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RESEARCH ARTICLE

Risks of multimodal signaling: bat predators attend to dynamic motion in frog sexual displays

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ABSTRACT

Many sexual displays contain multiple components that are received through a variety of sensory modalities. Primary and secondary signal components can interact to induce novel receiver responses and become targets of sexual selection as complex signals. However, predators can also use these complex signals for prey assessment, which may limit the evolution of elaborate sexual signals. We tested whether a multimodal sexual display of the male túngara frog (*Physalaemus pustulosus*) increases predation risk from the fringe-lipped bat (*Trachops cirrhosus*) when compared with a unimodal display. We gave bats a choice to attack one of two frog models: a model with a vocal sac moving in synchrony with a mating call (multisensory cue), or a control model with the call but no vocal sac movement (unimodal cue). Bats preferred to attack the model associated with the multimodal display. Furthermore, we determined that bats perceive the vocal sac using echolocation rather than visual cues. Our data illustrate the costs associated with multimodal signaling and that sexual and natural selection pressures on the same trait are not always mediated through the same sensory modalities. These data are important when considering the role of environmental fluctuations on signal evolution as different sensory modalities will be differentially affected.

KEY WORDS: Multimodal communication, Eavesdropping, Signal evolution, Co-option, Motion detection, *Trachops cirrhosus*

INTRODUCTION

Courting animals often produce elaborate multimodal signals that are perceived by both conspecifics and eavesdroppers through a variety of sensory systems (Hebets and Papaj, 2005; Moller and Pomiankowski, 1993; Partan and Marler, 1999). Many of these multimodal signals may have evolved through co-option, a process whereby a primary sexually selected signal is coupled with a secondary component, producing a novel display (Ryan and Cummings, 2013; Taylor and Ryan, 2013). Such incorporation of a signal component into a multimodal sexual display may increase the accuracy of signal transfer or enhance overall signal efficacy (Higham and Hebets, 2013; Johnstone, 1996). Combining multimodal components can lead to novel receiver responses, a unique feature that demonstrates why multimodal signals should be favored by sexual selection over unimodal signals (Higham and Hebets, 2013; Taylor and Ryan, 2013). However, the evolution of multimodal displays can be influenced by a combination of natural and sexual selection pressures that act directly or indirectly on the composite signal or its individual components (Bro-Jørgensen, 2010; Hebets and Papaj, 2005). Thus, understanding how multimodal signals evolved requires a detailed assessment of the costs and benefits of the production and perception of these signals.

A large body of work has focused on the benefits derived from multimodal signals. Numerous studies have provided experimental evidence for receiver preferences for multimodal over unimodal signals (see Holldobler, 1999; Narins et al., 2003; Taylor et al., 2008; Uetz and Roberts, 2002). Responses to multimodal signals also can favor receivers, as multimodal perception is thought to increase the accuracy of processing cues, which is particularly useful under complex environmental conditions (Bro-Jørgensen, 2010; Higham and Hebets, 2013; Munoz and Blumstein, 2012; Wilson et al., 2013). Multimodal signals are therefore more likely to be detected and accurately localized compared with unimodal signals (Gordon and Uetz, 2012; Uetz et al., 2011). However, potential costs associated with the production of multimodal signals have received limited empirical attention (Roberts et al., 2007; Smith et al., 2011). Costs can be imposed on multimodal signals through production or perception constraints, such as increased energetics, or cognitive limitations (Gomez et al., 2011; Hebets and Papaj, 2005). Alternatively, multimodal displays may recruit novel receivers, such as parasites or predators, or aid already known eavesdroppers (Ratcliffe and Nydam, 2008; Roberts et al., 2007). Eavesdroppers may also benefit from multimodal signals, utilizing similar increased detection and localization performance as intended receivers, although not necessarily using the same sensory modalities to acquire these benefits (Halfwerk et al., 2014).

In our study species, the túngara frog [*Physalaemus pustulosus* (Cope 1864)], males gather in shallow seasonal ponds and attract females with mating calls (Ryan, 1985). Like many other frog species, males possess a conspicuous vocal sac, which is inflated and deflated during the production of the call (Taylor et al., 2008; Taylor and Ryan, 2013). The frog's vocal sac has presumably evolved to aid the recycling of air, but has been co-opted into a multimodal display that is attended to by females (Taylor et al., 2008; Taylor and Ryan, 2013). When given a choice, females show a threefold preference for a frog model containing a dynamically moving vocal sac over a control model. Male frogs risk being detected by the fringe-lipped bat (*Trachops cirrhosus*), an eavesdropping predator that uses frog calls to detect, localize and assess its prey (Page and Ryan, 2005; Tuttle and Ryan, 1981). These bats tend to rely primarily on acoustic cues to find prey, but may also make use of additional, non-acoustic cues to fine-tune their prey assessment (Halfwerk et al., 2014; Page et al., 2012).

