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Visual sensitivity to a conspicuous male cue varies by reproductive state in *Physalaemus pustulosus* females

Molly E. Cummings[1,*], Ximena E. Bernal[1], Roberto Reynaga[1], A. Stanley Rand[2] and Michael J. Ryan[1,2]

[1]Section of Integrative Biology, University of Texas, Austin, TX 78712, USA and [2]Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Panama

*Author for correspondence (e-mail: mcummings@mail.utexas.edu)

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SUMMARY

The vocal sac is a visually conspicuous attribute of most male frogs, but its role in visual communication has only been demonstrated recently in diurnally displaying frogs. Here we characterized the spectral properties of the inflated vocal sac of male túngara frogs (*Physalaemus pustulosus*), a nocturnal species, and túngara visual sensitivity to this cue across reproductive state and sex. We measured the spectral and total reflectance of different male body regions, including inflated and non-inflated vocal sacs, along with samples of the visual background against which males are perceived. Inflated vocal sacs were the most reflective of all body parts, being one log unit more reflective than background materials. We utilized an optomotor drum with black stripes and stripes that mimicked the spectral reflectance of the inflated vocal sacs with various nocturnal light intensities to measure the visual sensitivity thresholds of males, non-reproductive females and reproductive females. All three groups exhibited visual sensitivities corresponding to intensities below moonless conditions in open habitats or at the edge of secondary tropical forests. Reproductive females exhibited the greatest visual sensitivity of all groups, and were significantly more sensitive than non-reproductive females. Though the mechanism for this physiological difference between reproductive and non-reproductive females is unknown, it is consistent with previously observed patterns of light-dependent phonotaxic behavior in túngaras. We suggest that the visual ecology of the vocal sac, especially in nocturnal frogs, offers a rich source for investigations of visual ecology and physiological regulation of vision.

Key words: visual sensitivity, reproductive status, visual cue, túngara frog, nocturnal vision, vocal sac.

INTRODUCTION

Most frogs are nocturnal, and the primary mode of reproductive communication is the advertisement call. Calling in many species of frogs is facilitated by large vocal sacs that recycle air during calling, and which also make the calling male more conspicuous (de Jongh and Gans, 1969; Martin, 1972; Gans, 1973; Dudley and Rand, 1991; Pauly et al., 2006). Although diurnal species typically call, mounting evidence shows that many use both body coloration and vocal sac pulsation to attract mates or repel rivals (Lüddecke, 1999; Summers et al., 1999; Hödl and Amézquita, 2001; Narins et al., 2003; Hirschmann and Hödl, 2006). Less expected is that in at least one species of nocturnal frog, the túngara frog (*Physalaemus pustulosus*), vocal sac inflation serves as a dynamic visual cue used by females when choosing mates (Rosenthal et al., 2004; Taylor et al., 2008). There is, however, relatively little understanding of how visual communication in this frog can take place 'in the dark'. Here we explored the conspicuousness and detectability of this visual cue in a nocturnal animal that has a well-developed auditory communication system.

Túngara frogs breed during the rainy season in Panama, from about May to December. Males join in choruses that range in size from a few animals to over a hundred. These breeding sites can be under the forest canopy or in open fields. The frogs breed under a range of light intensities. From an anthropocentric perspective 'it is too dark to see your hand in front of your face is the literal truth in the middle of the jungle on a cloudy night' (p. 166 of Ryan, 1985) while on a cloudless night with a full moon you could read a newspaper. Jaeger and Hailman (Jaeger and Hailman, 1981) reported that most activity of túngara frogs on Barro Colorado Island, Panama, occurred under 0.01 lx (~1.5×10^{-9} W cm^{-2}), the lowest light level they could measure. These frogs are prey for a number of predators, some of which find them by orienting to the frogs' vocalization while others seem to rely on visual cues (Ryan, 1985).

The vocal sac of túngara frogs is a rather conspicuous feature to the human observer. It differs in color from the surrounding area in being generally lighter than the rest of the dark-bodied frog. Through buccal pumping a male túngara frog can force pulmonary air into this distensible cavity, and this enlarged feature can increase the male's size by nearly 100% (Savitzky et al., 1999; Dudley and Rand, 1991). This extension of the vocal cavity clearly serves a vocalization role – allowing the males to attract females through a higher calling rate *via* recycling of air (Pauly et al., 2006) and thus reducing the cost of re-inflation (Bucher et al., 1982), and, as in all frogs, enhancing the coupling of sound to the environment (Ryan, 1985). Yet the light coloration, coupled with the large size, may serve as a visual cue for females trying to assess males under night-time skies. Hence, the objectives of our study were threefold: (1) to characterize the spectral properties of the putative 'vocal sac' visual cue, (2) to determine whether túngara frogs can detect this cue under nocturnal light conditions, and (3) to determine whether sensitivity to visual cues varies by reproductive state or sex.

MATERIALS AND METHODS

Reflectance and moonlight irradiance measurements

In June 2000, in Gamboa, Panama, 15 male *P. pustulosus* (Cannatella and Duellman, 1984) were collected from the field and measured for spectral reflectance within 6 h of capture. Head, ventral surface, dorsal surface and non-inflated vocal sac regions were measured on hand-held, non-anesthetized frogs with an Ocean Optics S2000 spectrometer and reflectance probe (Ocean Optics R400-7 UV/VIS, Dunedin, FL, USA) with an Analytical Instrument Systems deuterium–halogen light source (AIS model DT 1000, Flemington, NJ, USA). Directly following these measurements, males were killed by an overdose of MS222 for spectral reflectance measurements of inflated vocal sacs. The nares and mouth were sealed with Krazy Glue (Columbus, OH, USA), and a syringe was inserted into the posterior of the vocal sac; air was blown into the frog until the vocal sac was fully inflated (no wrinkles present). Four to six spots were measured per region (head, dorsum, ventrum, non-inflated vocal sac and inflated vocal sac) and each spot was measured at least 3 times (N=12–18 reflectance spectra collected per body region per male). Background substrate (mud, stone, dead leaves), where male frogs were collected, was also brought back to the lab for immediate reflectance measurements.

