Faux frogs, foam nests, and females: Mate choice as an evolutionary driver of conspicuous

mating signals

by Olivia Rose Marie Hamilton
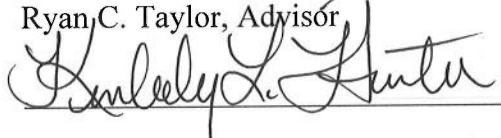
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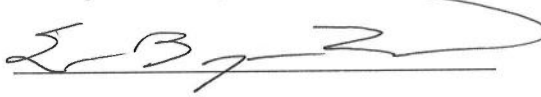
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Faux frogs, foam nests, and females: Mate choice as an evolutionary driver of

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By

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A thesis submitted to the Department of Biological Sciences of

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Dedication

This achievement would not have been possible without the love and support from those closest to me. My parents, Pamela Lauer-Hamilton and Matthew Hamilton have given me the space and acceptance to follow dreams a younger version of myself could have only imagined. (Not many parents would let their daughter travel alone to Panamá for nearly a combined nine months across three years.) They have been with me through everything, and I will be eternally grateful for their support even through the darker periods of my life. My best friend of three years and personal graphic designer, Christopher "Topher" Maness has been my rock through this degree. From brainstorming experiments to listening to me go on about evolution (and frogs) for hours on end, he has been a never-ending source of love and support that I can never repay him for; perhaps marriage will suffice.

All of that being said, I must dedicate this thesis to my younger self. Thank you for hanging on and not giving in. All those times you questioned if you were good enough or if your life meant anything are just memories now. Your future is so much better than you could have ever imagined and, for once, you are glad to be on this Earth. It was worth the wait.

To my future self: I hope you look back on this thesis and all of your early accomplishments and smile.

Acknowledgements

I must start by thanking the people who helped with the experiments themselves: my field assistants, Jorge Lopez and Leslie Barría; my lab's postdoc, Logan James; my lab's resident engineer, Paul "Paulie" Clements; and, of course, my PIs Kimberly Hunter and Ryan Taylor. Without the support of this group, I would not have been able to collect the data I present in this thesis. In the field, Gregg Cohen and Luke Larter were amazing people to have on my team during crunch time. I also thank Kelsey Owens for 3D printing of the faux foam nests I use for experiments in Chapter 3. Of course, I am also grateful to the Smithsonian Tropical Research Institute as well as the Republic of Panamá itself for hosting my stay. Most of all, I thank the combined 1,086 túngara frogs I collected over my two field seasons. I hope your toes grew back.

Chapter 1: If memory serves: A Multisensory signal improves memory for signaler location in the túngara frog *Physalaemus pustulosus*

Abstract:

Across many taxa, males gather in leks to perform multisensory courtship displays for females. Changes in the sensory scene over the course of mate evaluation are inevitable during a lek. This dynamic nature makes a female's ability to recall the location of individual signalers an important component of female mate choice. It is hypothesized that complex (especially multimodal) signals may improve a female's ability to remember, and thus discriminate among potential mates. To test this hypothesis, we employed robotic frogs and a blinding system in playbacks with female túngara frogs (*Physalaemus* (=*Engystomops*) *pustulosus*). Specifically, we asked if the visual component of a multimodal signal improves a female's ability to remember the location of a signaler. Females' memory for a multimodal signal was examined after an initial presentation period followed by a holding period and/or silent period. Females were only able to remember a signaler's location after the introduction of a silent period. They were still able to remember even after a combined 25 s after the obstruction of the visual stimulus (robotic frog). Silence is common in choruses and our data suggest that memory instantiation for multisensory stimuli occurs as a result of this silence.

Introduction:

Animal communication signals constitute some of the most diverse traits observed in nature, many of which function in mate attraction (Bradbury & Vehrencamp 2011; Rosenthal 2017). Signaling reproductive availability can be achieved through a variety of behaviors and sensory modalities. The calls of crickets on a warm summer evening (Olvido & Wagner 2004); the low frequency "bellows" of a koala (Ellis et al. 2011); and the incredible architectural design of a male pufferfish's nest (Kawase et al. 2013) are all produced in efforts to procure mates. Because females often decide the outcome of a reproductively motivated interaction, their decisions have critical fitness consequences (Andersson & Simmons 2006). In order for any signaler's stimulus to be recognized, however, the nervous system of the receiver must integrate and analyze components of the signal and assign those components to their source (Bee & Miller 2016). Thus, the selective force of the receiver's psychology, and the choice that is made as a result, is an important factor driving signal evolution (Guilford & Dawkins 1991).

Yet, signals may not always be unimodal, and are often transmitted in more than one sensory modality (Higham & Hebets 2013). During courtship displays, male butterflies, for example, use both chemical and visual signals (Costanzo & Monteiro 2007), while wolf spiders rely on visual and seismic signals (Hebets & Uetz 1999; Kozak & Uetz 2019). Male sagebrush lizards combine chemical and visual signals to both defend their territory and attract females (Thompson et al. 2008). Similarly, females prefer the visual display of a wingspread paired with an acoustic song over

either stimulus alone in the brown-headed cowbird (Ronald et al. 2017). Multimodal

courtship displays have also been documented in bats where males use chemical,

acoustic, and visual signals (Voigt et al. 2008). Simultaneous communication

channels in different modalities are often presumed to have evolved in response to

providing "more" information, thereby improving a female's ability to make critical

mating decisions (Rosenthal 2017). It has therefore been predicted that the additional

information in a multimodal signal should aid the female's ability to distinguish

among potential mates (Rowe 1999). Multimodal displays may also be more

perceptible by the receiver, ultimately making the signal more detectable in the

environment. Utilizing a multimodal display may therefore confer benefits to males to

make themselves more memorable when females are choosing among multiple

options (Guilford & Dawkins 1991; Rowe 1999). Indeed, some studies have provided

evidence for multicomponent cues improving a receiver's memory both within and

outside the context of mate choice (Rowe & Guilford 1999; Pardasani et al. 2021). In

species with lekking behavior, the memorability of a signaler may be even more

pertinent as females sample multiple males and make a choice within a relatively

short time span (Backwell & Passmore 1996; Schwartz et al. 2004; Pauli &

Lindström 2021). There are also periodic lapses in signal production (usually

acoustic) (Greenfield 2015; Coss et al. 2020), so that during these quiet periods,

females may have to rely on memory to make a decision or delay their mate choice.

The evolutionary trend for increased signal complexity may therefore have been

driven by their ability to increase memorability by the receiver, referred to as the memory duration increase hypothesis (Zhu et al. 2021).

Before making a choice, females assess potential mates they have at their disposal, and because mating is not random, there is likely to be an aspect of learning and memory involved in female choice (Bateson & Healy 2005), even on short time scales. It is known that prior experience with conspecifics can influence mating decisions (Schlupp et al. 1994; Hebets et al. 2003; Coleman et al. 2004; Cheetham et al. 2007; Bailey & Zuk 2009). Though several review papers have alluded to the possibility of learning and memory playing a role in the evolution of complex signals (Bateson & Healy 2005; Hebets & Papaj 2005; Bro-Jørgensen 2009; Higham & Hebets 2013; Ryan et al. 2019b), few studies have experimentally tested this hypothesis. There are numerous studies across taxa that have investigated the role of spatial memory during mate searching in males (Jones et al. 2003; Goh & Morse 2010; Foley et al. 2015), but our knowledge about the general role of memory during mate searching in females is not as extensive (Healy & Hurly 2004). One aspect of female mate choice that has received less attention, however, is the role of signal components instantiating memory for the signaler. Females are likely to learn and retain information about a potential mate (location, size, signaling ability, etc.) so that they can recall it later. For instance, female house mice are able to recall the previous location of a male pheromone for 14 days (Roberts et al. 2012). For females that sample multiple males, especially over longer periods of time and across territories, it

may be advantageous to remember information about each signaler to make a later choice, whether that be seconds from an initial contact or years.

To our knowledge, the only studies that have investigated the role of complex signals on memory during mate choice have been performed on anurans. Anuran communication encompasses a diversity of signal behaviors, with some species exhibiting visual, acoustic, chemical, and/or seismic signals that females attend to (Höbel & Kolodziej 2013; Starnberger et al. 2014a; Robertson & Greene 2017; Caldwell et al. 2022; James et al. 2022). Despite this variety, acoustic signals remain the primary modality for most anuran communication, though these too can vary in their complexity. In one of the studies investigating memory, Akre & Ryan (2010) found that female túngara frogs (*Physalaemus* (=*Engystomops*) *pustulosus*) were able to recall the location of a more complex call for up to 45 s after cessation of the call. This may be indicative of a selective pressure for females to maintain a working memory for the location of individual male callers. However, this study focused on the unimodal, acoustic component of the otherwise multimodal túngara frog call (Taylor et al. 2008). In the more recent study, Zhu et al. (2021) investigated the role of a multimodal signal on the memory of the serrate-legged small treefrog (*Kurixalus odontotarsus*). Zhu et al. (2021) found that the multimodal signal of a vocal sac inflation (via video playback) and an acoustic signal increased the active memory of females compared to a unimodal signal. Because multimodal signals have been suggested to improve the memory of a receiver, these studies provide evidence that adding complexity to signals may improve the ability of a receiver to recall the last

detected location of a signaler. In the present study, we again rely on anuran communication to further our understanding of multimodal signals and female memory.

Túngara frogs are a Neotropical species, with males gathering in nocturnal choruses to form leks (Ryan 1985). The acoustic stimulus of their mating display involves a simple "whine", which may have up to seven additional "chucks" accompanying the whine (Ryan 1985). Females show a strong and consistent preference for a more complex call (whine plus chuck) (Ryan et al. 2019a). In addition to their acoustic stimulus, males of this species display in other modalities to attract females. Consisting of an acoustic call, a visual cue, and a seismic component, the multimodal signal of a male túngara frog is preferred over the acoustic call alone (Ryan 1985; Taylor et al. 2008; James et al. 2022). The visual component of the male's vocal sac inflating likely evolved to maximize calling efficiency, and its inflation secondarily became integrated into their mating display as the visual stimulus (Pauly et al. 2006; Taylor et al. 2008). The inflation of the vocal sac occurs simultaneously with the acoustic call (Taylor et al. 2011). A túngara frog's vocal sac is exceptionally large, and when inflated, is nearly the size of the male itself (Dudley & Rand 1991). In a complex environment like the rainforests of Central America, the motion of a visual stimulus may be a variable that females rely upon to receive accurate information about a caller's location (Tan & Elgar 2021).

It is known that túngara frogs will stop calling in response to a predation threat (Dapper et al. 2011). Interruptions in the chorus are therefore common, with

inter-chorus intervals having an average duration 25 s (Akre & Ryan 2010). Though eavesdropping predators attend to the multimodal signals of males at a túngara frog lek (Halfwerk et al. 2014), females are also vulnerable to predation while mate sampling (Bernal et al. 2007). It may be beneficial then, for a female to evaluate males and quickly make a choice to mitigate predation risk. Experiments in both laboratory and natural settings seem to corroborate this (Rand et al. 1997). Female anurans tend to assess males and choose within a very short time period, with gray treefrogs (*Dryophytes (=Hyla) versicolor*) assessing potential mates for only about two minutes (Schwartz et al. 2004; Feagles & Höbel 2022). In such a dynamic environment as a lek, however, a male's signal may become interrupted or go silent during a female's assessment. In that scenario, does she wait and restart her assessment once the chorus resumes, or does she rely on memory to make a choice? As Akre & Ryan (2010) demonstrated, female túngara frogs are able to remember the location of a complex call for up to 45 s, spanning the average inter-chorus interval shown by the same study. It has also previously been shown that túngara frogs can associate a visual cue with a place-learning task (Liu & Burmeister 2017; Ventura et al. 2019). It is possible, then, that the visual cue of an inflating vocal sac improves a female's ability to remember a male's call and, therefore, his location.

Here we tested whether the visual stimulus of a male's vocal sac alters a female túngara frog's working memory after the occlusion of the visual stimulus. In addition, we also tested call playbacks with and without a silent period to understand how silence influences memory instantiation. By exploring how multimodal signals

influence a receiver's psychology during or after fluctuations in signal intensity, we may provide new insights into the evolution of complex signals.

Methods:

General Experimental Procedures

All behavioral experiments were performed at the Smithsonian Tropical Research Institute in Gamboa, Panamá. We collected amplexed pairs of frogs from wild choruses between 19:30 and 23:00 h during the rainy season in 2021 and 2022. After transporting them to our lab for testing, frogs were stored in total darkness for at least one hour prior to trials to allow their eyes to dark-adapt. This dark adaption is necessary after collecting frogs in the dark using flashlights. For each trial, we separated a female from her mate and placed her under a visually and acoustically transparent mesh funnel, in the center of a sound chamber (ETS-Lindgren, Austin, TX, USA). Light inside the chamber was produced via a GE brand nightlight to mimic light conditions within the range of natural breeding conditions (Taylor et al. 2008). The acoustic stimulus of each treatment was broadcast by a pair of Orb Audio speakers (Sherman Oaks, CA, USA) that were placed 80 cm away from the release point of the female. For all experiments, the speakers antiphonally broadcast identical, synthetic calls (whine-chuck=WC). We used Adobe Audition v22.2 to play call files. Once the female was placed inside the funnel, we presented the stimuli of each treatment for two or three minutes before the female either, 1) was released

immediately or 2) exposed to additional testing conditions and then subsequently released. After the female was released, we monitored her choice from outside the sound chamber as it occurred live using infrared cameras. We also recorded her choice and saved the video files for a blind observer to later analyze. We defined a choice as a female remaining within 8 cm of the base of a speaker (the "choice zone") for at least 3 s. If a female failed to move from her initial "funnel zone" within 4 minutes or did not make a choice within 10 minutes, we removed her from the chamber. After a period of at least 15 minutes, the female was re-tested, and if she failed a second time, she was set aside and not tested again in that experiment. After completion of the trial, we reunited the female with her mate and later toe-clipped the pair both to mark for recapture (avoiding retesting the same individual) and to obtain a genetic sample. Snout-vent-length and mass were also recorded for each frog. At the end of the night, all pairs were returned to their respective collecting site and released.

