

# Walk or swim: The substrate for movement affects female treefrog mate choice behaviors but has little effect on their preferences

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## Abstract

Semiaquatic animals breed in environments with a mix of aquatic and terrestrial features, each requiring appropriate types of locomotion with differential energetic demands and containing different suits of predators. We surveyed calling locations of male Eastern Gray Treefrogs (*Hyla versicolor*) at our study pond to assess average composition of land/water substrates available to females during mate choice. We also conducted acoustic playback trials to examine whether the mate choice behavior of females was affected by approach substrate; that is, whether females had to swim or walk towards a potential mate. We found that mate preferences were not strongly affected by the substrate of movement: the preferred call duration (“peak preference”) was similar in both treatments, but females showed somewhat stronger discrimination against values deviating from their peak preference (higher “preference selectivity”) in the water treatment. By contrast, other aspects of phonotaxis behavior were clearly different and consistent with females perceiving water as a more dangerous environment: in the water treatment, females took longer to leave the dry release point, jumped further when leaving the release point, and took advantage of features of the playback setup (i.e., the aquatic arena was surrounded by walls) to leave the water and approach the speaker “on land”. This suggests that females do not compromise mate preferences but adjust associated behaviors to minimize risk, and that environmental heterogeneity has little influence on sexual selection regimes.

## KEYWORDS

call duration, *Hyla versicolor*, mate preference, preference selectivity

## 1 | INTRODUCTION

The study of mate choice, and its sources of variation, provides insight into the driving forces behind the evolution of reproductive strategies and their consequences for population dynamics (Jennions & Petrie, 1997; Rosenthal, 2017). Mate choice causes nonrandom mating success among males (Andersson, 1994), and can be a key determinant of the genetic quality and fitness of

offspring (Kempnaers, 2007; Reynolds & Gross, 1992; Welch et al., 1998). Mate choice variation can also contribute to the process of speciation, as differences in mate preferences between populations can lead to reproductive isolation, ultimately resulting in the formation of new species (Hohenlohe & Arnold, 2010; Verzijden et al., 2005). Finally, variation in mate choice decisions can shape the genetic diversity and adaptability of populations, influencing the maintenance of genetic variation, which is crucial for

population resilience and adaptation to changing environments (Jennions & Petrie, 1997).

Variation in female mate choice behavior may arise from a range of sources and is often context-dependent (reviewed in Ah-King & Gowaty, 2016). Resource availability, sex ratio, or the presence of competitors and predators may modify female mate choice decisions (Feagles & Höbel, 2022a; Janetos, 1980; Neelon et al., 2019; Partridge & Halliday, 1984). Physical features of the environment can also be potential source of variation in mate choice. For example, different substrate types affect the efficacy of seismic courtship signals and mating success of male *Habronattus dossenus* jumping spiders and *Schizocosa retrorsa* wolf spiders (Elias et al., 2004; Hebets et al., 2008), increased structural complexity limiting visual contact between mates and rivals changes the mating behavior of *Gobiusculus flavescens* goby fish (Myhre et al., 2013), and the availability of elevated perches or cover objects that reduce predation risk changes mate choice in *Hyperolius marmoratus* reed frogs and *Gryllus integer* crickets (Backwell & Passmore, 1990; Hedrick & Dill, 1993). Moreover, most habitats are quite heterogeneous. For example, many semi-aquatic animals live in environments with a mix of aquatic and terrestrial features. These environments differ in many aspects, the two most obvious being that they require different types of locomotion (swimming vs. walking) and that they contain different suits of predators (Burne & Griffin, 2005; Indermaur et al., 2010; Silva et al., 2011).

Movement typically consumes a large proportion of an animal's energy budgets, but the energetic cost of locomotion varies with environmental conditions. The metabolic cost for walking is greater than for swimming across a range of semiaquatic animals, including *Bufo marinus* toads, *Emydura macquarii* turtles and mammals such as *Hydromys chrysogaster* water rats and the platypus, *Ornithorhynchus anatinus* (Baudinette et al., 2000; Bethge et al., 2001; Fish et al., 2001; Fish & Baudinette, 1999). Hence, different environments impose different energetic costs on movement, and energy savings accrued from moving in the metabolically more economical substrate may allow females to invest more into discriminate mate choice decisions.

Predators represent an inherent threat to survival, and compromising mate choice may be worthwhile when dangers become too severe (Gowaty & Hubbell, 2009; Lima & Dill, 1990; Sakaluk & Belwood, 1984). This leads to the general expectation that higher predation risk results in less discriminate mate choice decisions (Crowley et al., 1991; Real, 1990; Rosenthal, 2017; Sih, 1994), which has indeed been observed in a number of species. For example, predation threat leads to female *Poecilia reticulata* guppies and *Achroia grisella* wax moths reversing their mate preference and now preferentially approaching the less attractive yet less conspicuous male (Edomwande & Barbosa, 2020; Godin & Briggs, 1996; Gong & Gibson, 1996), and female *G. integer* crickets forgoing more attractive mates when less attractive ones can be approached under aerial coverage (Hedrick & Dill, 1993). Other species maintain discriminating mate choice decisions in the presence of predators but adjust their behavior in a way that reduces their own conspicuousness. For

example, *Ostrinia nubilalis* moths adjust the conspicuousness of their mate-seeking behavior to the degree of predation risk; pheromone release is strongly reduced under high predation risk but only slightly so under low-risk conditions (Acharya & McNeil, 1998). And female *Hyla versicolor* treefrogs maintain their willingness to invest in obtaining a more attractive mate (quantified as “choosiness”) under predation threat, but approach the male more cautiously, slower, and seeking out available cover (Feagles & Höbel, 2022a). Whether aquatic or terrestrial habitats pose greater predation risk likely varies with the focal species, but mate choice should be less discriminating and/or less conspicuous in the more “dangerous” environment.

