

RESEARCH ARTICLE

Female gray treefrogs, *Hyla versicolor*, are responsive to visual stimuli but unselective of stimulus characteristics

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ABSTRACT

The visual ecology of nocturnal anurans is poorly understood, but there is growing evidence that vision plays a role in important behaviors such as mate choice. While several recent studies have demonstrated that females are responsive to visual cues when selecting mates, the forces responsible for these preferences are unknown. We investigated the responsiveness of female gray treefrogs, *Hyla versicolor*, to video playbacks of calling conspecific males in which we varied attributes of the vocal sac, a conspicuous visual characteristic of calling males and a target of female choice in other species. Females responded surprisingly strongly to the video playbacks, but did so indiscriminately with respect to variation in vocal sac characteristics. We followed up on these results with a series of additional tests that examined female responses to abstract stimuli. Females continued to respond to such stimuli, leading us to conclude that their behavior was related to a generalized phototactic response. Because of this, we were unable to make conclusions regarding female preferences for vocal sac characteristics. Nonetheless, our results are significant in two respects. First, we illustrate that despite much effort into improving video playback methodologies, challenges remain, and we offer our experimental design as a method to ensure that appropriate conclusions can be drawn from such studies. Second, we argue that the female phototactic response has potentially significant behavioral implications and in general the consequences of anuran visual preferences deserve further investigation.

KEY WORDS: Video playback, Vision, Anuran, Multimodal, Phototaxis

INTRODUCTION

The visual ecology of nocturnal anurans is relatively poorly understood (Roth et al., 1998). While an understanding of nocturnal vision is well advanced in other taxa (Warrant, 2004), studies of anurans have focused primarily on the use of acoustic cues for important processes such as orientation and mating (Gerhardt and Huber, 2002). Nevertheless, many nocturnal anurans are responsive to visual cues, for example by showing positive or negative phototaxis (e.g. Hailman and Jaeger, 1974; Jaeger and Hailman, 1971; Jaeger and Hailman, 1973). Furthermore, evidence is accumulating that vision plays a role in the expression of behaviors such as orientation, predation and predator avoidance, and mate selection in nocturnal anurans (e.g. Landreth and Christensen, 1971;

Aho et al., 1993; Buchanan, 1998; Gomez et al., 2009). The study of vision is challenging in any system because it is difficult to present stimuli that replicate the salient visual features of objects in nature as perceived by the study organism (Bennett et al., 1994; Endler, 1990). The generalized phototactic response of anurans presents significant challenges in this respect (Jaeger and Hailman, 1971), and experimental lighting conditions have been shown to alter frogs' activity levels, their capacities to perform vision-mediated behaviors and their responses to stimuli in other modalities (Buchanan, 1993; Jaeger and Hailman, 1981; Rand et al., 1997). Nevertheless, a variety of approaches to the study of visually mediated behaviors in anurans have been utilized successfully, and many studies have focused in particular on the effects of visual cues and signals on female mate choice (Hödl and Amézquita, 2001). In this study, we measured the responses of reproductive females to video playbacks in the gray treefrog, *Hyla versicolor* LeConte 1825. Video playbacks are a widely used but difficult to implement methodology for the study of animal vision, and we illustrate particular hazards to their use that remain underappreciated, even after many reviews of this methodology (e.g. Oliveira et al., 2000).

Our initial motivation for this study was to measure female preferences for visual stimuli in *H. versicolor*. We based our experimental design on the findings of several previous video playback studies in anurans that females attend to characteristics of male vocal sacs, which are inflated concurrently with sound production (Gomez et al., 2009; Richardson et al., 2010; Rosenthal et al., 2004). The large size and rapid inflation of vocal sacs may improve females' abilities to localize potential mates (Preininger et al., 2013; Rosenthal et al., 2004; Taylor et al., 2008). The understanding of female attraction to visual cues such as vocal sac inflation has important consequences for sexual selection (e.g. Richardson et al., 2010; Gomez et al., 2011), and we therefore designed our study to measure female preference functions for different vocal sac characteristics. Because the vocal sac is inflated for the duration of the call, the duration of visible inflation of the vocal sac could provide redundant information to females on call duration, a frequent target of female choice in the acoustic modality (Gerhardt et al., 2000). In addition, the size of the vocal sac is likely to be correlated to overall male body size, a characteristic that is often correlated with the dominant frequency of acoustic signals (Gingras et al., 2013), but also potentially assessed visually by females. Finally, certain characteristics of vocal sacs may be signals that are entirely independent of the qualities assessed in the acoustic modality. In particular, vocal sacs can differ in color and brightness, and this quality, while not obviously tied to any acoustic characteristic, may also be involved in female attraction (Gomez et al., 2009; Sztatecsny et al., 2010).

We also performed an extensive series of control tests designed to evaluate female responsiveness to visual stimuli in general. As with any experimental paradigm, inferences on the relevance of a behavior in nature that are based on the response of an animal to

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experimental stimuli are strengthened when it can be confirmed that the responses are not artefacts of the experimental design itself. This problem is particularly challenging in video playback studies because the technology used to present video stimuli to animals may not match the intended properties of the stimulus to the animal's visual system (Fleishman and Endler, 2000; Fleishman et al., 1998). Nonetheless, many studies have used video playbacks successfully to elicit natural behaviors (e.g. Trainor and Basolo, 2000; Ord et al., 2002; Robinson-Wolrath, 2006), including some on nocturnal anurans (e.g. Rosenthal et al., 2004; Gomez et al., 2009). Another reason that a large number of controls were needed relates to a specific interaction between the properties of the anuran visual system and the video playback procedure. In particular, the majority of anuran species studied show a generalized, usually positive, phototactic response (Jaeger and Hailman, 1973). Because video monitors present images by projecting light, it is important to experimentally separate females' responses to the specific video stimuli of interest from their responses to the glowing monitor itself. These controls are crucial, but are not always performed in video playback studies. Thus, the significance of this study goes beyond the understanding of sexual selection to encompass generalized aspects of anuran visual ecology and the appropriateness of a commonly used experimental design.