The visual system operates by color-blind rod photoreceptors at night, and consequently visual targets are assessed in terms of lightness and darkness rather than color, which in daylight would be processed by comparing the output of different cone photoreceptors. The conspicuousness of nocturnal visual signals is therefore a function of how much light a target can produce relative to background, as opposed to the specific color contrast. To assess the conspicuousness of a given body part of a túngara frog, we calculated the total reflectance flux for each region:

$$\log\left[\sum_{\lambda=300}^{700} R(\lambda)\right], \qquad (1)$$

and compared this with the total reflectance flux of background materials. We also compared the specific increase in conspicuousness a male would achieve by inflating *versus* not inflating his vocal sac. For six of the 15 males, spectral reflectance measurements were collected from the left, middle and right areas of the vocal sac in a non-inflated condition and also measured in the inflated condition. Pair-wise comparison of reflectance intensities (Eqn 1) between inflated and non-inflated states was conducted using Student's paired *t*-test.

Nocturnal irradiance measurements were collected in Gamboa, Panama, in different areas along the edge of a secondary forest with túngara frogs present, using an International Light (Peabody, MA, USA) IL1700 Research Radiometer and calibrated PM271C photomultiplier detector with a 200–675 nm sensitivity range. Measurements were made by pointing the photomultiplier detector directly up in the sky (90° zenith angle) and towards background vegetation (horizontal to Earth's surface, 0° zenith angle) in June 2007 near midnight on three evenings: June 13th (00:06 h, light overcast sky, 1 day from new moon), June 23rd (23:02 h, light overcast sky, 1 day greater than quarter moon) and June 28th (22:00 h, light overcast sky, 2 days prior to full moon; Table 1).

Table 1. Optomotor illumination settings and nocturnal measurements (collected in Gamboa, Panama, in June 2007) using the IL1700 radiometer and PM271C photomultiplier detector

Trial setting	Optomotor illumination W cm^{-2} [log(W cm^{-2})]	Date: moon phase: detector orientation Nocturnal illumination measurement W cm^{-2} [log(W cm^{-2})]
1	1.2×10^{-13} [–12.9]	–
2	5.9×10^{-11} [–10.2]	–
3	7.3×10^{-10} [–9.1]	–
4	3.2×10^{-9} [–8.5]	13th June 2007: new moon – 1 day: horizontal 5.1×10^{-9} [–8.3] 23rd June 2007: quarter moon + 1 day: horizontal 3.7×10^{-9} [–8.4]
5	8.8×10^{-9} [–8.1]	–
6	1.7×10^{-8} [–7.8]	13th June 2007: new moon – 1 day: vertical 2.5×10^{-8} [–7.6] 28th June 2007: full moon – 2 days: horizontal 1.3×10^{-8} [–7.9]
–	–	23rd June 2007: quarter moon + 1 day: vertical 8.7×10^{-8} [–7.1]
–	–	28th June 2007: full moon – 2 days: vertical 2.4×10^{-7} [–6.6]

Vertical measurements were taken with the detector directly up towards sky (90° zenith angle), and horizontal measurements were taken parallel to the ground towards the background vegetation. The left and center columns indicate the optomotor trial setting and illumination measurement for each setting. The right column aligns the nocturnal measurement most similar to each optomotor trial setting.

Optomotor device

An optomotor device estimates visual sensitivity by measuring the optokinetic response of an animal, and this response is exhibited by a wide range of taxa, from fish (Lyon, 1904; Schaerer and Neumeyer, 1996), to insects (Hassenstein and Reichardt, 1956; McCann and MacGinitie, 1965), to frogs (Cronly-Dillon and Muntz, 1965). The optokinetic response is eye orientation followed by head and body movements coincident with vertical features in a moving environment. An optomotor device usually involves a rotatable striped drum with an interior stationary chamber that houses the focal animal and a means to monitor the animal's movement with the movement of the stripes. The intensity of the illuminating light can be modified to determine threshold sensitivity levels (the minimal intensity at which the animal follows the movement of the drum).

Typical optomotor devices incorporate a black and white striped drum that is monochromatically illuminated to test sensitivity functions across a span of wavelengths (e.g. Maan et al., 2006). Our question, however, was at what light intensities can túngara frogs see the inflated vocal sac? Thus we replaced the white stripes with a color that matched the reflectance spectrum of the inflated vocal sac. To do so, we placed a combination of spectral filters (Lee 185, Lee Filters, Burbank, CA, USA; GamColor 305, GamColor 1516 Gamproducts Inc., Los Angeles, CA, USA) above a white background for the 'vocal sac' colored stripes, and used electrical tape for the black stripes. All stripes were 2 cm in width. Fig. 1B shows the approximate matching between the reflectance of a male's inflated vocal sac and that of the 'vocal sac' drum stripe. Instead of using monochromatic light, we employed a light source with a spectrum mimicking that of

moonlight (see Fig. 1B). Reflectance measurements of both of the optomotor stripes (black tape and the combination of spectral filters) were made to calculate the contrast of the visual task. The Michelson contrast $(I_{max}–I_{min})/(I_{max}+I_{min})$, where I is the estimated radiant flux, between the black (I_{min}) and 'vocal sac colored' (I_{max}) stripes is 0.74. This level of contrast is of the same order estimated between inflated vocal sac and different natural backgrounds (from Fig. 2; contrast of vocal sac to stone, 0.73; to mud, 0.59; and to dead leaves, 0.67).