Many amphibians spend a significant portion of their lives in terrestrial foraging habitats, but return to aquatic habitats for mating and laying eggs (Johnson & Semlitsch, 2003). The environment within the ponds themselves is often heterogeneous, not only comprising open water but also floating and emergent vegetation that provide “terrestrial-like” substrate for them to walk on (da Silva et al., 2011). We conducted acoustic playback experiments with *H. versicolor*, Eastern Gray Treefrogs, to investigate whether the approach substrate influences mate choice decisions. We had two overall aims for this study: (i) survey calling locations of male frogs at the pond to establish whether female mate choice involves mostly aquatic or terrestrial approach; (ii) compare mate preferences and associated approach behaviors of female Gray Treefrogs in aquatic and terrestrial environments.

We aimed to test two hypotheses related to whether the substrate for movement influences female mate choice behavior. Energy savings when swimming may allow females to invest more in mate choice (Baudinette et al., 2000). The “energy hypothesis” therefore predicts that when tested in water, females will show more discriminating preferences than when tested on land. Given the preference for longer duration calls in this species (Feagles & Höbel, 2022b; Reichert & Höbel, 2015), we predicted that female Gray Treefrogs will prefer longer calls and/or accept less deviation from their preferred call value (here quantified as selectivity, see methods). Quantitative studies on the predators of Gray Treefrogs are lacking, but a wide range of predators have been reported to consume this species, including predacious diving beetles, giant water bugs, bullfrogs, snapping turtles, snakes, raccoons, opossums, skunks, herons, owls and hawks (Cannizzaro, 2024; Dodd, 2023; Hamilton, 1951; Hinshaw & Sullivan, 1990; Kapfer & Brown, 2022). Predation by *Nerodia* water snakes, and aquatic giant water bugs (Fam. Belostomatidae) is especially prevalent in frogs including Gray Treefrogs (de Luna et al., 2022; Hinshaw & Sullivan, 1990; Toledo, 2005), and is regularly observed in our study population (pers. obs.). The increased predation risk in water may force females to compromise mate choice decisions. The “predation risk hypothesis” therefore predicts that when tested in water, females will show less discriminating preferences than when tested on land. Specifically, we predicted that females will accept shorter calls and/or accept more deviation from their preferred call value. An additional prediction associated with this hypothesis is that females may not change their preferences, but show behaviors that mitigate or minimize exposure to aquatic predators.

## 2 | METHODS

### 2.1 | Study species and study site

Eastern Grey Treefrogs (*H. versicolor*) are an arboreal species inhabiting woodlands in the eastern and central United States. These frogs spend most of their lives in terrestrial foraging and overwintering habitats. Telemetry data indicate that the terrestrial habitats they use can extend more than 300m around the breeding pond (Johnson et al., 2007). During the breeding season (late spring to early summer), frogs aggregate around small woodland ponds. Males may remain at the pond for several days, but generally do not participate in the chorus for the entire season (Bertram et al., 1996). Females only enter the pond when they are ready to mate, and leave towards their foraging grounds in the forest after oviposition. Males may call from anywhere in or around the pond (Sullivan & Hinshaw, 1992). Females approach males guided by their mating call, a behavior termed phonotaxis; on average, they prefer male calls with longer duration (Feagles & Höbel, 2022b; Gerhardt et al., 1996, 2000; Reichert & Höbel, 2015; Sullivan & Hinshaw, 1992). After pair formation, the female carries her mate to her chosen oviposition site in the water where she deposits multiple small batches of eggs until the full complement is laid. Eggs may or may not be attached to vegetation and initially float on the water surface (Vogt, 1981).

We studied a population of Gray Treefrogs at the University of Wisconsin's (UWM) Field Station in Saukville, WI. Breeding seasons at our study site last 3–6 weeks, but chorusing and breeding is intermittent and determined by temperature variation (Höbel, pers. obs.). During late May to early June 2023, we surveyed male calling locations at our study pond ( $n=124$ ), and collected females ( $n=50$ ) for acoustic playback trials. We collected females in amplexus with a male to assure female receptivity to the playback trials. We transported frogs (each pair in an individual small plastic container with water) to our lab at the UWM campus and placed them on melting ice to prevent oviposition (females do not respond to calls after laying eggs). We tested females within 3 days and subsequently released them at their home pond. We did not mark frogs, but we are confident that the risk of testing females multiple times is low. In our study population, only 14% of females breed more than once per

season, and it takes them on average 20 days to produce a second clutch (Höbel et al., 2021). The female collection period for the present study spanned 14 days.