Our study species, *H. versicolor*, is a common treefrog in eastern North America. Males gather in nightly choruses around small ponds during the breeding season. Males produce conspicuous advertisement calls with a single subgular vocal sac. Interestingly, the spectral composition of the throat region is sexually dimorphic in this species. The throats of males tend to be dark gray to black, while the throats of females tend to be white or light gray (G.H., personal observation). A previous study found that male courtship behavior can be elicited by visual cues (Reichert, 2013). Thus, we hypothesized that females would be responsive to the visual cues of a simulated calling male. Our results, while demonstrating that females show a consistently high responsiveness to visual stimuli, serve to underscore the complexities associated with the use of video playbacks in animals.

RESULTS

Responses to variation in vocal sac characteristics

We first tested whether females would respond differently to video playbacks that were animated to simulate calling males with different vocal sac characteristics. Three separate characteristics were varied, and we found no evidence that females' responses to the videos were based on their preferences for certain characteristics of vocal sacs. We first tested whether females were differentially attracted to brighter vocal sacs by presenting them with stimuli in which the appearance of the vocal sac of a calling male frog animation was manipulated along the grayscale (white, light gray, medium gray, dark gray, black). Female gray treefrogs readily approached these video animations, but the proportion of females responding ($N=110$ observations of 22 females, $Z=-1.70$, $P=0.09$; Fig. 1A), as well as the latency to respond ($F_{4,48,2}=2.03$, $P=0.10$; Fig. 1B), did not depend on the brightness of the male's vocal sac. Females remained unselective to vocal sac brightness when videos were played along with chorus noise ($N=105$ observations of 21 females; proportion of females responding: $Z=1.49$, $P=0.14$; response latency: $F_{4,57,1}=0.37$, $P=0.83$; Fig. 1C,D). However, females were more likely to respond, and responded more quickly, when chorus noise was played with the videos compared with videos alone ($N=200$ observations of 20 females; proportion of females responding, brightness: $Z=-1.56$, $P=0.12$; acoustic treatment: $Z=-2.00$, $P=0.045$; response latency, brightness: $F_{4,115,6}=1.85$, $P=0.12$; acoustic treatment: $F_{1,123,5}=7.30$, $P=0.008$). Two-choice trials corroborated the results of single-stimulus trials (Fig. 2). Although latency to respond was significantly shorter for the medium versus light gray vocal sac stimuli (Fig. 2B), the effect was weak and the lack of differences in all other comparisons suggests that females do not show strong preferences for vocal sac brightness.

We then tested whether females exhibited preferences for vocal sac size by presenting them with stimuli in which the size of the vocal sac of the calling male animation was manipulated (no vocal sac, small vocal sac, medium vocal sac, large vocal sac and extra-large vocal sac). In single-stimulus trials, females readily approached these video animations, but there were no differences in

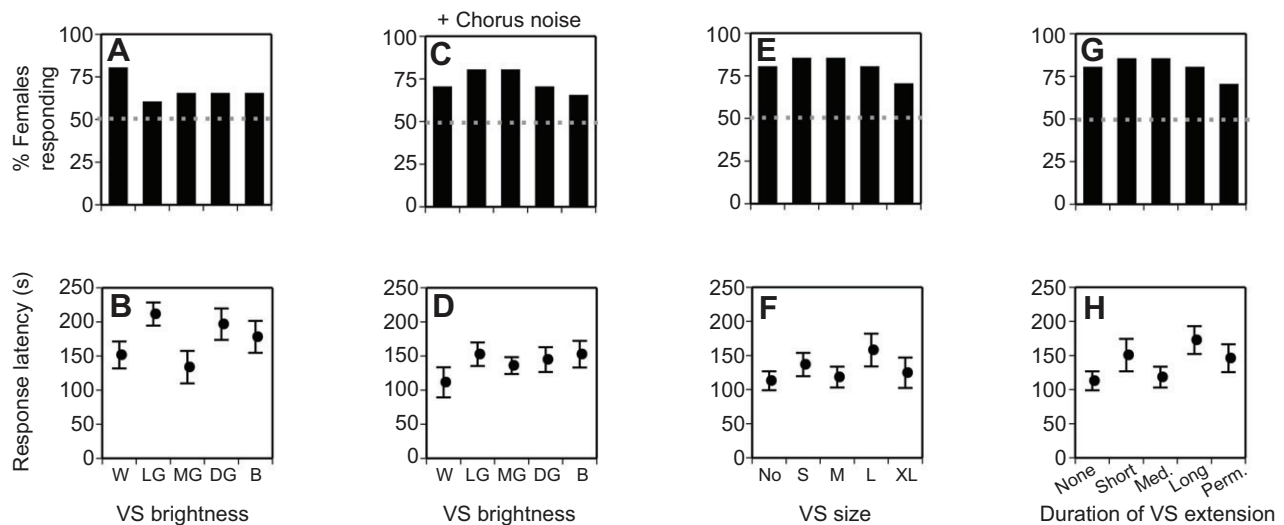
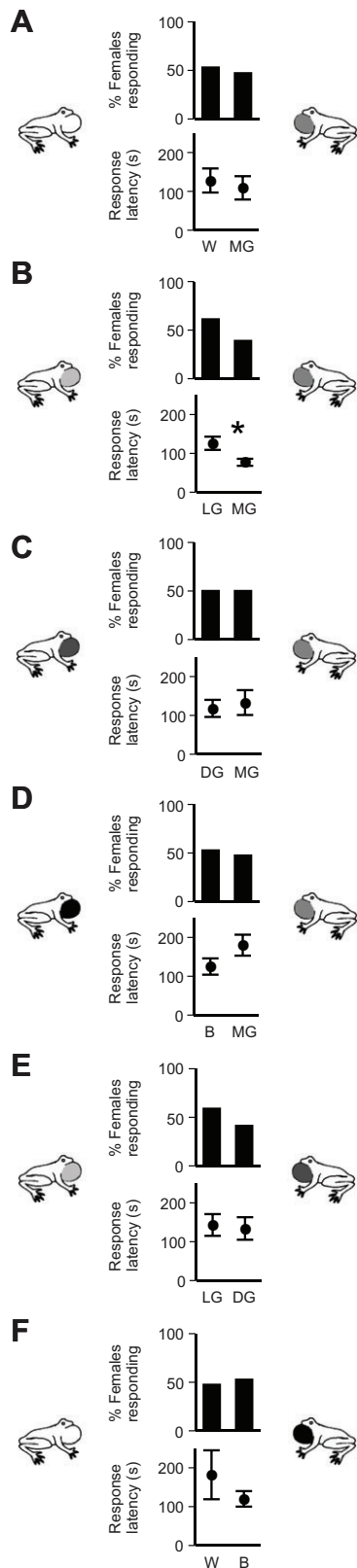