We constructed our optomotor device using a Grainger Gearmotor (50 RPM 90VDC model no. 4Z538 with DC speed control model no. 6A191; Roanoke, TX, USA) and ultra violet-transparent interior chamber for the frog. We used a PL-900 Dolan-Jenner Fiber-Lite (Boxborough, MA, USA) light source with Lee no. 138 filter over the end tip to simulate a moonlight irradiance spectrum, illuminating the chamber from below. We used a StellarNet Inc. (Tampa, FL, USA) charge coupled device (CCD) portable spectrometer (EPP2000C-UV+VIS) with a 400 μm fiber optic cable and CR-2UV cosine collector to measure the spectral irradiance of the optomotor illumination spectrum. CCD spectrometers are relatively insensitive to lower intensities of light, and thus we were not able to measure the spectral irradiance at the low light settings used during our optomotor trials that mimicked nocturnal conditions. However, to test whether the spectral distribution of our light source was a sufficient mimic to that of previous reports of moonlight spectra, we measured spectral irradiance of the Dolan-Jenner light source at its highest setting with the Lee 138 filter (Fig. 1B). While this illumination sufficiently mimics the spectral distribution of moonlight from 500–700 nm, it is relatively deficient in flux below 450 nm. We investigated optomotor response at six different light intensity settings, with intensity controlled by a rheostat. Trial setting intensities were measured using an International Light IL1700 Research Radiometer with a calibrated PM271C photomultiplier detector with a 200–675 nm sensitivity range placed in the center of the optomotor unit (see Fig. 1A) and directed towards the striped chamber at the same height and distance from the striped drum as viewed by the tested frogs. The six light-intensity levels for the optomotor trials represented a 5-fold change in log intensity (Table 1). The entire optomotor device was enclosed behind an opaque curtain to control the light environment. We placed an infrared camera (Panasonic WV-BP330 camera, WV-LFZF61/2 TV lens; Secaucus, NJ, USA) stationed directly above the optomotor drum. The inside of the drum was illuminated with infrared light (25 W halogen + B+W 58 mm 0.93 IR lens) connected to a Panasonic monitor (model no. 1420) outside the enclosure area to observe frog movement during the optomotor trials. The monitor screen was sectioned into 360° regions, and observers monitored the direction (with or against the drum rotation) and distance (number of degrees covered, with 5°≈1 cm at the edge of the cylinder) for each movement the frog made during the 2 min trials.

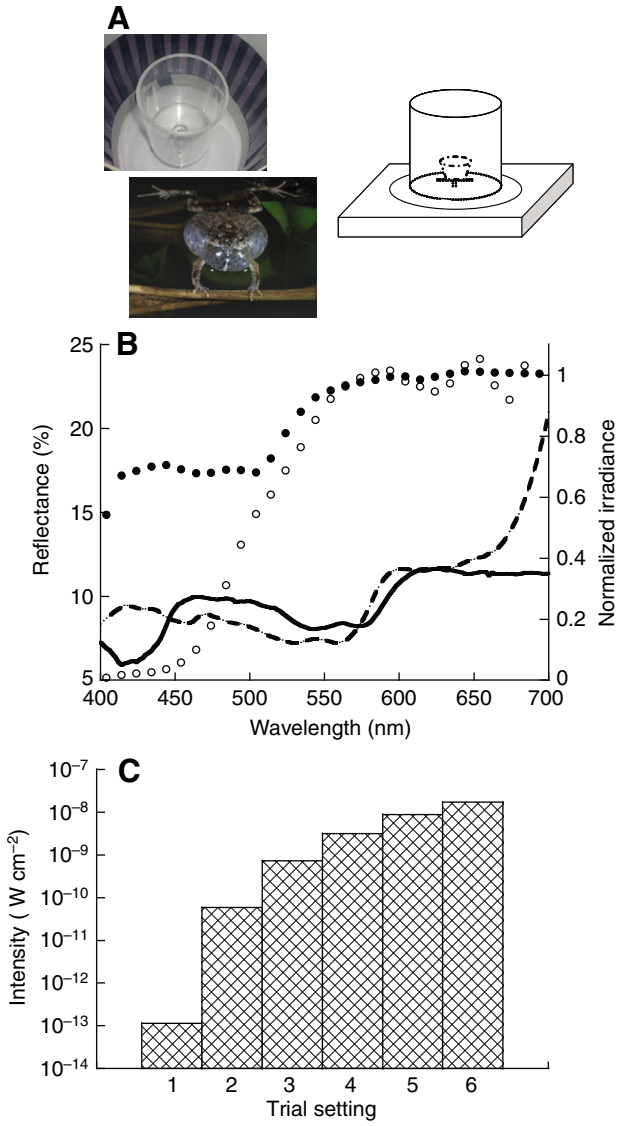

Fig. 1. (A) Optomotor device and male túngara with inflated vocal sac. (B) Optomotor stimuli and illumination spectra. Túngara-specific optomotor stripe reflectance: representative male inflated vocal sac spectral reflectance (solid line) and inflated vocal sac imitation stripes (spectral filters: Lee 185 + GamColor 305 + GamColor 1516 above white construction paper; broken line). Moonlight irradiance measurements (filled circles) in air and under clear skies [recreated from figure 4C in McFarland (McFarland, 1991)], and spectral irradiance measurements of our optomotor moonlight mimic illumination (open circles; Dolan-Jenner + Lee 138 filter). Both spectra were normalized to the flux at 600 nm. (C) Optomotor illumination intensities for each of the six trial settings.