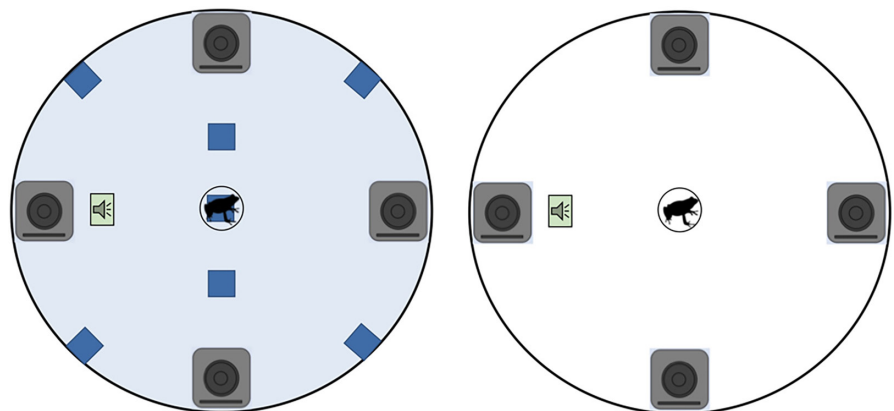
### 2.2 | Survey of male calling locations

To assess variation in approach substrate females have to cover to reach a mate, we surveyed the calling positions of male treefrogs at the same pond and during the same nights when we collected females for playback experiments. All males were within the water's boundaries of the pond, which is the typical distribution of calling males in our study population. We slowly walked circular laps inside and along the shore of the pond, looking for actively calling males. Once we located a calling male, we determined the proximity of the calling perch to the shoreline. We used a Foneso F100 Digital Handheld Rangefinder to measure the distance from the calling male to the closest shoreline location (in cm). We also estimated the relative proportions (in %) of walkable substrate (i.e., logs, floating algae mats and emergent vegetation) and swimmable substrate (i.e., open water and areas covered in duckweed) (see Figure 2 for examples) between each male and the shoreline. Females seeking mates at the pond are arriving from their surrounding foraging habitat in the forest and have traveled some unknowable distance by the time they reach the chorus/pond; we therefore attempted to standardize the male calling perch distance measure by setting the shoreline as the start line. We also noted the relative date of the breeding season, to assess whether there were seasonal changes in pond features and male calling locations.

### 2.3 | Female mate choice behavior

#### 2.3.1 | Approach substrate treatments

We assessed variation in female mate choice behavior (female preferences and approach behavior) using two substrate treatments: land and water. Both the land and water arenas were constructed out of large foldable dog pools (1.8 m diameter; 30 cm high; Jasonwell-B01I3DTB2S, Jiaxin).



**FIGURE 1** Two diagrams showing the water (left) and land (right) arenas. Three of the four speakers were “dummy” speakers to avoid providing females with visual cues associated with the sound source. The squares in the water arena indicate the position of the “posts” that were supporting the speakers and the base of the land arena.

The water arena was filled with water to a depth of 5 cm; this was deep enough that the frogs could not touch the bottom with their hind feet and had to swim to move around. The water was changed every 3 days. We placed 11 “posts” constructed out of small plastic containers filled with aquarium gravel inside the water arena (Figure 1). Four posts were distributed evenly around the arena perimeter and supported the speakers. One post was placed in the center of the arena and supported the female release box. Six were placed around the arena perimeter or halfway between center and perimeter and served as resting places for females during the water trials. All posts also served as support structures for the base of the land arena.

To convert from water to the land arena, we inserted two semi-circle foam boards (3 cm thick) on top of the water arena posts to create a level base, and then placed an identical folding pool on top of the foam boards (i.e., elevated but fitted inside the water pool). In the land arena, the speakers were placed in the same position, but we omitted the “posts” under speakers, release box and for resting (Figure 1).

### 2.3.2 | Playback setup

The experiments were conducted inside a semi-anechoic room; the arenas were set up in the center of the room. Inside the respective water/land arena, we placed four JBL Control 1Xtreme speakers, equally spaced from each other along the outer edge of the arena. Speakers were flush with the substrate (dry floor or water level, respectively). One randomized speaker played the stimulus, and the other three speakers were placed in the arena to eliminate potential confounding visual cues associated with the sound source. The call stimuli were played from a laptop computer and amplified using a Behringer Reference Amplifier (Behringer, A500 Model). The call amplitude was set to 85 dB SPL at the release point of the female using a Lutron SL-4001 Sound Level Meter (fast RMS, ‘C’ weighting).