Robofrog Control

Following Taylor et al. (2008), we replicated a multimodal stimulus for mate choice experiments using speakers and robotic frogs with artificial vocal sacs that are inflated by an electromechanical pump. The robofrogs provided the visual component of the multimodal display while the call was produced by the speaker immediately behind the robofrog. The pump was programmed to shunt air into tubing connected to the robofrog so that a silicone vocal sac inflated at a 19 kHz tone and deflated at a 16 kHz tone. These tones were placed at the beginning and end of the synthetic call in

Adobe Audition. This ensured that the robofrogs inflated in time with the acoustic stimuli, as occurs in nature (Ryan 1985). These tones are imperceptible to frogs (Ryan & Rand 1990). We calibrated the sound-pressure level (SPL) to be 82 dB (re. 20 μPa, fast C-weighting) from the female's position within the funnel. In this experiment a robofrog was placed in front one of the speakers (right or left), and its position was switched between experiments to control for side bias. During a two-minute presentation period, the robofrog inflated as the complex (WC) acoustic stimuli played from each speaker. As both the acoustic and visual stimuli continued to play, the funnel was gently lifted from outside the chamber so that the female was free to make a choice. In addition to the definition of a choice used earlier, we also defined a choice in this experiment as the female approaching the robofrog itself within 5 cm or if she touched the robofrog. As the robofrog was placed in front of a speaker, it extended the radius of the "choice zone" beyond that of a bare speaker.

Blind Cloth Tests

For the next set of experiments, we tested how a visual stimulus of a calling male (robofrog) that is suddenly obstructed from view influences a female's ability to recall the last known location of that mating signal. An opaque, black cloth (hereafter referred to as BC for blind cloth) was used to block the visual cue from all angles (Figure 1). The cloth wrapped around both the speaker and the robofrog (if present) 360° to completely obscure the female's ability to see the speaker/robofrog, irrespective of her position in the test chamber. We designed the cloth to be as tall as the speakers (13 cm) so that the females could not see anything that was hidden

behind them. The cloth was hand-sewn to a metal ring, which was tied to monofilament fishing line. Hanging from the ceiling of the chamber via magnetic hooks, the fishing line was accessible to the outside of the chamber through a small port that also allowed access for speaker cables. With the cloth surrounding the speakers, we measured the SPL at the female release point at 76 dB; a decrease of 6 dB from a bare speaker without the surrounding cloth. This SPL is still within the range of what females experience in nature and this level has been used in previous studies (Stange et al. 2016). In addition, both speakers were always enclosed with the blind cloths, thus the drop in SPL was held constant for both speakers. During the presentation period, the blind cloths hung unmoving from the ceiling of the chamber. After this, we then lowered the cloths to obscure the robofrog. Color-coded with tape corresponding to each speaker, the cloths were lowered as an observer at the live camera feed monitored their rate and speed to ensure they were even. In each trial, the cloths touched the floor of the chamber at the same time. The lowering of the cloths took approximately 7 s. Because the original choice zone used for the speaker was engulfed by each blind cloth, we defined a choice as a female approaching within approximately 8 cm of a blind cloth and remaining there for at least 3 s. Once the cloths touched the floor, the female was immediately released from the funnel and nothing else changed. We refer to this experiment as "BC Control" because the blind cloths (BC) were utilized. For our side bias control, each speaker played the identical WC call. We did not utilize a robofrog as we were simply verifying that there was no aversion or bias for one cloth over another.

BC Robo

The "BC Robo" experiment began with a presentation period. During the presentation period, a female was held within the funnel for 2 min while the speakers antiphonally broadcast identical WC calls. The robofrog inflated in time with the call and was visible to the female during this period. After the presentation period, the BCs were lowered. As soon as the BCs touched the floor, a 20 s holding period began. During this holding period, we continued to broadcast the calls uninterrupted, so there was no silent period. We did stop the inflation of the robofrog, however. To do this, we immediately switched the call file in Adobe Audition to a robo-less file (i.e. did not have the 19kHz or 16kHz sounds to activate the robofrogs). The females remained inside the funnel until the 20 s holding period was over and they were released. Again, we switched the location of the robofrog at the speakers, between trials, to control for side bias.

BC Robo + Silence

As previously, each speaker broadcast the same WC call. Here, instead of a holding period, the "BC Robo + Silence" experiment had a silent period. First, females were exposed to a 3 min presentation period prior to the BCs being lowered. Once the BCs were lowered, all sound was muted from the speakers for 5 s. After this 5 s silent period, the robo-less calls (i.e. no inflation of the robofrogs during playback) began and continued to play until the female made a choice.

BC Robo + Silence + Holding Period

For our final experiment, "BC Robo + Silence + Holding Period", we wanted to test how a female's memory was affected by all three periods (presentation, silence, and holding) during a single exposure. This experiment was the longest test of memory: 25 s total time before females were released from the funnel. To accomplish this, we first gave females a presentation period of 3 min where the calls played and the robofrog inflated as normal. During this period, the females were held under the funnel. As with the previous experiments, females could both hear the calls and see the robofrog display. After 3 min, the BCs were lowered and a silent period of 5 s immediately began. In those 5 s, the robo tones were muted. After the silent period ended, a holding period began with the speakers broadcasting the WC call without the robofrogs inflating. After a holding period of 20 s where the now robo-less calls were playing, we lifted the funnel and allowed the female to make a choice.

Statistical Analyses:

All analyses were performed in R (R Core Team 2020). We first tested the hypothesis that the proportion of choices for each treatment were different from chance by using a binomial test. We reported the mid-P-value to smooth the drastic jumps in significance with additional data that can be seen in these sample sizes. We used ANOVA to compare the mean latency to choice across treatments. A Levene's test for equal variance was also performed. Graphs were generated in R (R Core

Team 2020) using the packages: tidyverse (Wickham et al. 2019) and ggplot2 (Wickham 2016).

Ethics Note:

Experimental procedures were conducted with approval by the IACUC protocol from Salisbury University and the Smithsonian Tropical Research Institute (IACUC: SU-0052; SU-0052R and STRI 2018-0411-2021; SI-21012). The Ministry of the Environment of Panamá (MiAmbiente) approved our collection of animals and issued collecting permits for our team (ANAM: SE/A- 39-2020). We aimed to minimize our effect on the animals and their environment. Females that were used in experiments and the males they were collected with were toe-clipped. If females did not participate in an experiment, they were not toe-clipped. Toe-clipping was used to identify individual frogs and avoid their use in future experiments. Toes were preserved in ethanol for future genetic analyses. We followed regulations from the American Society of Ichthyologists and Herpetologists regarding toe-clipping procedures (Beaupre et al. 2004). Frogs are not adversely affected by toe-clipping as individuals are often recaptured/observed in the field weeks or even months after we first captured them. All frogs were released at their original collection site. Pairs were reunited and released together so that our handling did not interfere with the female's initial choice.

Results:

We tested female túngara frogs in several conditions to determine their memory capabilities when presented with multimodal versus unimodal stimuli. We ran two controls and through the use of blinding cloths (BC), we obstructed the visual stimulus (robofrog) from the view of the female in three treatments.

In our first control experiment, we aimed to determine the preference of females (N=46) to choose a robofrog (multimodal signal) when both speakers were playing the same, attractive call (whine-chuck). As in previous studies (Taylor et al. 2008, Stange et al. 2016), females expressed a significant preference for the multimodal signal over the unimodal acoustic call (binomial test: 33 robofrog:13 unimodal P=0.0024; Fig. 3).

Through the use of a blind cloth, we then tested how the presentation and subsequent removal of a visual cue influenced a female's choice under several experimental conditions. We first determined that there was no side bias for either blind cloth under equivalent conditions in the "BC Control" experiment (N=13) (binomial test: 6:7 ratio; P=0.8953; Fig. 3). Once that baseline preference had been established, we then added a robofrog to one of two speakers broadcasting identical, WC calls in the "BC Robo" experiment (N=38). After lowering the cloths to investigate how a multimodal signal affected a female's memory, we found that after the 20 s holding period, females did not approach the multimodal speaker

significantly more often than random (binomial test: 23:15 P=0.2277; Fig. 3),
although there was a trend in favor of the robofrog.

Next, we ran females in trials where there was a silent period before they were
released from the funnel. In the first experiment, "BC Robo + Silence," (N=25) after
a presentation period, a 5 s silent period began as soon as the visual stimulus was
hidden. After that, the females were released and the acoustic stimulus began playing
again. Interestingly, this 5 s of silence was sufficient in inducing the females'
memory (binomial test: 18:7 P=0.0361; Fig. 3). To investigate the effects on females
with all three conditions, we tested females first with the usual presentation period
followed by a silent period of 5 s in the "BC Robo + Silence + Holding Period"
experiment (N=25). After the silent period ended, a holding period began where the
female was held under the funnel for an additional 20 s while calls continued to play.
After the acclimation period ended, the female was released and allowed to make a
choice. Even after a total of 25 s since the cessation of the multimodal signal, females
were still able to remember its location (binomial test: 18:7; P=0.0361; Fig. 3).

We also investigated whether the latency of a female to make a choice was
affected by treatment type (Fig. 4). Treatment type did not have a significant effect on
choice latency (ANOVA: P=0.964; mean for Robofrog Control=82.76 s; mean for all
BC experiments=81.26 s).

Discussion:

To determine the effect of a multimodal signal on the memory of female túngara frogs, we asked if females could remember where a "calling male" was after the visual cue of a robotic frog was no longer visually accessible. To do this, we developed a unique pulley system for the lowering of two opaque black cloths that visually obscured both speakers (and the robofrog) from the females' view. After a presentation period, we then gave females either a holding period of 20 s, a silent period of 5 s, or both. Once those periods ended, we released the female from an acoustically and visually transparent funnel and allowed her to make a choice.

Our results provide evidence that the addition of a visual stimulus to a male frog's courtship call instantiates memory for the caller's location, but, interestingly, only after there is a period of silence. In our first memory treatment, a substantial proportion of females did not recall the location of a calling male after last being exposed to the multimodal signal 20 s before release in the "BC Robo" experiment. In the next memory treatment, "BC Robo + Silence," a significant proportion of females did recall the robofrog's location, but only after being exposed to 5 s of silence before being released. Because they did seem to remember the location of the signal with exposure to 5 s of silence, we were interested in knowing if the ability for the females to remember was simply a product of the very short, 5 s delay since their last exposure to the robofrog. In the "BC Robo + Silence + Holding Period" test, females were still able to remember even after a 25 s delay (5 s of silence plus 20 s delay under the release funnel). These findings demonstrate a potential mechanism wherein

more complex signals may improve a receiver's ability to remember a signaler, as proposed by Guilford & Dawkins (1991). This mechanism does not appear to be species specific, as Zhu et al. (2021) demonstrated a similar effect with a species of treefrog native to Southeast Asia. In that study, researchers were interested in learning how long a multimodal signal could instantiate memory, similar to the methods employed by Akre & Ryan (2010) for complex calls in túngara frogs. They found that females were able to recall a caller's location for 30 s which was closely related to the 45 s of túngara frogs (Akre & Ryan 2010; Zhu et al. 2021). Both of those studies utilized silent periods to titrate out the length of time for which the frogs could retain their memory. Here, we showed that the silent period itself, even when it is only 5 s long, appears necessary for instantiating the retention of spatial information.

Why a silent period seems to be a crucial aspect to memory instantiation could be due to, at least in part, the temporal updating that female túngara frogs appear to do. When female túngara frogs make mate choice decisions in our experiments, we commonly observe them moving and reorienting towards a speaker each time it broadcasts a call. The female túngara frog's sensory scene in the mating chorus is dynamic; as the sensory scene changes, they appear to rapidly update the options available to them among competing males. Silence itself is a natural part of many chorus dynamics (Schwartz 1991; Wilson et al. 2014; Greenfield et al. 2016). Male signalers commonly go silent in response to perceived predation threats, making themselves less conspicuous (Faure & Hoy 2000), but in doing so, they may also be unintentionally activating the female's ability to remember caller locations. When

male túngara frogs continuously duet with their neighbor (or two duetting speakers in an experiment) females seem to ignore previously available information and make rapid assessments of their options in real-time. The temporary cessation of sound, on the other hand, appears to trigger females to access memories of callers they previously sampled. The visual component of the sexual advertisement signal (inflating vocal sac) was the component that allowed females to remember a caller's location, but only after being triggered by a short period of silence. In the absence of silence, females seem to continue to update the acoustic signal information in real time and ignore where they had previous access to the visual component.

What remains unknown is how the length of the silent period influences memory for caller location. The silent period we employed in this study was 5 s; in nature, males go silent for highly variable periods of time, with mean inter chorus intervals of 25 s (Akre & Ryan 2010; Dapper et al. 2011). When males go silent it is often associated with a predation risk. Females may respond strongly to this cue and as a result, alter their behavior while mate sampling. When danger is sensed, instead of completely "starting over," females may continue with their decision-making, albeit with more caution (Bernal et al. 2007; Edomwande & Barbosa 2020; Feagles & Höbel 2022). Indeed, female túngara frogs have been shown to make faster decisions when there is a perceived threat (Baugh & Ryan 2010), and a silent period may provoke females to hasten their decisions, relying on previously acquired information of caller location. This may be a contributing factor to the short timeframe for mate evaluation that is observed both in nature (Schwartz et al. 2004) as well as in this

study. Despite 5 s being extremely short, to a frog in a high-risk scenario like a lek, 5 s is a biologically relevant timeframe.

