# Phonotaxis to advertisement calls by midwife toads (*Alytes muletensis*) is not necessarily related to mating

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**Abstract.** Previous studies with the Mallorcan midwife toad (*Alytes muletensis*) found that gravid females show inconsistent phonotactic preferences for male calls unless they are highly motivated to mate; and, non-gravid females will occasionally show phonotaxis to male advertisement calls. These results suggest that sometimes phonotaxis may not be related to seeking a mating partner. In this article we report on experiments that give further support to a non-mating function of phonotaxis in this species. Males and metamorphs were played conspecific male and female advertisement calls. Males that were expected to be unmotivated to mate were not less likely to respond to, or less consistent in their choice of, female calls than males who were expected to be motivated to mate. Metamorphs who were not yet in breeding condition nevertheless showed positive phonotaxis. We suggest that Mallorcan midwife toads may sometimes show phonotaxis simply to seek out refuges where toads aggregate.

## Introduction

Positive phonotaxis in anurans is a clear and unequivocal behaviour that involves travelling towards and localising a sound. For the most part, anurans receive the vocalisations of others through the tympanic membranes on either side of their heads. The tympanic membranes and middle ear cavities are linked together by air-filled channels. Anurans are thought to use pressure differentials between the inside and outside of each tympanic membrane to determine the direction of the sound (Wilczynski, 1992). Thus, positive phonotaxis involves repeatedly turning sideways, exposing each tympanum alternately to the sound, and hence results in a characteristic zig-zag pattern of movement.

In many anuran species, males call to advertise their sexual receptivity, competing vocally to attract a mate (Wells, 1977). Often, males form a chorus and Wells (1977) has suggested that one of the functions of this is to advertise the location of the breeding site to migrating females. However, acoustic cues, even when they are an obvious component of

reproduction, are perhaps not essential for migration to the breeding site and many other sensory cues may be used (Able, 1980; Sinsch, 1990, 1992). Females of many species do, however, readily show positive phonotaxis in call playback experiments, clearly using acoustic cues to find conspecific males (or speakers). This behaviour pattern has been extensively used to examine female preferences in terms of conspecific mate recognition and sexual selection (e.g. in midwife toads: Marquez, 1995; Bush et al., 1996; Marquez and Bosch, 1997a, b; Dyson et al., 1998; Lea et al., 2000; and other species reviewed in Gerhardt, 1994). These experiments were generally conducted during the breeding season, and the females used were gravid and assumed to be ready to mate. In all of these experiments phonotaxis was analysed in the context of reproduction.

Mallorcan midwife toads, *Alytes muletensis*, live communally in a few isolated ravines in the mountains of Mallorca, Spain. Small, widely dispersed groups of toads (both sexes and all ages) aggregate in narrow cracks and fissures throughout the year. Males brood the eggs terrestrially, and, at the end of the brooding period, carry them down the cliff faces to temporary pools where the tadpoles will hatch out. Females breed repeatedly over a very extended season (April-September in the field, and all year in captivity, Bush, 1993; Lea, 2000). However, the availability of sexually receptive males is often limited because males usually brood only one clutch of eggs at a time, and they take longer to brood them than females take to produce new clutches (Bush, 1993, 1996). Females may therefore need to leave their groups to find sexually receptive males and they will thus show phonotaxis to male calls.

Both sexes produce calls when they are sexually receptive although male calling is more common (Bush, 1993), and both sexes show phonotaxis (in the laboratory) to the calls of the opposite sex (Bush, 1993; Bush et al., 1996; Dyson et al., 1998; Lea, 2000, Lea et al., 2000). Several interesting behaviour patterns have been observed over the course of the above experiments. First, although gravid females readily respond to male calls, they only exhibit consistent call type preferences when they are ovulating eggs and (therefore) highly motivated to mate (Lea et al., 2000). Gravid females that are not ovulating eggs still show phonotaxis, but preferences are not repeatable. Also, females who have no ripe eggs in their ovaries occasionally exhibit positive phonotaxis (Lea, 2000) although they are obviously not ready to mate. These observations suggest that phonotaxis may not always be related to reproduction. Below we set out the rationale for experiments designed to examine if toads that are unable or unmotivated to mate will exhibit phonotaxis.