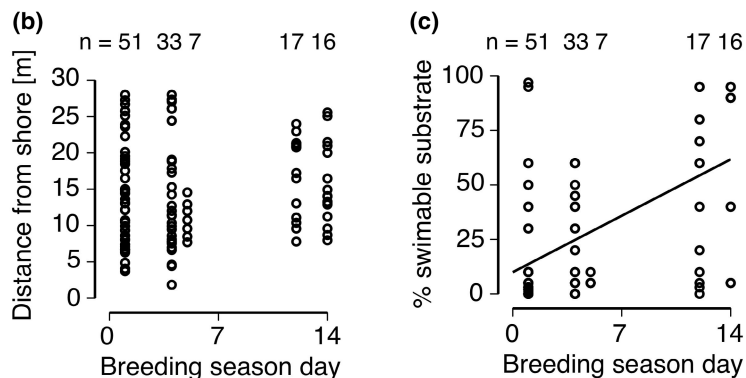
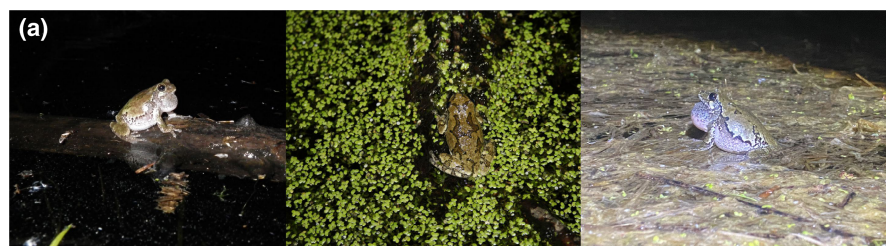
Females were tested in both the land and water arenas, but we randomly assigned half the females to start with the land treatment and half to start with the water treatment. Females were initially confined in a small release container in the center of the arena. After a stimulus had played three times, we remotely released the female by pulling a string that lifted the lid of the release container.

### 2.3.3 | Stimulus generation

The Gray Treefrog advertisement call consists of a series of short pulses. At 20°C, the average temperature of chorus formation of Gray Treefrogs, as well as our testing temperature, pulses are approximately 25 ms in duration, and are repeated after a pause of 25 ms (i.e., the duration of the call can be expressed in number of pulses or in milliseconds). We used the Seewave package (Sueur et al., 2008) in R (ver. 3.1.0) to generate a total of eight stimuli that differed only by the number of pulses (6–27 pulses, increasing by 3; the average in our study population is 18 pulses/call). All of the other characteristics of the synthetic advertisement calls mimicked the average call characteristics of the study population: call period (time from onset of one call to onset of following call) was 6 s. The call is composed of two frequency peaks: the high-frequency peak was 1071 Hz, and low-frequency peak was 2142 Hz (10 dB louder than low-frequency peak; Reichert & Höbel, 2015).

### 2.3.4 | Assessing call preferences

To test for female call duration preferences, we used a single-speaker design. We presented each female with eight stimuli varying in call duration (6, 9, 12, 15, 18, 21, 24 and 27 pulses, presented in random order). We used *approach latency* as the measure of



**FIGURE 2** Results of male call perch survey. (a) Representative examples of male calling locations showing variation in approach substrates. (b) Male perch-to-shoreline distances were variable but did not change across the breeding season. (c) The proportion of swimmable substrate required to traverse in order to reach a calling male increased over the course of the breeding season. Sample sizes are indicated in top of each panel; they reflect typical changes in chorus size across the season, not sampling effort. There were several cold spells during which no chorus formed.

preference, assuming that a faster approach indicates that the call is deemed more attractive (Bush et al., 2002). Approach latency, the time from the moment the top of the release container is lifted and the female is free to move to the time the female has reached the choice zone around the speaker, is the standard measure of preference in anuran mate choice trials (see Bosch et al., 2000; Gerhardt, 1992; Reichert & Höbel, 2015). In addition, we noted how long the females took to leave the (dry) starting platform after the lid had been lifted and they were free to move (“*exit time*”). From these we also calculated the “*active time*” [= approach latency – exit time], that is, the time between starting movement and reaching the speaker.

Call preferences are function-valued traits, that is, curves that describe female responses as a function of variation in call traits (i.e., Kilmer et al., 2017; Meyer & Kirkpatrick, 2005; Ritchie, 1996; Rodríguez et al., 2006). With the function-valued approach, the entire preference function is considered the trait of interest. We visualized preference functions using the program PFunc (Kilmer et al., 2017), which uses generalized additive models (GAMs) to fit nonparametric curves – termed cubic splines – to the data. This method makes no assumption about the shape of the functions other than that they should have some level of smoothness (e.g., it does not pre-specify a linear or quadratic shape but allows each function to be determined by the responses of the individual females). Smoothing parameters were determined empirically by manually adjusting smoothing to maximize the fit of the curve with the underlying data points. Note: In our assay, a strong preference for a given stimulus is expressed by a fast approach (a short approach latency). However, because interpretation of female preferences is more intuitive if a female's most preferred value is shown as the highest point in a curve, not the lowest, we converted raw latency data (in seconds) to a response score using the formula (Response score = 200s – approach latency) before generating preference functions in PFunc.