Previously, a female túngara frog's memory during mate evaluation had only been tested in one modality: auditory. Akre and Ryan (2010) tested female túngara frogs' memory for both a simple, one-chuck whine-chuck call and a complex, three-chuck whine-chuck call. They showed that females were only able to remember the location of a speaker when the complex call (wine plus three chucks) was broadcast. In the present study, a synthetic whine and one-chuck call was used for all acoustic stimuli, and yet, we were still able to induce memory in the females. What both our study and Akre & Ryan (2010) demonstrate is that in order to instantiate memory for signal location, the signal needs to be complex in nature - whether that complexity comes from additional acoustic stimuli (chucks) or from additional modalities (visual). What our findings also highlight is the role that vision plays during mate choice in anurans. It has been proposed that visual stimuli may act to attract the attention of a receiver to that signaler (Ord & Stamps 2008). In túngara frogs, not only does the visual stimulus attract the receiver's attention, but it also acts to correlate the signaler's location in her memory. Most studies on anuran communication focus on the importance of the acoustic component of their multimodal signal, but there is increasing evidence that while the acoustic signal is a necessary component, the visual stimulus of the vocal sac also serves an important function in mate signaling (Taylor & Ryan 2013; Starnberger et al. 2014b; Höbel et al. 2022). Females themselves become more visually sensitive when in a reproductive

state, which may have evolved to aid them in mate searching and evaluation (Leslie et al. 2020). In the absence of a call, the visual component of a male's vocal sac does not induce mate searching behavior (Taylor et al. 2011), but here we show that it is vital to induce memory in tandem with an otherwise "forgettable" call.

Though the focus of this study was not to investigate how long a female túngara frog is able to remember, it is important to note that the length of memory for the location of a calling male in the present study correlates with the previous findings of Akre & Ryan (2010). In that study, the average inter-chorus interval where males would become silent was 25 s. In our study, the longest duration after the multimodal stimulus was last presented was also 25 s, a combination of the previous two memory experiments' treatment periods. When presented with a complex acoustic-only stimulus, female túngara frogs were able to remember its location for up to 45 s (Akre & Ryan 2010); how that duration compares to a multimodal stimulus remains unknown. Zhu et al. (2021) showed that multimodal stimuli generate a longer working memory in a treefrog species, though future studies are needed to further compare the memory capabilities between unimodal and multimodal stimuli and across additional taxa.

Our understanding of the active role that females play in sexual signal selection has exponentially increased since the time of Darwin (Rosenthal & Ryan 2022). There are various hypotheses to explain why females generally prefer more complex stimuli; the receiver psychology hypothesis has provided a simple and powerful explanation for the mechanism of female choice for multimodal signals

(Rowe 1999). Interestingly, despite being proposed more than two decades ago, the role of multimodal signals instantiating memory in receivers has received little attention. If an evolutionary goal of signalers is to be chosen, then it follows that the more complex their display is, the more memorable it will be for the receiver and therefore be under positive selection. In the present study, while we found that multimodality was important for memorability, in order for memory to be induced, a potential disturbance/predation risk (silent period) had to be provided. In a lekking scenario, females continue to make a choice despite this risk, likely so as to not lose their reproductive investment (Bernal et al. 2007). We know that females dynamically assess males (Baugh & Ryan 2010) and will circle back to them once a "final" choice has been made (Dale & Slavsgold 1996). An association in a female's brain between one particular male and his location must therefore exist. Multimodal stimuli have been shown to improve both memory and learning outside of the context of mate choice (foraging: Leonard et al. 2011; Gil-Guevara et al. 2022; aposematic signals: Speed 2000; Rowe 2002; and song-learning: Hultsch et al. 1999). By utilizing a multimodal signal, signalers may therefore increase their chances of being remembered. Thus, how a signaler is chosen may be determined not by how well they advertise quality, but simply by how memorable they are during fluctuations in a dynamic signaling environment. Here, we used túngara frogs to better understand how the use of multiple sensory modalities may influence a female's memory and subsequent mate choice. Our results reflect the complex decisions females must make during mate evaluation, especially when multiple communication modalities must be

integrated and later remembered. The power in a female's choice must not be underestimated. A receiver's cognitive ability to adapt to rapidly shifting environments and still retain the ability to weigh attractiveness is both impressive and meaningful. A female's choice has direct evolutionary consequences, and furthering our understanding of the process of decision-making is critical for understanding diversity and evolution as a whole.

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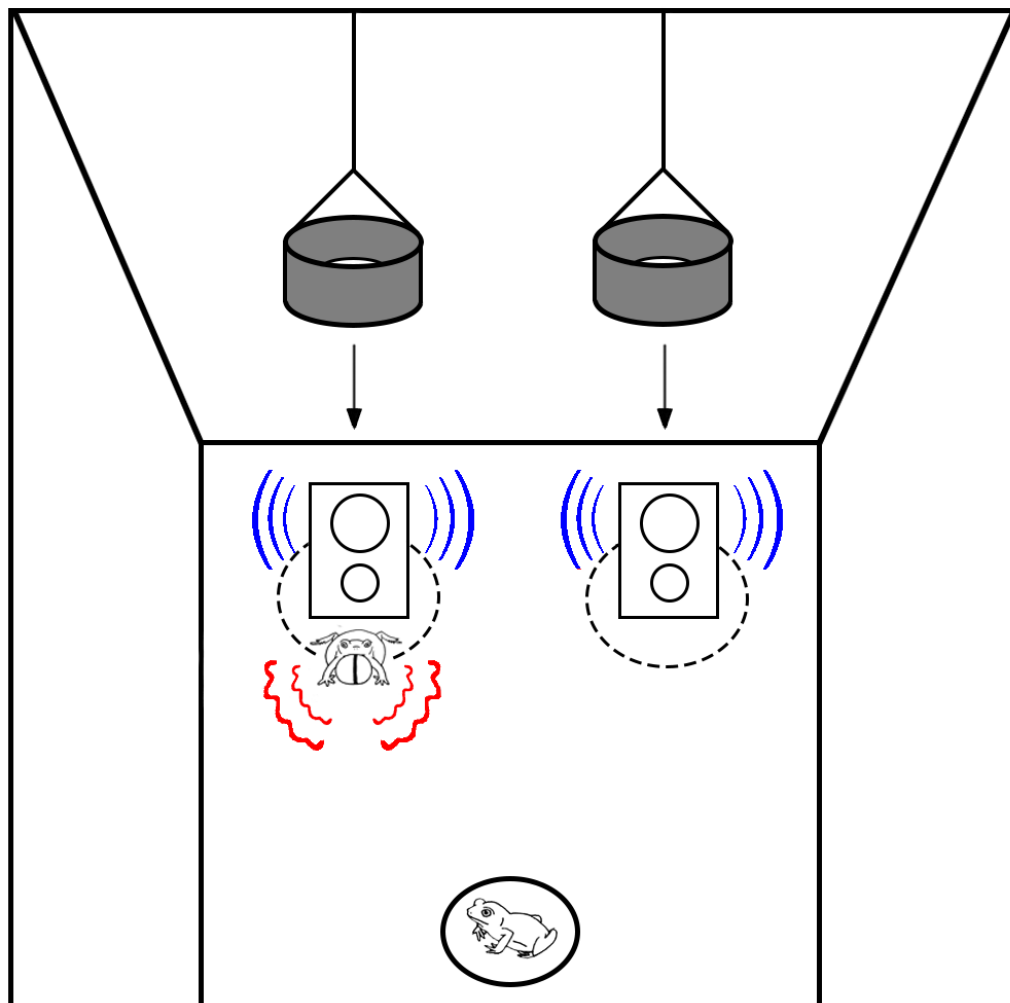
List of Figures:

Figure 1. The internal arrangement of the sound chamber for blind-cloth (BC) phonotaxis experiments. This diagram is not drawn to scale. A female frog is placed in the center of the sound chamber in the funnel zone (solid black circle) and is presented with multimodal stimuli. The auditory signal (solid blue crescents) is broadcast by speakers while the robofrog simultaneously inflates a silicone vocal sac to serve as the visual stimulus (curvy red crescents). After a presentation period of 2 or 3 min, the blind cloths are gently lowered to obscure the visual stimulus from the view of the female, still held within the funnel zone. If she approaches within approximately 8 cm of the cloths (choice zone: dotted circle) and remains there for a minimum of 3 s, we record that as her choice.
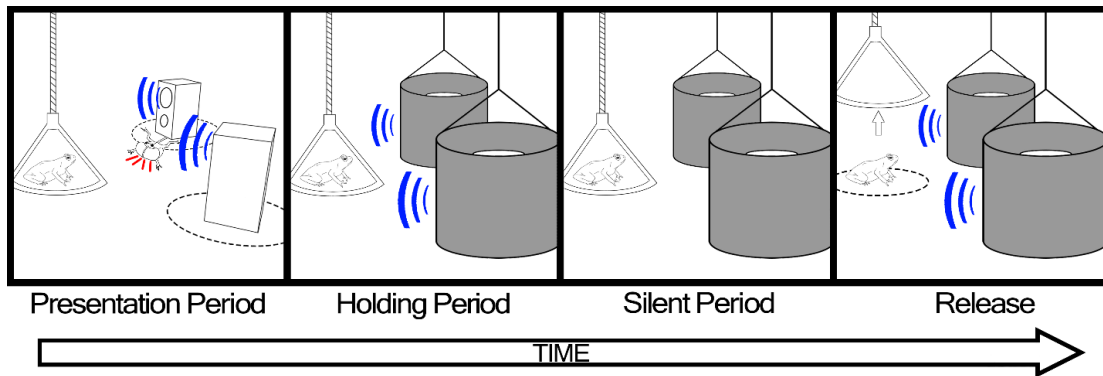
Presentation Period   Holding Period   Silent Period   Release

TIME

Figure 2. Periods of stimuli for female frogs in our experiments. All experiments first involved a presentation period of 2 or 3 min where the female is held in the funnel and speakers antiphonally broadcast attractive (WC) calls (depicted by the blue crescents). There is a robofrog (visual stimulus) at one speaker (depicted by the red lines) that has a silicone vocal sac inflating in time with the call at that speaker (multimodal speaker). The holding period then begins immediately after 1) the presentation period and 2) the lowering of the BCs. Here, the visual stimulus is no longer presented to the female, but the acoustic stimuli do not stop. The holding period lasts for 20 s. Next, some experiments would add a silent period of 5 s where the cloths were lowered and no stimuli (acoustic or visual) was presented to the female. Immediately following one or several of these periods, the acoustic stimuli began to play (if a silent period preceded it in that experiment) and the female was released from the funnel. From here, the female was free to make a choice between speakers.
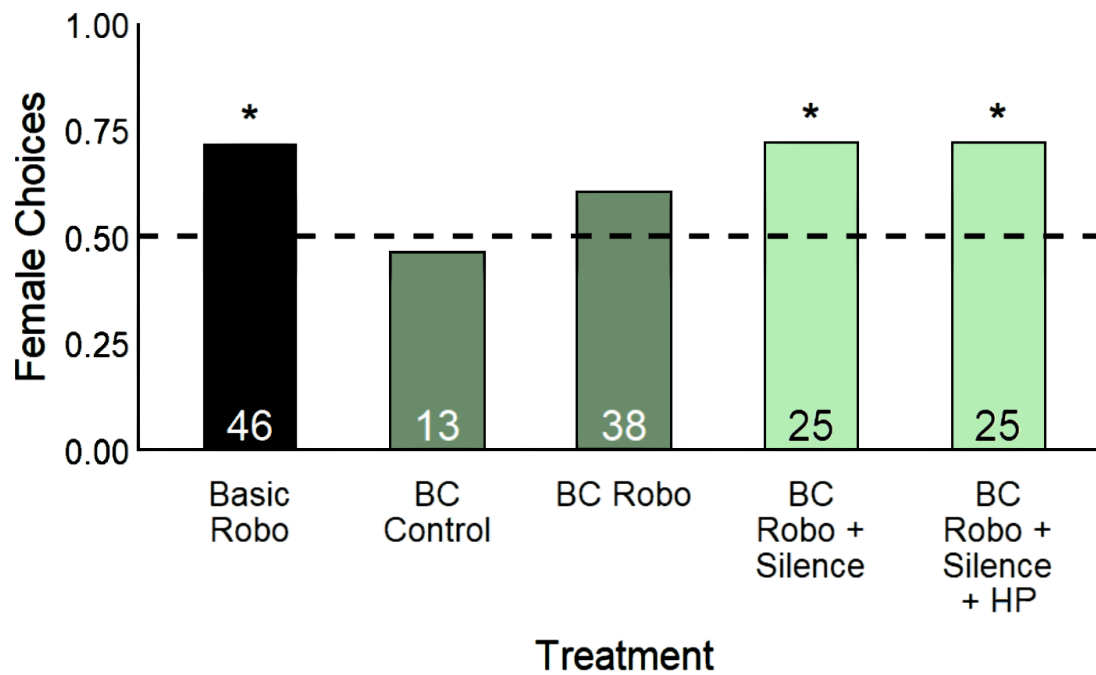
Figure 3. Proportion of female choice across treatments. Dotted line represents a random preference (50%). Asterisks (*) above bars indicate a significant difference from the base preference where $P < 0.05$. Numbers inside bars represent sample sizes for each respective treatment. The light green bars are treatments with a silent period while the dark green bars are treatments that do not have a silent period. "HP" is short for holding period.
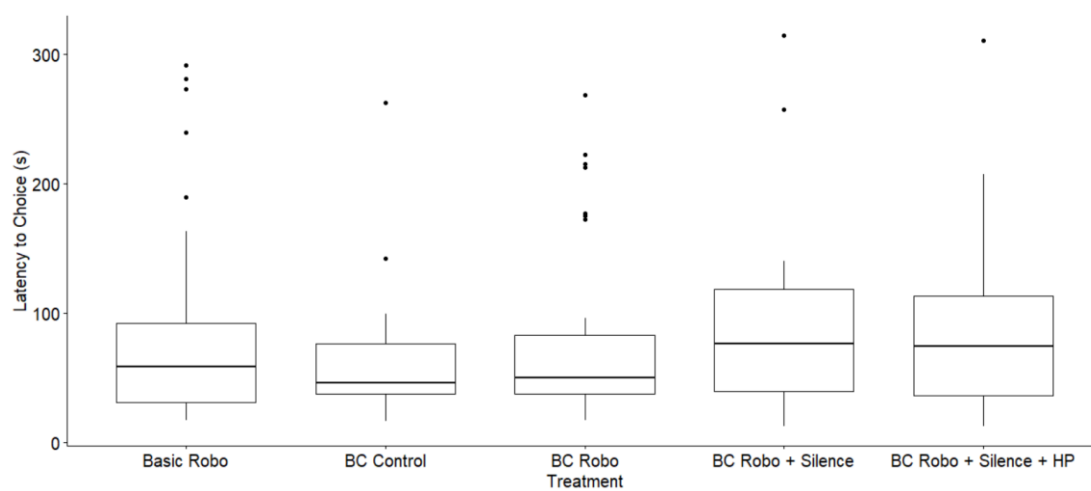
Figure 4. Mean latency to choice across treatments. The ANOVA did not report a significant difference between any of the treatments' latencies (*P*=0.964)

Chapter 2: Within hopping distance: Preference for a male visual stimulus deteriorates with increasing distance in female túngara frogs

Abstract:

Vision can play a vital role in a receiver's response to a signal. Often used in tandem with other sensory stimuli, vision is commonly used as a modality for signaling to potential mates. In nocturnal species, however, the neural processing of an image with a limited light source may degrade the resolution and/or details of that scene and the signalers within it. Túngara frogs (*Physalaemus (=Engystomops) pustulosus*) are a Neotropical species that perform nocturnal multimodal courtship displays (visual, acoustic, and seismic) to attract females. Females have been shown to have a consistent preference for a multimodal display in laboratory settings at a fixed distance. Here, we tested how the distance between a female and a displaying "male" (robotic frog and speaker) influenced the decisions of female túngara frogs. We compared the response of females to a robotic frog at three distances (80 cm, 90 cm, and 110 cm) from the release point of females. At the established 80 cm distance, females significantly chose the multimodal speaker over the unimodal, but this preference vanished at 90 and 110 cm. These data suggest that female túngara frogs are unable to recognize the visual stimulus of a male's multimodal display as it becomes more distant. This is consistent with observations of natural pairings wherein females sample and choose males at close distances. We suggest that female

túngara frogs efficiently integrate visual stimuli in nocturnal conditions, but their distance to a stimulus limits this ability.