Male *A. muletensis* will not re-mate after 3 days following a previous mating (perhaps because of developmental constraints on the tadpoles or energetic constraints on the males; Bush, 1996). So, if male phonotaxis functions to locate and mate with females, then one would not expect males that had mated more than three days previously to approach female calls. If these males do exhibit phonotaxis, then this is probably for purposes other than mating, and one might therefore expect them to show little discrimination between alternative call types. As such, we set out to test if there was a difference in phonotactic response, towards male or female calls, between males that had mated more than three days

previously and males that had mated within the last three days. Additionally, if phonotaxis to advertisement calls in general only serves mating functions then one would not expect metamorphs, who cannot mate, to show phonotaxis. We tested immature metamorphs to see if they would exhibit phonotaxis to conspecific advertisement calls.

## Methods

*The Experimental Animals.* All experimental animals were part of a large captive-bred colony at the Open University, Milton Keynes, UK. They were housed indoors, fed ad libitum on house crickets dusted with vitamin powder, and maintained throughout the year on an artificial photoperiod (12 hrs. white light / 12 hrs. red light) and a constant temperature ($22 \pm 2°C$). Toads were kept in mixed sex and cohort groups (approx. 50 animals) and allowed to mate freely; they bred all year round without showing any seasonal fluctuations in mating activity (Lea, 2000). Individuals were identified from their dorsal patterns. Sexually immature metamorphs and two groups of males were tested. All metamorphs were less than eight months old (the earliest breeding ages are 10 months for males and 13 months for females; Bush, 1993). Sexes could not be identified. The first group of males (called 'brooded <3 days') was tested within three days of mating, and the second group (called 'brooded >3 days') was tested five to seven days after mating.

*Call Synthesis.* Calls were synthesised using 'Sound Edit 16' (version 2, Macintosh$^©$). Pure sinusoidal tones were generated at a sampling frequency of 22 kHz and a resolution of 16-bits, and an amplitude envelope was then fitted to match that of recorded calls from the same population. Synthesising calls is preferred to using natural recordings because it eliminates variations in recording quality and background noise. The calls of both sexes are acoustically simple in structure, consisting of nearly a pure tone with few or no harmonics, no obvious frequency or amplitude modulation, and no pulses (Bush, 1997). These calls are easily and accurately synthesised and females readily respond to them in phonotaxis trials (Bush, 1993; Dyson et al., 1998; Lea, 2000; Lea et al., 2000). Females show no difference in frequency of phonotactic response to recorded or synthetic (male) calls, indicating that synthetic calls are an adequate replacement for natural calls (Lea, 2000).

All call parameters were based on mean values for the population (Bush, 1997) (table 1). Mean male and female calls are very similar in frequency but differ in duration, the female call being shorter than the male call (see table 1). Naturally, males and females also differ in the intensity and frequency with which they call (table 1). Therefore, two types of test were conducted on the metamorphs. In the first type of test the mean male and female calls were matched for SPL (equalised at 76 dB re: 20 $\mu$Pa at 35 cm) and call rate (both stimuli set at 18 calls/minute), whereas in the second type of test the same calls were played at more natural amplitude and calling rates (female calls were set at 36 calls/min and 60 dB, male calls at 18 call/min and 76 dB). Thus in this test (called the 'unmatched' condition) the female call was shorter and slightly lower in frequency and intensity than the male call, but repeated at a faster rate. In the 'matched' condition the only differences between the stimuli was that the female call was shorter and slightly lower in frequency than the male call. Each metamorph was tested only once in each experiment and there was a gap of one week between experiments.