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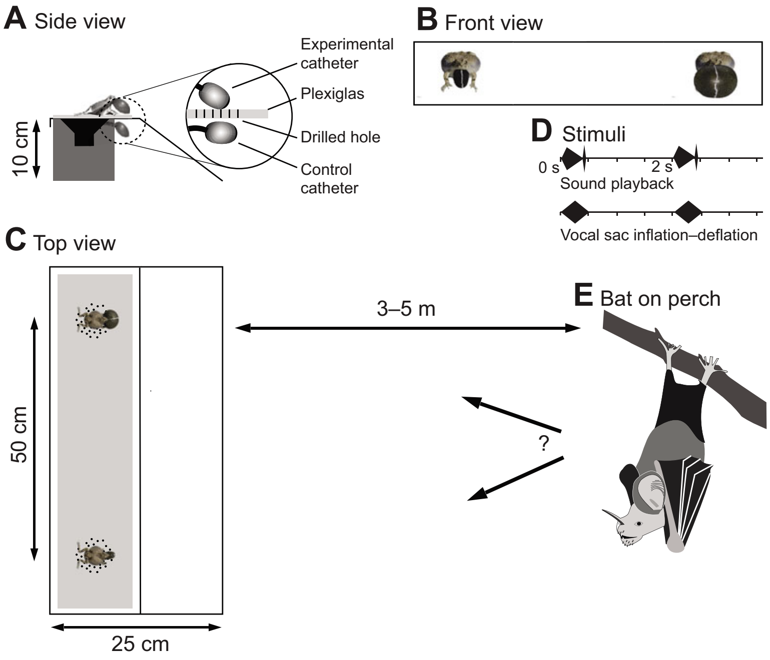

Fig. 1. Experimental setup of the two-choice tests. (A). Side view of the experimental setup showing the speaker used to broadcast acoustic call component with a Plexiglas screen plus frog model on top. Both the experimental catheter mimicking the túngara frog vocal sac as well as the catheter controlling for sound produced during catheter inflation are shown in the enlargement. (B) Front view showing the robofrog with inflated vocal sac on the right and the control model on the left. (C) Top view showing the two frog models, the Plexiglas screen and the holes underneath the models to allow for sound transfer. (D) Stimulus presentation showing sound playback (whine plus chuck) in the top channel and the inflation–deflation of the vocal sac in the bottom channel. Maximum inflation was reached 200 ms after sound and roughly 150 ms after peak amplitude of the whine. (E) Bat on the perch, 3–5 away from the experimental platform.

We tested whether bats are able to perceive the frog's vocal sac and whether the vocal sac is used to select prey. We presented bats with a choice to attack two model frogs, one emitting acoustic cues and one emitting acoustic cues plus cues coming from vocal sac inflation (Fig. 1). We varied stimulus presentation to assess whether bats prefer dynamic vocal sac cues over static ones and whether they use dynamic vocal sac cues that are continuously present or only available to them while hanging from their hunting perch. Furthermore, we examined the sensory system (echolocation or vision) used by bats to detect the vocal sac in a cue isolation experiment and quantified the detection limits of *T. cirrhosus* visual and echolocation systems.

RESULTS

Experiment 1: naive responses

Wild-caught bats were given a choice to attack a frog model with a dynamic vocal sac present (inflating–deflating in synchrony with sound) or a control model (with a deflated vocal sac). All bats made their very first attack on the model with the dynamic moving vocal sac [generalized linear mixed model (GLMM); N=10, intercept=1, z-score=2.79, P=0.005)].

Experiment 2: effect of stimulus presentation

For each bat we continued testing preference to attack the vocal sac model, but we varied the presentation of sound and vocal sac to test whether bats perceived the vocal sac from their perch and whether the vocal sac had to be dynamically inflated and deflated (see Table 1 for a detailed description of stimulus presentations). Sound and vocal sac were continuously presented until a bat flew from its perch towards the platform with the frog models. During two of our playback treatments we switched off the sound and in one treatment we halted vocal sac inflation as soon as the bat left the perch (see Table 1). Bats made an attack flight on average after 2.8±1.8 (mean ± s.d.) stimulus presentations. However, individual variation was large, with some bats almost always leaving the perch upon the first presentation, whereas others would wait up to 10 presentations before making their attack (mean individual range=1.1–7.0 presentations). Across all treatments the average attack rate on the vocal sac model was 56% higher compared with that of the control model. Furthermore, the attack rate depended on the type of presentation (GLMM; treatment effect, N=10, χ^2=8.32, d.f.=3, P=0.04; Fig. 2). Bats preferred to attack the frog model with a dynamic inflating–deflating vocal sac, even if they could detect the dynamic vocal sac only from their perch (Table 1, Fig. 2). Bats showed no attack preference with a static inflated vocal sac (Table 1) and direct comparison confirmed that bats only preferred to attack the vocal sac model when it was dynamically inflated and deflated (*post hoc* Tukey's test comparing dynamic and static treatment: z-score=2.44, P=0.041; Fig. 2).