Frog selection

From July 2003 to November 2004, 25 male and 40 female túngara frogs were selected from our colony population (in Austin, TX, USA) to be tested for visual sensitivity. Prior to testing, all individuals were measured (snout-to-vent length, SVL) and their reproductive state recorded. All males were of unknown reproductive readiness, although none had recently mated. Females were classified into two categories: reproductive (having oviposited within 48 h preceding test) or non-reproductive (no oviposition within 5 days of testing). After an initial optomotor test, females were isolated in containers or small colony tanks to monitor reproductive state. They were then retested in the alternative state. If females were first tested in a non-reproductive state, they were then placed in a chamber with a male and monitored daily for reproductive activity. Most females that were initially tested while reproductive were then retested 2 weeks later for their non-reproductive state measurement.

To induce oviposition a small subset of females were injected with either 100 or 500 i.u. of human chorionic gonadotropin (HCG; Sigma, St Louis, MO, USA). This is a ligand for luteinizing hormones that increases gonadal hormone production, and has been used previously in this species to induce reproductive-state behaviors

(Lynch et al., 2006). Females were injected with HCG and then placed with a male for 24 h; if a female oviposited she was tested in the optomotor chamber within 24 h of injection.

Optomotor procedure

Frogs were dark adapted for 60 min and then placed in a moistened optomotor inner chamber and allowed to acclimate for 5 min. After acclimation, a trial began by rotating the outer striped drum for 2 min at 3.3 r.p.m. in either the clockwise or counterclockwise direction while the frog's movements relative to the motion of the drum were monitored with an infrared video system. Angular movement of the frog was characterized by placing a transparency with 360° marked in 5° degree partitions above the inner chamber image on the monitor. Observers recorded the angle of the frog's initial position and then cataloged the direction (clockwise or counterclockwise) and the ultimate position (in degrees) for each movement the frog made during the 2 min observation period. Full 360° revolutions and jumping behavior were also noted. After 2 min, the drum rotation was reversed and frog movements and orientations were quantified for the opposite direction. After the frog completed a 2 min observation period in each direction, the light intensity level was increased to the next level and the frog was given another 5 min acclimation time before the testing process was repeated. All frogs were initially exposed to the lowest light intensity level (1.16×10^{-13} W cm^{-2}) and subsequently tested with incremental increases in light intensity (level 1, 2, 3, etc.) and all frogs were exposed to all six light levels. The sequence of tests was not randomized, which was necessary to maintain dark adaptation.

A pilot study was conducted to determine whether movements in the optomotor drum were random, by including a control trial prior to each optomotor trial. A control trial involved observing the total movements of the frog for 2 min with no motion of the drum. Comparison between control trials and optomotor trials indicated that movement in the optomotor trial was significantly greater than movement in the control trial (mean ± s.e.m. total degrees moved in 2 min for control, 72.5±11.9°; total degrees moved in 2 min optomotor trial period, 210.0±15.3°; d.f.=225, paired t=7.88, $P=1.4\times10^{-13}$); with 38% of the control trials exhibiting no movements over the 2 min observation period. Given the great variation in response across individuals, we employed a criterion response similar to that employed with other highly mobile taxa, e.g. zebrafish (Krauss and Neumeyer, 2003). A positive optomotor response was scored only if two criteria were met: (1) the frog showed a net positive movement in both 2 min trials in the same direction as the drum rotation, and (2) the proportion of all movements made by each frog in the 4 min observation (distance in angles where 5°≈1 cm at the outer edge of the optomotor cylinder) was >65% in the direction of the drum's rotation. This latter criterion was more stringent than some other studies that evaluated all movements of a subject during optomotor trials [e.g. 60% of all movement in the drum's direction (Krauss and Neumeyer, 2003)]. If an animal did not meet both criteria in any of the six light-intensity levels, they were not used in further analyses. An individual frog's threshold response was defined as the lowest trial setting in which a positive optomotor response was recorded. Each individual's 'threshold response' was used for all statistical analyses.

RESULTS

Reflectance

Mean reflectance spectra for each body region (Fig. 2A) represent the average calculated from 147 (head), 195 (dorsal), 198 (ventral), 189 (inflated vocal sac) and 159 (non-inflated vocal sac) reflectance spectra. Background reflectance averages were calculated from 36 (mud), 21 (dead leaves) and 9 (stone) reflectance spectra. In general, the inflated vocal sac represents a conspicuous visual target in that it exhibited nearly 1 log unit greater reflectance intensity than the background material: mean ± 1 s.e.m. reflectance intensity (see Eqn 1) of inflated vocal sacs, 3.05±0.05; stone, 2.18±0.07; dead leaves, 2.23±0.08; mud, 2.32±0.04. Fig. 2B illustrates the difference in reflectance spectra of individual males with and without inflated vocal sacs. Pair-wise comparison of reflectance intensity (Eqn 1) between the left, middle and right areas of male vocal sacs showed a significant increase in total reflectance in inflated relative to non-inflated vocal sacs (mean inflated vocal sacs, 3.05±0.05; non-inflated vocal sacs, 2.73±0.07; N=18 paired spectra, 6 males, 3 vocal sac areas per male; t=5.89, P=0.00002).