Males were played the mean male call *versus* the mean female call, both set at natural SPL and calling rates (as in the 'unmatched' condition above). We used this condition alone because males show a higher overall level of phonotaxis when presented with the 'unmatched' female call as opposed to the 'matched' call (Lea, 2000). The 'unmatched' condition therefore increases the likelihood that males will show phonotaxis, and presents a more natural situation. Eggs were removed from the males in the morning and tests were conducted in the evening. The eggs were removed by carefully cutting the connecting string with sharp scissors and sliding the egg mass down the male's legs. Each male was tested three times on the same evening with a gap of about 20 minutes between trials. Only males that responded in all three trials were used in the analysis, so that the observed responses could be compared to an expected (null-model) response distribution. Repeated tests on individuals were conducted to examine whether potential differences in reproductive motivation between the groups resulted in a difference in the likelihood of phonotaxis or the consistency of preferences. Figure 1 shows synthetic mean male and female calls played at the same amplitude (as in the 'matched' test).

**Table 1.** Call parameters for the sample population (as determined by Bush, 1997). Synthetic calls used in the experiments were based on the mean values.

| Parameter | Mean | $s$ | Range |
|---|---|---|---|
| Dominant frequency | | | |
|   Males | 1.8 kHz | 0.14 | 1.5-2.2 kHz |
|   Females | 1.7 kHz | 0.16 | 1.5-2.0 kHz |
| Call duration | | | |
|   Males | 102 ms | 17 | 75-134 ms |
|   Females | 62 ms | 15 | 33-79 ms |
| Inter-call interval | | | |
|   Males | 3.3 sec (or 18.3 calls/min) | 0.5 | 2.5-4.1 sec |
|   Females | 1.4 sec (or 43.8 calls/min) | 0.2 | 0.9-1.8 sec |
| SPL @ 35 cm | | | |
|   Males | 76.3 dB re: 20 $\mu$Pa | 5.8 | 62-86 dB re: 20 $\mu$Pa |
|   Females | 59.9 dB re: 20 $\mu$Pa | 7.7 | 48-70 dB re: 20 $\mu$Pa |
| Amplitude modulation | | | |
|   Males | rise time = 7 ms, fall time = 17 ms | (data are approximate, variances are not given) | (data are approximate, range is not given) |
|   Females | rise time = 4 ms, fall time = 13 ms | (data are approximate, variances are not given) | (data are approximate, range is not given) |
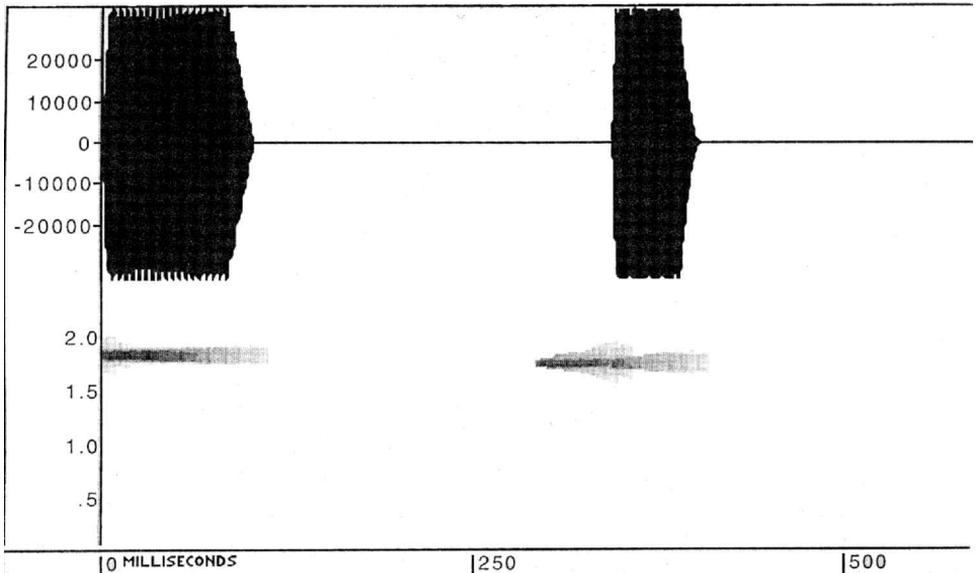


**Figure 1.** Oscillogram (top) and sonogram (bottom) of a synthetic male *A. muletensis* call (to the left) followed by a synthetic female *A. muletensis* call (to the right). Y-axis units are kHz (bottom) and amplitude in mV (top).