Table 1. Description of the different stimulus presentations used in Experiment 2 and their effect on attack preference

Treatment	Dynamic/static presentation of vocal sac	Sound off on leaving perch	Vocal sac off on leaving perch	Preference for vocal sac model
1. Continuous	Dynamic	No	No	z-score=1.96, P=0.051
2. Perch only	Dynamic	Yes	Yes	z-score=2.53, P=0.012
3. Dynamic	Dynamic	Yes	No	z-score=5.21, P<0.001
4. Static	Static	Yes	No	z-score=1.16, P=0.11

Each trial started with sound playback at both frog models and presentation of the vocal sac as well as the control condition. Treatments were as follows: (1) continuous*:* treatment was the same as used during Experiment 1 and consisted of simultaneous presentation of sound and the dynamic inflation–deflation of one of the frog models until the bat had finished its attack; (2) perch only: we stopped sound playback and vocal sac presentation as soon as the bat flew from its perch in the direction of the platform; (3) dynamic: sound was switched off as soon as bat left its perch, but vocal sac inflation–deflation continued; and (4) static*:* the vocal sac was inflated to its full size throughout the whole trial and sound was turned off as soon as the bat left its perch. Statistics reported under preference for the vocal sac model show estimates from a generalized linear mixed model.

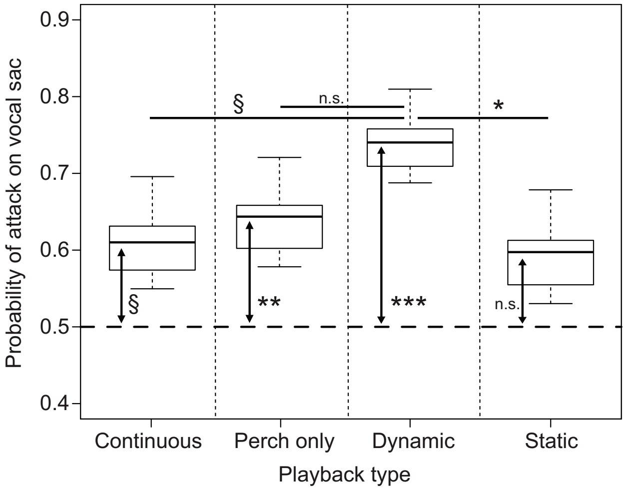

Fig. 2. Bats prefer to attack a frog model with a dynamically moving vocal sac. Shown are boxplots derived from the model estimates per playback treatment (Experiment 2). Bats preferred to hover over and attack the frog model with a dynamically inflated–deflated vocal sac under all playback conditions. Attack preference did not differ from chance with a continuous inflated vocal sac (static treatment). Dashed line indicates chance level at 50%. n.s., not significant; $^{\S}P<0.1$, *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Experiment 3: use of echolocation to detect vocal sac

We presented bats with dynamic vocal sac playback and manipulated the sensory environment to test what sensory systems (vision or echolocation) they used to assess vocal sac cues. The presence or absence of different sensory cues had a significant effect on bat attack preference for the vocal sac (GLMM; N=8, χ^2=34.18, d.f.=3, $P<0.001$; Fig. 3). During Experiment 3, bats showed a significant attack preference for the vocal sac when both echolocation and visual cues were present (z-score=4.31, $P<0.001$) and when trials were carried out in the dark so only echolocation cues were present (z-score=4.02, $P<0.001$). Bats showed no attack preference when echolocation was blocked by visually transparent spheres covering the vocal sacs of the model so only visual cues were available (z-score=0.32, P=0.75). Bats also showed no preference when neither visual nor echolocation cues were present (z-score=–0.28, P=0.98).

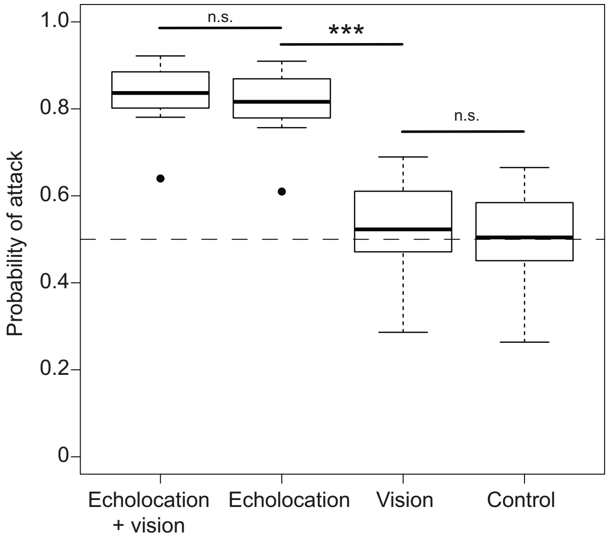

Fig. 3. The cue isolation experiment reveals that bats use echolocation to detect vocal sac movements. Shown are boxplots derived from the model estimates per cue isolation treatment (Experiment 3). Bats preferred to attack the robofrog when they had only access to echolocation cues coming from the vocal sac, but not when they only had access to visual cues. The control condition refers to the treatment in which bats had no access to visual or echolocation cues. Filled circles are outliers. n.s., not significant; ***$P<0.001$.

Optomotor response test of visual perception

We tested bats on optomotor responses in a rotating drum with varying light levels and stripes of different widths and contrast. Only two of the six bats that we tested in our optomotor setup showed clear responses. These two bats showed consistent head movements, only under the highest light levels (25 W red light), with the highest contrast (black stripes on white printing paper) and lowest spatial resolution (minimal separable angle of 3 deg).

