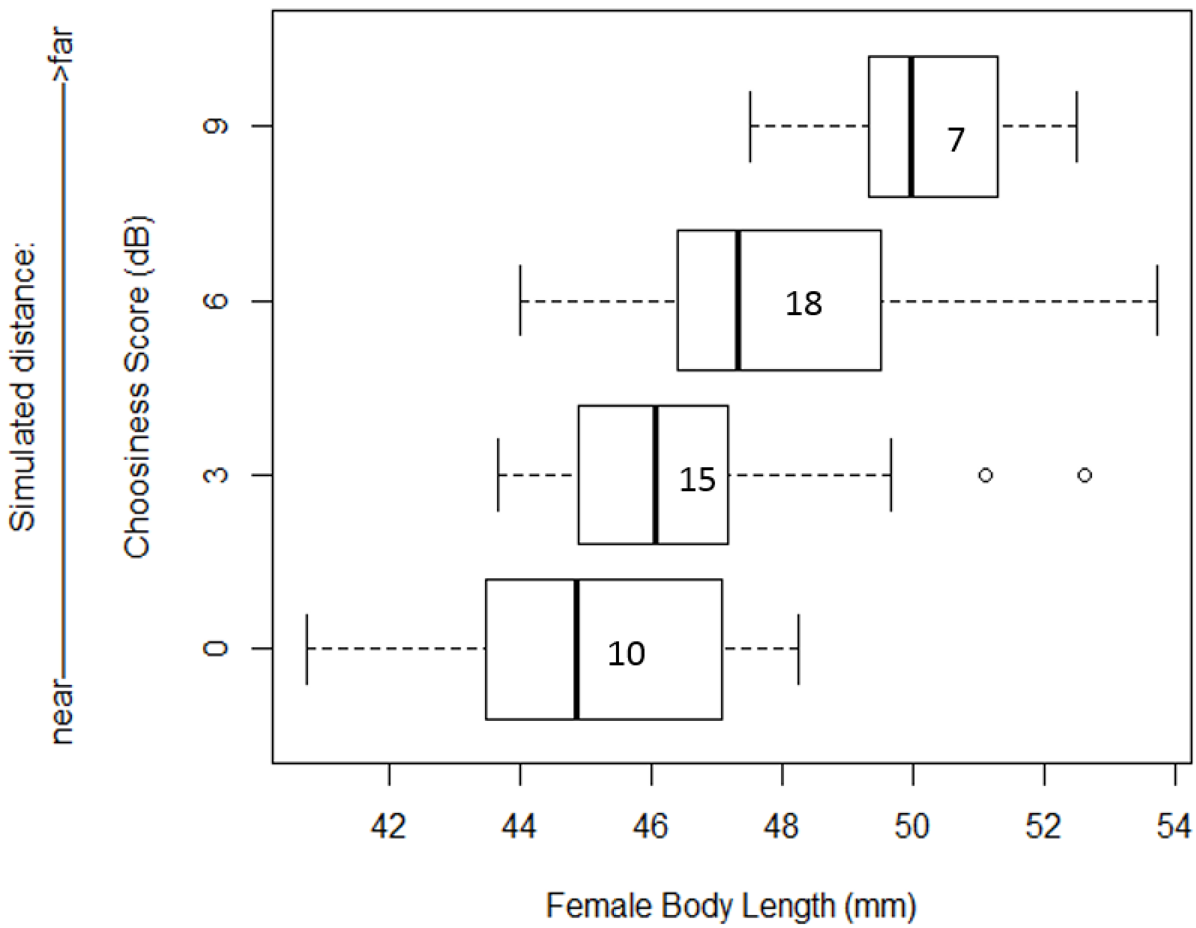

Figure 7. Relationship between female body length and choosiness. Larger females were choosier and hence willing to travel greater simulated distances to reach an attractive mate than their smaller counterparts. The number of females with each choosiness score is depicted inside each box.