A total of 50 females contributed to the data set; each female was tested with eight call duration stimuli under each of two substrate treatments. Thus, each female provided two preference functions (one for each substrate treatment). We extracted two important values from the individual preference functions that describe the shape of the function in independent ways (Feagles & Höbel, 2022b; Fowler-Finn & Rodríguez, 2012). *Peak preference* is the call duration estimated to elicit the strongest female response. *Selectivity* summarizes variation in the shape of the preference other than peak, and describes how strongly females favor the preferred (peak) call duration relative to other values. PFunc extracts four preference function traits from the functions: peak (the most preferred stimulus value), strength (the difference in attractiveness between the peak and the least-preferred values), tolerance (the spread of acceptable values outside the peak), and responsiveness (the mean magnitude of response across the entire function). We used peak to describe Peak preference, and ran a principal component analysis to summarize strength, tolerance and responsiveness into a single trait corresponding to Selectivity (PC1; eigenvalue = 2.29, explaining 76% of

the variation; eigenvectors: tolerance = .60, strength = -.59, responsiveness = .54). Note: because of the way tolerance, strength and responsiveness load on the selectivity PC, negative values correspond to higher selectivity. Because interpretation of selectivity is more intuitive if positive values indicate higher selectivity, we changed the sign of the PC scores before plotting the data.

### 2.3.5 | Phonotaxis behavior

In addition to constructing preference functions from approach latency data (above), we obtained data on variation in three aspects of phonotaxis behavior: (i) *Exit time*, measured as the time it took for the female to leave the release point and start walking/swimming towards the speaker. We collected exit time data live from all trials using a stopwatch. (ii) *Jump distance*, defined as the distance (in cm) of the first jump the female took away from the release platform. To obtain the jump distance data, we video-taped the trials with the 18 pulse duration stimuli (from both the land and water treatments). We later took a screen shot at the point where the females took the first jump away from the release point, and used ImageJ (Schneider et al., 2012) to measure the jump distance from the screenshots. We opted to use the 18 pulse duration trials (i.e., not shorter or longer stimuli) for this analysis because this corresponds to the average call duration in our population. (iii) *Wall climbing*, defined as when a female placed all four feet on the arena wall and ascended or climbed horizontally. We collected wall climbing data live from all trials using notes and sketches of female movement trajectories.

## 2.4 | Statistical analysis

### 2.4.1 | Male calling locations

We provide descriptive statistics on the average ( $\pm$ SD) distance between male calling locations and the shoreline of the pond, as well as the proportion of walkable and swimmable substrate along this distance. We calculated Pearson correlation coefficients to test whether distance and substrate distributions changed over the course of the breeding season.

### 2.4.2 | Preference functions

We examined variation in female mate preference functions in response to variation in approach substrate in two ways. We first used a “whole data set” approach that included all female individual responses to each playback. For this approach, we used a linear mixed model (implementing REML) with the *approach latency* of each female to each stimulus as the dependent term. The model included the following fixed explanatory terms: treatment (land/water); linear and quadratic components for call duration; and the interactions



between treatment and these linear and quadratic call duration terms. In addition, we included trial sequence (1–16; two treatments  $\times$  eight trials) to account for potential fatigue effects, and days since capture (1–3) to guard against effects of having females wait on ice before being tested. We also included female identity (Fem ID) as a random term, to account for each female having provided 16 data points. In this model, the main term for treatment tests for overall differences in intercept (or elevation) between mate preferences across treatments. The main terms for call duration (linear and quadratic) test for overall linear slope and curvilinear shape components in the mate preferences. The interactions between treatment and the call duration terms (linear and curvilinear) test for differences in the shape of the mate preferences across treatments (i.e., differences in slope with the linear term, differences in curvature with the quadratic term). This approach allows only limited options for describing variation in the shape of mate preferences (assuming linear or quadratic shapes), but allows powerful significance testing with the whole data set (cf. Fowler-Finn & Rodríguez, 2012). We ran the same model for *exit time* and *active time* to test whether different aspects of phonotaxis were affected differently. Inspection of quantile–quantile plots showed that the *exit time* data did not meet normality assumptions. We therefore used a square root transformation for the *exit time* data.

Second, we used the peak preference and preference selectivity values from the individual mate preference functions generated with PFunc (see above) as dependent variables. Because peak preference and preference selectivity vary independently in Gray Treefrogs (Feagles & Höbel, 2022b), we ran separate models for each. Inspection of quantile–quantile plots and residuals against fitted values showed that the peak preference data did not meet normality assumptions, and we found no transformation that could improve the fit. We therefore used a model with a Poisson error distribution—we considered this appropriate because the lack of normality in these data arose from the playback stimuli being bracketed between 6 and 27 pulses (see above). We fit a GLMM model with treatment (land/water) as fixed effect, and Fem ID as a random term. For preference selectivity, we used a linear mixed model (implementing REML), with treatment (land/water) as fixed effect, and Fem ID as a random term. We entered female ID because each female had contributed two data points (one function tested on land and one in water).

### 2.4.3 | Phonotaxis behavior

To test whether aspects of the phonotactic behavior are affected by the approach substrate, we calculate a series of standard least squares models. To test how exit time was affected, we entered treatment as predictor variable and female ID as a random term. To test how jump distance was affected, we entered treatment as predictor term and female ID as a random term. To test whether more females climbed up the arena walls during one of the treatments,

and to test whether trials with wall climbing were more frequent in one of the treatments, we used  $\chi^2$  test. We conducted separate tests to examine the proportion of females performing wall climbing and the proportion of trial with wall climbing, to ensure that the result of the latter was not biased due to some females showing unusually high rates of climbing behavior. All statistical tests were implemented in JMP 11 (SAS Institute Inc., Cary, NC).

