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RESEARCH ARTICLE

Evidence for individual vocal recognition in a pair-bonding poison frog, *Ranitomeya imitator*

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ABSTRACT

Individually distinctive vocalizations are widespread in nature, although the ability of receivers to discriminate these signals has only been explored through limited taxonomic and social lenses. Here, we asked whether anuran advertisement calls, typically studied for their role in territory defense and mate attraction, facilitate recognition and preferential association with partners in a pair-bonding poison frog (*Ranitomeya imitator*). Combining no- and two-stimulus choice playback experiments, we evaluated behavioral responses of females to male acoustic stimuli. Virgin females oriented to and approached speakers broadcasting male calls independent of caller identity, implying that females are generally attracted to male acoustic stimuli outside the context of a pair bond. When pair-bonded females were presented with calls of a mate and a stranger, they showed significant preference for calls of their mate. Moreover, behavioral responses varied with breeding status: females with eggs were faster to approach stimuli than females that were pair bonded but did not currently have eggs. Our study suggests a potential role for individual vocal recognition in the formation and maintenance of pair bonds in a poison frog and raises new questions about how acoustic signals are perceived in the context of monogamy and biparental care.

KEY WORDS: Anuran, Monogamy, Phonotaxis, Dendrobatid, Choice test

INTRODUCTION

Acoustic communication plays a prominent role in territory defense, mate choice and social cohesion across animals (Simmons et al., 2003). Being both long range and information dense, acoustic signals confer distinct advantages over other types of signals and in many cases it is valuable for individuals to have their own 'vocal signature' that distinguishes them from others. While acoustic communication is ancient in vertebrates (Jorgewich-Cohen et al., 2022), individual vocal recognition has evolved independently multiple times, including in birds, mammals, fish and amphibians (reviewed in Carlson et al., 2020). Yet, the importance of individual vocal recognition depends on a species' social system, and the ability to produce and recognize distinct vocalizations should only evolve when more general recognition systems will not suffice (Carlson et al., 2020). Even within populations, vocal recognition abilities have been shown to vary according to sex (Freeman and Ophir, 2021; Insley et al., 2003), age (Balcombe, 1990; Leonard et al., 1997; Sieber, 1986) and reproductive status (Pultorak et al., 2017; Shave and Waterman, 2017), and may be context dependent. For instance, single male prairie voles are able to discriminate between male conspecifics but not between female conspecifics (Zheng et al., 2013), yet pair bonded males can discriminate conspecifics independent of their sex (Blocker and Ophir, 2015). Thus, evaluating how animals respond to acoustic stimuli and discriminate between them is a good way to test predictions about the selective context in which signals evolve.

One social context expected to strongly influence the evolution of individual recognition is pair bonding (Prior et al., 2022). Both the formation and maintenance of a pair bond depend critically on an individual's ability to recognize and preferentially associate with a specific partner, which may be facilitated by distinctive individual vocal signatures (Prior et al., 2022). As pair bonding frequently co-occurs with biparental care, acoustic signaling may also facilitate the coordination of complex care to ensure offspring survival (Boucaud et al., 2016, 2017; Moss et al., 2023). Taken together, the ability to distinguish and respond rapidly to partner vocalizations may contribute directly to fitness in monogamous and biparental systems, providing a selective context favoring the evolution of individual vocal recognition.

Support for this idea can be found in monogamous birds, among which use of acoustic cues to recognize mates is widespread (e.g. Beer, 1971; Curé et al., 2011, 2016; Dentressangle et al., 2012; Lengange et al., 1999; Szipl et al., 2014; Wooller, 2010). In possibly the most well-studied example, zebra finches form remarkably enduring pair bonds, which are maintained via individuals' strong preference for the songs of their mates (Hernandez et al., 2016; Miller, 1979), even in the absence of visual contact (Silcox and Evans, 1982) and when presented only with unlearned calls (D'Amelio et al., 2017). Discrimination of partner vocalizations is also observed in mammals (Snowdon and Cleveland, 1980; Snowdon et al., 1983), but evidence from other taxa remains limited despite multiple independent origins of monogamy and parental care (Emlen and Oring, 1977).