Introduction:

Vision is a vital sensory system that is nearly ubiquitous in the animal kingdom (Nilsson 2021). Though many species have adapted to life with limited or no vision, for most, it is necessary for their survival (Cronin et al. 2014). For animals with a well-developed vision, this sensory system is commonly recruited for the use of communication signals (Miles & Fuxjager 2018). Aggressive displays (Pryke et al. 2001, birds; Heathcote et al. 2018, fish; Anderson et al. 2021, frogs), aposematic coloration (Ximenes & Gawryszewski 2020, spiders; Willadsen 2022, hymenopterans), and mating displays (Yorzinski et al. 2013, birds; Liotta et al. 2021, fish; McGinley et al. 2022, spiders) are just some of the examples in which animals communicate by exploiting a receiver's ability to see.

Courtship displays, in particular, provide some of the most diverse examples of visual communication. In the blue crab, *Callinectes sapidus*, males prefer females with red claw dactyls (Baldwin & Johnsen 2009), while female golden-collared manakins, *Manacus vitellinus*, choose mates based on the performance of males during a highly acrobatic display (Barske et al. 2011). Multimodal mating displays using more than one sensory modality are also common across taxa, and visual signals are commonly combined with acoustic or seismic components (Higham & Hebets 2013). The African cichlid, (*Astatotilapia burtoni*), combines low frequency

sounds with body quivers to attract females (King et al. 2022). While visual and seismic cues are used in in wolf spider, *Schizocosa*, during mate attraction (Hebets et al. 2013; Stafstrom & Hebets 2013). The integration of stimuli in multiple senses can manipulate a female's attraction to or recognition of the male signalers. However, the response of females to visual-only displays may be diminished or even nonexistent, if it is not coupled with a seismic or acoustic component (Elias et al. 2005; Taylor et al. 2011; O'Loghlen & Rothstein 2012). This may be because the visual signal was co-opted into the communication system secondarily to another component (e.g. acoustic). Although a combined, multisensory stimulus may be more attractive to receivers than a unimodal one, variability in the physical environment may restrict a female's field of vision, thereby causing a signal in another modality to be more easily transmitted and received than a visual stimulus alone (Hebets & Papaj 2005). In anurans, for example, acoustic signals are often the most important component in a multisensory display (Starnberger et al. 2014). Environmental heterogeneity may therefore enforce selection for multiple modalities (Hebets & Papaj 2005; Vásquez & Pfenning 2007).

Anurans are one of the most well-studied taxa for nocturnal visual and multimodal communication (acoustic, visual, and seismic) (Starnberger et al. 2014; James et al. 2022). Anurans are known to use visual stimuli during mate choice, even in nocturnal, starlight conditions (Taylor et al. 2007; Gomez et al. 2010; Laird et al. 2016; Robertson & Greene 2017; Zhu et al. 2021; Hamilton et al. in prep). Despite the wealth of information available on low-light visual sensitivity (Aho et al. 1993;

Leslie et al. 2020) and visual signaling in anuran courtship (Lindquist & Hetherington

1998; Montanarin et al. 2011; Zhao et al. 2022), relatively little is known about

anuran visual acuity (but see Aho 1996; Caves et al. 2018). Anurans and other

nocturnal species have specialized adaptations for scotopic (dim light) vision or

visual displays in dim light (Lewis & Cratsley 2008; Penteriani & Delgado 2017;

Zhang et al. 2018; Alonso et al. 2021; Warrant & Somanathan 2022). To compensate

for the reduction in available light (photons), an animal's eye can sum the photons

that are available, thereby improving visual sensitivity in dim light (Warrant 1999;

Cronin et al. 2014; Stöckl et al. 2016). This summation can occur temporally and/or

spatially. Though this improves visual sensitivity, it also reduces either acuity

(resolution of fine detail: spatial summation) or the ability to resolve motion

(temporal summation) (Cronin et al. 2014). Irrespective of whether nocturnal animals,

such as frogs, sum photons temporally or spatially, target acuity declines with

increasing distance between the receiver and the object itself.

Indeed, for animals that signal visually, there may be an ideal viewing

distance during mate sampling to effectively differentiate males and recognize the

entirety of their mating displays. When there are dozens of males present at a lek

(Ryan 1985), however, this task can become increasingly more difficult. When at

varied distances, females may choose males based on their relative attractiveness

(Akre & Ryan 2010; Murphy 2012) or alternatively, based on the distance they are

located from the female (Meuche et al. 2013). The visual stimulus of inflating vocal

sacs may aid in the differentiation of individual callers and their locations (Taylor &

Ryan 2013; Starnberger et al. 2014b; Höbel et al. 2022; Hamilton et al. in prep). The bright colors and conspicuous patterns of many anuran species may also act to increase the detectability of males against their background environment (Gomez et al. 2009; Starnberger et al. 2014; Höbel et al. 2022; Robertson et al. 2022). Even so, an individual caller's location may be challenging for females to discern in larger choruses. Anurans are one of only two vertebrate groups with evidence of nocturnal color vision (Roth & Kelber 2004; Yovanich et al. 2017). While females can and do use vision for mate assessment in nocturnal conditions (including color, pattern, and motion), acuity limitations are likely to place an upper bound on the distance over which they are useful. If females are simply too far away to resolve a visual target, then they cannot use it when mate sampling.

The ability to identify individual displays is especially important in lekking species such as túngara frogs (*Physalaemus (=Engystomops) pustulosus*) (Ryan 1985). Female túngara frogs attend to both acoustic signals and visual cues from males at nocturnal leks that can host upwards of 100 males. The acoustic component of their signal consists of a simple "whine" with the optional addition of up to seven "chucks" appended after the whine (Ryan 1985). While calling, males inflate an elastic vocal sac in their throat. This inflation is biomechanically constrained to occur during sound production and has been shown to be a visual stimulus in this species. In multiple studies, females have been shown to preferentially approach a speaker with a robotic frog (inflating a vocal sac) over a speaker playing the same call alone (Taylor et al. 2008; Taylor et al. 2017). The vocal sac originally evolved to maximize calling

efficiency in males (Pauly et al. 2006), and it was later recruited for use by females searching for mates. In laboratory settings with a robotic frog, the visual stimulus of a static vocal sac does not influence a female's decision, but rather it is the movement of an inflating/deflating vocal sac, when coupled with an acoustic call, that is critical for eliciting a response (Taylor et al. 2008; Taylor et al. 2011). A male túngara frog's inflated vocal sac differs in color from the rest of his body and is conspicuous against the natural background they call against (Cummings et al. 2008). It is also extremely large, approaching the body size of the entire frog when fully inflated (Dudley & Rand 1991) (Fig. 1). Because the visual sensitivity of females increases as they enter a reproductive state, the contrast in color between the inflated vocal sac and the environment is visible to females even on moonless nights (Cummings et al. 2008; Leslie et al. 2020). This increase in visual sensitivity is likely to aid females in mate sampling (Leslie et al. 2020).

Female túngara frogs respond to the motion of a calling male's vocal sac (Taylor et al. 2008; Stange et al. 2016), but the influence of distance on the female's ability to see, and hence use the vocal sac in mate assessment is unknown. In previous studies, Taylor and colleagues used a fixed distance of 80 cm, in which females respond to the movement of the vocal sac of a robotic frog coupled to a speaker producing a call (Taylor et al. 2008; Taylor et al. 2011; Stange et al. 2016; Cronin et al. 2019; Coss et al. 2022). These studies have all performed choice tests with a light source that is equivalent to a moonless night, typical of what this species experiences in nature (Taylor et al. 2008). When the temporal synchrony between vocal sac

inflation and the acoustic call was manipulated, Taylor et al. (2011) showed that females respond negatively when there was less than 50% overlap between the inflating vocal sac and the acoustic stimulus. Thus, females changed their response to the robofrog from positive (approach when vocal sac and acoustic signal are synchronous) to negative (avoid when vocal sac and acoustic signal are asynchronous). This indicates that females can resolve this temporal motion on time scales at least as short as 250 ms. If túngara frogs were relying on a temporal summation to maintain low-light sensitivity, they would be unlikely to discriminate these time delays, as temporal summation requires increased visual integration periods (Cronin et al. 2014). Another anuran, the European toad (*Bufo bufo*) demonstrated an integration period of 2 s, indicative of substantial temporal summation (Aho et al. 1993). The ability of túngara frogs to resolve timing differences between vocal sac movement and sound production, suggests that túngara frogs perform spatial, rather than temporal summation to preserve nocturnal visual sensitivity. This preserves temporal aspects of the frog's visual motion detectors at the expense of spatial acuity.

The present study aimed to investigate how increasing distance influences the utility of the visual cue (vocal sac) in mate choice decisions when females rely on scotopic vision. We tested the preferences of female túngara frogs for a multimodal signal (robofrog plus call) at varying distances from a fixed starting point. We tested the hypothesis that increasing distances will change female response rates to the

robofrog. Specifically, we predicted that increasing distances should make the robofrog more difficult to see, and therefore female responses rates would decline.

Methods:

Animal Care

We collected pairs of frogs in amplexus from sites in Gamboa, Panamá. Collection at wild choruses between 19:30 and 23:00 h during the rainy season in 2021 and 2022. We then transported the amplexed pairs back to our lab at the Smithsonian Tropical Research Institute where they were dark-adapted for at least one hour prior to testing. Dark adaption was necessary after the frogs were exposed to flashlights during collection, and allowed their eyes to adjust to the experimental conditions. All tests were conducted inside a hemi-anechoic sound chamber (ETS-Lindgren, Austin, TX, USA). Within the chamber, a GE brand nightlight provided illumination that was within the range of natural nocturnal conditions (ca. 5.5×10^{-10} W/cm^2; Taylor et al. 2008). After we recorded a female's choice in a trial, we placed her back with her mate. If a female did not respond during the trial, we did not retest that individual for at least 15 min. If she did not respond a second time, she was not included in that experiment. At the end of the night, we recorded morphological data (snout-vent length (SVL) and mass) and toe-clipped both the female and the male she was captured with. Toe-clipping provided a method of recapture as well as a genetic sample for later use. All pairs were subsequently released at their site of collection.

Acoustic and Visual Stimuli

Before each trial, a female was separated from her mate and was then placed into an acoustically and visually transparent funnel. The funnel was positioned in the center of our sound chamber (Fig. 2). Near the front of the sound chamber, we placed a pair of Orb Audio speakers (Sherman Oaks, CA, USA) at varying distances from the release point of the female. Speakers were placed at distances of: 80 cm, 90 cm, and 110 cm, from the female's release point, depending on the treatment. These distances were measured from the center of the funnel zone (Fig. 2). We used Adobe Audition v22.2 to antiphonally broadcast identical, synthetic calls (whine-chuck) from the speakers. Regardless of distance, we calibrated the sound-pressure level (SPL) to be 82 dB (re. 20 µPa, fast C-weighting) from where the female was held inside the funnel. To create the visual stimuli, we used robotic frogs with silicone vocal sacs (Taylor et al. 2008; James et al. 2022). These vocal sacs connected via pneumatic tubing to an electropneumatic pump outside of the chamber. The tubing exited the sound chamber through a small opening. We verified that there was no light leakage into the chamber several times throughout each field season. To inflate the vocal sac, a solenoid valve shunted compressed air through pneumatic tubing at a 19 kHz tone and would then deflate at a 16 kHz tone. Because male túngara frogs inflate their vocal sacs as they call, we aligned these tones with the beginning and end of the synthetic call to ensure that inflation/deflation of the vocal occurred synchronously with the call. These tones are above the anuran hearing threshold and have no effect on the frogs (Ryan & Rand 1990). The robofrog and its inflating vocal

sac was placed in front of one speaker and we switched its location (right or left speaker) between trials to control for side bias.

Data Collection

Once we placed a female under the funnel, we began playing both the acoustic and visual stimuli. After an acclimation period of 2 min, the funnel was lifted from outside the chamber to release the female. The stimuli continued to play until either a choice was made or a female failed to respond. To monitor her choice, infrared cameras streamed a live feed to a computer outside of the chamber. For a bare speaker (no robofrog) to be chosen, females had to be within 8 cm from the base of the speaker ("choice zone"). However, because the robofrog sat in front of a speaker, the radius of the choice zone was extended so that if a female approached within 5 cm of the robofrog or touched the robofrog for at least 3 s, we deemed that as a choice. A female failed to respond if she did not move from the starting point (funnel zone; Fig. 2) after 4 min or if she failed to make a choice after 10 mins.