Fig. 1. Female responses to single-monitor video playback tests. Top row shows the proportion of females tested that met our response criteria (see Materials and methods). Bottom row shows the mean \pm s.e. of response latency for those females that responded to each stimulus. (A,B) Response to stimuli varying in the brightness of the vocal sac (referred to by color, see Materials and methods: W, white; LG, light gray; MG, medium gray; DG, dark gray; B, black). (C,D) Response to stimuli varying in the brightness of the vocal sac presented simultaneously with overhead chorus noise. (E,F) Response to stimuli varying in vocal sac size (No, no vocal sac; S, small vocal sac; M, medium vocal sac; L, large vocal sac; XL, extra-large vocal sac). (G,H) Response to stimuli varying in the duration of extension of the vocal sac (none, no vocal sac extension; short, short duration; med., medium duration; long, long duration; perm., permanent extension of vocal sac).



their responses to different-sized vocal sacs (proportion responding: $N=114$ observations of 23 females, $Z=-1.06$, $P=0.29$; latency to respond: $F_{4,57.6}=0.99$, $P=0.42$; Fig. 1E,F).

Finally, we tested whether females exhibited preferences for the duration of inflation of the vocal sac of the animated calling frog (no vocal sac, short duration, average duration, long duration,

Fig. 2. Female responses to two-monitor video playback tests. The brightness of the vocal sac of the male on each monitor was varied. For each pair of stimuli tested, graphs show the percentage of females that chose each monitor and their response latency. Drawings flanking the graphs illustrate the particular combination of vocal sac brightnesses tested; data points on the left side of graphs correspond to the stimulus depicted on the left, those on the right side correspond to the stimulus depicted on the right (W, white; LG, light gray; MG, medium gray; DG, dark gray; B, black). Asterisks indicate female responses that were significantly different between the stimuli (ANOVA; LG versus MG, $P=0.047$; all other cases $P\geq 0.12$).

permanently extended vocal sac). Females also responded readily to these single-monitor video animations, but again showed no significant differences in their responses to these stimuli (proportion responding: $N=114$ observations of 23 females, $Z=-0.78$, $P=0.44$; latency to respond: $F_{4,53.3}=1.9$, $P=0.13$; Fig. 1G,H). The two-choice trials corroborated the results of single-stimulus trials: similar proportions of females responded to stimuli differing in vocal sac extension duration (Fig. 3A). However, in the trial testing a moving vocal sac with an average inflation duration against a permanently inflated one, females showed a faster response to the moving stimulus (Fig. 3B).

Responses to abstract images and playback monitor

To better understand the pattern of female responses observed during the previous experiment, we performed additional trials to determine the range of video stimuli to which females are responsive. These trials revealed that females approach abstract shapes, and that the glowing video monitor itself also elicits responses. During single stimulus trials, 45–76% of females approached the various abstract shapes that were presented. Static and moving shapes were equally effective at attracting females (Fig. 4). Compared with the standard frog video stimulus, females were equally likely to approach a black glowing screen, but fewer females approached a plain white screen, and females almost never met our response criteria when the monitor was powered off (Fig. 4). The response to the powered-off monitor did not differ from the proportion of females expected to enter the choice area by chance, calculated based on the diameter of the choice area relative to the circumference of the arena (binomial test: $P=0.12$). Females that approached the control stimuli did so at latencies that were comparable to those of their approach to frog videos (Fig. 4).

During two-choice trials there was no significant difference in the likelihood of females approaching the frog video or a glowing blank screen, although there was a tendency for females to favor the frog video stimulus (Fig. 5A). In contrast, females showed a shorter latency to respond when approaching a blank screen compared with an animation of a calling frog (Fig. 5A). Further, a white square was preferred over the frog video, but a gray square was not (Fig. 5C,D).

DISCUSSION

Our results clearly demonstrate that female gray treefrogs oriented towards the video playback stimuli. The strength of this response was surprising because most tests did not include an auditory stimulus. The use of auditory stimuli for mate localization by female anurans is an important and well-developed component of their reproductive behavior (Gerhardt and Huber, 2002; Wells, 2007). In contrast, the role of vision in mating behavior in nocturnal anurans has only recently been emphasized (e.g. Taylor et al., 2008; Gomez et al., 2009), and it is unclear whether vision contributes significantly to mate localization. Nonetheless, visual cues may be of general importance for orientation in this and other contexts (Sinsch, 2006). The results of our experimental manipulations of

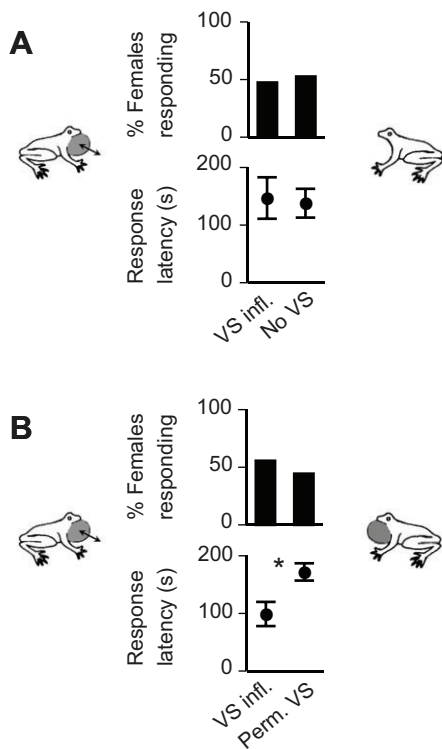


Fig. 3. Female responses to two-monitor playback tests. (A) An inflating vocal sac (VS infl.) versus an absent vocal sac (no VS). (B) An inflating vocal sac versus a permanently inflated (i.e. non-moving) vocal sac (perm. VS). Asterisks indicate female responses that were significantly different between the stimuli (ANOVA; $P=0.016$). Interpretation of axes and drawings as in Fig. 2.

vocal sac characteristics, when considered along with the control tests, were such that we are unable to make conclusions on the importance of visual cues in mate selection or localization. Below we discuss other potential explanations for these results and explore the methodological challenges associated with studying behavioral responses to visual cues in anurans.