Anurans (frogs and toads) have long served as models for the study of acoustic communication. While the contexts in which frogs vocalize are varied and numerous, the power of individual discrimination remains poorly resolved. For example, only some species appear to discriminate between calls of neighbors and strangers (Bee et al., 2016; Lesbarrères and Lodé, 2002; Tumulty et al., 2022) – a pattern that has been linked to living in more complex social environments with strict territorial boundaries (Tumulty and Bee, 2021). Moreover, almost nothing is known from this group about individual vocal recognition in other social contexts, such as pair bonding.

The mimic poison frog, *Ranitomeya imitator* (Schulte 1986), is a monogamous species in which males and females form exclusive

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pair bonds lasting many months, defend shared territories, and cooperate over the care of eggs and tadpoles within those territories (Brown et al., 2008, 2010). Acoustic communication plays a key role in coordinating these behaviors, with males (the sole vocalizers in this species) calling to attract, court and solicit cooperative parental care from females, which follow male partners at a close clip during egg laying and egg feeding interactions (Brown et al., 2008; Tumulty et al., 2014). Despite intimate exchanges surrounding reproduction, physical contact between partners is otherwise rare, with males dedicating much of their time to territory defense whereas females tend to forage in seclusion (Brown et al., 2008). Home territories are small (5–14 m^2) but composed primarily of vertical space with dense understory vegetation (Brown et al., 2008, 2009). Taken together with the small size of their inhabitants (13–20 mm snout–vent length, SVL), visual contact between partners is unlikely to be constant. Recent work revealed that cooperation between pair-bonded partners, specifically coordinated biparental egg feeding, has contributed to the diversification of male signals (Moss et al., 2023). However, considerable overlap in spectrotemporal properties between call types and individual callers in the same study leaves open questions about whether receiver (female) recognition and/or preference has co-evolved alongside acoustic signals.

With this study, we tested whether females of *R. imitator* (1) are attracted to male calls in general (i.e. when not pair bonded), and (2) preferentially associate with calls of a mate over calls of a stranger. We combined single-stimulus (no-choice) and two-stimulus (two-choice) test paradigms to evaluate responses of virgin and pair-bonded females to male acoustic stimuli. Choice tests are a standard method for evaluating female preference in anurans (Bush et al., 2002; Gerhardt, 1995) and other taxa (Wagner, 1998), and use of simple playback stimuli ensures subjects are responding to acoustic cues alone. Our results provide a first glimpse into how vocalizations and the use of individual vocal signatures may facilitate pair bond formation and maintenance in a biparental frog species.

MATERIALS AND METHODS

Subjects

All experimental frogs were sourced from a captive poison frog colony at the University of Illinois Urbana-Champaign. Founders of our colony descended from hybrids of five localities and were reared in admixed social groups. Although color morph variation has been suggested to influence mate choice in geographically isolated populations in the wild (Twomey et al., 2014, 2016), based on the breeding success of these laboratory-established pairs, we conclude that any effect of morph differences on preference formation and maintenance in our study is negligible. Adults were housed in opposite-sex pairs in ∼30.5×30.5×46 cm glass terraria (Exo Terra, Mansfield, MA, USA) in an animal room containing 30 tanks along each of two walls. Each home tank contained a bioactive substrate layer (mix of soil, clay and orchid bark), sphagnum moss, live plants, dry film canisters for egg laying and water-filled film canisters for tadpole deposition. Froglets were group-housed (up to 10 frogs in a plastic container with the same bioactive substrate and live plants) until 6–8 months of age, at which point they were transferred to adult terraria and housed in same-sex pairs or trios during the experiment. Animal rooms were temperature controlled (21–23°C) on a 12 h:12 h light:dark cycle, and terraria humidity was maintained at >80%. Adults were fed wingless *Drosophila* fruit flies dusted with multivitamin and calcium supplements three times per week. Froglets were fed springtails and (after attaining sufficient body size) fruit flies three times per week. All animal care and experimental procedures were approved by the University of Illinois Animal Care and Use Committee (IACUC protocol #20147).

Acoustic stimuli

The advertisement calls of 12 adult, breeding males were used as stimuli for choice tests. These calls are best characterized as high-pitched trills lasting approximately 1–2 s and containing anywhere from 18 to 46 pulses (Mayer et al., 2014; Moss et al., 2023; Twomey et al., 2015). Advertisement calls show weak or no differences between color morphs (Twomey et al., 2015) but contain significant identity information relative to other call types in the repertoire of *R. imitator* (e.g. courtship or egg-feeding calls; Moss et al., 2023). Specifically, dominant frequency, spectral bandwidth, and pulse number and duration are variable among individuals but highly consistent within individuals (Moss et al., 2023). Therefore, we expected advertisement calls to be a reliable signal for distinguishing individuals.