*Call Playback.* Calls were pasted onto separate channels of a digital sound file, recorded onto DAT tape, and played back through an HHb PDR1000 Portadat, an H.H. 'VX300 Mos-Fet' amplifier and two Realistic Minimus 7 speakers. A perforated black grill on the face of each speaker obscured the movements of the diaphragm so that visual cues were minimised. Playback was conducted in a semi-anechoic chamber, hexagonal in shape, 1.4 m high and 1.9 m between opposing faces (see Lea, 2000, for a full description). The chamber was housed in a room away from the rest of the colony and the sound of other calling toads. A grid (10 × 10 cm squares) was painted on the floor in order to map the movements of the toads. Observations were made via a video camera fitted in the ceiling which relayed pictures to a VHS recorder and monitor outside the chamber. Speakers were placed opposite each other; the face of each speaker was 85 cm from a central release point.

The toads were placed in the centre of the arena, underneath an acoustically transparent mesh lid (10 × 10 × 2 cm), and in a random orientation with respect to the speakers. For 30 seconds they were played both stimuli. The lid was then carefully lifted off and they were free to move. A positive result was scored only if they moved in the zig-zag pattern characteristic of anuran phonotaxis and then reached within 10 cm of a speaker. Each animal was allowed five minutes to make a choice and if no choice was made within this period the trial was terminated.

Sound pressure level (SPL) was set using a Brüel and Kjær SPL meter (type 2231), microphone (type 4155-fast weighted, frequency response flat to 20 kHz ± 1 dB), and band pass filter set at a centre frequency of 1.6 kHz. This apparatus was placed flat on the floor facing each speaker at a distance of 35 cm, and a Gould (DSO1425) oscilloscope was attached to the output of the amplifier to confirm the peak to peak amplitude of each channel. Also, at the start of each 'matched' experiment the Brüel and Kjær meter was placed in the centre of the arena to verify that there was equality of SPL at the release point. In order to control for directional bias within the chamber, the channels broadcasting each call were switched half way through each experiment or between trials using the same animal. On each evening the experiments took place between 1900 and 2400 hrs. Experiments were conducted during the summer of 1999.

*Statistics.* For the metamorphs, a two-tailed binomial exact test was used to compare the observed response pattern of the group to a pattern of no preference for either call. Significance values were calculated using SPSS for Windows 1995. For the two groups of males, the observed frequency distribution of responses to the three trials was compared to the expected distribution based on an equal likelihood of response to either call. The expected frequency distribution of responses to three consecutive trials is given by the binomial expansion of
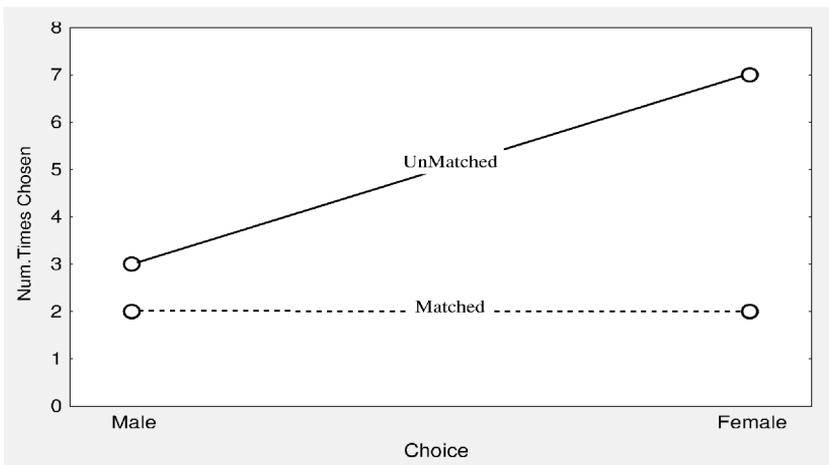


**Figure 2.** Phonotactic responses of *A. muletensis* metamorphs. 'Matched' and 'Unmatched' refer to whether SPL and call rates of the stimuli were equal. The endpoints of the lines represent the alternatives presented, and the number of toads that chose that alternative (see text for more details). There were no significant preferences for any of the calls ($n = 32$ for each experiment).