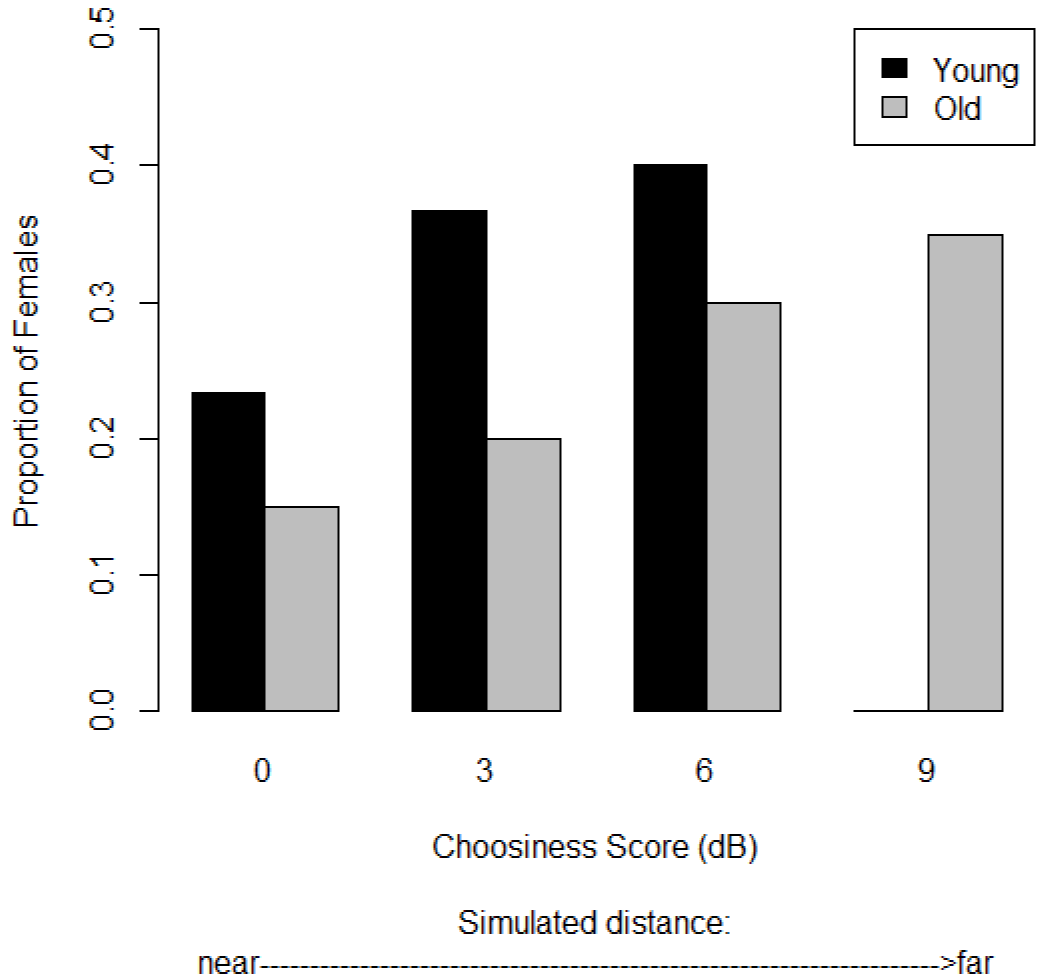

Figure 8. The proportion of young vs. old females with each choosiness score. Although older females appear to be choosier and willing to travel greater simulated distances to reach attractive mates, this pattern was driven by a significant relationship between length and age; age itself was not a statistically significant predictor of female choosiness (see results).