We followed our previously established protocol for recording frogs in the laboratory (Moss et al., 2023). Briefly, subjects were recorded in the animal facility during daytime hours using a digital audio recorder (H4n Pro, Zoom, Tokyo, Japan) and shotgun microphone (K6/ME66, Sennheiser, Wennebostel, Germany) positioned directly above the focal terrarium (i.e. atop acoustically transparent screen mesh lids, <0.5 m from subjects). Calls were recorded in WAV format at a sampling rate of 48 kHz/16 bit. These methods have been shown to be effective in capturing and isolating calls of focal individuals, via cross-validation with video recordings (Moss et al., 2023). To generate playback files, we used the open-source audio editing software Audacity v.3.0.4 (https://www.audacityteam.org/). Recordings were filtered to remove background noise using the Noise Reduction, High-Pass Filter (<2000 Hz with 24 dB roll-off), and Low-Pass Filter (>20,000 Hz with 24 dB roll-off) tools. Ten representative calls from each male were selected to generate playback files, which consisted of 10 calls spaced 10 s apart in a randomized sequence (Fig. 1A). The constant inter-call interval was applied to control for variation in signal perception introduced by call period or sampling variance. Given that most spectral and temporal properties of advertisement calls except for inter-call interval have been shown to have high potential for individual coding in *R. imitator* (Moss et al., 2023), this protocol captures the range of meaningful within-individual variation in calls. All stimulus files were matched for peak amplitude prior to playback using the Normalize tool.

Experimental overview

Phonotaxis apparatus and procedures

All trials were conducted inside a sound isolation booth (SE 2000, WhisperRoom, Inc., Knoxville, TN, USA) furnished with ∼5 cm of acoustical foam to minimize ambient noise. The apparatus used for the choice tests was a long corridor measuring 74×12.7×18 cm L×W×H with a central starting point (13 cm long) and two equal-length arms (30.5 cm long) running in opposite directions (Fig. 1B). A wooden frame provided the main scaffold for the structure while acoustically transparent black mesh enclosed the walls. The floor was lined with the same substrate mixture and moss used in the frogs' home terraria, and was misted in between each trial to maintain consistent humidity levels. To facilitate video recordings, the top of the arena was cut from Plexiglas and the interior was softly illuminated with LED tape.

Acoustic stimuli were broadcast from speakers (Mod1 Orb speaker, Orb Audio, New York, NY, USA) located just outside the end of the choice arms. The frequency response of the playback

