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Original Article

Environmental constraints on size-dependent signaling affects mating and rival interactions

Judith A. H. Smit,[a] Hugo Loning,[a] Michael J. Ryan,[b,c] and Wouter Halfwerk[a,b,]
[a]Department of Ecological Science, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081 HV, Amsterdam, The Netherlands, [b]Smithsonian Tropical Research Institute, Apartado 0843-03092 Balboa, Ancón, Republic of Panama, and [c]Department of Integrative Biology, University of Texas, 1 University Station, Austin, TX 78712, USA

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Advertisement signals can convey information about a sender's characteristics, such as body size. The reliability of signals, however, can be reduced when signal production is partially dependent on the environment. Here, we assess the effect of display-site properties on the production, attractiveness and honesty of sexual signals. We recorded male túngara frogs (*Physalaemus pustulosus*) while manipulating water levels in order to constrain calling. We found that water level affected male call properties in a size-dependent manner, with call amplitude being less affected in smaller males when forced to call in shallow water. Next, we tested how size-dependent and display-site-dependent signaling affected female choice and rival competition. Both males and females showed the strongest response to the call of a large male when he was calling at the deepest water levels. However, females showed no preference for large over small males when both were recorded in shallow water levels, or, depending on the call rate and timing of calls, even preferred small males. Likewise, males responded equally to large and small rivals recorded calling during shallow water level trials. Our experiments show that display-site properties can influence signal production and attractiveness in a size-dependent manner. These results can have important consequences for the evolution of signaling, as small males may be able to use their size to their advantage when selecting appropriate display sites and thereby outcompete large males.

Key words: **display site, honest signaling, production constraints, *Physalaemus pustulosus*, sexual selection.**

INTRODUCTION

Communication signals aid animals in functions crucial for reproduction and survival, such as mate attraction, territory defense, and predator avoidance (Bradbury and Vehrencamp 2011). Communication systems, however, can differ in the reliability of information that is transferred from senders to receivers (Bradbury and Vehrencamp 2011). Signal reliability, often anthropomorphically referred to as "honest" communication, is evolutionary stable in situations of shared mutual interest, for example, during group foraging (Gil and Gahr 2002; Hollén et al. 2008; Bradbury and Vehrencamp 2011). Signal reliability may be less stable in situations where senders and receivers have conflicting evolutionary interests, such as during sexual communication between males and females (Johnstone 1997; Kokko and Johnstone 2002).

Some signals are constrained to be reliable (Johnstone 1997; Smith and Harper 2003). These so-called "index signals" are thought to be unfakeable due to physical production limits (Fitch and Hauser 2003; Searcy and Nowicki 2005) and thereby provide reliable information regarding sender properties, such as size, body condition, or available resources (Smith and Harper 2003; Biernaskie et al. 2014). For example, morphological traits can impose constraints on signal design such as the temporal patterning or the range of frequencies that can be produced (Podos 1997; Cooper and Goller 2004; Podos et al. 2004). A well-known example comes from the biomechanical constraint on sound production that determines the relation between the signaler's size and the frequency and amplitude it can produce (Gerhardt 1975; Gingras et al. 2013; Reber et al. 2017). Receivers interested in the size of the signaler thus benefit by attending to the signal's frequency and amplitude characteristics.

The signaling environment can also play an important role in signal reliability. Signals traveling through the environment typically degrade with distance, losing specific frequencies and signal structure (Morton 1975; Wiley and Richards 1978). Environments can differ greatly in transmission properties and the location of the sender therefore determines the signal content that arrives at the receiver (Wiley and Richards 1978; Schwartz et al. 2016). Signals traveling through dense vegetation attenuate faster than signals traveling through more open habitat, affecting both signal amplitude and frequency. Variation in call sites of male tree

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Address correspondence to W. Halfwerk. E-mail: w.h.halfwerk@vu.nl.

frogs, for example, affects how mating signals arrive at females and thus how attractive males are, irrespective of their size or body condition (Schwartz et al. 2016). The calls of male frogs advertising from burrows may also not provide reliable information about sender properties, as acoustic cues are often related to the burrow's size-dependent resonance properties (Cui et al. 2012; Muñoz and Penna 2016). On the other hand, a burrow's size might be useful information for a female that seeks adequate space to deposit her eggs. Receivers can thus only obtain reliable information when paying attention to both the effect of the environment as well as sender properties (e.g., size and weight) on acoustic cues (e.g., frequency and amplitude) that reach them (Ringler et al. 2017).

Signal reliability may also depend on the effect of display sites on signal production, which can be more difficult to assess by signal receivers. Display sites can set limits on how signals can be produced (Halfwerk et al. 2017; Mhatre et al. 2017). Crickets displaying from burrows, for example, need to match the frequency of their calls to the resonance properties of their surroundings to optimize signal transmission (Bailey et al. 2001). Furthermore, tree crickets only make holes, or so-called "baffles" in large compared with small leaves, to enhance the amplitude of their calls (Mhatre et al. 2017). Environment-dependent production constraints can even interact with morphological structures of the signaler. Many cricket species show a size–frequency relationship due to mechanical properties of their signal production mechanism. When calling from burrows, smaller crickets may have to call from smaller burrows compared with larger males, to exploit the resonance properties optimally. Display-site characteristics and signalers' characteristics can thus interact in order to produce the most effective and attractive mating display, which may also provide signalers with opportunities to "cheat" (Halfwerk et al. 2017; Mhatre et al. 2017).