Statistical Analyses:

Analyses were performed in R (R Core Team 2020). We used a binomial test to test the hypothesis that the proportion of choices for each treatment were different from chance (50%). For these binomial tests, we reported the mid-P-value to smooth the often large jumps in significance with sample sizes like those we collected. We used ANOVA to compare the mean latency to choice across treatments. To control for the use of 9 frogs between two treatments, we used generalized linear mixed

effects models (GLMMs) with frog ID as a random effect to compare the experiments. To compare to chance, permutation tests were run 10,000 times with the same GLMM. The package lme4 was used (Bates et al. 2015) to perform this analysis and the median P-value is reported. R (R Core Team 2020) was again used to produce graphs using the packages: tidyverse (Wickham et al. 2019) and ggplot2 (Wickham 2016).

Ethical Note:

Experiments followed the approval of IACUC protocols from Salisbury University and the Smithsonian Tropical Research Institute (IACUC: SU-0052;SU-0052R and STRI 2018-0411-2021; SI-21012). The Ministry of the Environment of Panamá (MiAmbiente) also approved and issued collecting permits for our team (ANAM: SE/A- 39-2020). Experimental procedures aimed to minimize our effect on the animals and their environment. Following their use in experiments, we toe-clipped females that participated in an experiment(s) as well as the mate they were collected with. We used toe-clipping to identify individuals in the field and avoid their use in subsequent experiments. We followed regulations from the American Society of Ichthyologists and Herpetologists for our toe-clipping procedures (Beaupre et al. 2004). All toes were preserved in ethanol for use as a genetic sample in future analyses. Toe-clipping does not adversely affect frogs as we very often recapture individuals of both sexes weeks or even months after their first capture. When we released frogs back into the wild, we ensured to release them where they were

initially captured. We also released pairs together after reuniting them so that we did not interfere with the female's original mate choice.

Results:

We tested the preference of female túngara frogs choosing a multimodal signal (robofrog and speaker) over a unimodal signal (speaker only) when both speakers were placed at varying distances: 80 cm, 90 cm (N=32), and 110 cm (N=36).

We first refer to the study by Hamilton et al. (in prep) for the response rate of females to choose the robofrog at 80 cm over a bare (unimodal) speaker. They reported that 71.7% of females preferred the multimodal, robofrog speaker over the unimodal speaker (N=46). This preference rate is consistent with similar studies evaluating a bare speaker and a robofrog speaker at 80 cm (Taylor et al. 2008, 80%; Taylor & Ryan 2013: 70%; Stange et al. 2016, 70%). The 71.7% response rate in the Hamilton et al. (in prep) study was significantly different from random (binomial test: 33 robofrog:13 unimodal; P=0.0024; Fig. 2) and was used as the expected preference for the present study.

Here, we placed the speakers first at 90 cm and found that females' preferences for the multimodal speaker were not significantly different from random (binomial test: 19:13; P=0.3367; Fig. 2). We then placed the speaker placed 110 cm away from the release point and females again failed to show a significant preference for the robofrog speaker (binomial test: 20:16; P=0.5646; Fig. 2).

We also tested if females' latency to choice differed between treatments; there were not significant differences (ANOVA: P=0.373; Fig. 3). In the 80 cm and 110 cm experiments, 9 frogs were shared and all other frogs regardless of treatment were independent. To account for this overlap, we performed a GLMM to confirm the results of the previous binomials and these results reported similar significances (80 cm: P=0.001; 90 cm: P=0.3407; 110 cm: P=0.5661).

Discussion:

All else being equal, female túngara frogs preferentially respond to the multimodal signal of a calling male (visual + acoustic) over the same acoustic stimulus alone (Taylor et al. 2008; Coss et al. 2022). Prior to this study, the range of distances over which females can detect and respond to this visual stimulus was unknown. Under nocturnal-level lighting conditions of the sound chamber, we placed a robotic frog at one speaker (multimodal) while the other speaker was left bare (unimodal). When both speakers antiphonally broadcast identical attractive calls (synthetic whine-chuck), we found that the preference for the multimodal stimuli decreased as the initial distance from the female increased (Fig. 2).

These data demonstrate that female túngara frogs become less likely to perceive the motion of an inflating vocal sac as it becomes proportionally smaller in their field of vision with increasing distance. Numerous studies have demonstrated that a significant proportion of female túngara frogs (ca. 70%), respond preferentially

to a multimodal stimulus over a unimodal one (Taylor et al. 2008; Taylor & Ryan 2013; Stange et al. 2016; Taylor et al. 2021; Hamilton et al. in prep). When we increased the distance between a female and the robofrog by an additional 10 cm (from 80 cm to 90 cm), response rates declined, with females effectively choosing at random. To a human observer, 10 cm does not seem like a large difference, but for a túngara frog, that has an average snout-vent length (SVL) of 27 mm (Hamilton et al., unpublished data), 10 cm is almost four times their body length. We re-tested females again for the multimodal speaker at an additional distance of 110 cm. Again, females chose nearly at random between the two stimuli.

Seeing in dim light is a challenge for any animal. The sparse photon rain necessarily curtails acuity; visual systems then must employ one or more strategies to maintain visual sensitivity in dim light. Aside from evolving increasingly large eyes (to gather more photons), animals can also employ physiological strategies to maintain sensitivity. Summing photons temporally and/or spatially increases overall sensitivity (Warrant 1999; Cronin et al. 2014; Stöckl et al. 2016), but it also degrades resolution in some capacity. With spatial summation, an animal can use multiple photoreceptors in the retina as a "single receptor;" that is, a ganglion cell can take inputs from multiple photoreceptors in an area as a single response. This effectively uses multiple photoreceptors as one receptor, but consequently decreases visual acuity because the multiple receptors occupy a larger are of the retina. Alternatively, with temporal summation, each photoreceptor provides information only after receiving multiple photons over time. This maintains spatial resolution, but comes at

the cost that moving objects become blurred (Cronin et al. 2014). In túngara frogs, it is not surprising that females are able to see the coloration of the vocal sac alone as its reflectance is well within the visual sensitivity range for reproductive females (Cummings et al. 2008). However, if we assume that the female túngara frogs in this study were unable to perceive the actual motion of the vocal sac inflation as it got farther away, it may indicate that their visual systems have limitations. While it has been established that female túngara frogs in a reproductive state are able maintain visual sensitivity even on moonless nights (Leslie et al. 2020), this remarkable night vision comes at the cost of resolution. There are three possible mechanisms by which túngara frogs are able visual sensitivity in dim light: 1) they employ spatial summation; 2) they employ temporal summation; or 3) they use a combination of both temporal and spatial summation to maximize the resolution of both, as was demonstrated in a species of hawkmoth (Stöckl et al. 2016). Future studies of the neurological underpinnings of scotopic vision would need to be performed to uncover the exact mechanism in this species.

As we have demonstrated, in nocturnal conditions, túngara frogs likely have a low visual acuity. When presented with a visual stimulus that is known to be attractive (robofrog) but was proportionally smaller in their field of vision, they exhibited a declining response with increasing distance. A distantly inflating vocal sac may be regarded by female túngara frogs as a static vocal sac perhaps due to either a reduced resolution of their sensory scene or an inability to resolve the inflating motion itself. These results likely reflect the natural history of this terrestrial

species wherein females are evaluating mates in close proximity to the advertising males at a lek (Ryan 1985). As Taylor et al. (2008) demonstrated, to elicit a response from females, the vocal sac (coupled with an acoustic call) must be inflating dynamically with the call. It only took a 10 cm increase in distance from a female's release point to the visual stimulus (90 cm) for their response rate of the robofrog to decline to 59%. At a distance of 110 cm, female response rates was 55%, indicating that females were effectively choosing at random. This suggests that the 80 cm distance that had previously been utilized may already be near the edge of a female's visual capabilities in terms of recognizing the vocal sac inflation.

Vision is an extremely useful and versatile sense in mating contexts. When paired with additional stimuli, visual cues within a multimodal signal can increase the female's sensory stimulation, which can bolster her preference for the mate she is evaluating (Taylor et al. 2008), and even aid a female in remembering the location of a calling male (Hamilton et al. in prep). The distance over which vision is effective, however, seems to be rather limited in nocturnal conditions. The acoustic stimulus of a male túngara frog's display may therefore become more important with increasing distances, especially those greater than one meter. Females commonly evaluate mate at distances of under a meter (Ryan 1985; pers obs). When in close proximity to multiple calling males, however, the visual stimulus of a vocal sac inflation is important for influencing a female's final decision in a mate. Prior to this study, there was no knowledge on the distance at which female túngara frogs could effectively recognize a visual stimulus. Our data have clear implications that female túngara

frogs have an effective nocturnal vision range within 80 cm. Whether this acuity range is same for túngara frogs of both sexes is not currently known. Because female túngara frogs have a significant increase in visual sensitivity when in a reproductive state in comparison to males, it may be possible that a male's acuity range is lower than the 80 cm we demonstrated with females. A sex difference in visual acuity has been documented in several species of diurnal fish, but not in anurans (Corral-López et al. 2017; Caves et al. 2021). Future research would benefit from testing for a sex difference. For nocturnal animals, movement-based stimuli present a problem due to the very nature of scotopic vision. Here, we present evidence that female túngara frogs' ability to perceive the movement of a male's visual display deteriorates with distance. Further, the effective distance of the male's visual display does correspond to distances at which females commonly conduct their mate assessment. As with many species, the evolution of conspicuous sexual signals is dependent on the receiver's sensory system and the environment that those signals evolved in.

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List of Figures:

Figure 1. When male túngara frogs display for mates, they simultaneously inflate their vocal sacs (visual stimulus) and call (acoustic stimulus). When fully inflated, the vocal sac is exceptionally large and nearly the same size as the male's body (Dudley & Rand 1991). Females have been shown to prefer the visual stimulus of an inflating vocal sac (via a robofrog) combined with an acoustic call versus an acoustic call alone (Taylor et al. 2008). The reflectance of the vocal sac itself is different from that of the rest of the male's body and may serve to increase a male's detectability to females in their environment (Cummings et al. 2008).
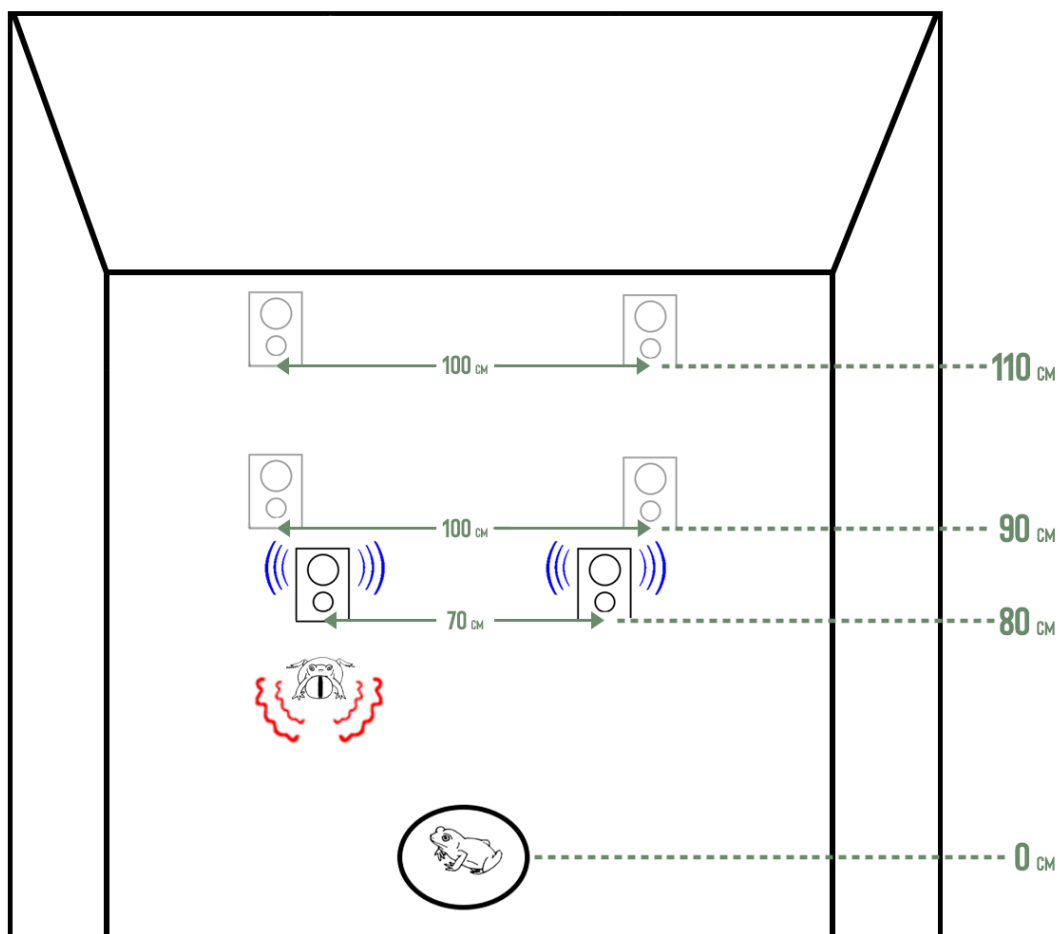
100 cm   110 cm