## 2.5 | Ethical note

All procedures were approved by the Animal Care and Use Committee of the University of Wisconsin Milwaukee (Protocol Number: 22-23#29). All frogs were released unharmed at site of capture.

## 3 | RESULTS

### 3.1 | Survey of male calling locations

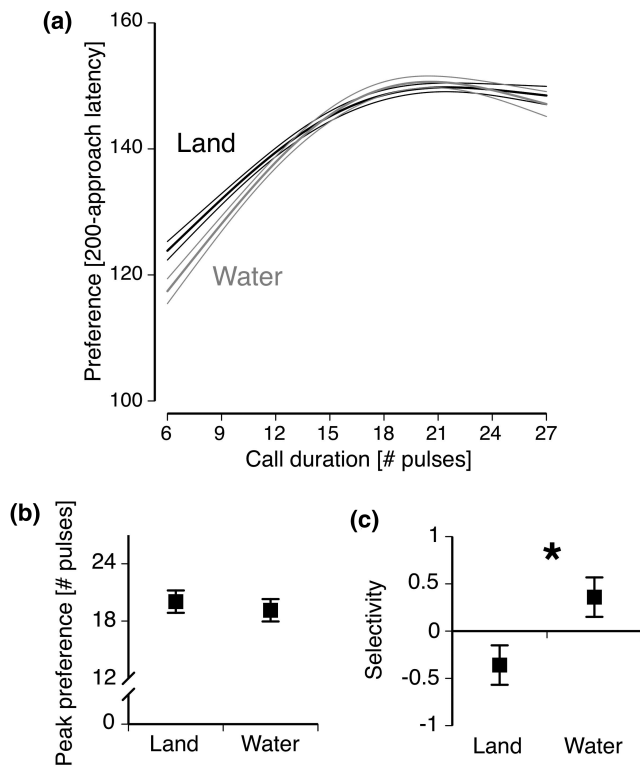
During this study, all males were within the water's boundaries of the pond. In previous years, we occasionally observed males calling from branches of trees at the margin (but overhanging the water), but this did not occur during our sampling period. Throughout the breeding season, males were calling from a variety of distances from the shore and almost all required a mix of swimming and walking to be approached by females (Figure 2). The average ( $\pm$ SD) distance from the shore at which males were calling was  $14.8 \pm 6.8$  m (range: 1.8–28 m), and this did not change over the course of the breeding season (Figure 2b;  $r(122) = .15$ ;  $p = .08$ ). The average ( $\pm$ SD) proportion of swimmable versus walkable substrate between the shoreline and calling males was  $29 \pm 33\%$  (range: 0%–97%) swimmable substrate (open water or duckweed). This aspect of the male call locations did change over the course of the breeding season: the relative amount of water to cross to reach a calling male increased (Figure 2c;  $r(122) = .54$ ,  $p < .0001$ ). This was because the floating algae mats (that supported frogs and allowed for walkable approach; see Figure 2a) that were very prevalent at the beginning of the season began to sink and disappear as the season progressed.

### 3.2 | Variation in females mate preferences

Females preferred longer duration calls (Figure 3a; Table 1, significant call duration terms), irrespective of approach substrate (Table 1, non-significant treatment term). Using approach latency or active time as the test variable yields similar results: call duration affects female preferences, but substrate treatment does not (Table 1, left and center).

Analysis of female preferences derived from individual preference functions showed that average peak preferences did

not differ between treatments (GLMM:  $F_{1,42.0} = .004$ ,  $p = .95$ ; Figure 3b), but that females were on average more selective when approaching the sound source in water (LSM:  $F_{1,49} = 7.51$ ,  $p = .009$ ; Figure 3c). The somewhat steeper slope of the water-treatment preference function (see Figure 3a) is consistent with this difference in selectivity.



**FIGURE 3** Female preferences as a function of different approach substrates. (a) Females prefer longer duration calls, irrespective of whether they approached the speaker on land (black line) or in water (gray line); Shown are average  $\pm$  95% CI. (b) Average ( $\pm$ SE) peak preferences did not differ between treatments. (c) Average ( $\pm$ SE) selectivity was higher during the water treatment. Significant differences indicated with asterisk.

**TABLE 1** Results of three linear mixed model (implementing REML) examining the effect of variation in approach substrate on preference for call duration (using approach latency or active time data) or on phonotaxis behavior (using exit time) in female Eastern Gray Treefrogs.