Anurans (frogs and toads) are useful taxa to study the role of the environment in driving signal evolution. In the vast majority of anurans, the dominant mode of communication involves males calling from particular display sites to defend and attract females (Wells 2010). Females sometimes benefit from selecting larger than average males and rely on the male's advertisement calls for their selection process (Ryan 1980, 1983; Fairchild 1981). In many anurans, larger males call louder, slower and at a lower pitch compared with smaller males (Zweifel 1968; Davies and Halliday 1978; Arak 1983, 1988). Females base their choice on the variation in these size-related signal components (Ryan 1980; Marquez and Bosch 1997). Males also attend to the amplitude and frequency of their rivals and respond more strongly to calls from large rivals that impose a greater threat compared with small rivals (Davies and Halliday 1978; Arak 1983). Call production does however not only depend on a male's size, but also on the location from which he is advertising. In particular, frogs that call while floating on a water surface can be constrained in producing loud and attractive calls. In many species, males shuttle a large volume of air from their lungs to a vocal sac and back (Dudley and Rand 1991; Jaramillo et al. 1997). Floating frogs depend on sufficient water levels to support lung and/or sac inflation and shallow water levels thus limit optimal calling, as vocal sac inflation and/or lung volume is proportionally related to call amplitude (Halfwerk et al. 2017). Optimal display-site properties for signal production may however also depend on male characteristics, such as body size (Zweifel 1968; Ziegler et al. 2011).

We focused on the túngara frog (*Physalaemus pustulosus*), a neotropical species that calls while floating on the surface of small, temporal water bodies, typically a few centimeters deep. A calling male produces a multi–harmonic frequency sweep known as a "whine" that is often followed by an amplitude-modulated element known as a "chuck" (Ryan 1985). Males can flexibly add up multiple chucks to the whine, thereby increasing the complexity of their calls (Rand and Ryan 1981; Ryan 1985; Gridi-Papp et al. 2006). Males can also increase their call rate and call amplitude in response to the presence of a female, as well as to rival male competition (Bernal et al. 2009; Akre and Ryan 2011; Halfwerk et al. 2016). Both males and females respond more strongly to calls that are on average more complex, delivered at faster rates and at higher amplitude (Bernal et al. 2009; Akre et al. 2011; Halfwerk et al. 2014). Males call by shuttling air from the lungs to a relatively large vocal sac and need to float in sufficient levels of water in order to inflate the lungs to their full extent (Halfwerk et al. 2017).

Here, we tested whether call-site characteristics can influence signal reliability and whether this in turn affects mating and rival interactions. We focused on the effect of water level at call sites on signal production of males of different sizes and tested whether females as well as rival males paid attention to both a signaler's size and call-site characteristics. We recorded male túngara frogs of different sizes in our custom-built arena that allowed us to manipulate water level in between recording trials without disturbing the focal individual. Males were stimulated to call during deep water levels when they could float and inflate their vocal sacs to the full extent, as well as during shallow water levels that constrained floating as well as vocal sac inflation. We expected larger males to be more constrained by low-water treatments compared with smaller males and thus overall signal reliability to receivers to decrease with water level. Next, we tested whether females preferred the calls of large males to the calls of small males and assessed whether this preference was influenced by water level conditions as well as call timing (an important alternative strategy to increase signal attractiveness). Finally, we carried out a field playback experiment with males to assess whether rivals respond to their opponents depending on the difference in size as well as water level conditions under which the calls were recorded. We expected females to prefer large males to small males, but that this preference depends on signal reliability and thus call-site properties. Likewise, we expected rivals to respond more strongly to large compared with small males, but that the difference in their response would decrease with water depth.

MATERIALS AND METHODS

Study site and species

The study was carried out using túngara frogs (*Physalaemus pustulosus*) and was situated in the rainforest of Soberanía National Park in the Republic of Panama. We recorded males in our lab in Gamboa, in August 2014, and carried out experiments with males and females in May to July 2017. We collected calling male frogs or pairs in amplexus from various locations in the vicinity of Gamboa 1–3 h after sunset. Frogs were brought to the lab in small plastic containers and were kept in a plastic cooler in between experiments. Males and females were either toe-clipped or their belly pattern was photographed for individual recognition. All individuals were measured after the recordings and experiments (mass in grams and snout-vent length, SVL, in mm) and released at the capture site the same night. All experiments were licensed and approved by STRI (IACUC permit: 2014-0805-2017) and the Autoridad Nacional del Ambiente de Panama (SE/A-82–14 & SE/A-38-17).

Experimental recording setup

Twenty males were recorded in a hemi-anechoic chamber under IR-lighting in a setup that allowed us to manipulate water levels.