Video playbacks in anurans

We designed this study to measure female preferences for visual characteristics of male vocal sacs. Previous video playback studies in anurans had demonstrated female attendance to vocal sac characteristics. For instance, in *Physalaemus pustulosus*, females presented with a video of a calling male along with acoustic playback of advertisement calls preferred videos in which the male inflated its vocal sac over those in which the male did not call or in which a rectangle was animated to mimic the size and temporal characteristics of a vocal sac (Rosenthal et al., 2004). In *Hyla arborea*, females in two-choice video playback tests showed preferences for more colorful vocal sacs (Gomez et al., 2009), and female preferences for acoustic characteristics were sometimes modulated by characteristics of males' vocal sacs in video playbacks (Richardson et al., 2010).

Thus, it was unexpected that our study found no evidence for female preferences for vocal sac characteristics in *H. versicolor*. It may be that females simply do not exhibit such preferences in this species. One key methodological difference between our study and previous studies is that most of our playback stimuli did not include an acoustic component. We purposely excluded acoustic stimuli because we were interested in females' responses to visual stimuli

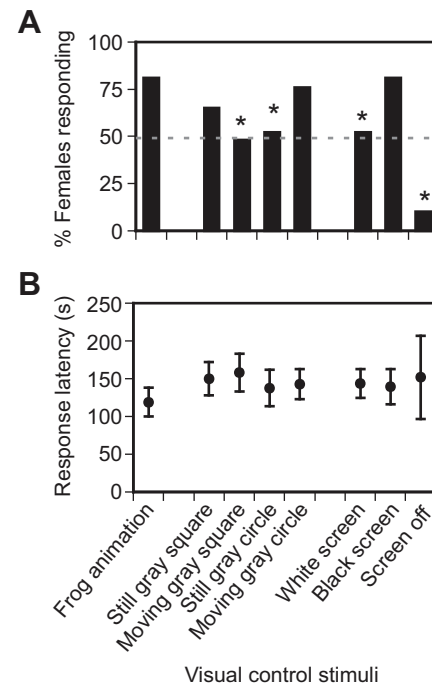


Fig. 4. Female responses to single-monitor control playbacks. Variables as in Fig. 1. Control stimuli are described in the Materials and methods. For comparison, a female response to the standard frog animation is also shown. Asterisks indicate female responses that were significantly different (ANOVA, $P<0.05$) between control stimuli and the standard frog animation.

alone, to provide the baseline for an understanding of multimodal signal interactions. A study of multimodal signaling in *Bombina orientalis* found that playing only a video of a frog elicited no response, although a video combined with an acoustic stimulus was effective (Zeyl and Laberge, 2011). Thus, the coupling of the video with an acoustic stimulus may be necessary for females to pay attention to the visual stimulus and perhaps to recognize it as a calling male. In *H. versicolor*, as in most frogs, vocal sac inflation is an automatic byproduct of acoustic signaling. Thus, if there is a visual signaling function of vocal sac inflation, the resulting multimodal signal is a fixed composite signal (Partan and Marler, 2005) and is potentially unrecognizable to females in the unimodal, visual form. Additional tests with video stimuli coupled with call playbacks would be useful to resolve this issue. However, we note that in our experiment females did not discriminate between different visual stimuli when these were accompanied by conspecific chorus noise. Our control tests provide a more likely explanation. That is, because females were essentially equally responsive to any video image presented to them, including images of not only male frogs but also abstract objects and glowing but otherwise blank screens, the responses we measured were probably unrelated to mate localization per se. Rather, the responses of females in these trials probably reflect the general tendency of anurans to approach a light source (Hailman and Jaeger, 1974; Jaeger and Hailman, 1971; Jaeger and Hailman, 1973). We consider it unlikely that we failed to find evidence for female discrimination of visual stimuli because our protocol did not simulate an appropriate mating context. In all cases we used gravid, and thus motivated, females that were initially tested in response to advertisement calls. Furthermore, the results of the visual cues in chorus noise experiment demonstrate that even with the auditory stimulation of a mating chorus, females showed phototaxis but failed to discriminate between visual stimuli. There are many other

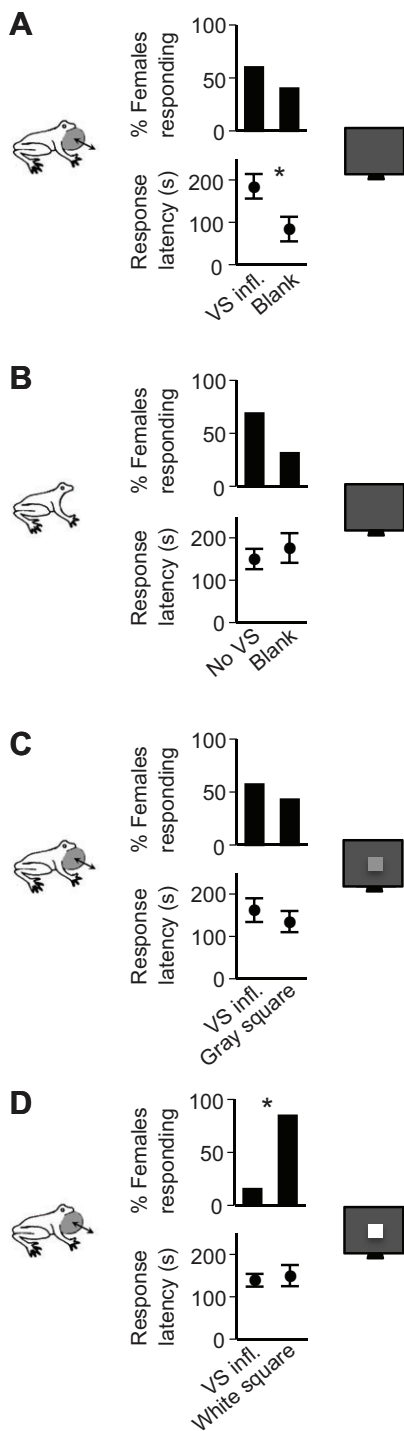


Fig. 5. Female responses to two-monitor control playbacks.