100 cm   90 cm

70 cm   80 cm

0 cm

Figure 2. An internal view of the sound chamber over various treatments. The image is not drawn to scale. A female frog was placed in the center of the chamber under a funnel. For 2 min, the frog was acclimated to the stimuli of an attractive male call (WC) via speakers (represented by blue crescents) and the simultaneous vocal sac inflation of a robofrog (represented by the red curvy crescents). After 2 min, the funnel was lifted and the female was allowed to make a choice. If the female did not leave the funnel zone (solid black circle) after 4 min, she was not re-tested. We recorded a choice if a female 1) approached within 8 cm of the base of either speaker and/or 2) within 5 cm of the robofrog and remained there for at least 3 s. The three distances (80, 90, and 110 cm) were measured from the center of the funnel. The distances between speakers are also shown. For all three distances (80, 90, 110 cm), the robofrog was placed on one speaker (multimodal signal) while the other was left bare (unimodal signal).
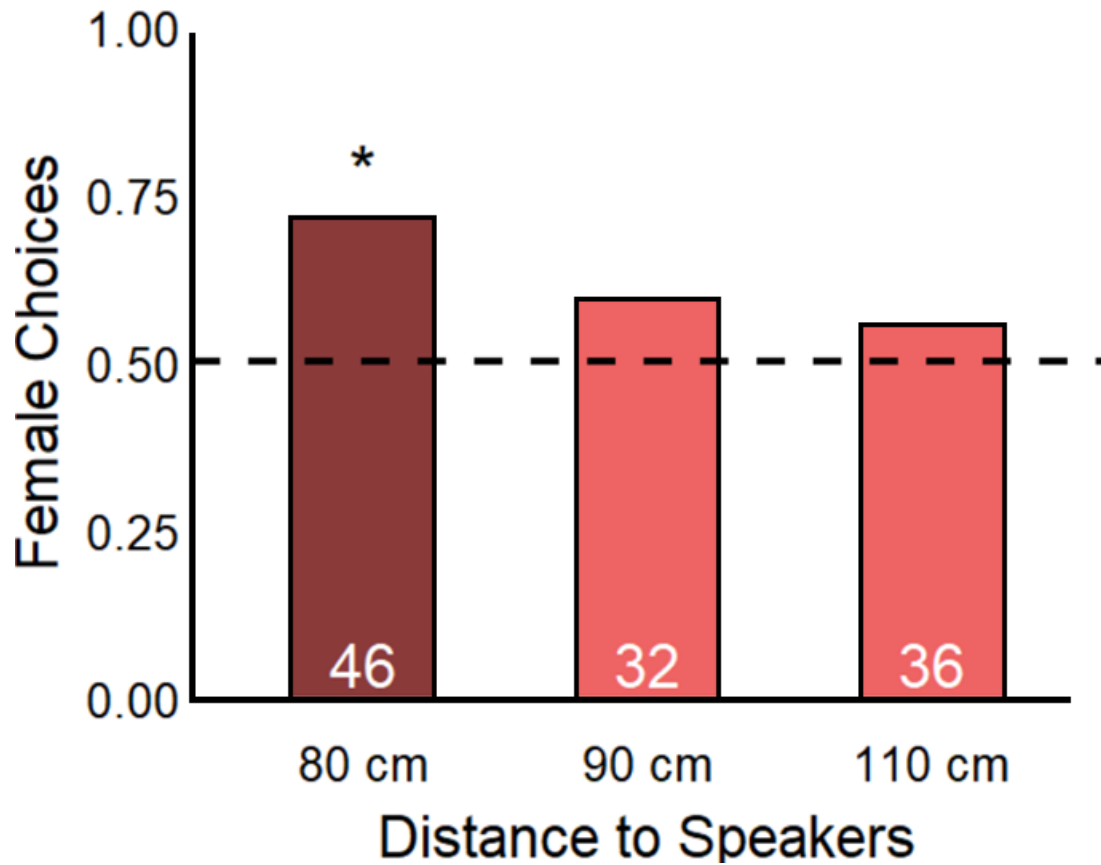
Figure 3. The proportion of females choosing the robofrog (multimodal speaker) for each treatment's distance. The light red bars represent the experiments performed in the present study. The dotted black line represents a random preference (50%). The asterisk (*) above the 80 cm bar indicates a significant difference from random where $P < 0.05$. The dark red bar represents the 80 cm data reported by Hamilton et al. (in prep) and is used as a baseline preference for comparison. Numbers inside bars represent sample sizes for each treatment.
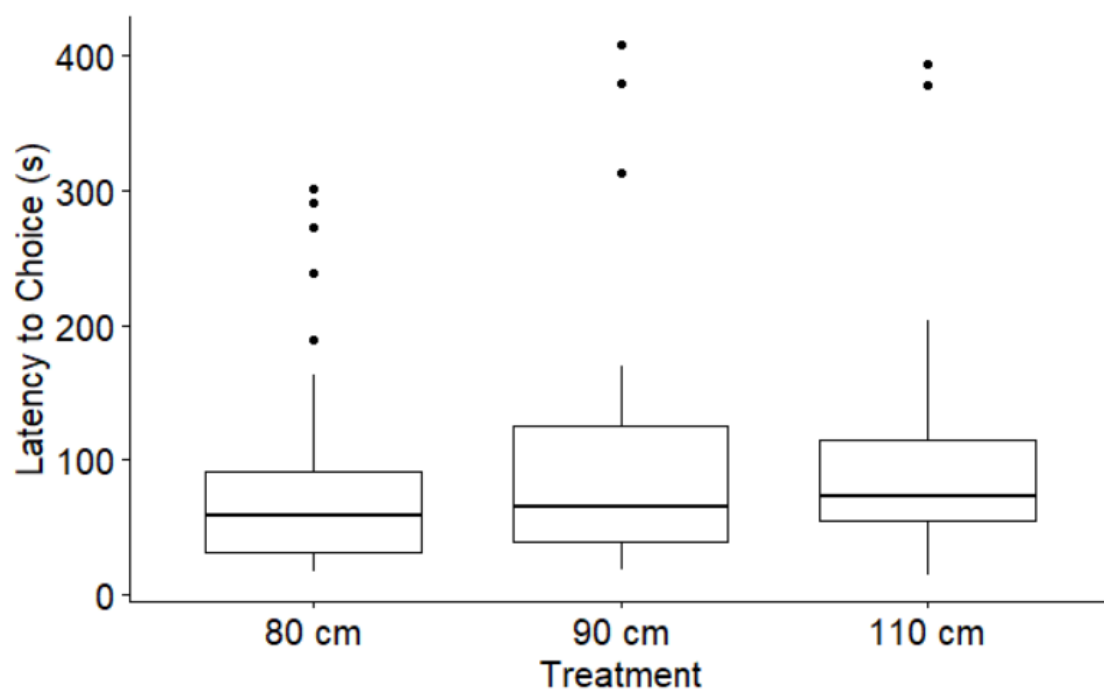
Figure 4. The mean latency to choice for each treatment's distance. Based on an

ANOVA, there was no significant difference between distances (*P*=0.373).

Chapter 3: Foam nests do not influence mating decisions in a female leptodactylid frog

Abstract:

Anurans exhibit extreme diversity in their reproductive strategies. During fertilization, species from several lineages produce foam nests that buffer developing embryos to their external environment. There have been many proposed functions for the production of these nests, but one function that has received little attention is how foam nests may act as an environmental cue to influence female mating decisions. In the túngara frog (*Physalaemus (=Engystomops) pustulosus*), males commonly call near foam nests when they are displaying for females. Previously, it was demonstrated that females of this species prefer a male call when it is associated with a real, field-collected nest (Martin et al. 2011). Amplexed pairs also often form communal nests, and it has been hypothesized that foam nests may act as a cue for an ideal oviposition site to amplectant females. Here, we tested the preferences of females using both real and faux, 3D printed foam nests in various treatment conditions. We were unable to find any evidence that females respond to the visual stimulus of foam nests during mate evaluation or that foam nests act as a possible cue for an oviposition site. We did, however, show that females had identical responses to the faux and real foam nests. Our results demonstrate that even in a well-studied

organism like túngara frogs, we still have much to learn about their fundamental

ecology.

Introduction:

Anuran evolution is largely characterized by the ability to utilize both a

terrestrial and aquatic environment. Their ancestral reliance on water for reproduction

prevented some lineages from venturing into drier habitats, but many were able to

overcome this restriction (Crump 2015). There are currently 42 recognized

reproductive strategies for which eggs are laid and offspring develop, reflecting the

incredible diversity within Anura (Haddad & Prado 2005; Malagoli et al. 2021). One

of the modes that has allowed some lineages to expand into more terrestrial

environments is the production of foam nests. Foam nests evolved independently in

three major groups across continents, and the diversification of the largely South

American family, Leptodactylidae, can be attributed, in part, to their ability to

produce foam nests (Pereira et al. 2017). Some leptodactylids produce foam nests that

encase fertilized eggs and their developing embryos until the larvae hatch into

exotrophic tadpoles (Haddad & Prado 2005; Pereira et al. 2017). To produce a nest, a

male in amplexus scoops up eggs from a female's cloaca, fertilizes them, and whips

the egg-jelly of the female together with water (Dalgetty & Kennedy 2010) (Fig. 1A).

Though the placement of these nests can range from entirely terrestrial to entirely

aquatic depending on the species, there are several proposed functions for their

construction apart from preventing desiccation including improving oxygen supply to embryos (Seymour 1999). It also includes: prevention of microbial growth (Fleming et al. 2009), predator protection (Fleming et al. 2009), UV protection (Hissa et al. 2008), and thermoregulation (Méndez-Narváez et al. 2015).

In one leptodactylid species, the túngara frog (*Physalaemus (=Engystomops) pustulosus*), foam nests have previously been proposed to have an additional function: mate attraction. In the field, it is commonly observed that males advertise for females, calling adjacent to a foam nest produced by another frog pair (Martin et al. 2011; O.R. Hamilton, pers. obs.) (Fig. 1C). Typically, males of this species gather in large nocturnal choruses to produce a multimodal courtship signal (composed of acoustic and visual stimuli) to attract females (Ryan 1985; Taylor et al. 2008). Túngara frogs produce a simple whine call and may append up to seven optional "chucks" onto the end of the whine (Ryan 1985). Female túngara frogs consistently show an 85% preference for the complex whine-chuck over the simple whine call (Gridi-Papp et al. 2006; Ryan et al. 2019). Martin et al. (2011) tested the preference of female túngara frogs for the visual stimulus of a foam nest at a speaker broadcasting an attractive call. The researchers played identical attractive calls (whine + 1 chuck) on two speakers and placed a real, field-collected foam nest next to one of them. The study found that females preferred the speaker with the foam nest over the acoustic-only speaker at a 2:1 ratio (Martin et al. 2011). Though the acoustic call has primacy over the visual cue in a male túngara's courtship display, the visual component of the male's vocal sac inflation is known to modulate females' responses to the acoustic

call (Taylor & Ryan 2013). When in a reproductive state, visual sensitivity increases in female túngara frogs, but not in males (Leslie et al. 2020). As females are the sex that actively conduct mate searching behavior, their increased visual sensitivity allows them to sample potential mates and their visual courtship display even in light levels equivalent to a moonless night (Cummings et al. 2008). Indeed, the white reflectance of the túngara frog foam nest is conspicuous against the tropical forest background, even to a human observer at night (Fig. 1A). Already highly sensitive to visual stimuli, females in a reproductive state may simply have a positive phototactic response towards any source of brighter light/reflectance within their optimum ambient illumination (Jaeger & Hailman 1976). Despite other leptodactylid species exhibiting maternal care (Rodrigues et al. 2011), foam nests of túngara frogs are abandoned shortly after their production (Ryan 1985). The behavior of males to call near foam nests is not a form of parental care, as males presumably only mate once per night (Ryan 1985). Given that females are known to respond to the visual stimulus of a male's inflating vocal sac (Taylor et al. 2008), it may be possible that these "bachelor" males are actively exploiting the bright reflectance of a foam nest to attract females. Alternatively, both male and female túngara frogs may simply be exhibiting a phototactically positive response to the highly reflective foam nests, instead of exploiting them for any material benefits. It is currently unknown if foam nests are purposefully used by túngara frog males to bolster their attractiveness, but males of another anuran species - the Bornean tree-hole frog (*Metaphrynella sundana*) - are known to exploit the environment for their benefit when displaying for

mating opportunities (Lardner & bin Lakim 2002). Despite data showing a positive response to a foam nest by females (Martin et al. 2011), virtually nothing is known about the influence of these nests on the courtship behavior of túngara frogs.

The prominent white coloration of túngara frog foam nests has been proposed to aid in UV protection (Hissa et al. 2008), but predators may also use this coloration to visually locate nests. Though foam nests possess some anti-predator defenses such as possessing a high viscosity and anti-oophagy proteins (Fleming et al. 2009), instances of predation in both aquatic and terrestrial environments, while somewhat rare, do occur (Gould et al. 2021). Predators of foam nests range from wasps, snakes, and even other anurans (Ryan et al. 1985; Ribeiro et al. 2014; Starr et al. 2020; Hamilton et al. in press). Because females carry males during amplexus, they alone are responsible for choosing where to oviposit (Ryan 1985). Foam nests can become more or less vulnerable to desiccation and/or predation risks based on the female's decision. Though such risks are often related to water depth (Gould 2021), they can be mitigated by nesting communally (Zina 2006). It has long been observed that pairs of túngara frogs often construct their nests communally to create a single, large mass that can be the product of up to 8 pairs' efforts (Ryan 1985; O.R. Hamilton pers. obs.) (Fig. 1B). The bright reflectance of foam nests may therefore serve an additional function as a cue for an ideal oviposition site to other amplectant females. One study showed that females prefer to nest communally, perhaps as a method to reduce desiccation with the lowered surface area to volume ratio (Zina 2006). Alternatively, Marsh and Borrell (2001) documented that females did not necessarily use foam nests

as a cue and instead had very flexible oviposition strategies. Yet another study found that female túngara frogs did not choose to oviposit in a location with conspecific nests, though it is to be noted that these nests were over a day old and the experiment took place in complete darkness (Dillon & Fiaño 2000). We do not know what cue(s) initially attract females to oviposit, let alone how those cues may impact females still searching for mates. Additional research into the role of foam nests and oviposition site preference is therefore needed.

Female preferences for properties of male advertisement signals are well known (Ryan et al. 2019; Taylor and Ryan 2013), but the use of external, environmental cues to exploit underlying biases and/or attraction of females is poorly understood (Lardner & bin Lakim 2002; Muñoz & Penna 2016). If females are attracted to foam nests as a cue for an oviposition site, perhaps there is a two-fold attraction to foam nests wherein females are first attracted to males calling near nests, as shown by Martin et al. (2011), and while in amplexus, females may again be drawn to foam nests for oviposition. Few studies have investigated the role of túngara frog foam nests outside of the context of larval development and we aimed to provide evidence of an additional function of foam nests in mate choice. In the present study, we tested the preferences of females using both a real, field-collected foam nest and faux, 3D printed foam nests in various treatment conditions. Investigating the role of this bright reflectance in mate choice may allow for further insight into the variables that influence mate choice.