At the start of the experiment males were placed in a small circular cage (8 cm diameter; 12 cm height) consisting of a ring of evenly spaced nylon monofilament (diameter of 0.05 mm fishing line every 0.5 cm). The cage was placed in a pool (diameter of 50 cm) containing a tube that allowed the experimenter to either add (using a funnel) or subtract (using a 50 mL syringe) water to manipulate water levels at the position of the frog. Males were stimulated with a low-amplitude chorus recording until they were readily calling for 1 min. We assessed male calling behavior at 2 different water levels, ranging from 0.25 to 0.5 cm for the shallow water level treatment and 1.0–2.0 cm for the deep water level treatment (see for additional information on recording conditions Halfwerk et al. 2017). Next, males were stimulated with a low-amplitude chorus sound, a single call (whine + chuck) played every 2 s, or with silence. Males were recorded during 12 consecutive trials during which the water level and sound stimulation was altered in a randomly but balanced manner. Each recording trial lasted for 1 min, followed by a 2-min break during which the water level was altered (starting 1 min before the next trial). Male calls were recorded with a microphone setup (G.R.A.S. 40 BF microphone amplified by 20 dB by G.R.A.S. 26 AC amplifier, G.R.A.S. Sound & Vibration A/S, Holte, Denmark, connected to an Avisoft 116Hm Ultrasound gate, Avisoft Bioacoustics, Glienicke, Germany) onto a desktop PC, using a sampling rate of 50 kHz. The microphone was placed at a 45° angle and at a distance of 50 cm from the frog. The recording setup was calibrated prior to each experiment using a tone generator (G.R.A.S. 42 AB, 114 dB at 1 kHz). We analyzed the call rate and call complexity by counting the number of calls and the number of chucks emitted during the 1-min trials using Avisoft SASLab Pro (Avisoft Bioacoustics, version 5.2). In addition, we selected three calls from the start, middle and end of each recording trial and measured the RMS (root-mean-square) and peak-to-peak amplitude of the whine part of the call.

Stimulus preparations

We selected recordings from the single call stimulation trials for the five smallest and five largest males (SVL min 23.73 mm, max 29.00 mm) that emitted at least 6 calls in both the shallow (0.25 or 0.5 cm, depending on when most calls were produced) and the deep (2.0 cm) water treatment. From each recording we selected the 15 s in which the male produced the most calls and we replaced the stimulating calls by silence using Audacity(R) recording and editing software (version 2.0.6, Audacity Team 2017). Furthermore, we filtered out noise below 50 Hz and added a fade in and fade out of 0.02 s to each call to prevent clicks produced by the speakers. We created ten combinations by selecting recordings from a small and large male with the largest possible size difference. Recordings of each male were used in combination with recordings of 2 other males.

For our first female preference experiment, we looped the 15-s recordings, in which we did not modify the naturally recorded call rate (ranging from 4 to 11 calls per 15 s; see Table 1). Furthermore, since we expected that call timing might influence female preference (Tárano 2015), we repeated the experiment using a standardized call rate of 1 call per every 2 s. For these follow-up experiments, we created a new set of stimulus pairs by selecting one call per 15-s recording (the call with the highest complexity and amplitude). In addition to standardizing the call rates, we altered the timing of the large and small male stimuli so that they were either alternating or overlapping each other. In the call overlap treatment, the lagging call started at 50% of the leading call (Greenfield and Rand 2000; Tárano 2015). For the overlapping stimuli, we created 2 sets, one in which the large male was leading and one set in which the small male was leading. For all stimuli, we maintained the natural variation in frequency, complexity, and amplitude (recordings were obtained by using a calibrated microphone, see above). See also Table 1 for an overview of the number of calls and mean call complexity of the stimuli.

Female preference tests

We assessed whether water level, male size, call timing, and the interactions between these factors influenced a male's attractiveness to females in a hemi-anechoic chamber under IR-lighting. Individual females were placed under an acoustically permeable funnel in the center of the room (2.7 × 1.8 × 1.78 m, L × W × H; Acoustic Systems, Austin, TX) and were stimulated with a playback from two opposing speakers (Mirage Nanosat, Klipsch Group Inc. Indianapolis) placed 80 cm away from the female. The speaker volumes were calibrated with a reference recording of an artificial whine set to 90 dB SPL (re. 20 μPa at 50 cm, measured with an SPL-meter, type 407764, Extech Instruments, Massachusetts, set to C-weighting, fast and max). The amplitude of the reference signal was 6 dB greater in peak-to-peak value than the highest amplitude call used in the preference test. One speaker played the calls from the large male and the opposite speaker the calls from the small male, with the locations of the males (left or right) randomized and balanced. We raised the funnel after 2 min from the start of the playback and scored the female's choice (defined as staying 2 s within a 10 cm radius of the center of a speaker). We also noted the latency until this choice. If the female did not approach one of the speakers within 10 min after raising the funnel, or if the female remained immobile the first 5 min or any subsequent 2 min we ceased the test and no choice was scored. Also, when a female started to climb the walls we stopped the test, since we interpreted this as an indication of no interest in the stimuli (85 out of 273 females did not choose a speaker during the tests). We tested each female twice on its preference for large or small males recorded at both water level treatments. Thus, when a female made a choice in the first trial, we immediately subjected her to the second trial.