Interpretation of axes and drawings as in Fig. 2. (A) Frog with animated vocal sac (VS infl.) versus a blank but powered-on screen (blank). (B) Frog with no vocal sac (no VS) versus a blank but powered-on screen. (C) Frog with animated vocal sac versus a gray square. Asterisks indicate female responses that were significantly different between the stimuli (proportion of females responding: binomial test; $P=0.023$ for D, all other cases $P\geq 0.21$; latency: ANOVA; $P=0.023$ for A, all other cases $P\geq 0.30$).

combinations of auditory and visual stimuli that could be tested, and interactions between these modalities are known to be important in anurans (Narins et al., 2003; Taylor et al., 2011). However, the

primary aim of our study was to determine whether females responded to, and discriminated between, visual stimuli alone. Females' discrimination abilities may also have been weakened by our use of red light to search for them between trials (Buchanan, 1993). However, we consider this an unlikely explanation for our results because females were clearly able to rapidly detect the rather dim visual stimuli, in contrast to Buchanan's (Buchanan, 1993) finding that some measures of foraging performance were reduced following exposure to red light in the closely related *Hyla chrysoscelis*.

This phototactic response provides significant challenges to the interpretation of video playbacks because even if females exhibit a differential response towards certain stimuli, they may do so for reasons related to general perceptual biases towards video images that do not necessarily share anything in common with the natural features the videos are supposed to represent (Oliveira et al., 2000). An important difficulty with video playbacks is that it is challenging to present the relevant features of a signal to the study species because playback devices are generally designed for the human visual system (D'Eath, 1998; Fleishman and Endler, 2000; Fleishman et al., 1998). Thus, the interpretation of responses to video playbacks must be made with great care (Schlupp, 2000). We suggest that, although these challenges have been discussed repeatedly, many video playback studies continue to provide insufficient controls. Our results suggest that an essential control for any video playback experiment involves measuring the responses of animals to a powered-off monitor and to a powered-on monitor displaying irrelevant stimuli or a blank, glowing screen. We note that while not all previous video playback studies in anurans utilized these controls, the results for both *P. pustulosus* and *H. arborea* appear to be robust because later studies in both species obtained similar results when visual preferences were measured in response to physical models that simulated a calling male (Gomez et al., 2010; Taylor et al., 2008), and because in *H. arborea* the effects of phototaxis were specifically ruled out by the pattern of female response (Gomez et al., 2009).

Phototaxis and its consequences

While we are unable to make conclusions about sexual selection on vocal sac characteristics based on our findings, we found that females showed strong phototactic tendencies under our experimental design. These tendencies were demonstrated most clearly in the control trials, in which females almost never oriented towards a powered-off monitor but frequently responded to the monitor anytime it showed a video stimulus. Because these tests were conducted in an otherwise darkened room, the clearest explanation for our results is that females were attracted to the light of the glowing monitor. While the visual acuity of most anurans remains poorly understood, some previous studies have measured their behavior in response to varying illumination levels and provide a context for the interpretation of our results. For instance, Cummings et al. (Cummings et al., 2008) measured visual sensitivity of reproductive female *Physalaemus pustulosus* using the optomotor response without accompanying acoustic stimulation and found a median visual sensitivity of 5.9×10^{-11} W cm⁻². Most of our composite stimuli were one to two orders of magnitude brighter than this value (Table 1). Thus, while the minimum visual sensitivity of *H. versicolor* is unknown, if it is similar to that of *P. pustulosus*, then the stimuli presented should have been easily perceptible by the females, which our results confirmed. Females responded frequently to the dimmest stimulus we were able to measure, the frog with a black vocal sac. The brightness of this stimulus was 7.2×10^{-12} W cm⁻², which implies a minimum visual sensitivity equal

Table 1. Brightness of the experimental stimuli

Experiment	Stimulus	Brightness (nW cm ⁻²)
1 – VS brightness	Frog + black VS	0.00721
	Frog + dark gray VS	0.07629
	Frog + medium gray VS	0.30106
	Frog + light gray VS	0.47929
	Frog + white VS	1.2046
	Black VS	–
	Dark gray VS	0.00735
	Medium gray VS	0.10291
	Light gray VS	0.30614
	White VS	0.54993
2 – VS size	Frog + small VS	0.23171
	Frog + large VS	0.42252
	Frog + extra-large VS	0.5585
Control	Gray square	0.60603
	Gray circle	0.57052
	White screen	2.07854
	Black screen	–

Brightness measured five times per stimulus and then averaged and extrapolated to a 1 m viewing distance (see Materials and methods). Values for frog stimuli are representative values measured for one of the three body exemplars used per stimulus. For two stimuli (black vocal sac and black screen), our spectrometer was not sensitive enough to obtain a brightness measurement. VS, vocal sac.

to or lower than this value. Jaeger and Hailman (Jaeger and Hailman, 1973) measured responses of individual *H. versicolor* to light of varying intensities, again in the absence of acoustic stimulation. Their stimuli varied in brightness over a much greater range than those used in our study, and the results suggested a preference for increased stimulus brightness within the range of experimental stimuli. Jaeger and Hailman (Jaeger and Hailman, 1973) found a sharp increase in responsiveness between a stimulus intensity of 2.6×10^{-11} W cm⁻² and the next brightest stimulus intensity, 1.8×10^{-7} W cm⁻². Nearly all of our stimuli fall within these values, and although the strong slope of the response curve presented by Jaeger and Hailman (Jaeger and Hailman, 1973) suggests that these should be discriminable by *H. versicolor*, our results indicate that the brightness differences between stimuli were insufficient to result in differences in female responses.