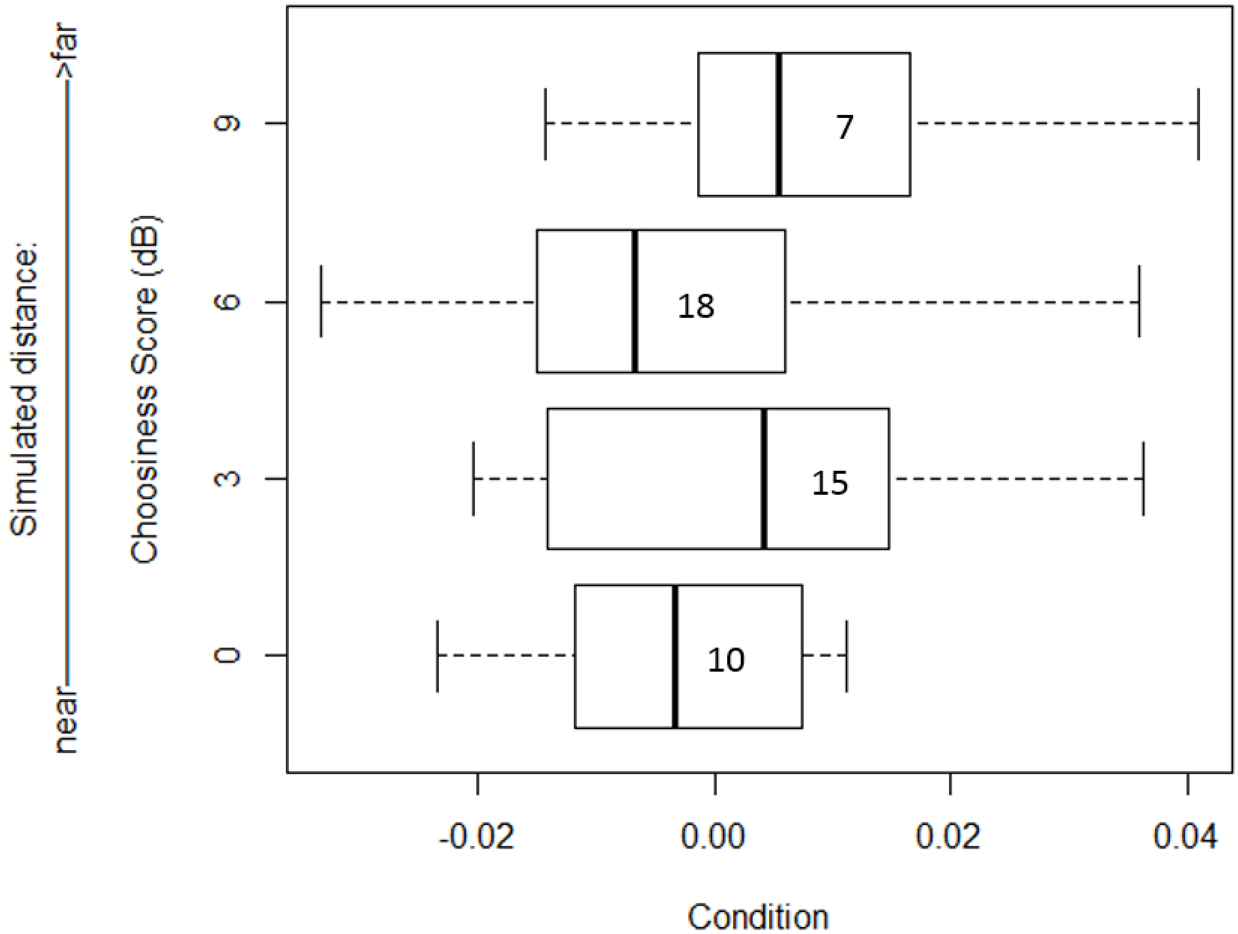

Figure 9. Female condition did not predict female choosiness in *Hyla versicolor*. The number of females with each choosiness score is depicted inside each box.

DISCUSSION

We found that large female gray tree frogs were more likely than small ones to choose longer (more attractive) calls at increasing simulated distances. Contrary to our predictions however, female age and physical condition were not significantly predictive of choosiness, after statistically controlling for the length effect. Below we provide several, non-mutually exclusive, potential explanations for why larger females may be choosier.