Table 1

Description of the number of calls and chucks ± SD of the stimuli used

	Mean number of calls per 15 s	**Mean number of chucks/call** unstandardized call rate	**Mean number of chucks/call** standardized call rate
Shallow water—Small male	7.8 (SD = 2.7)	0.5 (SD = 0.5)	0.6 (SD = 0.5)
Shallow water—Large male	7.2 (SD = 3.6)	0.2 (SD = 0.4)	0.2 (SD = 0.4)
Deep water—Small male	8.0 (SD = 2.3)	1.0 (SD = 0.7)	1.0 (SD = 0.7)
Deep water—Large male	7.8 (SD = 1.5)	1.0 (SD = 0.1)	1.0 (SD = 0)

Each female was subjected to one of the call timing treatments: natural call rate ($N = 30$), alternating calls ($N = 30$), or overlapping calls ($N = 40$ small leading, $N = 40$ large leading). We conducted the analyses on the females that made choices in both tests (140 out of 273 females). To avoid biasing our group of females by the more responsive individuals, we also analyzed the data including the females that only chose once (48 females chose only once). The results of the latter followed the same patterns.

Male response tests

We carried out a field playback experiment to test how males respond to the calls of rivals of different sizes calling from shallow or deep water levels. We used 20 males that we found calling without any nearby males (at least more than 8 m away). In a few cases, we translocated nearby males to avoid them responding to our playback. Prior to the start of a playback experiment, we placed a speaker (UE Roll 2, Ultimate Ears, Irvine) and sound recorder (Tascam DR-40 Linear PCM, Montebello) mounted on 2 tripods at a distance of approximately 0.8 m from the focal male. The speaker volume was calibrated using a 1 kHz reference tone set to 87 dB SPL as measured with a RadioShack SPL-meter at 0.5 m distance (33–2055, Fort Worth, set to fast response, C-weighing, max). The peak-to-peak amplitude of this tone was equalized to the amplitude of the loudest call from our stimulus set. The stimuli used in this experiment ranged from 69 to 85 dB SPL in amplitude as measured at 0.5 m with the SPL-meter. We initiated each experiment with a playback of 1 min of a chorus of túngara frogs. If males called in response to the chorus we continued the experiment with 30 s of silence after which we started with the playback of one of four stimuli. Males that failed to respond to the chorus were assumed to lack motivation and were not used for further tests ($N = 15$). We tested each focal male on its response to the playback of a large or small male calling from either deep or shallow water. The four playback trials were presented in a randomized and balanced order with 30 s silence in between and consisted of a call that was repeated for 1.5 min (1 call per 2 s). If a male moved away or was not fully inflated during the test, we excluded these data and repeated the test with a different male. One of the playbacks of one experiment was disturbed by noise and therefore we excluded this playback from the analyses, making the total number of conducted playbacks 79.

For each trial, we counted the number of calls as well as the number of chucks made per call using Avisoft SASLab Pro (Avisoft Bioacoustics, version 5.2). In addition, we measured the call timing of the focal male in relation to the stimulus timing. We noted for each call whether it overlapped with the closest stimulus call. For the overlapping calls, we noted whether the focal male call was the leading call or the lagging call. Next, we calculated the timing interval (time between the start of the focal male call and the preceding stimulus call). In case a male produced two calls in between two stimuli, we excluded the second call from the analyses on timing interval. From the timing intervals we calculated the coefficient of variation to test whether the focal male called more or less regular depending on stimulus type.

Data analyses

We assessed the effect of water level on male calling behavior, female preferences and rival communication general and generalized linear mixed models (GLMM and GzLMM using the *lme4* package (Bates et al. 2014) in R (version 3.5.1, R Core Team 2016). Significance of fixed effects as well as interactions between multiple fixed effects were assessed using likelihood (ML) ratio tests. All models were tested for a deviation from normality, overdispersion, and heteroscedasticy.

For the data on calling behavior, we modeled random variation in male response by fitting random slopes or intercepts and selected the best random structure using Akaike Information Criterion (AIC) scores. The models on call rate, call complexity, peak and RMS amplitude all contained water level, and male size as fixed effects and used a Gaussian distribution with identity link function. Additionally, we added the interaction between male size and water level as fixed effect. All models contained trial number as well as stimulation (chorus/single call/no call) as random intercept and the models on peak amplitude contained call order (start, mid, or end section of a trial) as an additional random intercept.

To test for the effects of water level treatment and call timing on female preference (for small or large male calls), we ran generalized linear mixed models (using a binomial distribution and logit link function). Female identity and stimulus pair number were added as random intercepts. Additionally, we tested whether female preference deviated from 0.5 by 1-tailed binomial tests. Latency until choice was investigated by taking the logarithm of the latency (the data were right-skewed) as response variable and water level and call timing treatment and their interaction as fixed effects in a linear mixed model with Gaussian distribution and identity link function. We ran these models for the data including the females that made no decision within 10 min (latency set to 10 min) or made only a decision in one of the 2 trials ($N = 190$) and excluding those females ($N = 139$).