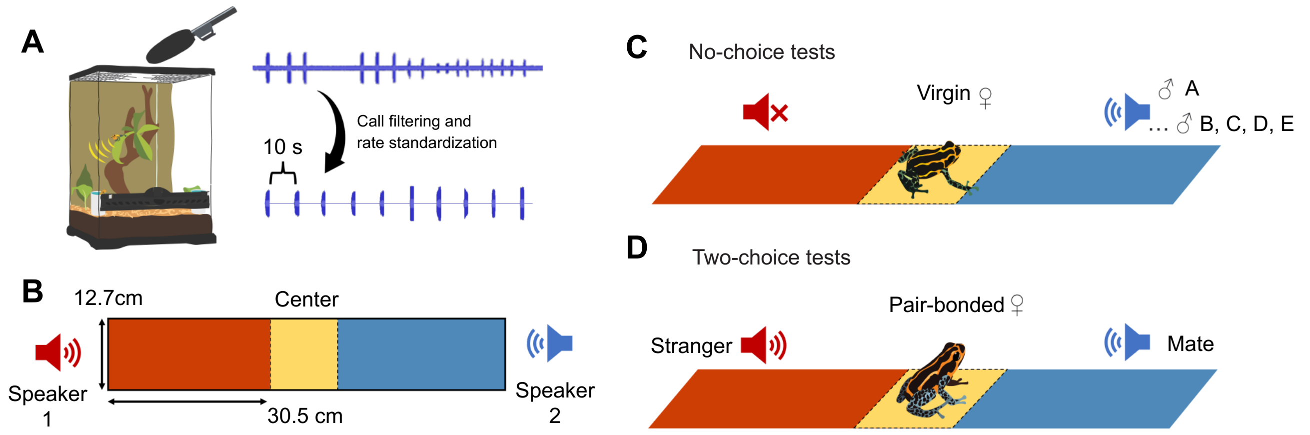

Fig. 1. Experimental overview of choice tests. (A) Acoustic stimuli to be used for playback were generated from advertisement calls of adult male *Ranitomeya imitator*, which were recorded in the laboratory and filtered to remove background noise and standardize call rates. (B) Trials were conducted in a two-arm maze inside a sound isolation booth, with subjects released in the center of the arena. (C) No-choice (single-stimulus) tests (*n*=40) were conducted by broadcasting male acoustic stimuli from one (randomized) side of the arena. Subjects (virgin females; *n*=9) were presented with five male acoustic stimuli in a randomized order. (D) Two-choice (two-stimulus) tests (*n*=28) were conducted by broadcasting competing acoustic stimuli from opposite (randomized) sides of the arena. Subjects (pair-bonded females; *n*=9) were presented with acoustic stimuli of their mate and an unfamiliar male.

system was flat ±2.5 dB over the range of interest (2–6 kHz). Stimulus files were stored on individual USB flash drives such that at the end of each 10-call sequence, playback restarted from the beginning and looped for the duration of the trial. Calls were broadcast through amplifiers (MAMP1, MouKey, Solihull, UK) at 60–70 dB, which approximates the sound pressure level (SPL) of calling males measured from a distance of ∼30 cm. Speakers were calibrated to the desired SPL at the beginning of each testing day using a digital sound level meter (RisePRO) positioned ∼30 cm away from the speaker at the level of the arena. At the start of each trial, the focal female was placed under a clear plastic cup in the center of the arena and playback was initiated from one or both speakers. For trials that utilized both speakers, competing stimuli were offset by 5 s to minimize interference and we counterbalanced which call was starting. After an acclimation period, we used a pulley system – a string threaded through the Plexiglas and operated by the observer concealed behind a privacy blind – to lift the cup, marking the start of the choice test. While tests of phonotactic behavior in anurans have traditionally involved species that orient and move rapidly to the first attractive sounds they hear (Gerhardt, 1995; Wells, 2010), the breeding biology of *R. imitator* (a pair-bonding species with prolonged reproductive and courtship periods) is unlikely to have selected for such vigorous responses. To account for this, subjects were afforded 10 min to move throughout the arena and their behavior was quantified throughout, rather than as a single choice response. All trials were recorded using remote-controlled video cameras (Brave 7, AKASO, Frederick, MD, USA).

Experiment 1: no-choice tests

To evaluate female receptivity to male acoustic stimuli in females that had never pair bonded, we exposed virgin females (all 8–9 months old at the time of testing) to a single acoustic stimulus in a no-choice test. Subjects (*n*=9) were chosen from among female F1 offspring of colony founders. These individuals were reared in the lab without access to deposition pools and were never housed with an adult male, and thus we could be confident that attraction to acoustic stimuli was unaffected by a prior pair bond. Nevertheless, female response speed and/or strength may depend on the perceived quality of an individual male stimulus. To account for this possibility, we sequentially tested each subject's response to each of five acoustic stimuli, each from a different, unfamiliar male (Fig. 1C).

We assayed each female over two consecutive days during the experimental period (June–July 2022) in alternating sets of two or three tests spanning 30–45 min. Because of mortality, only seven of nine subjects completed all five trials, while two completed only two or three trials. A single speaker was used for the stimulus and its placement was randomized between sides for each trial, such that the stimulus was broadcast from the left or right side in equal numbers. Tests for each male were conducted in a randomized order between 10:00 h and 13:00 h. Each test included a 5 min acclimation period immediately prior to the 10 min trial.

Experiment 2: two-choice tests

To test whether pair-bonded females preferentially associate with mate calls, we exposed adult females to competing acoustic stimuli, one of their mate and one of a stranger, in a two-choice test (Fig. 1D). Trials were conducted over two time blocks, 9 weeks apart. Subjects were chosen from proven breeding pairs, defined as pairs that had successfully cared for at least one clutch (i.e. reared eggs to hatching and transported tadpoles). Three proven pairs could not be included in block 2 owing to mortality of one or both partners. These were replaced with new breeding pairs which, in turn, were tested only in block 2. In sum, *n*=9 subjects experienced between two and four tests (total *n*=28 trials). Breeding status at the time of testing (i.e. whether the pair currently had eggs or not) was recorded throughout.