Echolocation recordings and assessment of detection distances

We recorded echolocation behavior during attack sequences with an ultrasonic microphone placed in front of the two frog models. We selected calls produced from the perch, directed at the models, measured their amplitude and frequency content and created a artificial echolocation call that we used for an ensonification and transmission experiment to assess the echo amplitude and frequency information available to bats on their perch. Six out of eight recorded bats produced echolocation calls almost immediately with the start of each trial (see example in Fig. 4). At least one vocalization always overlapped with stimulus presentation and thus vocal sac inflation. Individual echolocation behavior was highly consistent across trials, with some bats, for instance, always producing two calls overlapping with the vocal sac inflation (assessed from ultra-sonic recordings), shortly before an attack flight. Interestingly, the two individuals that did not call from the perch also showed little or no preference (probability of 0.5 and 0.56) during trials in which the vocal sac could only be detected from the perch.

We recorded a total of 28 suitable perch calls from six different bats that were produced at a mean (±s.d.) distance of 3.78±0.69 m from the platform, shortly before an attack flight. These calls had a mean (±s.d.) amplitude of 50.1±3.46 dB SPL, a peak frequency of 72.3±4.49 kHz and a frequency bandwidth of 53.8±4.8 kHz at the location of the frog models. Target strength (loss in amplitude of returning echo, measured at 20 cm from the target) of the models was 12.3±0.1 dB SPL and attenuation between perch and target was −32.2±0.18 dB SPL. Together, these measurements resulted in average estimated echo amplitude of 6.1 dB SPL at the perch.

We carried out an ensonification experiment with artificially created echolocation calls to assess the spectral information of returning echoes. Echoes returning from the fully inflated vocal sac model showed substantial changes in spectral composition compared with echoes returning from the fully deflated vocal sac models (Fig. 4C).

DISCUSSION

Our experiments reveal a substantial cost of multimodal communication, namely increased predation pressure on sexually displaying frogs by the fringe-lipped bat. When given a choice, all bats preferred to attack a model with a vocal sac that was dynamically inflated and deflated in synchrony with acoustic call production on their very first approach. The attack preference depended on the dynamic movement of the vocal sac and not just increased size alone, as bats did not prefer to attack a model with an

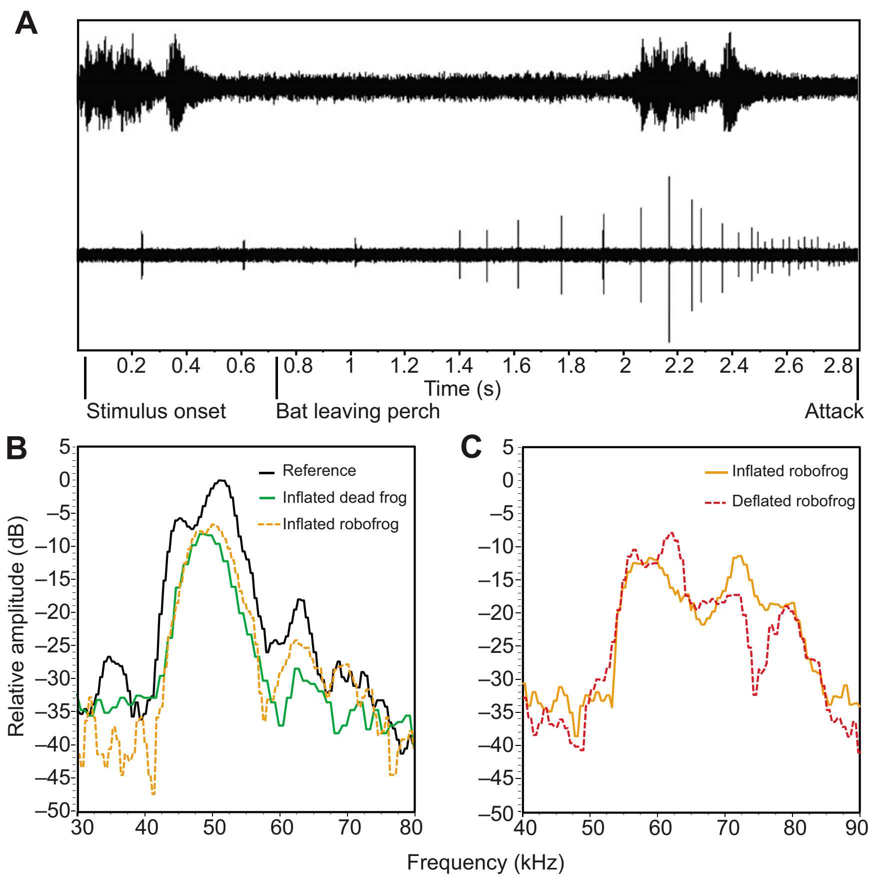

Fig. 4. Echolocation behavior and echo-cue information. (A) Waveforms showing the acoustic stimulus presentation (top) and echolocation calls (bottom) during an experimental trial. In this example, the bat produced a signal shortly after onset of the call (and vocal sac inflation) and left the perch shortly after call offset. The bat attacked the model with the vocal sac ~2 s after leaving the perch. (B). Powerspectrographic echo profiles recorded during an ensonification experiment using a synthetic flight call. Comparing the reference signal with echoes returning from dead or robotic frogs illustrates the loss of acoustic energy (target strength is roughly −10 dB in this example). Spectral profiles of an inflated dead frog and our robofrog with inflated vocal sac do not show major differences. (C) Comparison of spectral profiles of echoes from inflated and deflated robofrogs using synthetic call based on perch calls recorded during the experiment.

inflated static vocal sac over models with a deflated vocal sac. Bats relied on echolocation to detect the vocal sac and had access to sensory information from returning echoes when perched 3–5 m away from the frog models.