Responses evoked in males were analyzed in terms of the probability of calling back, call rate, mean call complexity, proportion of overlapping calls, and mean and variation in call interval. For the probability of responding and the proportion of overlapping calls, we used a linear mixed model with binomial distribution and logit link function and for the other models we used a Gaussian distribution and identity link function. In all models, we added the water level treatment, size of the rival (small/large), and their interaction as fixed effects. In the models on the call interval, we also corrected for the number of emitted calls by the focal frog by adding this as a fixed effect. In all models, we accounted for repeated measures by fitting male ID as random intercept and/or slope (with water level treatment or male size), and selected the random structure with the lowest AIC value that was able to converge.

RESULTS

Effect of water level on male signaling

Water level treatment had a strong effect on calling behavior as male frogs in the deep water treatment called at higher rates compared with the shallow water treatment (GLMM; $N = 20$; $\chi^2 = 447.7$, $P < 0.0001$), with higher amplitude ($\chi^2 = 20.47$, $P < 0.0001$) and with greater complexity ($\chi^2 = 309.5$, $P < 0.0001$). Male body size had no overall effect on call rate ($\chi^2 = 0.41$, $P = 0.52$), amplitude ($\chi^2 = 0.08$, $P = 0.77$), or complexity ($\chi^2 = 1.23$, $P = 0.26$), but did show a significant interaction effect with water level treatment for call rate ($\chi^2 = 4.13$, $P = 0.042$) and amplitude (RMS; $\chi^2 = 5.47$, $P = 0.019$), but not call complexity ($\chi^2 = 1.80$, $P = 0.18$). In the deep water treatment, we found a positive relation between male size and call amplitude (Figure 1A), whereas in the shallow water treatment, we found the opposite relationship (Figure 1B). Call rate showed no clear relation with size in the deep water treatment but

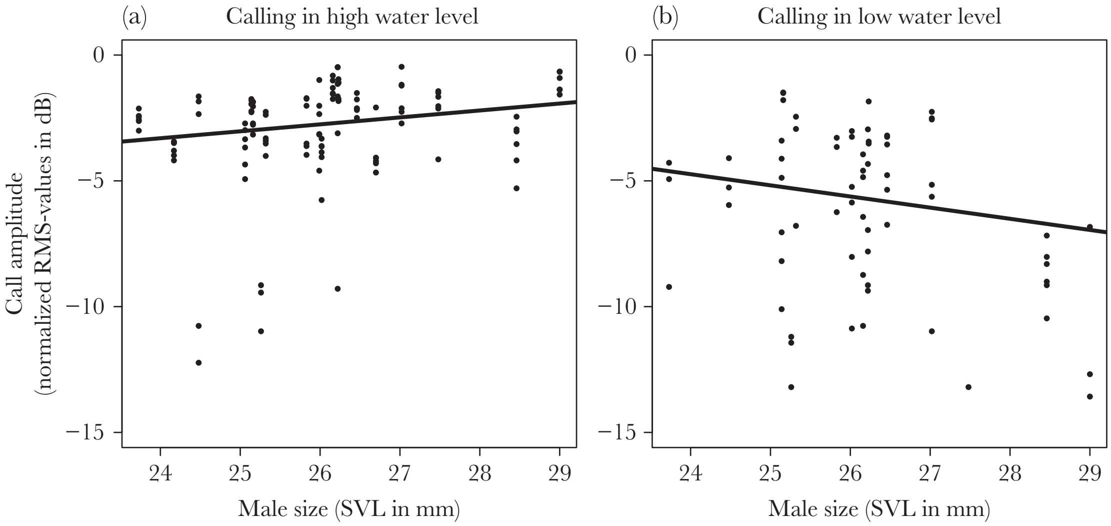

Figure 1

Effect of water level treatment and male size on call amplitude. (A) In high-water levels (1.0–2.0 cm), larger males call at louder amplitudes than smaller males do. (B) In low-water levels (0.25–0.5 cm), the pattern is reversed. Graphs depict amplitude values on a normalized dB-scale of the whine part of calls that were selected from the start, mid and end of a 1-min trial. Twenty males were treated to 2 high- and 2 low-level water treatments and 3 different acoustic stimulation treatments resulting in a maximum of 12 data points per male. Only data from trials in which males were stimulated with a rival call are shown, as these calls were also used in the female preference and rival competition experiments. Regression lines are based on effect sizes from a general linear mixed model that tested for an effect of size, water level, and their interaction (see main text for statistics). Data on peak amplitude of the calls showed a similar pattern as well as a significant interaction effect between water level and body size.

showed a positive relation with size in the shallow water treatment. This effect was probably caused by call cessation in some of the larger males during the shallow water treatment. Body mass did not have any significant effect on call behavior (all $P > 0.5$).