For each block (February–April 2022 and May–June 2022), subjects experienced a series of choice tests. Tests were conducted in sets of three in a randomized order between 10:00 h and 17:00 h. One of the tests involved white noise broadcast from one or both speakers; however, because of imperfect replication across blocks, these results are not shown. We report here only on two tests that were performed with perfect replication: (1) a mate versus stranger test, in which calls of the mate were broadcast from the left side of the arena and calls of a stranger were broadcast from the right side; and (2) a stranger versus mate test, in which the same stimuli were used as in step 1 but speaker positions were reversed. This procedure was repeated in the second experimental period but with a different stranger stimulus. Thus, our design accounted for potential side preference as well as stranger identity preference. Each test included a 10 min acclimation period in addition to the 10 min recorded trial. The reason for this extended acclimation time was to account for the delayed exit from the center by pair-bonded females relative to virgins in our preliminary trials.

Behavioral analysis

We analyzed video recordings of trials using the behavioral analysis software BORIS (Friard and Gamba, 2016). Target behaviors were coded to reflect time spent (in seconds) in each arm of the phonotaxis arena (Fig. 1B). Entrance into a region was defined as the entire body of the subject crossing a superimposed threshold corresponding to the edge of the central starting point. Scoring was conducted blind with respect to treatment group and stimulus side.

We imported ethogram outputs from BORIS into R Studio v.3.386 (http://www.R-project.org/) for statistical analysis. For each trial, we quantified the total duration of time frogs spent in each arm of the phonotaxis arena. For the no-choice tests, the two arms represented ‘towards stimulus’ and ‘away from stimulus’. For the two-choice tests, the arms represented ‘towards mate’ and ‘towards stranger’. To assess whether subjects showed an initial preference between arms and how quickly this judgment was made, we also extracted the first movement out of the center for each trial. The distribution of initial orientations in either direction was compared against chance levels using one-way chi-squared tests. We compared time spent between arms and the latency to first move into either arm by fitting mixed effects models in lme4 (Bates et al., 2015), where breeding status was included as a covariate for the two-choice tests and female ID, speaker position and block (for the two-choice tests) were included as random effects. Because some subjects failed to respond (i.e. did not exit the center) for the full 10 min, not all trials could be included in models of time spent and latency to exit. We compared the number of non-responsive trials between no-choice and two-choice tests and between subjects that were breeding and non-breeding using two-way Fisher’s exact tests. To understand how phonotaxis behavior varied as a factor of male ID in the no-choice tests, we fitted a mixed effects model as above and significance was evaluated via ANOVA. To visualize changes in space use over time (i.e. directional movement towards or away from a given stimulus), we extracted the position of frogs for each second of the trial across all trials and plotted total occurrences in either arm as a time series.

RESULTS

Experiment 1: no-choice tests

Virgin females spent more time in the arm with the stimulus than without (Fig. 2A; $F_{3,71}$=6.806, P=0.011) and there was no significant side bias. Identity of the male caller had no significant impact on either the latency of females to approach the stimulus ($F_{2,22}$=0.885, P=0.496) or the time females spent in the arm broadcasting the stimulus ($F_{2,35}$=1.025, P=0.410). This pattern of attraction was relatively constant over time, with frogs consistently spending more time towards the stimulus than away from it (Fig. 2B). While in the majority of trials (n=29) the first movement was directed towards the stimulus as opposed to away from it (n=13; χ^2=3.270, P=0.071), initial orientation did not influence the speed of approach ($F_{2,22}$=0.009, P=0.925; mean latency to exit center 144 s, range 2–548 s). In three of 40 trials, the focal female did not move from the center for the full 10 min. Random effects (female ID and speaker position) together accounted for <10% of residual variance in the overall distribution of time spent in either arm (<0.005) or in latency to exit the center (0.086).