Perceptual mechanism to detect vocal sac movement

Bats in our experiment were able to detect the moving vocal sac from their perch and occasionally made an attack after one or two stimulus presentations (see Fig. 4A). During trials, bats typically produced a few echolocation calls (always at least two) in response to and shortly after sound onset. Processing of acoustic cues thus precedes processing of echolocation cues [see Page et al. (Page et al., 2013) for a discussion on sequential prey cue assessment]. Furthermore, the time window for this sequential assessment decreases with distance and may limit detection of the vocal sac. On most of our echolocation recordings, the first call overlapped with the time period that the vocal sac was inflated (between 100 and 300 ms after call onset, see also Fig. 1D and Fig. 4A), although two bats were never recorded to produce calls overlapping with vocal sac inflation (and also did not prefer to attack the vocal sac model).

Bats could have used the Doppler shift information of a single echo returning from an expanding or retracting vocal sac. However, the broad-band, short FM pulse of the fringe-lipped bat probably provides insufficient information to assess the associated frequency change (Surlykke et al., 2013; Simmons et al., 1979). Thus, the most likely mechanism involves a comparison of two echoes from an inflated and a deflated vocal sac shortly after each other. Such a comparison either provides information on spectral differences (see Fig. 4C), or information on a change in delay between call production and reception (Goerlitz et al., 2010).

Predation pressures in a variable sensory environment

Our cue isolation experiment showed that bats use echolocation cues and not visual cues to detect the frog's vocal sac. The predation pressure imposed on the frog's multimodal signal will thus depend on the sensory conditions that affect the bat's echolocation behavior. The fringe-lipped bat hunts along streambeds in the forest understory under echo-acoustically complex conditions (Kalko et al., 1999). In particular, echoes returning from vegetation create background clutter that can severely interfere with object detection by echolocation (Arlettaz et al., 2001; Schmieder et al., 2012; Schnitzler and Kalko, 2001; Siemers and Schnitzler, 2004). Bats will likely benefit from the acoustic mirror effect of smooth-surfaced water bodies from which male frogs typically call and which makes any object conspicuous to the bat's echolocation system because of reduced background clutter and/or increased target strength (Siemers et al., 2005; Siemers et al., 2001). However, frogs mostly call close to the edge of a pond or close to rocks or vegetation inside ponds (Ryan, 1985), and bats would likely have to create a detailed picture of the perceptual scene before they can pick out prey cues among the high levels of background clutter. The vocal sac may, in particular, aid bats under these conditions as echoes returning from the sac movements will make the frog stand out against a stationary background (Simmons et al., 1979; Schnitzler and Kalko, 2001). We recently showed that bats also rely on water ripples produced by the calling frogs (Halfwerk et al., 2014), and it would be interesting to test how the combination of vocal sac and ripple cues affect attack preference under varying environmental conditions.

Our optomotor response tests revealed that bats had relatively low visual sensitivity, as bats showed no response under normal nocturnal light levels. Furthermore, spatial resolution was low, but comparable to some other bat species with similarly small eyes

[*Myotis lucifugus* and *Phyllostomus hastatus* (Suthers, 1966)]. Bats do not always show clear optomotor responses (Suthers, 1966), and only two out of six bats showed a response during our tests and we should therefore be careful in drawing strong conclusions. However, the physiological response matches the behavioral data that showed that our bats do not use vision to detect and localize prey.

Thus, the light environment is not likely to alter predation pressures on the multimodal signal.

Benefits from perch hunting

Radio-tracking data indicate that fringe-lipped bats hunt from perches in the vicinity of frog calling sites (Kalko et al., 1999). Hunting from a perch allows bats to scan their surroundings and to build a picture of the environment from which objects of interest could be discerned (Neuweiler et al., 1987; Surlykke et al., 2013). Most bats can flexibly alter both amplitude and directionality of their echolocation signals, which allows them to scan the environment sequentially and to reduce the amount of clutter that is off-axis from the scanning direction (Jakobsen et al., 2013; Surlykke and Kalko, 2008). The fringe-lipped bat has also been shown to scan its surroundings from its perch before making an attack (Surlykke et al., 2013). This behavior probably enables a bat to observe ponds with calling túngara frogs without being visually or acoustically detected by the frogs (Bernal et al., 2007). Furthermore, it has been argued that detection thresholds of returning echoes are lowered for bats on a perch (0 dB) compared with threshold for bats in flight (20 dB), because of the high noise levels generated by passing of air when flying (Surlykke and Kalko, 2008). We estimated that the maximum echo amplitude returning from the vocal sac to bats on their perch was approximately 6.1 dB, much lower than the presumed detection threshold for flying bats (Surlykke et al., 2013; Surlykke and Kalko, 2008). Lowered echo detection thresholds additionally help explain why perch hunting is such a widespread foraging strategy, particularly for bat species hunting among or close to vegetation (Fenton, 1990).