Female preference for large males depends on water level of the call site

We tested females for their preference for the calls of males that differed in size and that were calling from either deep or shallow water in three experiments differing in call rate and/or timing. Female preference was significantly affected by the interaction between male size and the water level treatment from which males called at naturally recorded rates (GzLMM, $N = 30$ females; $N = 10$ male stimuli: $\chi^2 = 8.95$, $P < 0.005$). When males called in deep water, two-thirds of the females preferred to approach the speaker broadcasting the call of the large male ($P = 0.049$, 1-sided binomial test, Figure 2A). Alternatively, when males called in shallow water, two-thirds of the females preferred the small male to the large male call ($P = 0.049$, Figure 2A).

Water level treatment also had a significant effect on female preference for the calls of small versus large males across all different call timing strategies, i.e., alternating ($\chi^2 = 8.27$, $P < 0.005$, $N = 30$ females, Figure 2B), small leading ($\chi^2 = 3.92$, $P = 0.048$, $N = 40$ females, Figure 2C), and large leading ($\chi^2 = 5.02$, $P = 0.03$, $N = 40$ females, Figure 2D). In deep water, females more often chose the calls of large males than of small males. The preference for large male calls significantly differed from 0.5 when the males were alternating (probability = 0.87, $P < 0.005$) or when the small male was leading (probability = 0.80, $P < 0.005$), but not when the large male was leading (probability = 0.60, $P = 0.13$). Alternatively, in shallow water, the calls of large males were only significantly preferred when the small male was leading (probability = 0.68, $P = 0.02$). Females did not have a significant preference when the large male was leading (probability = 0.48, $P = 0.68$) or when the males were alternating (probability = 0.57, $P = 0.29$). Small male calls were never preferred by females (Figure 2), contrary to the results obtained in the first experiment that used natural variation in call rate and timing.

In addition to female preference, we also scored the latency for females to make a choice and assessed whether these latencies were affected by water level and timing treatment. Females took on average 187.7 s (±11.15 SE, $N = 190$) to make a decision, but were 17% faster in reaching one of the speakers when the broadcast calls were recorded in the deep water treatment (females that made 2 choices; GLMM; $\chi^2 = 57.9$, $P < 0.005$, $N = 139$; all females; $\chi^2 = 70.34$, $P < 0.005$, $N = 190$). Choice latency did not significantly differ between the 4 call timing treatments nor did we find a significant interaction between timing and water level treatment (All $P > 0.19$).

Male responses depend on rival size and opponent's call site

In the field playback experiment, each focal male ($N = 20$) received 4 playback trials and in 56 out of 79 trials males produced at least one call in response. The probability of calling back in response to the playback did not depend on water level, or rival body size (All $P > 0.14$). Males responded with more calls to the small compared with the large rivals' calls, although this difference was not statistically significant (GLMM; $N = 20$ focal males, $N = 10$ rival stimuli; $\chi^2 = 2.37$, $P = 0.12$; Figure 3A). The number of calls did not differ depending on water level treatment ($\chi^2 = 1.96$, $P = 0.16$) or the interaction between water level and rival size ($\chi^2 = 0.18$, $P = 0.67$). Call complexity on the other hand was affected by water level treatment. Focal males produced more complex calls in response to a rival calling from deep versus shallow water ($\chi^2 = 6.47$, $P = 0.01$; Figure 3B), but call complexity did not differ with rival size alone