Thus, whether variation in female phototactic responses has significant consequences in nature remains an open question, and further studies should examine female visual sensitivities in a variety of contexts and across a wide range of stimuli. Both spatial and temporal variation in nocturnal illumination are high in breeding choruses (e.g. Cummings et al., 2008), and the consequences of this variation are potentially of great importance for the understanding of male mating success if females tend to orient towards especially bright locations. Indeed, some previous studies are consistent with the idea that the light environment or visual cues may modulate the expression of female choice for acoustic signals, although the effects in these studies are probably not related to phototaxis itself (Bonachea and Ryan, 2011a; Bonachea and Ryan, 2011b; Rand et al., 1997; Richardson et al., 2010). Indiscriminate orientation towards bright stimuli could aid a female in mate localization, particularly if males are brighter than their surroundings (e.g. Sztatecsny et al., 2010). However, the significance of such behavior may be unrelated to mating. Several studies showed that anurans use celestial light as a cue for orientation (Ferguson et al., 1967; Ferguson et al., 1968; Landreth and Christensen, 1971). It is possible that our experimental design revealed a similar effect and females used the light for some form of orientation unrelated to the

localization of specific mates. For instance, such visual orientation may be used along with acoustic cues (Bee, 2007; Gerhardt and Klump, 1988) to help females localize breeding ponds. The important point is that our results suggest that more attention be paid to the visual ecology of nocturnal anurans. Despite low light levels, they are clearly responsive to visual cues and it will be important to determine how the orientation behaviors we observed in our playback experiments correspond to female behavior in the field.

MATERIALS AND METHODS

We captured gravid female *H. versicolor* from two local ponds (University of Wisconsin-Milwaukee Field Station and Riveredge Nature Center, Ozaukee County, WI). All females were captured in amplexus and were brought to the laboratory on the night of capture, where they were held in containers placed in melting ice to prevent oviposition. Females were acclimated prior to testing by placing them in a light-safe box maintained at the testing temperature of 20°C for 30 min. All tests were performed during the natural breeding season of *H. versicolor* (May 2012). All experiments were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee (protocol no. 11-12 #01).

Stimulus preparation

We performed video playbacks with computer-animated stimuli generated with Motion 2.1 software (Apple Computer Inc., Cupertino, CA, USA). The standard animated stimulus consisted of a still image of a male *H. versicolor* with an appended image of a vocal sac that was animated to mimic the inflation and deflation that takes place during calling (see supplementary material Movie 1). We varied single properties of this animation to measure female responses to the stimuli described below. We were interested in testing whether females responded to the visual stimuli alone; thus, with the exception of Experiment 4 (see below), playbacks were performed without any accompanying auditory stimuli.

To create the animated stimuli, we obtained images of naturally calling male *H. versicolor* from video recordings. We placed males on a wooden dowel surrounded by a black background and video-recorded at least 10 calls using a Sony DCR-HC38 camcorder (Sony Electronics Inc., San Diego, CA, USA). Recordings were made from a lateral position so that one side of the frog's body was fully visible. We extracted still images from these videos of males ($N=14$) calling with vocal sacs maximally inflated and measured the area of the frog's body without the vocal sac and the area of the vocal sac itself using ImageJ software (version 1.46K; National Institutes of Health, Bethesda, MD, USA). We selected five images each of male bodies and vocal sacs that most closely approximated the average size. These images served as exemplars and ensured that our conclusions were generalizable across multiple stimuli. Unless otherwise specified below, for each characteristic we tested, there were a total of nine exemplars consisting of all combinations of three randomly selected body and vocal sac images.

These images were manipulated in the animation software to create stimuli that mimicked the natural movements of the vocal sac during calling. Images were scaled so that they would appear life-sized on the playback monitor. The background of all videos was a uniform black. Prior to the calling event, only the image of the frog body with no vocal sac was presented. Unless otherwise noted, the stimulus was animated so that the duration of the simulated call (i.e. the total duration of vocal sac inflation) was 1 s. The video was presented in a loop with a total duration (i.e. simulated call period) of 5 s. These temporal calling characteristics represent typical values for males in the study populations (G.H., personal observation). At the initiation of the calling event we animated the vocal sac so that the time it took to grow linearly from an infinitesimally small point to the size of maximum inflation was 0.167 s. The vocal sac remained fully inflated for 0.667 s and then deflated completely in 0.167 s. With the exception of experiments in which we manipulated vocal sac brightness, the vocal sac of the stimulus was a uniform medium gray (RGB=128,128,128). Thus, we refer to the 'standard' stimulus as an animation in which the male had a medium gray vocal sac, an inflation duration of 1 s, and an average area of 3.9 cm². All animations were saved as lossless plus alpha MOV files (30 frames s⁻¹, 1920×1200 pixel resolution, bit depth=8).

Playback and monitor adjustment

We played back videos using the VLC media player (VideoLAN) onto a Dell U2412M LCD computer monitor (Dell Inc., Round Rock, TX, USA). Videos were broadcast from a laptop computer located outside of the testing chamber to a monitor placed inside the chamber. Stimulus presentations using video monitors must be adjusted to account for the specific characteristics of the visual system of the study organism under relevant environmental conditions (Fleishman and Endler, 2000; Fleishman et al., 1998). Thus, we adjusted the monitor's output to ensure that the stimuli presented to females matched the spectral characteristics of natural male *H. versicolor* vocal sacs in a nocturnal breeding chorus.

To adjust the playback monitor, we first adjusted its brightness to its minimum, which was the value used during playbacks. We followed the methods of Gomez et al. (Gomez et al., 2009) to characterize and adjust our monitors' pixel outputs. Briefly, we determined the spectral output of each of the three pixel types of the monitor using an Ocean Optics USB 2000 spectrometer (Ocean Optics Inc., Dunedin, FL, USA) calibrated with an HL-2000 calibration lamp to measure the spectrum of uniform red, green and blue stimuli. The spectral output was collected with a fiber optic probe (QP400-2-UV-VIS) and cosine collector (CC-3-UV-S). Spectra were recorded using Ocean Optics SpectraSuite software and then interpolated in 1 nm steps and converted to quantal units ($\mu\text{mol s}^{-1} \text{m}^{-2}$) using Avicol software (Gomez, 2012).