In conclusion, our data reveal that sexual and natural selection pressures on the same trait are not always mediated through the same sensory modalities, a finding that has important consequences for models assessing the evolution of complex multimodal signals under dynamic selection regimes (Bro-Jørgensen, 2010). We know that female frogs assess the male's vocal sac in the visual domain (Taylor et al., 2007; Taylor et al., 2008) and we have shown that bats monitor frog vocal sacs mainly using echolocation. Changes to the light environment are thus likely to affect sexual selection pressures, but not natural selection pressures, whereas the opposite holds true for changes affecting echolocation cues such as background clutter. Models assessing the effect of environmental fluctuations on multimodal signal evolution thus have to take into account the different receiver modalities.

MATERIALS AND METHODS

Study system

We captured fringe-lipped bats (*Trachops cirrhosus*; *N*=10) from Soberanía National Park, Panamá, between May and December 2012. Bats were captured with hand nets from roosts during the day, or caught with mistnets set along streambeds, 0–2 h after sunset. Each bat was injected with a subcutaneous passive integrative transponder (Trovan, Ltd) for individual recognition, and released in a large outdoor flight cage (5×5×2.5 m) for testing [see Page and Ryan (Page and Ryan, 2005) for a more detailed description of the test arena]. The same bats were tested in three different experiments for up to six consecutive nights and released at their capture site after the experiment.

All research reported here complied with Institutional Animal Care and Use Committee (IACUC) protocols from the Smithsonian Tropical Research Institute. We obtained all required permits from the Government of Panama.

Playback setup

We used robotic frogs developed by Taylor et al. (Taylor et al., 2008) to mimic calling frogs emitting either unimodal (acoustic cue) or multimodal stimuli (acoustic cue plus additional cue derived from vocal sac movement). Our experimental setup consisted of two frog models, each placed 50 cm apart on a smooth-surfaced Plexiglas platform (10×60 cm), echo-acoustically mimicking a water surface (Siemers et al., 2001; Siemers et al., 2005) (Fig. 1A–C). The platform was placed 10 cm above the ground, on top of a speaker (Peerless, 2.5 inch). The platform had a 15 cm slope in front. Both models contained a catheter in front of the model (that mimicked a vocal sac for the multimodal frog) as well as a catheter underneath the Plexiglas platform that functioned as a control for the sound produced by the inflation of the vocal sac catheter (Fig. 1A). We drilled holes in the Plexiglas below the models to allow for sound transfer of the speaker and control catheter (Fig. 1A,C). Both catheters were connected with a 3 m tube to a custom-made gas-relay station, which released 20 ml of air from a compressor tank to the closed air system upon receiving a 19 kHz actuation signal from a laptop (Lenovo Thinkpad), enabling inflation and deflation of the artificial vocal sac in synch with the call playback. Upon gas release, catheters would inflate. Their maximum inflation was achieved ~200 ms following the onset of the 19 kHz signal (Fig. 1D). The excess gas was then released from the closed system and the catheters would deflate to their starting size.

Each catheter was attached to its own gas-relay station, which allowed us to control the maximum inflation size (and thus associated sound level) by loading catheters with an additional amount of air prior to each trial. We loaded the experimental catheter with 3 cc of air, which resulted in a maximum vocal sac diameter of 2.5 cm. The inflation to 2.5 cm produced a broad-band sound (10–40 kHz) with a peak frequency of 13.1 kHz and a peak amplitude of 24 dB SPL (measured at 20 cm with an amplitude calibrated G.R.A.S. microphone). We matched sound levels (at 20 cm above the experimental platform) between catheters by loading the control catheter with an additional 4 cc of air (7 cc total), resulting in a diameter of 4.0 cm. We checked catheter sound levels by ear prior to every trial and we regularly measured sound levels with the ultrasonic microphone. The control catheter ensured that bat choices were not influenced by different sound production between the two experimental stimulus presentations.

For trials in which visual cues were available, the experimental platform was illuminated by a single nightlight (GE model no. 55507; Fairfield, CT, USA), set to 7.8×10^{-7} W cm^{-2} (measured with an International Light IL 1700 research radiometer and PM271C photomultiplier detector), mimicking natural nocturnal light levels at túngara frog breeding grounds (Cummings et al., 2008).

Experimental design

Each bat was allowed to acclimate to the flight cage and to the experimental setup before trials began. Bats were given an 80 cm×80 cm×2 m shelter made from black cloth attached to the ceiling of the cage; this shelter served as the bat's starting point for each experimental trial. The shelter was located in one corner of the experimental cage, open to the test setup, which was situated 3–5 m away (Fig. 1E).

To acclimate the bats to foraging in the flight cage, we broadcast frog calls from beneath a model frog and offered prey rewards (small pieces of baited fish) on top of the model (in the absence of the vocal sac). Frog calls were only broadcast from beneath one model at a time, and to ensure that the bats did not develop a side bias, we alternated the presentation side with each trial. Once a bat readily flew to the platform and attacked the model frog, we started the first experiment.