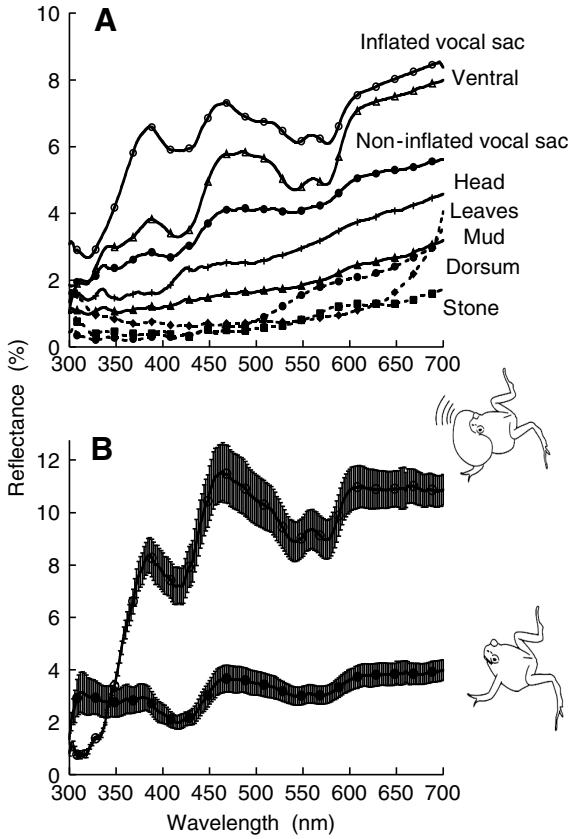

Fig. 2. Male túngara and background spectral reflectance. (A) Average spectral reflectance of body regions from 15 male túngaras (solid lines) including head (+), dorsal (▲), ventral (△), non-inflated vocal sac (●) and inflated vocal sac (○). Average background material spectral reflectance is also shown (broken lines) including mud (●), stone (■) and dead leaves (◆). (B) Average spectral reflectance of the vocal sac of a representative male túngara frog in non-inflated (●) and inflated (○) states. Mean ± s.e.m. reflectance data include replicates made in left, middle and right areas of the vocal sac.

Optomotor responses

Eighty-seven trials using vocal sac-specific optomotor stimuli (see Fig. 1) were conducted with 25 males and 40 females including numerous repeated measurements from the same individual in different reproductive states. Of the initial 65 individuals, only 23 males and 33 females that showed a complete optomotor response (i.e. met the criteria for an optomotor response in one of the six intensity levels) were included in further analyses. Optomotor

responses were quite strong among individuals, with movement in the direction of the rotating drum significantly greater than movements in the opposite direction [Student's paired *t*-test, mean ± s.e.m. distance (in degrees) moved with the direction of the rotating drum, 204.4±21.5°; mean degrees moved against the direction of the rotating drum, 24.0±7.2°; t=9.49, d.f.=54, $P=4.1\times10^{-13}$]. The mean ± s.e.m. per cent movement with the optomotor rotation was 93±2.5% of all movements across the 4 min observation period.

Of the 33 female túngara frogs, only 14 were tested successfully in both the reproductive and non-reproductive state. Of these 14 repeat females, eight were initially tested in the reproductive state and six were initially tested in the non-reproductive state. Mean optomotor threshold responses between reproductive and non-reproductive state among females were different (mean ± s.e.m. reproductive female threshold response, 1.86±0.23; mean non-reproductive female threshold response, 2.64±0.40); however, there was a significant order effect (ANOVA model on 14 repeat females with threshold response as the dependent variable, reproductive state as a factor, and trial order as covariate: reproductive state F=2.214, P=0.149; trial order F=7.17, P=0.013). Females showed greater sensitivity (lower threshold response) in their first optomotor test than in their second (Fig. 3; mean ± s.e.m. first optomotor threshold response, 1.64±0.17; mean second optomotor threshold response, 2.86±0.39; Wilcoxon signed-rank test, z=2.579, P=0.01).

Given the significant order effect, we compared only the initial testing trial between females first tested in the reproductive state (N=12) and those first tested in the non-reproductive state (N=20). The majority of the 12 reproductive females were tested within 24 h of laying foam nests (five tested on the same day as laying a foam nest; four on the next day; two on the second day, and one on the third day). We observed a significant difference in optomotor threshold response between females in the reproductive and non-reproductive states (mean ± s.e.m. reproductive state optomotor threshold response, 2.25±0.35; median, 2; mean non-reproductive state optomotor threshold response, 3.6±0.28; median, 4; Kruskal–Wallis, U=51, χ^2=7.535, P=0.006). Comparing responses between equivalent sample sizes yielded an even stronger difference (first 12 non-reproductive females' mean optomotor threshold response, 3.92±0.35).

Three of the 12 reproductive females had been injected with HCG to induce oviposition. Whether females oviposited naturally or with a 100–500 i.u. injection of HCG did not significantly affect their visual sensitivity (mean ± s.e.m. optomotor threshold for natural egg-laying females, 2.33±0.44; for HCG-injected females, 2.0±0.58; Kruskal–Wallis, U=15, P=0.77). Given the lack of significant differences between HCG and naturally ovipositing females, we pooled all reproductive females for comparison across groups (non-reproductive females and males). Reproductive females showed the greatest visual sensitivity across all three groups (Fig. 4; Kruskal–Wallis test, 10.74, P=0.005) and *post hoc* comparisons showed that the only significant difference was between non-reproductive and reproductive females (Tukey HSD probabilities: male *versus* non-reproductive females, P=0.43; male *versus* reproductive females, P=0.19; reproductive *versus* non-reproductive females, P=0.02).