Factor	Approach latency			Active time			Exit time		
	df	F	p	df	F	p	df	F	p
Treatment	1,713.8	.38	.54	1,714.2	.05	.82	<b>1,713.3</b>	<b>3.98</b>	<b>.046</b>
Call Dur	<b>1,713.7</b>	<b>23.65</b>	<b>&lt;.001</b>	<b>1,714.1</b>	<b>17.19</b>	<b>&lt;.001</b>	<b>1,713.3</b>	<b>11.45</b>	<b>.001</b>
Call Dur $\times$ Call Dur	<b>1,714.1</b>	<b>5.97</b>	<b>.01</b>	1,714.6	2.12	.15	<b>1,713.5</b>	<b>8.96</b>	<b>.003</b>
Treat $\times$ Call Dur	1,713.7	.68	.41	1,714	.47	.50	1,713.3	.07	.79
Treat $\times$ Call Dur $\times$ Call Dur	1,714.1	.41	.52	1,714.6	.16	.69	1,713.5	.17	.68
Sequence	15,713.8	.66	.82	15,714.1	.95	.51	<b>15,713.3</b>	<b>3.12</b>	<b>&lt;.001</b>
Days since capture	2,47.9	1.32	.28	2,48.2	2.01	.15	2,47.4	1.00	.38

Note: Approach latency = time from opening release cage to female reaching sound source; Active time = time from female leaving the starting platform to female reaching sound source; Exit time = time from opening release cage until female leaves starting platform; Approach latency = Exit time + Active time. Significant terms are set in bold.

### 3.3 | Differences in phonotaxis behavior

Overall, there was a clear effect of substrate treatment on phonotaxis behaviors. Females that had to swim instead of walk towards the speaker took significantly longer to leave the starting point (Figure 4a; Table 1, right; significant effect of treatment on exit time). They also took longer to leave when the stimulus was shorter (Table 1, right; significant effect of call duration), and during their first 1–2 trials (Table 1, right; significant sequence effect).

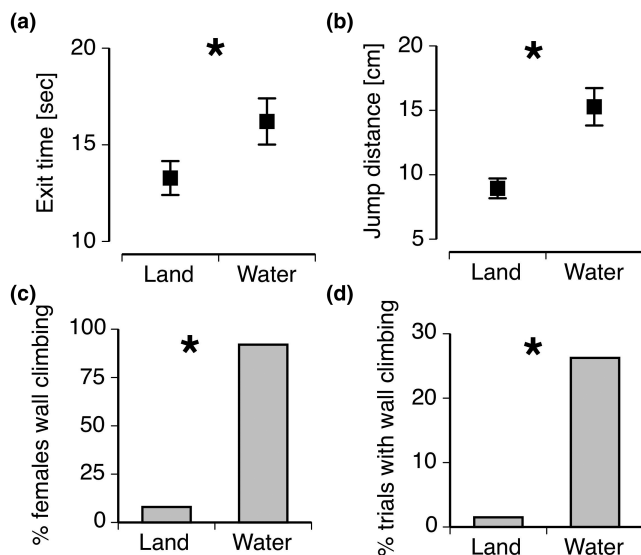
Females in the water treatment took longer leaps when leaving the starting area (Figure 4b;  $F_{1,49.9} = 16.2$ ,  $p = .0002$ ). The proportion of females that engaged in wall climbing was higher in water compared to land (Figure 4c;  $\chi^2 = 62.1$ ,  $df = 1$ ,  $p < .0001$ ), and the frequency of trials with wall climbing was also higher in water than on land (Figure 4d;  $\chi^2 = 102.5$ ,  $df = 1$ ,  $p < .0001$ ).

This means that the number of females that engaged in wall climbing at some point during the experiment was higher in the water treatment. It also means that there was an overall higher number of water trials where females showed wall climbing (each female participated in 16 total trials).

## 4 | DISCUSSION

The response of female Gray Treefrogs to variation in the features of the breeding habitat offers intriguing insights into the complex interplay between environmental context and mate choice. Approaching a potential mate in water may offer energy savings (Baudinette et al., 2000), but also carries increased predation risk (Hinshaw & Sullivan, 1990). How do female treefrogs balance these costs and benefits?

We tested two hypotheses related to whether the substrate for movement influences treefrog mate choice behavior: (i) the “energy hypothesis” predicts that energy savings accrued from approach via the energetically favorable locomotion mode of swimming will result in more discriminating mate choice (i.e., preference for longer calls and/ or higher selectivity) in the water treatment, while (ii) the



**FIGURE 4** Differences in phonotaxis behaviors during trials on land and in water. (a) Females took longer to leave the starting platform (exit time) during trials conducted in water (mean  $\pm$  SE). (b) Females took longer leaps from the starting platform when they had to swim to the speaker (mean  $\pm$  SE). (c) More females engaged in wall climbing in the water treatment. (d) Wall climbing was more prevalent during trials in the water treatment. Significant effects are indicated with asterisk.

“predation risk hypothesis” predicts that the abundance of predators in the aquatic environment will result in less discriminating mate choice (acceptance of shorter calls and/or lower selectivity) in the water treatment, or if females maintain discriminating mate choice in water, they will show behaviors that mitigate or minimize exposure to aquatic predators.