Experiment 1

The first experiment examined naive bat preference for the multimodal versus unimodal stimuli (none of the bats tested had any experience with our dynamic robofrog stimulus playback prior to Experiment 1). We offered the bats the option to attack a model with a dynamic vocal sac or a control model with a static, deflated vocal sac. We broadcast a synthetic call consisting of a whine plus one chuck at 0.5 calls s^{-1} and 76 dB SPL (re. 20 μPa at 1 m, measured with Extech SPL-meter type 407764, set to C-weighted, fast and max) simultaneously through both speakers. Sound playback was accompanied by inflation and deflation of a catheter in front of one model (Fig. 1D), which started with sound onset (hereafter referred to as the vocal sac model) and inflation–deflation of the control catheter underneath the other model (hereafter referred to as the control model). Bats were given up to four choice trials, with the side of the vocal sac alternated between each trial. In all trials we positioned small pieces of baited fish on top of both frog models and halted stimulus presentation once a bat obtained the reward. We stopped the experiment once the bat obtained a food reward during two consecutive trials.

Experiment 2

Experiment 2 examined whether bats perceived the vocal sac from the perch and whether they relied on the dynamic inflation–deflation as a cue, or simply on the size of the vocal sac when fully inflated. All of the bats received four different stimulus presentation treatments: (1) continuous, during which both sound and a dynamically inflated–deflated vocal sac were presented until a choice was made, identical to the first experiment; (2) stopped when leaving perch, during which both sound and a dynamically inflated–deflated vocal sac were immediately halted when the bat flew from its perch; (3) dynamic, during which vocal sac movement was continued, but sound playback was halted when the bat flew from its perch; and (4) static, during which vocal sac was in constant state of inflation, and sound was halted when the bat flew from its perch. Bats received 12 trials per stimulus treatment and treatment was randomly alternated every fourth trial. To keep bats motivated throughout the night, but not quickly satiated, we included a rewarded trial after every two unrewarded trials. The side with the robofrog was randomized and balanced for both rewarded and unrewarded trials.

Experiment 3

In Experiment 3 we tested how bats perceive the vocal sac by removing either visual or echolocation cues. Cue isolation treatments consisted of: (1) echolocation plus vision, a control condition similar to Experiment 2 during which echolocation cues were not blocked and nocturnal light levels were optimal; (2) echolocation, during which the light illuminating the platform was switched off, leaving only echolocation cues; (3) vision, during which echolocation cues were blocked by placing a visually transparent plastic sphere (Ø 4 cm) over the vocal sac; and (4) control, during which the plastic sphere was painted black from the inside, allowing no visual or echolocation cues. The rewarding scheme was the same as in Experiment 2 and the stimulus was presented dynamically, with sound turned off when bats left their perch, as this showed the biggest effect of vocal sac presentation in Experiment 2.

We moved the platform to a different location to avoid potential side biases when bats attacked the same side four times in a row. Nevertheless, five out of 10 bats developed a side bias during Experiments 2 and 3 (defined as choosing a particular side more than six times in a row) and were subsequently rewarded on the non-preferred side only (in the absence of vocal sac presentation) until they would consistently fly to that side. These additional training trials were not included in the analyses. All bats started with Experiment 1, followed by Experiments 2 and 3. Two bats had to be released after Experiment 2 because they were not maintaining proper body mass in captivity.

Behavioral measurements

We assessed the behavior of bats with two cameras (Sony Nightshot DCR-SR45 camcorder, illuminated with two Wisecomm IR045 lights), one recording the front view of the experimental platform and one recording the side view. Bats were also observed on their perch using night vision goggles. An attack sequence started with a bat flying from the perch to the platform, hovering over one of the models before making an attack on the model itself or taking the food reward. Throughout the experiments, bats stopped attacking the models when no reward was present, but still hovered over the models for inspection. Bats almost always attacked the side over which they hovered and we therefore used hovering as our measure of attack preference (the subset of trials with actual attacks did not show different results, nor did we detect a difference in preference between rewarded and unrewarded trials).

Optomotor response tests

We tested visual sensitivity and spatial acuity of six bats by measuring their optomotor response (Eklöf and Jones, 2003; Suthers, 1966). Each bat was placed in a transparent round box (Ø 20 cm, 30 cm in height) in the center of a transparent rotating drum (Ø 80 cm). By offering perching material only on the ceiling of the box, we ensured that the bats would hang only from the box ceiling, facing the drum. The drum was covered on the inside with evenly spaced black stripes (using electrical black tape, 0.5, 1.0 or 2 cm in width, corresponding to 0.75, 1.5 or 3 deg). The outside of the drum was either covered with white printing paper to create high contrast, or left transparent to create lower contrast of black against the background spectrum of the room (mainly wooden material). The drum was automatically rotated with a gear motor (Grainger, type 1LPW4, 47 r.p.m.) clockwise or counterclockwise, at a speed of 4 or 8 rotations min^{-1} (DC-controlled). The test chamber was illuminated with one of two types of light positioned 2 m above the optomotor device: a 25 W red light bulb, the standard light used in our flight cage, or the GE night light that we used in the experiments described above, a light that mimicked the natural nocturnal light spectrum of a full moon on a forest edge (7.8×10^{-7} W or 5.3 lx). Additionally, we covered the GE light with black tape to reduce light levels (by 50, 75 or 87.5%).