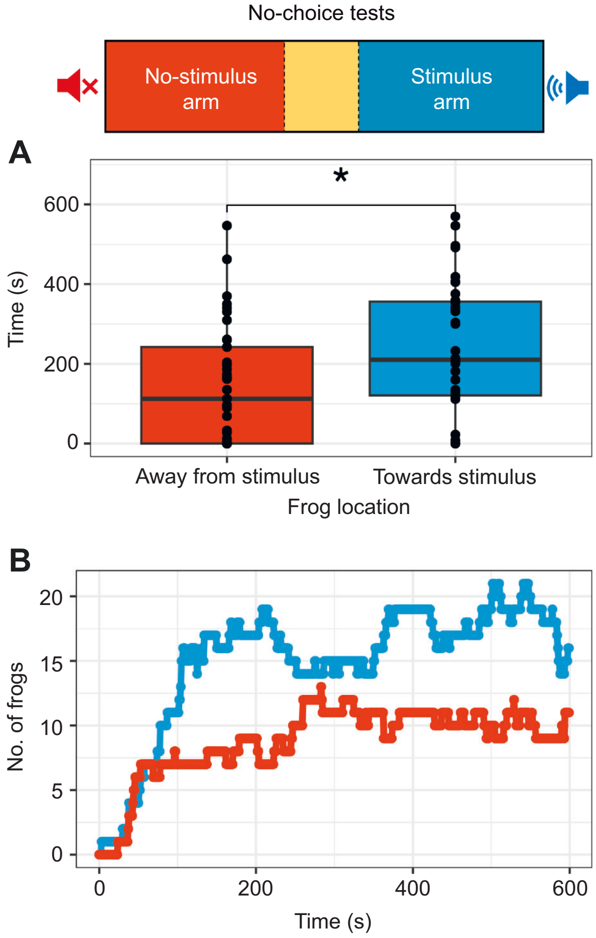

Fig. 2. Summarized results from the no-choice (single-stimulus) tests of virgin female *R. imitator*. (A) Cumulative time (s) spent in the arm without an acoustic stimulus (red; n=37) versus the arm with an acoustic stimulus (blue; n=37), excluding three non-responsive trials. Trials were repeated up to five times for each of nine subjects using different male stimuli and randomizing the position of the speaker. Boxes depict the median and interquartile range, and the asterisk denotes a significant difference in a one-way ANOVA (P=0.011). (B) Changes in frog location over time for the same 37 trials, depicted as the total number of frogs in the no stimulus (red) versus the stimulus (blue) arm at each second of the trial across trials.

Experiment 2: two-choice tests

Pair bonded females spent significantly more time in the arm broadcasting calls of their mate than in that playing acoustic stimuli of a stranger (Fig. 3A; $F_{4,34}$=5.340, P=0.027). Closer inspection of the time course of female behavior revealed that subjects favored the mate arm for the first 400 s of the trial but were more likely to be found in the stranger arm in the final 200 s, diminishing overall differences between stimuli (Fig. 3B). Initial orientation was more frequently directed into the mate arm (n=12) than into the stranger arm (n=7), albeit not significantly so (χ^2=1.316, P=0.251). Initial orientation had no bearing on the latency to first movement ($F_{4,15}$=1.945, P=0.185); however, subjects that initially oriented towards the mate arm spent cumulatively more time there ($F_{3,16}$=1.945, P=0.005) as did subjects with shorter latencies to exit the center ($F_{3,16}$=16.425, P=0.0008).

Females that had eggs at the time of testing were significantly faster in exiting the center ($F_{4,15}$=6.80, P=0.020) than females that were pair bonded but did not have eggs or tadpoles at the time of testing. Random effects (female ID, block and speaker position)