Comparing visual sensitivities to nocturnal irradiance measurements, all three groups exhibited visual sensitivities at intensities that were below the near new moon vertical measurement as well as quarter moon horizontal measurements (Table 1). This

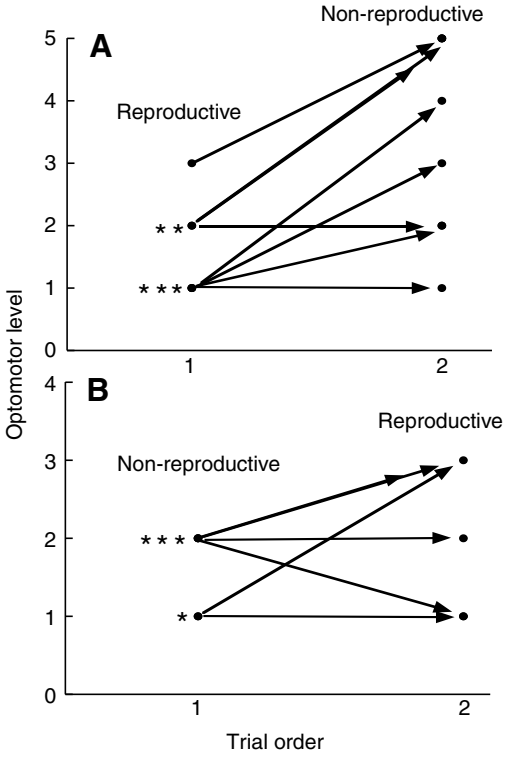

Fig. 3. Repeat testing of 14 túngara females and the optomotor trial setting of their threshold response. (A) Females initially tested in the reproductive state (N=8), and (B) females initially tested in the non-reproductive state (N=6). Each line connects the threshold responses for a single female with arrowheads indicating the direction of sensitivity change between the first and second testing dates. Asterisks indicate additional females exhibiting the same initial threshold response.

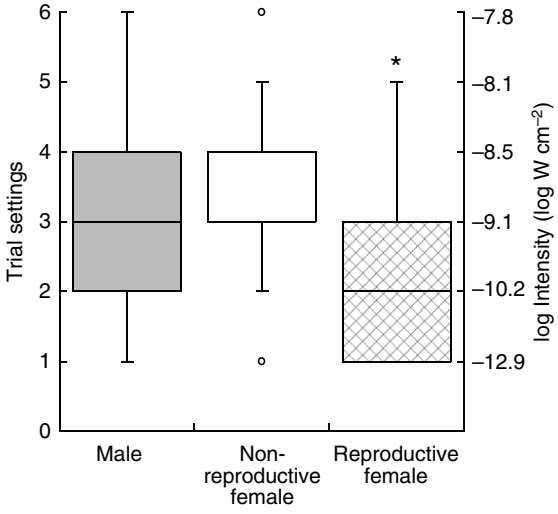

Fig. 4. Box and whisker plots of visual sensitivity thresholds (trial setting of threshold response and corresponding intensity levels) across three testing groups: males (gray, N=22); females in a non-reproductive state (white, N=20) and reproductive state females (cross-hatched; N=12). The center horizontal line marks the median of the sample, and the length of each box shows the range containing 50% of all values with the box edges representing the first and third quartiles. The open circles represent far outside values or outliers (>first or third quartile ± 3×interquartile range). Each group includes data from individuals obtained on their initial test only. *P<0.05.

result suggests that all groups have visual thresholds allowing detection of the inflated vocal sac under nearly all nocturnal sky conditions in an open environment.

Size differences

Body size was also a significant covariate in accounting for variation in optomotor responses among individuals [ANOVA model on all tested individuals (N=53) with threshold response as the dependent variable, group ID (N=3, male, reproductive female or non-reproductive female) as a factor, and body size (SVL) as covariate: group ID F=8.011, P=0.001; body size F=4.5, P=0.039]. Across all groups, body size did not explain a significant amount of the variation in optomotor response [Fig. 5A, r^2=0.009, N=53 (note two individuals were not measured), t=0.665; P=0.509]. Within each group, however, the relationship between size and response varied. Both males and reproductive females showed no significant relationship between SVL and visual sensitivity (males r^2=0.105, t=1.53, P=0.14; reproductive females r^2=0.035, t=0.573, P=0.58), whereas non-reproductive females showed a significant relationship between size and optomotor threshold response (non-reproductive females r^2=0.212, t=2.2, P=0.04), with larger females showing a decrease in visual sensitivity. Importantly, reproductive females were slightly larger than non-reproductive females (mean ± s.e.m. SVL of reproductive females, 29.5±0.62 mm, N=12; non-reproductive females, 27.3±0.63 mm, N=20; t=2.25, P=0.03). Thus, differences in visual sensitivity due to size alone would suggest that the reproductive group of females should have lower visual sensitivity (higher optomotor thresholds). Of note, the initial 12 females tested in each condition showed no difference in mean size (mean ± s.e.m. SVL of reproductive females, 29.5±0.62 mm; first 12 non-reproductive females, 29.2±0.46 mm; t=0.437, P=0.67). Mean male SVL was 28.4±0.28 mm, which was not significantly larger than that of the non-reproductive females (t=1.64, P=0.11) nor smaller than that of the reproductive females (t=1.82, P=0.08).