Methods:

General Procedures

Experiments were conducted at the Smithsonian Tropical Research Institute in Gamboa, Panamá during the rainy season of 2021. Amplexed pairs were collected from wild choruses between 19:30 and 23:00 h. On nights when we tested the preference for real foam nests, we collected nests from the same sites where we collected pairs. We gently scooped a fresh foam nest (<1 day old; usually constructed the night of collection) and some of the pond/puddle water into a plastic cup. We transported the pairs back to our laboratory where we stored them for a dark-adaption period of at least one hour. Dark-adaption allows the females' nocturnal vision to be restored after exposure to flashlights during collection. Females were separated from their mates and placed under an acoustically and visually transparent funnel in the center of a hemi-anechoic sound chamber (ETS-Lindgren, Austin, TX, USA). A GE brand nightlight on the ceiling of the chamber produced light within levels similar to moonless nights at natural breeding ponds (Taylor et al. 2008). Two speakers antiphonally broadcast either a complex túngara call (synthetic whine-chuck (WC)) or a simple call (synthetic whine (W)); within an experiment, both speakers broadcast the same, identical call. We calibrated the sound-pressure level (SPL) to be 82 dB (re. 20 µPa, fast C-weighting) from the female's position within the funnel. We played call files using Adobe Audition v22.2. The speakers were placed 90 cm away from the center of the funnel zone and were 100 cm apart. The frogs were acclimated to the stimuli of their respective treatment (detailed below) for 2 minutes before being

remotely released from the funnel and allowed to make a choice between two

equidistant speakers. Using infrared cameras, we monitored the female's choice live

and recorded the video for later evaluations. We determined a female made a choice

if she was within 5 cm of the front of the speaker (the "choice" zone) for at least 3 s.

We also extended the choice zone to include the 5 cm that surrounded any foam nest

that was placed in front of or next to a speaker. If a female touched a foam nest, we

also recorded that as a choice. The position of foam nests (right or left speaker)

during each trial was switched between trials in all experiments. If after the raising of

the funnel the female remained in the initial "funnel zone" for 4 minutes or failed to

make a choice within 10 minutes, we removed her from the chamber. After at least 15

minutes, the female was re-tested, and if she did not respond a second time, we

removed her from the chamber and she was not tested again for that experiment.

After completion of a trial, we reunited females with their mates. At the end of the

night, we recorded morphological data (snout-vent length (SVL) and mass) on each

frog and later toe-clipped the pairs for both a genetic sample and a method of

recapture. We released all pairs and foam nests back at their respective collecting site

within 24 hours of collection.

Foam Nest Experiments

As natural foam nests may have variation in size, reflectance (muddiness),

and/or chemical cues, we designed a 3D printed foam nest to serve as a standardized

stimulus across trials (Fig. 1E). These artificial (faux) foam nests were printed at

Salisbury University's MakerLab (Salisbury, MD, USA) and had a bright white

reflectance to mimic that of real foam nests. Because the original Martin et al. (2011) study used natural foam nests (FN), however, it was necessary to first test a real foam nest against a faux foam nest to establish that there was no difference in preference. For our first experiment, "Real vs Faux FN," we placed a faux foam nest on one speaker and a real foam nest on the other speaker. After collecting a real nest from the field in a plastic cup, we gently transferred the nest onto the lid of the plastic cup along with enough tap water, treated with AmQuel brand dechlorinator (Kordon llc, Hayward, CA, USA) to cover the plastic lid (Fig. 1D). To ensure conditions were held equal for both the real and faux foam nest, the faux foam nest was also placed onto a plastic lid with the same amount of treated water as the real foam nest. The faux foam nests were slightly taller than the collected foam nests, so we placed both foam nests next to their respective speakers so that they did not block the speaker driver and interfere with sound production (Fig. 1D & Fig. 1E). This is reflective of field observations where males can either call in front of or adjacent to previously constructed foam nests. The complex whine-chuck (WC) call was broadcast antiphonally from both speakers during this treatment. We then repeated the methods of Martin et al. (2011) and placed a real foam nest on one speaker while leaving the other bare. For this experiment, "Real FN vs Bare" we placed the foam nests (still sitting on a plastic lid) in front of its respective speaker as Martin et al. (2011) did. The complex WC call was again broadcast from both speakers.

After conducting these control experiments, we then aimed to further our understanding of female preferences for foam nests under different experimental

conditions. First, we conducted the "FN vs Bare (WC)" experiment where females chose between a bare speaker without a foam nest or a speaker with a faux foam nest. Both speakers broadcast the WC call during this treatment and the faux foam nest was placed beside one speaker. Next, we wanted to test how females' preference for foam nests would change if a simple call (W) was broadcast. In our "FN vs Bare (W)" experiment we placed a faux foam nest beside one speaker and left its counterpart bare while both broadcast a whine (W) call. Finally, we wanted to test how the act of being in amplexus may influence a female túngara frog to move towards a foam nest, perhaps as an indicator of oviposition site. A previous study with amplexed females showed that females had an increased response to male stimuli (Lynch et al. 2005). A female's choice to move towards a conspecific foam nest may also be indicative of an underlying preference to communally nest with conspecific nests. Because pairs were often in amplexus after the 1-hour dark adaption period, we tested females in this experiment ("Amplexed + FN") at the beginning of the night. The faux foam nest was placed beside one speaker as the WC call played antiphonally from both speakers. All other procedures during the female-only choice experiments were applied to this experiment.

Ethical Note:

The Ministry of the Environment of Panamá (MiAmbiente) approved our collection of animals and issued collecting permits for our team (ANAM: SE/A- 39-2020). Experimental procedures were approved by the IACUC protocol from Salisbury University and the Smithsonian Tropical Research Institute (IACUC: SU-

0052;SU-0052R and STRI 2018-0411-2021; SI-21012). We aimed to minimize our effect on the animals and the environment they were collected from during experimentation. Both the females that were used in experiments and the males they were collected with were toe-clipped the night of capture. If females did not participate in an experiment, they were not toe-clipped. Toe-clipping was used to identify individual frogs when collecting so that females did not participate in an experiment more than once. Toes were preserved in ethanol for future use in genetic analyses. We followed regulations from the American Society of Ichthyologists and Herpetologists regarding toe-clipping procedures (Beaupre et al. 2004). Toe-clipping does not negatively affect frogs as we often recapture individuals throughout the field season weeks or even months are their initial capture. We were extremely gentle with our handling of foam nests during collection and when using them for our experiments. Foam nests always had a water source, and to replicate ambient temperature, our laboratory was never below 25° C during experimentation. When we returned foam nests to their original collection site, we ensured they were in the same position as when we encountered them (e.g. against a surface, not free-floating on the surface of the water). Similarly, we released all frogs back to their original collection site. Pairs were kept and released together so that we did not interfere with the initial decision of the female.

Statistical Analyses:

All analyses were conducted in R (R Core Team 2020). We tested the hypothesis that the proportion of females that chose a foam nest was different from

random chance (50%). For the Real vs Faux FN experiment, a binomial test was used to test if there was a difference in females choosing one foam nest over another. We reported the mid-P-values to smooth drastic differences in significance. We log-transformed the latency to choice data to normalize the distribution and then used ANOVA to compare the latency to choice between treatments. A Levene's test for equal variance was also performed. Graphs were generated in R (R Core Team 2020) using the packages: tidyverse (Wickham et al. 2019) and ggplot2 (Wickham 2016).

Results:

We first performed a control experiment to test the preference of females between a real and faux foam nest. For the Real vs Faux FN experiment, females (N=32) did not express a preference for either foam nest type (binomial test: 16:16; P=0.93; Fig. 2). This was an essential control that then allowed for the subsequent use of the 3D printed, faux foam nest in later experiments as opposed to real foam nests. We next wanted to repeat the experiment performed by Martin et al. (2011) to establish the preference of females for foam nests. Interestingly, in the Real FN vs Bare experiment, females (N=32) did not express a significant preference for the speaker with a real foam nest over a bare speaker (binomial test: 20 real FN speaker:12 bare speaker; P=0.189; Fig. 2). In the original paper, females chose the foam nest speaker at a 2:1 ratio, but we were unable to reproduce those results in this experiment (Martin et al. 2011).

Because we showed that females could not discern a difference between a real versus faux foam nest, we used faux foam nests to standardize any external factors that may have influenced females to choose against the perhaps inconsistent, natural foam nests in the reproduced Real FN vs Bare experiment. For our FN vs Bare (WC) experiment, we again found that females (N=32) did not show a preference for the foam nest speaker (binomial test: 18:14; P=0.542; Fig. 2). Knowing that females have a strong and consistent preference for the whine-chuck call over a whine (Ryan et al. 2019), we repeated the previous experiment, but played a whine call instead of a whine-chuck. We hypothesized that the attractiveness of the whine-chuck call may be confounding the females' underlying preference for the foam nests, causing females to care less for the visual stimulus. However, we found the results of the FN vs Bare (W) experiment to be similar to previous experiments, with females (N=24) showing no preference for the foam nest (binomial test: 13:11; P=0.764; Fig. 2). We lastly wanted to test the how females' preference for the visual stimulus of a foam nest is influenced by their reproductive state. We repeated the FN vs Bare (WC) experiment, but with amplexed females. Still, we could not demonstrate that reproductive females (N=32) had a preference for a foam nest (binomial test: 16:16; P=0.93; Fig. 2).

We also tested whether treatment type had an effect on latency to choice (Fig. 3). There was a significant difference in latency to choice for the Amplexed + FN experiment (ANOVA: P=1.32e-12; Fig. 3). No other treatment type had a significant difference in the females' latency to choice. The Levene's test did not show a significant difference in variance (P=0.579).

Discussion:

Though some species have direct development and lay their eggs on land (Westrick et al. 2022), many anurans rely on a water source during oviposition (Crump 2015). Often referred to as mud puddle frogs, túngara frog males call from ephemeral water sources ranging from ponds to divots in a sidewalk (Ryan 1985). During oviposition, the amplexed pair of frogs produce a foam nest that helps protect embryos from desiccation in these sometimes unreliable water sources (Zina 2006). As bachelor males are often seen calling in front of these foam nests, we wanted to investigate the possible role that foam nests may play in mate attraction. We used both field-collected, real foam nests and 3D printed faux foam nests to test female preferences when a male's call is broadcast. We manipulated several factors of our treatments (call type, reproductive state, real and faux foam nest) and yet we were unable to show that there is any indication of a female preference for the visual stimulus of a foam nest during mate choice.

In the original study by Martin et al. (2011), they showed that female túngara frogs had a 2:1 preference for the foam nest when an attractive, whine-chuck (WC) call was played. We replicated that experiment using a real foam nest, but our results were neither consistent with the original results nor statistically different than random. This was an interesting result given that túngara frogs tend to show consistent preferences for various stimuli over time (acoustic: Ryan et al. 2019; visual: Taylor et al. 2008; Hamilton et al. in prep.).

Because real foam nests can have several varying characteristics (size, age, reflectance, possible chemical cues, etc.), we next used 3D printed faux foam nests so that the visual stimulus was consistent across trials and nights. First, we verified that the females did not have either an aversion to or preference for a faux foam nest over a real foam nest in the Real vs Faux FN experiment. We had a 50/50 preference for each stimulus, which allowed us to move forward with our next experiments. Interestingly, females failed to show any preference for either foam nest, despite the faux foam nest being substantially larger than the real one. We repeated the methods of the Martin et al. (2011) study, but we used a faux foam nest in place of the real foam nest we had used in the Real FN vs Bare experiment. Just as we had before, a complex WC call was used in our FN vs Bare (WC) experiment, but we were still unable to demonstrate that females had a preference for a foam nest. We next considered that perhaps the attractiveness of the WC call nullified females' preference for a foam nest stimulus. In our FN vs Bare (W) experiment, we kept all variables the same as the previous treatment, but changed the calls of both speakers to be a simple whine (W) call. Even with the reduction in attractiveness of the acoustic call, females did not demonstrate a preference for a speaker associated with a foam nest.

Finally, we were interested if a female's reproductive state influenced her preference to move towards a bright visual stimulus. Keeping all variables equivalent to our FN vs Bare (WC) experiment, for our Amplexed + FN experiment, we used females that still had their mates on their back instead of separating the pair like we

had in the previous treatments. Despite a previous study demonstrating an increased responsiveness in females that were in amplexus (Lynch et al. 2005), amplexed females in our study did not preferentially move toward foam nests. This experiment did show a significant slower response in females' time to choice when compared to the other treatments, though this may simply be attributed to females having to carry the additional weight of the male when moving towards speakers.

Previous studies have provided mixed evidence for the influence of foam nests on females' oviposition site selection (Dillon & Fiaño 2000; Marsh & Borrell 2001; Zina 2006). Though this was not the main focus of this study, we were unable to provide evidence for what initially evokes females to choose a site adjacent to a previously constructed foam nest. The benefits of nesting communally in this species as well as others have been fairly well-studied (Zina 2006; Gould 2021), but the cue that initially attracts females to choose one oviposition site over another is still largely unknown. Results of previous studies do not support a chemical cue being the influence that draws other females to nest communally. The Martin et al. (2011) study blocked the foam nest from view and could only show the 2:1 preference when the foam nest was visually accessible. Likewise, Dillon & Fiaño (2000) performed their oviposition site preference study in complete darkness and found that females actually preferred to oviposit away from conspecific nests. With the results the present study, however, the visual stimulus alone is an unlikely influence on a female's decision on whether to nest individually or communally. It is possible that there is another cue(s) that has not yet been discovered for why females choose to

oviposit alongside other pairs (e.g. a visual or seismic cue of other pairs creating a nest). Future studies investigating oviposition site preference would benefit from investigating cues aside from the visual stimulus of the foam nests themselves.