## Males, Brooded < 3 Days
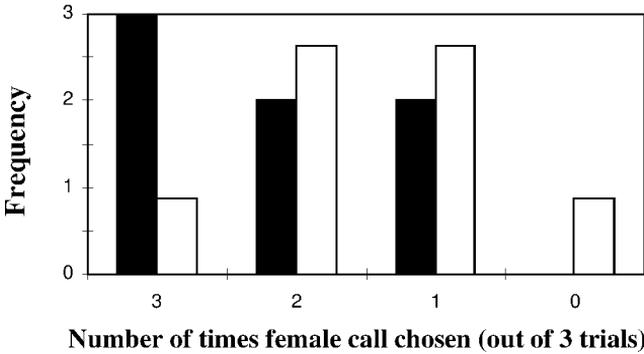


**Figure 3.** Phonotactic responses by males that had mated less than three days before: observed (■) versus expected (□) distribution. The responses did not differ from the pattern expected if there was no preference for either call; $G_{adj} = 1.40$, $P > 0.05$.

## Males, Brooded > 3 Days
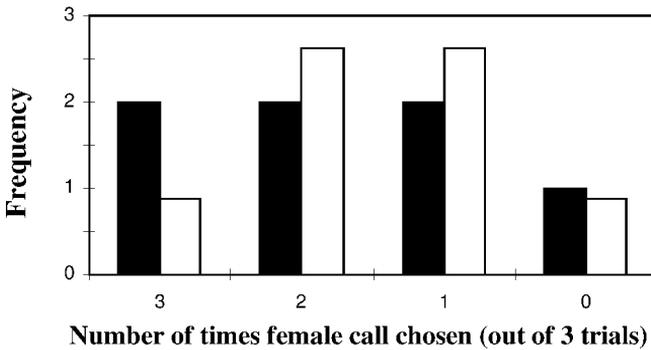


**Figure 4.** Phonotactic responses by males that had mated more than three days before: observed (■) versus expected (□) distribution. The responses did not differ from the pattern expected if there was no preference for either call; $G_{adj} = 0.21$, $P > 0.05$.

$(p + q)^3$; where $p$ is the probability of responding to the male call, and $q$ is the probability of responding to the female call, i.e. $p = 0.5$ and $q = 0.5$. Comparisons between the observed and the expected responses were made using the log-likelihood goodness-of-fit test (G-test) with Williams' correction (Sokal and Rohlf, 1980). Two response classes were combined when the expected frequency in any class was less than five.

## Results

Metamorphs showed clear phonotaxis, approaching both male and female calls. Their response rate, however, was low (4 out of 32 toads showed phonotaxis in the 'matched' experiment; 10 out of 32 toads showed phonotaxis in the 'unmatched' experiment; fig. 2). There were no significant preferences for any of the calls.

Thirty males that had mated >3 days previously were tested, but only seven of these responded in all three trials (23% response rate). Twenty males that had mated <3 days previously were tested, and seven of these responded in all three trials (35% response rate). The difference in response rates was not significant ($G_{adj} = 0.8$, $P > 0.05$). In neither group did the pattern of response differ significantly from that expected if there was no preference for either call. There was a trend, however, for the female call to be chosen slightly more times than was expected from the binomial distribution (figs 3 and 4).