To simulate grayscale stimuli, we adjusted the monitor's pixel outputs so that each photoreceptor would be stimulated equally by our stimuli. The spectral sensitivity functions of *H. versicolor* rods are unknown, so we used functions measured from the closely related congener *H. cinerea* (King et al., 1993). With these values and a moonlight irradiance spectrum (Warrant, 2004), we used eqn 2.1 in Gomez et al. (Gomez et al., 2009) to calculate the adjusted pixel outputs necessary to stimulate each of the two rods equally and thus to simulate the grayscale as seen in a breeding chorus. We held the blue and red pixel values constant at 100 and solved for the value of the green pixel output. We then adjusted the color output of the monitor to this value (15). Subsequent measurements of the spectral output of playback stimuli at these adjusted pixel values confirmed that the stimuli should have stimulated the two rod functions approximately equally. We were primarily interested in female responses to vocal sac characteristics. Thus, we accounted for spectral characteristics of the vocal sac but did not make additional adjustments to the frog's body. We covered the computer monitor with an opaque black cloth cut so that only the portion of the monitor displaying the frog image was visible (uncovered area=7.5 cm high, 10 cm wide). Between tests, we entered the chamber to reposition the female in the release cage. We ensured that females' eyes remained as dark-adapted as possible during this process by performing the tests in a darkened laboratory and searching for the female with a headlamp with a red filter, to which this species likely has minimal sensitivity (King et al., 1993; but see Buchanan, 1993).

We used the spectrometer to quantify the brightness of the experimental stimuli. Our spectrometer was insensitive to the stimuli at the female release distance of 1 m from the monitor. Thus, we measured brightness by placing the bare spectrometer probe (without cosine collector) 10 cm from the monitor and then extrapolating these values to the expected values at 1 m using the inverse-square law. Brightness was calculated from the average of five measurements of the spectra for screen output between 350 and 700 nm using Avicol software. We measured brightness for one exemplar of each experimental stimulus, the control stimuli, and for vocal sacs without the accompanying frog body image to confirm that the brightness of the vocal sac images in Experiment 1 differed (Table 1).

General testing procedures

All tests took place in a darkened semi-anechoic chamber (3.3×3.3×2 m). Within the chamber, we placed the female in the center of a circular arena (2 m diameter) surrounded by hardware cloth covered with an opaque black cloth. We restrained the female underneath a visually and acoustically transparent release cage in the arena's center. All females were first tested for responsiveness by presenting them with a synthetic *H. versicolor* call from a speaker positioned 1 m from the female along the border of the arena. The audio stimulus (16 bit WAV file, 20 kHz sampling rate) was generated with custom software provided by J. Schwartz and the call characteristics

were based on those of a typical *H. versicolor* advertisement call (call duration=1 s; call period=5 s; pulse rate=20 pulses s^{-1} ; low-frequency peak=1100 Hz; high-frequency peak=2200 Hz). We broadcast the stimulus for 1 min while the female was confined in the arena's center and then released her by pulling on a rope attached to the top of the release cage.

We monitored the female's movements remotely using an infrared camera placed directly above the female release point. In order for the female's behavior to be scored as a 'response', we required her to enter an area within a 10 cm radius of the stimulus after making deliberate movements towards the stimulus. If the female failed to leave the release cage or failed to meet our response criteria within 5 min of being released, we scored her as having 'no response' to the test stimulus. Females that did not respond to the initial audio control ($N=5$) were not tested further. For each female response, we measured its latency to respond, i.e. the amount of time from the trial's start to the moment the female first met our response criteria.

Video playbacks proceeded largely as above. We removed the speaker and replaced it with the video playback monitor, which was placed 1 m away from the release point facing the female along the arena's edge. We adjusted the monitor's position in the arena periodically between tests. We placed the female in the release cage and again exposed it to 1 min of the test stimulus before removing the cage. We measured female responses as above. Females that failed to respond in three consecutive trials were tested again with the audio stimulus to check whether their lack of response was due to a general lack of motivation. If females responded to the audio, we continued testing, while females that failed to respond at this point ($N=7$) were not tested further and are not included in the analyses presented here. Females were tested with up to 15 different video stimuli, presented in random order. After testing was completed, we marked the female with a subcutaneous fluorescent tag (VI Alpha Tags, Northwest Marine Technology, Shaw Island, WA, USA) to ensure individual identification and to prevent repeated testing of the same females. A total of 61 females contributed data in the experiments described below.

Experiment 1: vocal sac brightness

We first tested whether females' responses to the animated stimuli varied depending on the brightness of the simulated vocal sac. We used GIMP version 2.6 software (<http://www.gimp.org/>) to generate vocal sac images uniformly filled in with five different values along the grayscale. We note that because we varied images along the grayscale, the images varied only in intensity. Thus, we tested female preferences for vocal sac brightness, although we refer to stimuli by their color as they appeared to the human eye prior to monitor adjustment. The specific values used were: white (RGB=255,255,255), light gray (191,191,191), medium gray (128,128,128), dark gray (64,64,64) and black (0,0,0); this encompasses the range seen in natural vocal sacs in the study population (G.H., personal observation). We used the vocal sac inflation and deflation characteristics of the standard stimulus described above. We presented females with each of these playback stimuli, in a random order, on a single computer monitor. As noted above, we adjusted vocal sac characteristics but not those of the corresponding body image, and there were five different body image exemplars. Thus, the overall brightness of the screen output varied somewhat independently of the brightness of the vocal sac image, depending on the combination of vocal sac and body image. We assumed that the vocal sac is the relevant characteristic for female preferences, which would therefore be expressed irrespective of minor differences in the brightness of the overall video image.