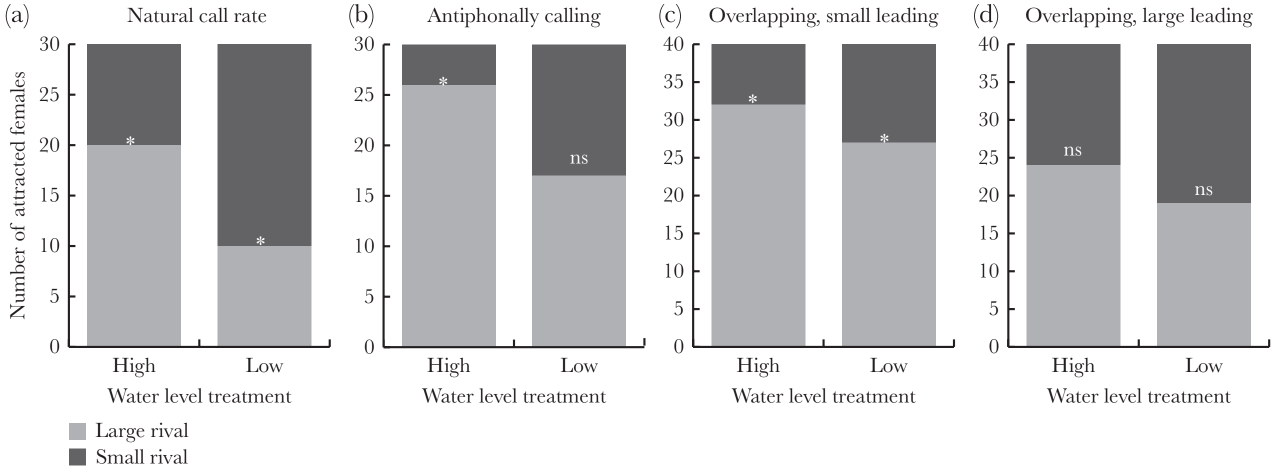

Figure 2

Effect of water level treatment on female preference for large over small males. (A) Under naturally recorded call rates, females prefer large to small males when both are calling from deep water, but in shallow water this pattern is reversed. (B). When male stimuli are broadcast antiphonally, at a standardized call rate and with no overlap, females prefer large over small male calls, but only under the high-water level treatment. (C) When male stimuli overlap, with the small male's call leading by 50% of the large male's call, females prefer large male calls under all water level treatments. (D) When large males are the leading stimuli, females do not prefer large nor small male calls irrespective of water level treatment. Shown on the y-axes are the number of females choosing for small or large male calls. Asterisks indicate significant deviations from 50% in female preference for large or small males.

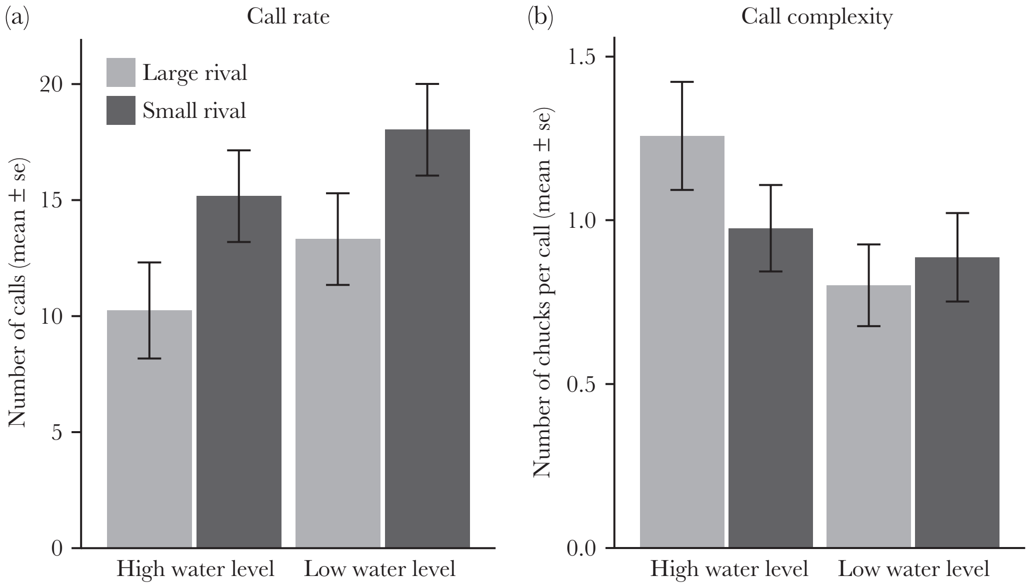

Figure 3

Effect of water level treatment and male size on rival competition. (A) Focal male vocal response in call rate in number of calls per 1.5 minute. (B) Evoked response in call complexity. Focal males called less in response to stimuli from a large compared with a small rival as well as to stimuli recorded during the deep water compared with the shallow water treatment, but this was not significantly different. Call complexity showed a significant interaction between rival size and water level treatment. Focal males only increased their complexity in response to a large male calling from deep water. See text for statistics.

($\chi^2 = 2.16$, $P = 0.14$). Interestingly, the response depended on the interaction between water level and size ($\chi^2 = 7.21$, $P = 0.007$). Males responded with higher call complexity to the calls of large compared with small rivals in deep water, whereas in shallow water these differences disappeared (Figure 3B).

In addition to call rate and complexity, we analyzed whether males altered the timing of their calls in response to the onset of the rival stimuli. Focal males produced 1125 calls during the four playback trials, out of which 142 calls overlapped with the playback stimuli. The focal male started in 88% of these overlapping events, thus producing the leading call. Males overlapped their calls more often when the rival frog was calling from deep water compared with shallow water (GLMM: $\chi^2 = 13.13$, $P < 0.005$), but this did not depend on the size of the stimulus frog ($\chi^2 = 0.05$, $P = 0.82$) nor their interaction ($\chi^2 = 2.41$, $P = 0.12$). Mean call interval ($\chi^2 = 22.98$, $P < 0.005$; defined as the time difference between the start of the stimulus and the start of the response), as well as the covariance in call intervals ($\chi^2 = 10.86$, $P < 0.005$) showed a similar significant effect of water level treatment.

DISCUSSION

We recorded males of different sizes calling under different water levels treatments and tested the effectiveness of their signaling behavior in attracting females as well as during rival interactions. We found that water level can constrain call production in a size-dependent manner, as large males called on average with lower amplitudes compared with small males in shallow water, but not in deeper water. This size-dependent signaling constraint had important consequences for a male's attractiveness: large males were almost always preferred by females when calling from deeper water levels, but when calling from shallow water levels this preference disappeared, or even reversed to a preference for small males. In shallow water, the call of a large male only attracted more females when it was overlapping the last part of the small male's call. Male responses to the calls of rivals differing in size and water level treatment showed a similar pattern as observed in female preference tests. During field playback experiments, males increased their signal complexity in response to a large male's call compared with a small male's call, but only under the deep water level treatment.