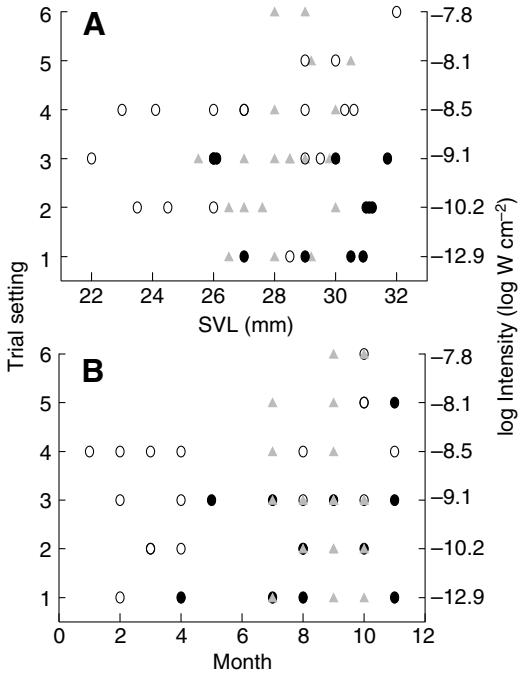

Fig. 5. (A) Optomotor threshold response by size (SVL, snout-to-vent length) across all three testing groups: males (gray triangles), non-reproductive females (open circles) and reproductive females (filled circles). (B) Optomotor threshold response by testing month across all three testing groups where 1 is January and 12 is December.

Seasonal differences

Overall, there was no significant relationship between season (month of testing) and optomotor threshold response (r^2=0.019, N=55, t=1.012; P=0.316; Fig. 5B). There was also no significant relationship between threshold response and month with either males or reproductive females (reproductive females: r^2=0.111, N=12, t=1.117, P=0.29; males: r^2<0.001, N=23, t=0.064, P=0.95). There was, however, a significant relationship between optomotor threshold and season within the non-reproductive females (r^2=0.286, N=20, t=2.687, P=0.015), with non-reproductive females showing the greatest sensitivity in the early spring months (February–April).

DISCUSSION

The vocal sac as a visual cue in frogs

One of the more distinguishing features of frogs is their distensible vocal sac. As mentioned previously, the vocal sac has numerous functions associated with calling. The vocal sac of anurans, however, can exhibit a stunning variety of colors, patterns, sizes and shapes. It is possible that the size and shape of the vocal sac could serve an acoustic function; both could influence how it couples the sound to the environment. But not so for the colors and patterns that adorn it. These adornments suggest the result of selection to be visually conspicuous. Many herpetologists have had the experience of attempting to find a calling male frog, but being unable to do so until the frog revealed itself by inflating its vocal sac. Thus it seemed the vocal sac might serve a visual cue, but it was not obvious that it could function as such in the dark. We have now shown that túngara frogs can see the vocal sac at light levels that are found under the darkest of nocturnal conditions (moonless sky), and that the vocal sac increases the visual conspicuousness of the frog against the background it calls against in the wild. We have shown that the visual sensitivity of túngara frogs allows them to perceive the inflated vocal sac under the majority of nights in the lunar cycle when the sky is clear or with light overcast, and when the frogs are in an open habitat or on the edge of secondary forest. We do not have data on nocturnal intensities under the forest canopy, where these frogs sometimes breed, and thus cannot assert that this form of visual communication is possible wherever the frogs breed. We do assert, however, that our data demonstrate that the female's behavioral response to the vocal sac as a visual cue that has been shown in the lab (Rosenthal et al., 2004; Taylor et al., 2008) can also function in the wild.

We suggest that the vocal sac initially evolved under selection for acoustic function and has been co-opted in túngara frogs, and probably many other frogs, as a visual cue. Although this was known in diurnal frogs (Lüddecke, 1999; Summers et al., 1999; Hödl and Amézquita, 2001; Narins et al., 2003; Hirschmann and Hödl, 2006), we have now shown it is true in at least one nocturnal species. There are other instances in which dynamic structures associated with acoustic signaling serve as additional cues for communication. One example is the McGurk effect (McGurk and McDonald, 1976), in which the movements of the lips associated with speech contribute to speech recognition in humans. A challenge in any form of communication is to produce signals that can be distinguished from background noise (Maynard Smith and Harper, 2003; Ryan and Cummings, 2005). Thus it comes as no surprise that such a conspicuous and dynamic structure as the frog's vocal sac, which is so intricately associated with vocalization, would come to serve a communication function. We refer to the vocal sac in this context

as a visual cue rather than a visual signal since it is an aspect of the phenotype that provides information to the receiver but did not evolve under selection for signaling (e.g. Hauser, 1996). We do suggest, however, that the colors and patterns that sometimes adorn vocal sacs probably have, in many circumstances, evolved for a communication function and thus might be considered visual signals rather than visual cues. The striking variation in vocal sacs among frogs offers a rich, untapped resource for studies of visual and multimodal communication.

We also suggest that the particular coloration of the inflated vocal sacs of túngara frogs appears to have been selected for detectability by frog viewers. When the vocal sac of male túngara frogs is inflated it has higher reflectance, and therefore is likely to be more conspicuous, against the dark background than any other body region measured (Fig. 2). This increase in reflected light is predominantly in the middle wavelength region (450–550 nm), which corresponds to the visual sensitivity of most anuran rods (reported λ_{max} values, 498–529 nm) (Liebman and Entine, 1968; Hárosi, 1975; Donner et al., 1990; Fyhrquist et al., 1998; Palma et al., 2001), and consequently the increase in reflected light is likely to be detectable by conspecifics.

Dimorphic response

We found that reproductive females exhibited a visual response at lower light intensities than did males or non-reproductive females (Fig. 4). Reproductive females, on average, exhibited a 1 log unit increase in sensitivity over non-reproductive females, and a 0.5 log unit increase in sensitivity over males. If the vocal sac is a component of the male calling signal, it is relevant that reproductive females showed a mean response to the vocal sac coloration under the darkest of night sky conditions. Rand et al. (Rand et al., 1997) showed that female mate choice behavior is affected by light intensity, with significantly more female túngara frogs exhibiting phonotaxis toward male calls under darker light conditions (mimicking moonless nights). Their interpretation was that females are more likely to make mate choice decisions when there is a lower risk of predation from visually orienting predators, which are quite common (Ryan, 1985). We have found that females are capable of seeing a male vocal sac under these conditions where mate choice seems to be most common.