In addition to single-monitor tests, we also assessed female responses to stimuli with different vocal sac brightness in a series of two-monitor choice tests. In these tests, we placed two monitors at 180 deg from one another on opposite sides of the arena. We designed playback stimuli as a single video in which each frog image was visible on only one of the two monitors by splitting the monitor feeds using a Matrox DualHead2Go display adapter (Matrox Electronic Systems Ltd, Dorval, QC, Canada). The stimuli were animated so that the simulated frogs appeared to call antiphonally with one another (i.e. the vocal sac of a given frog image began to inflate 2.5 s after the initiation of vocal sac inflation of the frog on the other monitor). The female was released in the center and given 5 min to meet the response criteria towards one of the two monitors. We tested females with the following pairs of vocal-sac brightness stimuli, presented in random order:

medium gray versus black, medium gray versus dark gray, medium gray versus light gray, medium gray versus white, white versus black and light gray versus dark gray.

Experiment 2: vocal sac size

We tested whether female responses depended on the size of the vocal sac stimulus. In these single-monitor tests, we manipulated the area occupied by the vocal sac at its maximum extension. In all cases, the size of the frog body image remained constant, the inflation and deflation times were those of the standard stimulus and the vocal sac was medium gray. The specific sizes used were as follows: no vocal sac (here we used all five body image exemplars), small (3.0 cm²), average (3.9 cm²), large (4.8 cm²) and extra-large (5.9 cm²). Sizes of vocal sacs were selected based on the measurements of naturally calling males used to prepare the video stimuli (see Stimulus preparation, above) and correspond to a non-calling frog, the mean size minus 1 s.d., the mean vocal sac size, the mean size plus 1 s.d. and the maximum size measured in our recordings, respectively.

Experiment 3: vocal sac inflation duration

We tested whether female responses depended on the duration of inflation of the vocal sac. We used the standard stimulus, and manipulated the amount of time that the vocal sac was extended. Rise and fall times were kept constant at 0.167 s each. The specific durations used were as follows: no vocal sac, short duration (0.5 s), average duration (1 s), long duration (1.5 s) and permanently extended (i.e. statically displayed at maximum extension throughout the playback). The stimulus without a vocal sac represents a non-calling male, the three intermediate duration stimuli were chosen to encompass the approximate range of call durations in *H. versicolor* (Gerhardt et al., 1996), and the permanently extended vocal sac has no natural analogue but was designed to test whether females respond to the vocal sac in the absence of the movement cues associated with its inflation and deflation. In this experiment, we also tested females with two-choice tests as in Experiment 1. Here the stimuli were the standard stimulus versus a still image of a frog with no vocal sac, and the standard stimulus versus a still image of a frog with a permanently inflated vocal sac.

Experiment 4: visual cues in chorus noise

We focused this study on females' responses to visual stimuli, which we initially predicted would be expressed in a mate-choice context. However, because acoustic signals are a prevalent component of mating aggregations in this species, it is possible that acoustic stimulation is required for females to become selective towards visual stimuli. Thus, for this experiment, we repeated the methods of Experiment 1 but in this case included an auditory stimulus of a recording of an *H. versicolor* chorus. The chorus recording was broadcast at 85 dB SPL at the female release point from a speaker located directly overhead. Thus, we provided the general auditory context of a mating aggregation but the speaker's position did not provide any localization cues that would bias females' movements.

Controls

Our method of using video playbacks to measure female responses to vocal sac characteristics depended on the assumption that video playback stimuli effectively simulate a calling male from a female's perspective. While this assumption was implicit in several previous video playback studies in anurans (Gomez et al., 2009; Rosenthal et al., 2004; Zeyl and Laberge, 2011), caution must be taken in interpreting the results of these experiments because the emitted light from a computer monitor, even after correction to match the properties of the study species' visual system, may not necessarily be perceived by the test subject as the physical object being simulated (reviewed by D'Eath, 1998). Thus we performed a series of control playbacks of abstract images, allowing us to test whether females respond to monitor brightness irrespective of the stimulus presented.

We performed the following single-monitor tests: (1) a non-moving medium gray square of approximately the same area as the average vocal sac, (2) a medium gray square that did not change in size, but whose movements mimicked those of the standard stimulus (i.e. the displacement and temporal characteristics of movement were the same as the leading edge of the standard vocal-sac stimulus), (3) a non-moving medium gray circle, (4) a medium gray

circle that moved continuously across the screen (during the stimulus period, the circle moved approximately the same distance as the maximum displacement achieved by the vocal sac of the standard stimulus, and was reset every 5 s), (5) a completely white screen, and (6) a completely black screen. The completely black screen still broadcast some light; thus as an additional control we measured female responses to (7) a powered-off monitor.

In addition, we used a series of two-monitor tests to determine whether females preferentially responded to the animated frog stimuli over general abstract images. We performed the two-monitor tests as in Experiment 1, above. We measured female responses to the following stimulus combinations: the standard stimulus versus a white screen, the standard stimulus versus a black screen, the standard stimulus versus a gray square, the standard stimulus versus an image of a frog with no vocal sac, the standard stimulus versus an image of a frog with a permanently extended vocal sac, and an image of a frog with no vocal sac versus a black screen.

Statistical analyses

We used linear mixed models (lme4 package in R 3.0.1 software) to test whether visual stimulus traits (separate models for vocal sac brightness, size, extension, and single-stimulus controls) affected whether females approached the stimulus (binary response variable, logit link). We ran standard least squares models (with restricted maximum likelihood estimation) in JMP version 8.0 (SAS Institute, Inc., Cary, NC, USA) to test whether visual stimulus traits affected the latency of response. Trials in which females did not respond to the stimuli were not included in the analyses of response latency. We included female identity as a random term in all models because we measured each female's responses to multiple stimuli.

To analyze the two-stimulus trials, we report the proportion of females responding to each alternative and calculated two-tailed binomial tests with the null hypothesis of equal responsiveness to either stimulus. We used ANOVAs to compare whether there were differences in the latency to respond to presented alternatives. All statistical tests were two-tailed with $\alpha=0.05$.

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.R. and G.H. conceived and designed the experiments; H.G. and M.R. performed the experiments; H.G. compiled the raw data; G.H. analyzed the data; and M.R. and G.H. wrote the paper.

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Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.106666/-DC1>

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