## Discussion

Anuran phonotaxis is a behaviour pattern that is typically associated with mating. Yet we have shown here and elsewhere (Lea, 2000) that toads who are not ready to mate, or not expected to be sexually receptive, nevertheless show positive phonotaxis to conspecific calls. In addition to this, gravid females readily show phonotaxis to male calls but only exhibit reliable preferences when they are likely to be highly motivated to mate (Lea et al., 2000). Thus, when a toad shows phonotaxis it may not be for the purpose of seeking a mating partner. *Alytes muletensis* are social throughout the year and not just during the breeding season. This propensity to aggregate may reflect a scarcity of suitable refugia or the need to reduce evaporative water loss in a dry terrestrial environment; or, it may be a 'selfish-herd' strategy (Arnold and Wassersug, 1978; Graves, et al., 1993; Heinen, 1993). Callers are not territorial (Bush, 1993), and, where there is a caller, there are likely to be conspecifics. It is therefore plausible that many toads show phonotaxis to calls simply to find others, or to seek out hiding places.

None of the animals tested showed any significant discrimination between alternative calls. There was a greater overall response to the natural ('unmatched') female call, but this may have occurred because the female call was played at a faster rate (although at a lower intensity). Preferences for faster call rates are common and can override preferences for other variables, either because they elicit a higher level of neural stimulation (Gerhardt, 1991) or because faster calls are easier to locate (Parker, 1983). Given the small sample sizes and low power of the statistical tests, however, it is possible that any discrimination between calls could have been missed. However, if toads were showing phonotaxis for reasons other than mating then perhaps they should not have been expected to discriminate between male and female calls because they were not seeking a mating partner. Certainly, metamorphs, and males that were expected to be unmotivated to mate, did not discriminate between male and female calls. However, males that had mated within the last three days,

and were expected to be motivated to mate, were no more responsive in the phonotaxis tests and did not show greater discrimination between the calls than males who were expected to be unmotivated to mate. This lack of a differential response between the two groups of males could have been influenced by the experimental procedure. For instance, it is possible that the males that had brooded for more than three days were stimulated to re-mate by the removal of their eggs earlier in the day. Or, the handling involved in removing the eggs could have produced stress effects that reduced any inclination to mate in the males that had brooded for less than three days. Thus the expected differences in motivation between the groups could have been nullified.

Other reasons for male phonotaxis can be envisaged. Males could be showing phonotaxis as a 'satellite' strategy. That is to say, silent males orient towards a calling male in order to intercept a female before she reaches the calling male. Satellite behaviour is usually a 'sit and wait' strategy rather than an active approach (satellites adopt characteristic low postures near territorial males) (Halliday and Tejedo, 1995). Male *A. muletensis* are not territorial, and no form of satellite behaviour has ever been reported in any midwife toad. It therefore seems unlikely that this is a good explanation for male phonotaxis.

The observation that immature metamorphs will exhibit phonotaxis is the most com-pelling evidence for a non-mating function of phonotaxis. Emerging metamorphs need to find their way up from the pools in which they lived their lives as tadpoles, to the rocky crevices where other toads are located. These toad groups are highly dispersed within the Mallorcan habitat, and as such the vocalisations of calling toads are probably a major cue to metamorphs searching for others. Wells (1977) noted that the breeding vocalisations of many male anurans have two functions, that is, in addition to attracting females, the calls can also function in territory maintenance or the maintenance of inter-individual dis-tance. Other work has shown that anuran calls affect the reproductive physiology of both signallers and receivers (e.g., Burmeister and Wilczynski, 2000; Lea, et al. 2001). In this study we have suggested that *A. muletensis* calls may also serve a social cohesion function that is not necessarily related to breeding (i.e. the calls facilitate aggregation in general). If animals that are usually communal suddenly find themselves isolated (in a phonotaxis chamber) they may well show phonotaxis simply to find others. These animals would prob-ably show poor levels of discrimination between alternative calls and as such drawing con-clusions about phonotactic preferences (especially mating preferences) would be invalid. Given the extensive use of phonotaxis in sexual selection studies, this result highlights the need to treat with caution apparent mate choice preferences, or indeed assumptions about the function of phonotaxis, in communally living species.

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