While reproductive females exhibited the greatest visual sensitivity of all three groups studied, there was no significant difference in visual sensitivity between non-reproductive females and males (Fig. 4). If the inflated vocal sac serves as a function in antagonistic interactions as it does in the diurnal frogs with conspicuous vocal sacs, such as *Phrynobatrachus krefftii* (Hirschmann and Hödl, 2006) or *Epipedobates femoralis* (Narins et al., 2003), then we would expect both males and females interested in mating to exhibit abilities to detect this cue under relevant conditions. Males, however, usually remain in the same place while calling, whereas females are coming to the breeding site to find a mate. It is possible that the vocal sac acts as a long-distance beacon to females for mate localization, a function that might be less important to males.

Our results suggest that the inflated vocal sac would be detected by all females and males on most, if not all, nights in a month. It is also worth mentioning that this vocal sac could possibly be detected in even darker environments (deep canopied forests) given that the inflated vocal sac can expand beyond the 2 cm width tested in our optomotor device (Dudley and Rand, 1991). Since scotopic (rod-based) vision operates by spatially pooling photoreceptor signals, a larger, reflective object such as an inflated vocal sac is more likely to be seen than a smaller object of equal reflectivity.

Reproductive state and sensitivity

Perhaps the most intriguing result of our optomotor experiment is that visual sensitivity differed significantly by reproductive state among females. This is interesting as neuroendocrinology studies in túngara frogs have shown that two female reproductive behavior decisions, discrimination (preference for one stimulus over another) and permissiveness (tolerance for unattractive signals), vary with reproductive cycle. Lynch and Wilczynski (Lynch and Wilczynski, 2005) showed that reproductive hormones varied across three reproductive states in female túngara frogs. Plasma concentrations of estrogen and progesterone in unamplexed and post-mated (non-reproductive) females were significantly lower than in amplexed (reproductive) females. Their study showed significant changes in the levels of plasma gonadal steroids despite the túngara frogs showing asynchronous oogenesis (constant production of oocytes), suggesting that the hormonal increase has roles beyond gamete production. Lynch and colleagues have also shown that female choice behavior varies across these same reproductive states with female túngaras showing the maximal receptivity and permissiveness during the amplexed stage or when estrogen levels are increased (Lynch et al., 2005; Lynch et al., 2006). Taken together, their studies suggest that surges in estrogen and progesterone change reproductive behavior in female túngara frogs.

Our findings, though only correlational, suggest that the surge in reproductive hormones may be having physiological effects on the sensory system that may contribute to surges in specific reproductive behaviors. A similar result was found in sticklebacks whereby optomotor tests of visual sensitivity showed that female sticklebacks were more sensitive to red wavelengths than were males during the reproductive season; yet there was no sexual dimorphism in visual sensitivity outside of the breeding season (Cronly-Dillon and Sharma, 1968). The similarity between our results and those of the stickleback study is that red wavelengths correspond to the nuptial coloration that male sticklebacks use to attract females during the breeding season. More recent work on estrogens and the human retina suggests that steroid hormones increase the transmission speed in the optical pathway by augmenting the effect of glutamate and dopamine (Drouva et al., 1988), and are argued to be responsible for the shorter latency values and higher amplitudes of evoked potentials in women (Celesia et al., 1987; Yilmaz et al., 1998; Yilmaz et al., 2000).

Steroid hormones have recently been shown to play a role in sensory tuning of the auditory system. Estrogen- or testosterone-treated midshipman (*Porichthys notatus*) females become more precisely tuned to the temporal cues of the male courtship song than control females (Sisneros et al., 2004a). Similar to túngara frogs and other vertebrates, the onset of reproductive behavior is associated with circulating levels of both estrogen and testosterone (Sisneros et al., 2004b). This group identified an estrogen receptor (ER_α) in the midshipman's saccular epithelium that is suspected to directly control the action of steroids on the sensory neurons. In humans, estrogen fluctuations during the menstrual cycle influence evoked neural responses to sound (Elkind-Hirsch et al., 1992). Estrogen receptors have been found in the inner ear of mammals as well (Stenberg et al., 1999) and are suspected to influence hearing. There have been few investigations of hormonal effects on the auditory system in frogs, but it seems that overall auditory sensitivity is increased, but not frequency shifted, in the breeding season (Yovanof and Feng, 1983; Penna et al., 1992).

CONCLUSION

Although the vocal sac is a visually conspicuous attribute of most frogs, its role in visual communication has only been demonstrated recently. Most studies are of diurnally displaying frogs, by far the minority in this order. To date, the only nocturnal frog in which the vocal sac has been documented as a visual cue is the túngara frog. We have shown here that inflation of the vocal sac greatly increases the frog's conspicuousness against its background in the wild. We have shown that in both males and females the sensitivities to the reflectance spectrum of the vocal sac under nocturnal illumination are such that this mode of communication should be effective for nocturnal communication for most of the frog's breeding season. Furthermore, reproductive females are more sensitive to this spectrum than males or non-reproductive females, mirroring an effect of hormones on vision that has been seen in fish and humans. We suggest the visual ecology of the vocal sac, especially in nocturnal frogs, offers a rich source for investigations of visual ecology and the physiological regulation of vision.

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