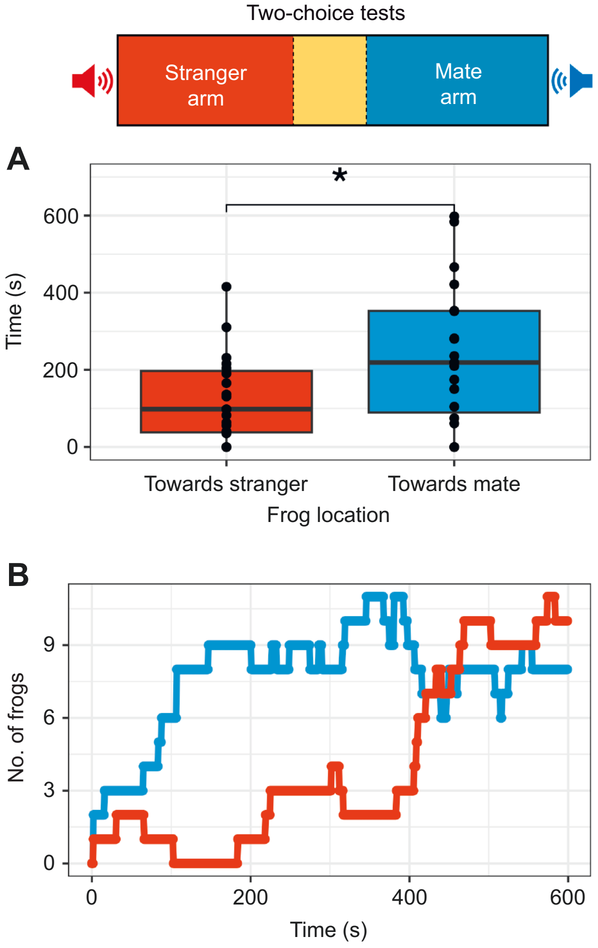

Fig. 3. Summarized results from the two-choice (two-stimulus) tests of pair-bonded female *R. imitator*. (A) Cumulative time (s) spent in the arm broadcasting a stranger stimulus (red; *n*=19) versus the arm broadcasting a mate stimulus (blue; *n*=19), excluding nine non-responsive trials. Trials were repeated up to four times over two blocks for each of nine subjects, swapping out the stranger stimuli and the position of the speaker. Boxes depict the median and interquartile range, and the asterisk denotes a significant difference in a two-way ANOVA with breeding status (*P*=0.027). (B) Changes in frog location over time for the same 19 trials, depicted as the total number of frogs in the stranger (red) versus the mate (blue) arm at each second of the trial across trials.

together accounted for <12% of residual variance in the overall distribution of time spent in either arm (<0.005) or latency to exit the center (0.112). We note that for nine of 28 trials in experiment 2, the focal female did not move from the center for the full 10 min, which is a significantly higher proportion than in experiment 1 (Fisher odds ratio=5.682, *P*=0.011). While the majority of responsive subjects (79%) were breeding at the time of the test, non-responsiveness could not be significantly attributed to breeding status (Fisher odds ratio=0.348, *P*=0.201).

DISCUSSION

In this study, we performed playback experiments in the laboratory to evaluate the scope for vocal recognition at the individual level in the pair-bonding poison frog, *R. imitator*. Through a series of no-choice experiments using virgin females as test subjects, we first demonstrated that acoustic signals elicit behavioral responses from inexperienced females and that male identity does not contribute significantly to variation in preference for unfamiliar individuals. Following the demonstration of attraction to conspecific males, we subjected pair-bonded females to two-choice tests involving acoustic stimuli of their pair-bonded mate versus a stranger. Females spent significantly more time near the speaker broadcasting playback of their mate versus that of a stranger, especially early on in the trial. However, subjects were also more reticent to explore the arena and many appeared to sample both stimuli rather than preferentially associating with one. Nonetheless, ours is the first study to show that females may recognize partners based on calls alone, suggesting a role for individual recognition in partner preference across poison frogs.

Although the primary motivation of our study was to investigate individual vocal recognition in the context of pair bonding, the first step in testing this was to determine whether non-pair-bonded females respond to male acoustic stimuli. To our knowledge, only one experiment to date has examined behavioral responses of *R. imitator* to acoustic playback, and this study focused on interactions between territorial males in the field (Mayer et al., 2014). Here, we show that virgin female *R. imitator* are attracted to conspecific male acoustic stimuli when the alternative is no sound. Behavioral responses did not vary significantly among subjects or between the five distinct male stimuli presented, suggesting that any advertisement call is sufficient to elicit interest from inexperienced females. Use of subtle variation in male advertisement calls to assess mate quality has been demonstrated experimentally in related poison frogs (Dreher and Pröhl, 2014; Forsman and Hagman, 2006; Peignier et al., 2022; Pettitt et al., 2019), although receptivity to calls may have little bearing on mate choice *in situ* (i.e. relative to practical factors, such as proximity; Meuche et al., 2013). Given the prolonged courtship of *R. imitator*, initial attraction to acoustic stimuli is only the first step in a more thorough evaluation of mate quality, which may involve visual and olfactory cues of which more experienced females may be more discerning. Future studies will be necessary to determine the role of acoustic signal variation in initial mate attraction in *R. imitator* and the influence of experience on choice.