We tested bats with the five different light conditions, two different rotation speeds, three different stripe widths and two different types of contrast. Light levels were increased stepwise in between trials for each stripe/contrast condition until a response was scored, after which light levels were gradually decreased again. Rotation speed and direction (clockwise/counterclockwise) were randomly alternated and we recorded the bats' behavior using the Sony Nightshot and Wisecomm IR045 lights. We scored an optomotor response when a bat made repeated head movements during a test trial and when the starting direction (clockwise/counterclockwise) of head movements switched with the rotation direction of the drum (see Eklof and Jones, 2003).

Echolocation behavior and assessment of detection limits

We recorded the echolocation behavior of all individuals during a subset of the trials using ultrasonic recording equipment (either a G.R.A.S. microphone amplified by 40 dB by G.R.A.S. amplifiers connected to an Avisoft ultrasound gate and Lenovo Thinkpad, or a SongMeter SM2Bat+ with a SMX-UX microphone). We combined measurements from these recordings with measurements derived from an ensonification experiment and a transmission experiment to assess amplitude levels of echoes returning from the vocal sac at the perch. The ensonification experiment was carried out in a hemi-anechoic chamber (ETS-Lindgren). Calls were broadcast in bouts of 0.2 s at a rate of 30 calls s^{-1} using ultrasonic playback equipment (Scanspeak ultrasonic speaker connected to an Avisoft sound gate and a Lenovo Thinkpad). We suspended objects of interest (a dead frog or a frog model both with and without a fully inflated vocal sac) on a string attached to the ceiling of our test chamber, 20 cm in front of the speaker. We used two dead frogs and inflated their vocal sacs by piercing a small metal tube through the membrane and by closing the puncture with superglue (the two specimens were acquired from an IACUC-approved frozen stock used to feed bats as part of their captive diet). Echoes were recorded with the ultrasonic G.R.A.S. microphone placed 5 cm above the speaker, directed at the object of interest. The playback and recording setup was covered with sound-absorbing foam to reduce the returning echoes other than from the objects of interest. For comparison between the frog model and the dead frogs we used a synthetic echolocation call, based on *T. cirrhosus* flight calls recorded earlier from two individuals. For assessment of target strength and attenuation rate we used a different synthetic echolocation call, based on average measurements of perch calls made during Experiment 2 (see below). Target strength (difference in dB between peak amplitude of echo and a reference signal) was estimated following Surlykke et al. (Surlykke et al., 1999) and we compared spectral profiles of dead frogs with our frog models, as well as models with and without an inflated vocal sac.

We recorded perch calls of six bats with an amplitude-calibrated G.R.A.S. microphone (amplified by 40 dB by G.R.A.S. amplifiers connected to a Avisoft ultrasound gate) placed 10 cm in front of the experimental setup, 10 cm from the ground. Calls were recorded on a Lenovo Thinkpad at a sampling rate of 300 kHz and we selected for each individual up to five calls that were recorded on axis (as confirmed by an additional camera, or with night goggles) for further analyses. We measured peak frequency, peak frequency amplitude (dB SPL), start and end frequencies of the second harmonic, call duration and frequency bandwidth (range of spectral energy present at 20 dB below peak frequency) using Avisoft (Saslab Pro).

Additionally, we used the echoes recorded in the ensonification experiment to assess attenuation rates between our robofrog model and the bat's perch. Echoes were broadcast with the ultrasonic playback device under environmental conditions that were similar to the conditions during testing (25°C, 80% relative humidity). We recorded echoes at 20 cm and 3.7 m (average distance between the perch and platform during perch call recordings) from the speaker with the G.R.A.S. microphone and assessed the attenuation rate in Avisoft.

We calculated whether the echo-acoustic information returning from the robofrog would be above a theoretical detection threshold of 0 dB (Surlykke and Kalko, 2008) [echo amplitude at the perch=amplitude at model + target strength + attenuation rate, following Surlykke and Kalko (Surlykke and Kalko, 2008)].

Statistical analyses

We compared the number of attacks on the vocal sac models with the number of attacks on the control model frog using generalized linear mixed models (GLMMs) in R v. 3.0 (Team, 2012), with a binomial error structure, a logit-link function and bat ID as random effect. We used the binding function in R and assessed whether bats preferred to attack the robofrog by testing for a significant positive intercept. Additionally, we added treatment as fixed effect for Experiment 2 (stimulus presentation) and Experiment 3 (cue isolation) and used Tukey's *post hoc* tests to assess difference between treatment groups.

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Competing interests

The authors declare no competing financial interests.

Author contributions

W.H., R.C.T., M.J.R., R.A.P. and P.L.J. conceived the research and wrote the manuscript. W.H. and P.L.J. designed the experiments. W.H., M.M.D. and K.J.O. carried out the experiments and analyzed the data.

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