Why we were unable to replicate the results from the Martin et al. (2011) study across multiple, varying treatments highlights the importance of repeatability in science. Although the probability is small, even well-designed experiments with good sample sizes can generate statistically significant results by chance. This speaks to the importance of repeating experiments when feasible. For example, in 2017, Liu & Burmeister showed that female túngara frogs performed better with a visual cue in a place-learning task than males. In 2019, however, Ventura et al. repeated the same experiment and found no sex difference in their performance. Infamously in the field of human health, a research paper hypothesized the cause of the impairment of memory in Alzheimer's patients, but was later accused of having fabricated data in their results (Lesné et al. 2006; Piller 2022). The original 2006 paper was used as basis for subsequent research studies and its results were largely not questioned until recently (Piller 2022). Had these methods been attempted and publicly questioned earlier, this field of research may not have been misled in a direction where there is, in reality, little evidence of the original paper's findings (Piller 2022). Results like those that were gathered in the present study may not provide evidence of a new discovery, but rather serve as a reminder that researchers should never assume the results of one study are constant or a reflection of an entire population.

   In our present study, we were unable to replicate previous findings of female preference for the visual stimulus of a foam nest. As male túngara frogs often call near foam nests, it has been speculated that these males were using this visual stimulus as an extended phenotype that may act to attract females (Martin et al. 2011). Here, however, we were unable to find any evidence for foam nests influencing the mate choice of female túngara frogs, and we failed to find evidence for this using several different variations of the stimuli. The bright white reflectance of foam nests may then serve only to protect developing embryos from UV damage (Hissa et al. 2008). Despite a likely increased predation risk, túngara frogs and other anurans that produce foam nests have not been hindered by this adaptation as foam nest development has independently evolved three times over several continents (Pereira et al. 2017). Foam nests have helped to relieve various lineages from their dependence on water sources, and likely provide benefits to developing embryos without any secondary behavioral functions.

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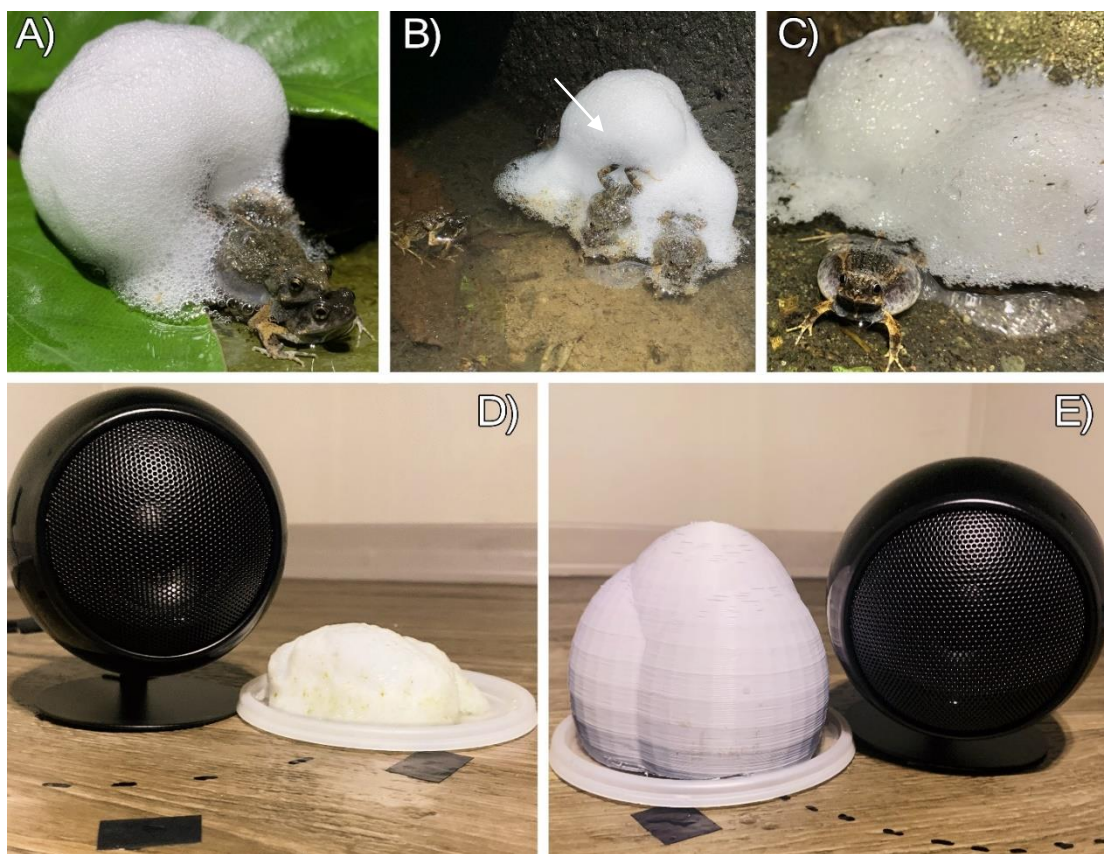
List of Figures:

Figure 1. Túngara frogs (*P. pustulosus*) produce bright white foam nests that incubate embryos until hatching. **A)** After choosing a mate, the amplexed female must then choose where to oviposit. As she oviposits, the male (on top) fertilizes the eggs and whips the secretions of the female (egg jelly) with his hind legs into a foam that will house the developing embryos. **B)** When choosing an oviposition site, pairs will often build nests communally. Pictured is a foam nest that is a combination of 3 pairs' efforts (third pair shown by arrow). Another pair that has presumably not yet produced a nest is spotted close by; the female possibly drawn to the reflection of the large foam nest. **C)** Male túngara frogs are often observed displaying for mates in front of or next to preconstructed conspecific foam nests. We tested females in experiments to better understand if they have a preference for this visual stimulus by using both a 3D printed faux foam nest **(D)** and real, field-collected foam nests **(E)**. Photo Credit: ORH.
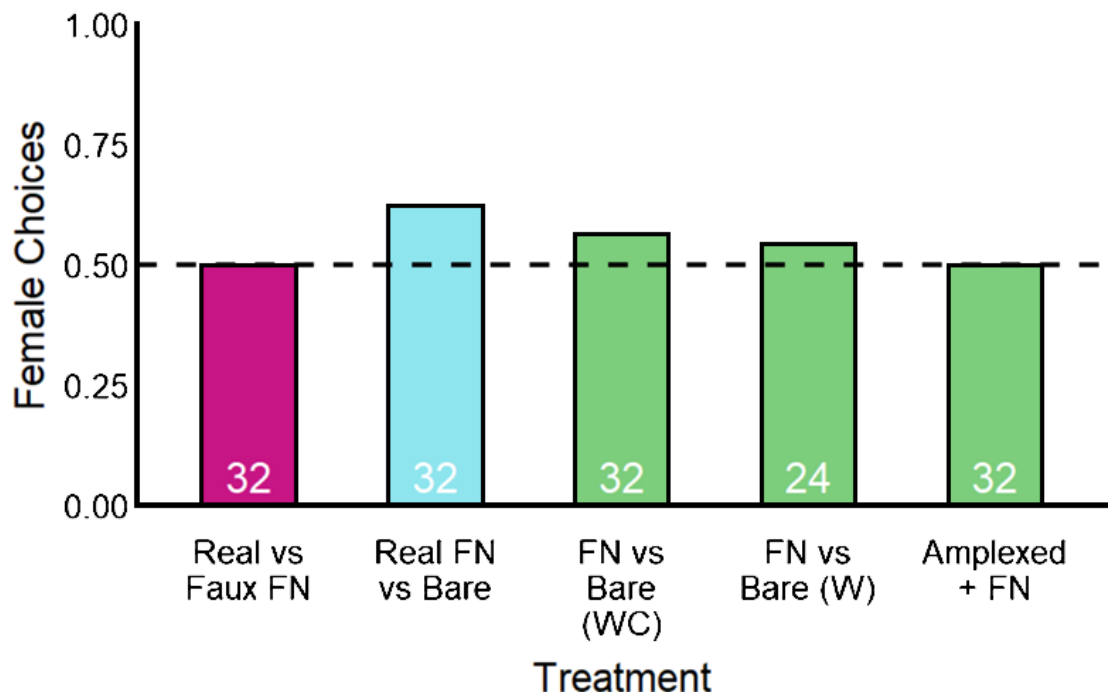
Figure 2. The proportion of female túngara frogs choosing the foam nest (FN) in each treatment. The blue bar is the experiment that replicated the methods of the Martin et al. (2011) study. The green bars indicate a 3D printed, faux foam nest was used. The magenta bar indicates the comparison experiment between a real foam nest versus a faux foam nest. The dotted line represents a random preference (50%).
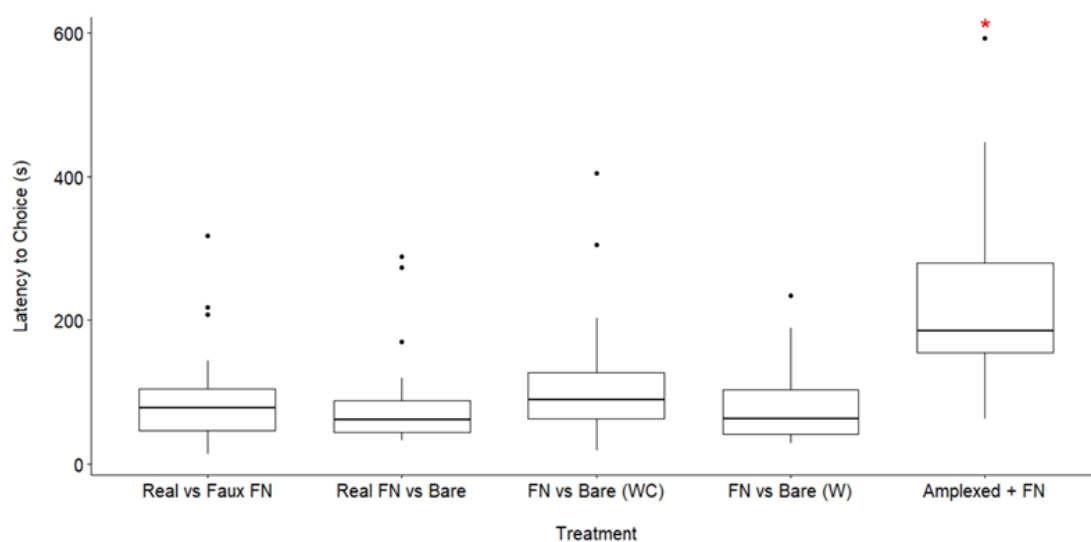
Figure 3. The mean latency to choice across treatments. The Amplexed + FN experiment was the only treatment to have a significant difference (indicated by the red asterisk (*) above its plot) in latency to choice based on an ANOVA (P=1.32e-12).

Chapter 4: Túngara frog egg predation by terrestrial snake

Abstract:

In this natural history note, we describe the first observation of a terrestrial vertebrate predator for the foam nests of túngara frogs (*Physalaemus (=Engystomops) pustulosus*) in the rainforest of Soberanía National Park, Panamá. The snake species we observed feeding on the eggs within the nest was documented as a juvenile *Erythrolamprus* (=*Liophis*) epinephelus and this is also the first account of oophagy for this species.

As a seasonally breeding anuran, the reproduction of *Physalaemus (=Engystomops) pustulosus* (túngara frog) coincides with the rainy season where females choose their mates at a lek of calling males. While still in amplexus, females also choose the oviposition site at a water source that can range from a pond to a divot in the road (Ryan 1985). During oviposition, *P. pustulosus* males use their hind legs to kick up the egg-jelly produced by the females. Along with the addition of water, this kicking action produces a foam structure that houses the developing embryos and protects them from desiccation, even if the initial water source evaporates. As the eggs are held near the core of the viscous foam, the egg-free outer portion provides an ideal environment for incubation while also buffering the embryos from external predators (Dalgetty & Kennedy 2010). However, if a predator is able to breach the nest, they have access to a nutrient-dense meal at the nest's core. At ca 0950 h on 11 August 2019, we observed a juvenile *Erythrolamprus (=Liophis) epinephelus* in Soberanía National Park near Gamboa, Panamá performing this behavior.

The approximately two-day old *P. pustulosus* foam nest was located on Pipeline Road. The puddle where it was originally oviposited had evaporated and the nest was resting on a mud surface. Repeated movement from within the nest drew our attention and we watched as the snout of a juvenile *E. epinephelus* emerged from the center. With its tail sticking out of the opposite side, the snake struggled to push itself out of the nest, likely as a result of the foam's viscosity. The physical effort to push into (or out of) the nest indicates that the snake made a concerted effort to gain access

to the interior of the nest. After exiting the foam nest, the *E. epinephelus* spread its neck in defense and remained unmoving until we left.

Prior to this observation, there have been no direct accounts of a terrestrial vertebrate predator for the foam nests of *P. pustulosus*. Aquatic predation by tadpoles of *Agalychnis callidryas* on *P. pustulosus* foam nests has been documented (Ryan 1985) along with an account of predation by wasps (Starr et al. 2020). However, in both of these instances, the foam nests were thoroughly degraded so that the eggs lay exposed, whereas the foam nest we observed was still fully intact. This suggests that a snake predator may more easily burrow into and take advantage of an intact foam nest than other potential predators. Snakes of species closely related to *E. epinephelus* (Lingnau & Di-Bernardo 2006) have been documented as oophagous to other Leptodactylid species, but until now, a terrestrial vertebrate predator has not been documented for the nests of *P. pustulosus* specifically. In addition, although *E. epinephelus* is a known predator to adult anurans (Savage 2002) it has not, to our knowledge, been documented as oophagous.

Foam nests likely fostered the diversification of Leptodactylidae so that these anurans could occupy new environments unhindered by their reliance on permanent water sources to reproduce (Pereira et al. 2017). Some researchers have therefore argued that the principal role for a foam nest is to prevent microbial growth and desiccation in an environment where water sources are sometimes unreliable (Fleming et al. 2009). Even so, the dissuasion of predators has likely been important for the evolution of foam nests as instances of oophagy remain fairly rare for species

that produce foam nests. This may be in part attributed to the lectins and cystatins found in a foam nest that, in tandem with its viscous consistency, are thought to largely be sufficient to deter oophagy (Fleming et al. 2009). While the nest structure likely discourages egg predation, our observation indicates that at least one snake species has been able to breach the physical defense of a *P. pustulosus* nest to gain access to the eggs.

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