Mate searching is less energetically costly for large females vs. small ones

Larger females might be willing to travel greater distances to reach attractive males if travelling incurs lower energetic costs. Smaller females, in contrast, may be more energetically constrained and therefore obtain lower residual benefits from costly mate searching. However, we do not think that direct travel costs are likely to explain our experimental outcomes, since the distances simulated in the experiment (~between 1 to 4 meters) are very small compared to the distances females typically travel between breeding ponds, foraging sites, and overwintering habitats (Johnson and Semlitsch; Johnson et al. 2007).

Mate searching is riskier for small females

It is also possible that mate searching incurs lower predation risk for larger females. Bullfrogs (*Lithobates catesbeianus*) and green frogs (*Rana clamitans*) are abundant sit-and-wait predators of gray treefrogs in our study ponds as well as others (Hinshaw and Sullivan 1990; Gerhardt et al. 1996). If larger females are less likely to be attacked, better able to avoid/escape predation, or too large for gape-limited predators, then they may be experiencing lower costs for traveling greater distances to reach attractive mates. This is a plausible explanation for why larger females are choosier than smaller females that will need to be tested with future studies.

Larger females derive larger marginal fitness returns from choosiness

In addition to lower costs for mate searching, larger females may be choosier if they gain greater marginal benefits from choosiness (Cotton et al. 2006). This may occur in species where larger or better condition females produce more eggs/offspring and being selective ensures choosing a high quality mate that can completely fertilize the clutch or successfully care for the young (Mazzi 2004; Cotton et al. 2006). This hypothesis however, seems unlikely to explain our findings from *Hyla versicolor*; even though larger anuran females often have higher fecundities (Duellman and Trueb 1986; Ritke et al. 1990), previous research has found no relationship between male *Hyla versicolor* call length and sperm number/viability (Doyle 2011), and there is no parental care in this species.

Differences in mate choice are not adaptive

Larger females may also exhibit bolder mate searching behaviors as a correlated byproduct of selection on non-sexual behavior. Previous research has demonstrated that ecological factors can generate differences in suites of traits that may carry over into sexual behaviors (Brown et al. 2007; Luttbeg and Sih 2010). In *Hyla versicolor*, larger females may be under selection to be more exploratory and less shy in general, which could explain their willingness to travel greater distances to seek out more attractive mates. Size dependent differences in sensory or perceptual abilities may provide an additional non-adaptive mechanism for among female variation in mate choice.

Future experiments should focus on determining the exact causes of increased choosiness in larger females. These studies should focus on quantifying the precise costs and benefits of choice for both large and small females. For example, the ability of females of different sizes to escape capture from predators could be investigated.

Implications of variation in choosiness

Regardless of why larger females are choosier in our study, we have clearly demonstrated the presence of systematic, size-dependent individual level variation in mate choice in this species. Individual variation in female preferences and choosiness can alter the strength and direction of sexual selection across the population, driving the evolution of male secondary sexual characteristics (Jennions and Petrie 1997; Pfennig and Tinsley 2002; Chaine and Lyon 2008), and potentially contributing to speciation (Turner and Burrows 1995). The variation in choosiness observed in this study may influence the strength of sexual selection on male call properties. Depending on the average choosiness in the population, this variation may either increase or decrease the strength of selection for long calls. In future research, it would be interesting to know how the variation in mating behavior observed in this study influences the variation in sexual signals observed in field settings (Kuczynski et al. 2015).

Variation in female mate choice is also necessary for female preference to evolve (Kirkpatrick 1982; Jennions and Petrie 1997; Widemo and Saether 1999). Differences in male calling behavior in *Hyla versicolor* can result from several environmental factors (Runkle et al. 1994; Schwartz et al. 2002; Kuczynski et al. 2016), and other studies have provided evidence consistent with genetic influences on male calling behavior (Gerhardt 1991; Sullivan and Hinshaw 1992; Gerhardt et al. 1996). Future studies exploring the role of genes and the environment and how they interact with intrinsic characteristics such as body size to shape female mate choice will be useful for understanding both male traits and whether female preference itself can evolve.

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