In our two-choice paradigm, we found that pair-bonded females spent more time associating with the mate stimulus than the stranger stimulus, that this was especially true immediately after stimulus presentation, and that this behavior was correlated with initial orientation towards the mate stimulus (Fig. 3A). A similar, non-significant trend was recently found in a study of a promiscuous dendrobatid (*Allobates femoralis*): that females tend to prefer familiar partners over novel partners as mates (Peignier et al., 2022). The authors suggest this result may be due to captive housing conditions and/or knowledge of males' territorial status. Alternatively, females may be picking up on acoustic indicators of prior paternal experience, as has been shown in *Dendrobates leucomelas*, *Epipedobates tricolor* and *Anomaloglossus beebei* (Forsman and Hagman, 2006; Pettitt et al., 2019). Compared with non-breeding females, females in our study that currently had eggs and/or tadpoles were quicker to exit the center, which raises the intriguing possibility that physiological changes associated with reproduction (i.e. hormonal changes) may enhance motivation to respond to stimuli (e.g. Dey et al., 2015). Even so, considerable variation within and between individual trials combined with the apparent erosion of preference over time leads us to conclude that females probably rely on multimodal cues (e.g. visual and olfactory) for accurate identity determination in most circumstances. This would be consistent with the mating and social system of *R. imitator*, as males and females cohabit small territories that are typically non-overlapping with other pairs (Brown et al., 2008, 2010), and the need to distinguish partners based on calls alone may arise infrequently.

An important caveat to the above findings is that in almost a third of trials, pair-bonded females did not move from the central starting point. When subjects did explore the arena, they spent overall less time outside the center than females in no-choice trials. A reduced effect size in two-stimulus tests relative to single-stimulus tests is not without precedent in studies involving anurans (Tanner et al., 2017) and may result from females 'sampling' both sides and obscuring choice (Bush et al., 2002). In line with this, inspecting the time course of frog locations in the arena revealed investigation of the stranger stimulus increased only at the end of trials (Fig. 3B), suggesting that a female's motivation to approach a stranger may stem from their failure to locate their mate or from a place of territory defense. For instance, monogamous California mice that use ultrasonic vocalizations for territory defense do not preferentially approach vocalizations of their partners but do approach vocalizations of a stranger (Pultorak et al., 2017).

In conclusion, our study shows that females of a pair-bonding poison frog are behaviorally responsive to male acoustic signals and, for the first time in an anuran, evaluates individual vocal recognition in the context of monogamy. Pair bonding is predicted to strongly influence the evolution of sensory perception and communication (Prior et al., 2022), although empirical tests of this prediction have been rare outside birds and mammals. Having previously shown that advertisement calls of *R. imitator* are reliable signals of male identity (Moss et al., 2023), we show in the present study that receivers in this system (pair-bonded females) recognize and preferentially associate with calls of their partner. However, long latencies to respond combined with the erosion of preference over time suggest that additional signal modalities and context cues are vital to the maintenance of pair bonds in this system. At the same time, behavioral motivation must be interpreted through the lens of species biology, and ultimately our results also highlight limitations of translating standard behavioral assays across taxa. Given *R. imitator*'s prolonged reproduction and courtship, variation in female assessments of male calls may be more evident at the hormonal or neural as opposed to behavioral level. Future investigations should explore the use of alternative assays for uncovering the role of acoustic and other modes of communication in maintaining pair-bond structures.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.E.P., J.B.M., E.K.F.; Methodology: M.E.P.; Formal analysis: J.B.M.; Investigation: M.E.P., J.B.M.; Resources: E.K.F.; Data curation: M.E.P., J.B.M.; Writing - original draft: M.E.P., J.B.M.; Writing - review & editing: M.E.P., J.B.M., E.K.F.; Supervision: J.B.M., E.K.F.; Funding acquisition: J.B.M., E.K.F.

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Data availability

All raw data and R scripts are available from the Dryad digital repository (Podraza et al., 2024): https://doi.org/10.5061/dryad.5hqbzkhd2

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