While our findings supported predictions from both hypotheses, the majority of the results favor the “predator avoidance hypothesis”. Females were more selective in the water treatment, which is one of the predictions of the “energy hypothesis”. However, in the water treatment females frequently approached the speaker while climbing on the arena walls (i.e., waking not swimming), calling into question whether the predicted energy advantage of swimming was consistently realized in this treatment. Further, while we had predicted that higher selectivity would be more connected to energetic benefits than predation risk, it is possible that females weighed those two factors differently and showed higher selectivity in response to increased perceived predation risk. This would be a novel response to perceived predation risk, as to our knowledge predation risk tends to result in less discriminating mate choice (i.e., Edomwande & Barbosa, 2020; Godin & Briggs, 1996; Gong & Gibson, 1996; Hedrick & Dill, 1993).

Findings related to changes in phonotaxis behavior are in line with predictions from the “predation risk hypothesis”, since they seem geared towards minimizing time spent in water. In the water treatment, females were reluctant to enter the water (longer exit time and further jump distance) and they took advantage of features of the playback setup (i.e., the swimming pools were surrounded by

walls) to leave the water and approach the speaker “on land” (more wall climbing behavior). Overall, the results suggest that rather than compromising on their preferences, females made subtle adjustments to their behavior that may mitigate the higher predation risk in water.

The consistency in average peak preference across both treatments indicates a robust acoustic preference in this species, underscoring the importance of call duration as a key determinant in female mate selection. This preference is consistent with previous literature on Gray Treefrog call preferences (Gerhardt, 2005; Gerhardt et al., 2000; Schwartz et al., 2001). It is also in line with the observation that variation in nocturnal light levels does not affect call duration preferences in this species (Underhill & Höbel, 2017, 2018). While the breeding season of Gray Treefrogs spans several weeks, chorus formation, and hence, reproduction, is predominantly determined by favorable temperatures and rendered intermittent in Wisconsin due to frequent cold spells. Environmental conditions that are unrelated to breeding temperature optima, like moon phases or pond water level/vegetation structure may be too stochastic to select for differential female mate choice behavior. The implications for sexual selection are that irrespective of environmental variation, female mate choice exerts directional sexual selection on male call duration, and males with longer calls are expected to have higher mating success.

Many treefrogs, including Gray Treefrogs, aggregate around ponds to advertise for mates and do lay eggs in water. Despite the pronounced reliance on water for reproductive activities in nature, laboratory mate choice trials are predominantly conducted on dry substrates in terrestrial setting. Phonotaxis is robustly expressed under such laboratory conditions, greatly facilitating the study of anuran mate choice behavior (i.e., Gerhardt et al., 2000; Höbel & Gerhardt, 2003; Ryan & Rand, 1990). Our results suggest that terrestrial trials are a suitable method to assess peak call preferences that can suggest the direction of sexual selection on male traits, but because selectivity was somewhat higher in the water treatment, terrestrial trials may underestimate the strength of selection.

Data on male calling perch distribution further elucidates the challenges females face in reaching mates, particularly the considerable distances they must cover. Males are, on average, 15 m from shore (equivalent to 300 times a female's body length) and frequently require crossing of large amounts of open water for females to reach their chosen mate. Certain structural elements like floating logs or emergent/floating vegetation may offer walkable approach avenues, but females likely always have to swim some distance to access potential mates. It will be interesting to explore whether attractive males are randomly distributed in the breeding habitat or occupy perches closer to the shoreline, as well as whether females take environmental features into account when approaching a mate. Do they take less direct routes if a “walkable” approach is possible? We used single speaker trials to assemble call duration preference functions, but another frequently used assay in anuran mate choice research is to use choice trials where females indicate their preference by approaching one of two presented alternatives (i.e.,



Bee, 2008; Gerhardt, 2005). A potential follow-up study could test whether “walkable” approach routes associated with one speaker modifies female preferences. This has been observed in *H. marmoratus* reed frogs. When females were presented with two identical call stimuli but one was associated with vertical wooden dowel sticks allowing for arboreal approach while the other speaker was only accessible by crossing an open area on the ground, females preferentially approached the stimulus associated with suitable perches (Backwell & Passmore, 1990). Moreover, the preference for calls broadcast at higher intensity (Dyson, 1985) as well as for calls of lower frequency (Dyson & Passmore, 1988) was abolished when the less attractive alternative was paired with suitable approach perches (Backwell & Passmore, 1990). Female Gray Treefrogs may show similar trade-offs between preferences for calls and preferences for habitat features.

Our study uncovered habitat-dependent variation in female Gray Treefrog mate choice behavior. The observed consistency in peak preference for longer duration calls suggests a stable acoustic criterion for mate choice, while the context-specific variations in selectivity and phonotaxis behaviors minimizing time spent in water highlight the dynamic interplay between mate choice and environmental challenges. These findings contribute to our understanding of the adaptive nature of anuran behaviors in response to diverse ecological contexts, shedding light on the intricacies of mate choice variation in Gray Treefrogs. Further research is warranted to explore the impact of ecological conditions on mate choice and male call site selection to elucidate their broader implications for sexual selection.

#### AUTHOR CONTRIBUTIONS

**Hannah Warner:** Conceptualization; writing – original draft; investigation; funding acquisition; visualization. **Gerlinde Höbel:** Conceptualization; data curation; formal analysis; methodology; supervision; visualization; writing – review and editing.

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#### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT

Data available from the FigShare Digital Repository <https://doi.org/10.6084/m9.figshare.26367268.v1>.

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