Morphological and environmental constraints on signaling

Male túngara frogs shuttle air from their lungs to a large vocal sac when calling and the higher the air flow or pressure, the louder the call they can produce (Dudley and Rand 1991; Halfwerk et al. 2017). Furthermore, when inflated, the vocal sac resonates strongly with the dominant frequency of the chuck, which thus seems to function in the coupling of sound between the air inside and outside the frog's body (Halfwerk et al. 2017). Previous work has shown that in shallow water (less than 1 cm), males are constrained in inflating their lungs and their vocal sac to the full extent, which directly reduces their call amplitude as well as complexity (Halfwerk et al. 2017). In the present study, we demonstrate that these call constraints are size-dependent, as male size showed a positive correlation with call amplitude when males were able to call unconstrained when tested in deep water, whereas in shallow water, male size showed a negative correlation with amplitude. Call-site characteristics and signalers' characteristics thus need to match in order to produce the most effective and attractive mating display. Similar interactions between ecological and morphological signaler traits are likely to occur in other species that signal from confined spaces, such as burrows, or along small twigs that have to match their body size or weight (Bailey et al. 2001; Ord et al. 2007).

The evolution of signaling strategies

Male túngara frogs produce louder and more complex calls when competing acoustically with their rivals for mating opportunities (Bernal et al. 2009; Halfwerk et al. 2014, 2016). The signal complexity of evoked responses in males matched with the attractiveness of the rival's signal, but not necessarily with rival size. Females preferred larger males to smaller ones, but mainly when males were able to call unconstrained in the deep water level treatment. In a competition context, males also showed the strongest increase in call complexity, a feature that is well known to increase their attractiveness to females, when confronted with the calls of a large male recorded in the deep water level treatment. Males did not differ in their signal complexity in response to calls of large versus small males when these were recorded during low-water treatment. This may indicate that male túngara frogs only perceive large opponents as a more competitive threat than small opponents when their rivals perform at their optimum.

The female preference tests also revealed that males can additionally increase their attractiveness by calling shortly after their rival. These results differ however from previous findings showing that females generally prefer the leading male to the lagging male (Tárano 2015). Furthermore, our field playback revealed that when the focal male occasionally overlapped the rival's call, he was leading most of the time. We do not have any clear explanation for these discrepancies.

Finally, males could adopt a signaling strategy that is based on call-site selection. In Fowler's toads (*Bufo fowleri*), large males usually call from water bodies, whereas small males call from the adjacent banks. Females prefer large males, but female assessment of size can potentially be influenced by the temperature of the display site, as call frequency and pulse rate are negatively related to body size, but positively to call-site temperature (Zweifel 1968; Fairchild 1981). By calling from the relatively cooler aquatic site, large males may be able to adjust for the potential negative effect of temperature on their attractiveness (Fairchild 1981). Alternatively, large males may be forced to display from water in order to be able to call unconstrained. Irrespective of the mechanism (thermal or physical constraints for larger males), the evolution of call-site selection could be driven by both large and small males. Small males, for example, may decide to call from locations where they can match, or even outcompete large males in terms of signal attractiveness. Furthermore, by calling at locations not accessible by large males (e.g., at the edges of ponds where water levels are usually very shallow), small males could be able to intercept females in search for a mate (Fairchild 1981).

Signal reliability depends on environmental signaling conditions

We found that females prefer large males over small males, but only under optimal signaling conditions of deeper waters. Similarly, males discriminated between calls of differently sized rivals only under the deeper water treatment. Under suboptimal conditions, when males were constrained in a size-dependent way, female showed no preference, or even preferred smaller males. These findings suggest that signaling in túngara frogs does not provide reliable information on sender size across all environmental conditions. Females may thus not necessarily prefer the largest mate, but simply the loudest, most complex calling male, irrespective of his noncommunicative properties (e.g., his size), or the location from which he calls.

Receivers may be able to incorporate information on environmental signaling conditions when extracting sender information. Females may for example weigh specific signal features differently depending on the type of habitat (and thus transmission properties) they find themselves in. Receivers may also incorporate the effect of transmission on more than one feature, such as the combination of amplitude and frequency profile of the signal, to assess sender distance, which may allow them to better estimate a sender's size (Ringler et al. 2017). Receivers, however, may have little or no information on specific call-site properties that influence signal production. In these cases, we predict that senders are able to "cheat" in providing reliable information on their physical properties to receivers.

In conclusion, we have shown that display-site properties can set limits to signal production that may not affect all individuals in a population equally. These findings have important evolutionary consequences, as they suggest that males that are unattractive under optimal signaling conditions can become attractive when environmental conditions change. Such alternative mating strategy may even drive the evolution of display-site selection, in particular when this is accompanied by changes in other phenotypic traits. When small males are favored through changes in other selection pressures, such as food availability, or predation pressure, a switch in display site, for example, from water to land, can potentially evolve rapidly.

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