

The reproductive biology of *Hypsiboas punctatus* (Anura: Hylidae): male territoriality and the possible role of different signals during female choice

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Abstract. Anuran amphibians are known to employ acoustic signals as the principal form to access potential mates, while recent studies have highlighted the importance of multiple cues during courtship. *Hypsiboas punctatus* is a hylid species in which males emit advertisement calls and have sexually dimorphic skin glands (SDSGs). However, knowledge on the reproductive biology of this species is very limited. We present the first systematic field-observational study on the behaviour of *H. punctatus*, which was complemented by observations under laboratory-controlled conditions. Males exhibit a territorial behaviour as is evidenced by site fidelity, aggressiveness against male intruders (usually involving fights), and signalling behaviour. We found that female choice would be operating at two different levels, which we defined as Initial Exploration (I.E.) and Proximate Exploration (P.E.). These levels would be guided by acoustic signals in I.E., while tactile and/or chemical signals (considering the presence of SDSGs) would be more important in P.E. We also provide descriptions of vocal interaction between males, amplexus, and egg clutch. We suggest that SDSGs located at the mental gland may be important in delivering sexual pheromones to the female during amplexus. Finally, our results are compared to similar behaviours that have been described for related species, and discussed in terms of the probable existence of multimodal communication.

Key words. Sexual selection, sexually dimorphic skin glands, courtship, amplexus, Cophomantini.

Introduction

DARWIN (1871) proposed two mechanisms of sexual selection: male–male competition and female choice. These forms of selection are evident in the analyses of mating systems, which are classified based on the level of polygamy (EMLEN & ORING 1977, SULLIVAN et al. 1995). However, an adequate description and explanation of any mating system should also consider the temporal and spatial distribution of reproductively interacting individuals, and their behavioural tactics (SULLIVAN et al. 1995). Based on temporal patterns, most anurans can be classified as either explosive or prolonged breeders (WELLS 1977a). In species with a prolonged breeding season, male–male competition takes the form of elaborate vocal interactions, defence of calling sites, and/or elaborate courtship. In turn, female choice is favoured because they have ample opportunities to move amongst calling males and compare the quality of potential mates or their territories (WELLS 2007).

Most of our knowledge of communication in anurans stems from the study of acoustic signals (GERHARDT & HUBER 2002, WELLS & SCHWARTZ 2006). However, visual, tactile, and chemical cues are known to play significant roles during mating and courtship in some anuran species (e.g., HADDAD & GIARETTA 1999, WABNITZ et al. 2000, HÖDL & AMÉZQUITA 2001, GRAFE et al. 2012). For example, in the hylid subfamily Hylinae, courtship behaviour that includes the female contacting her snout to the flanks and gular region of the male, has been described for *Scinax ruber* (BOURNE 1992), some species of *Aplastodiscus* (HADDAD & SAWAYA 2000, HADDAD et al. 2005, HARTMANN et al. 2004, ZINA & HADDAD 2007), *Bokermannohyla ibitiguara* (NALI & PRADO 2012), and species of the *Hypsiboas faber* group (KLUGE 1981, MARTINS & HADDAD 1988). Chemical communication by way of sexual pheromones has been described for only two species of anurans (PEARL et al. 2000, WABNITZ et al. 2000), but recent studies suggest that they might be more ubiquitous than previously thought (POTH

et al. 2012, WILLAERT et al. 2013). These different modalities of communication (visual, tactile, chemical, and acoustic) may be interlinked, which led to an increased interest regarding multimodal components in courtship signals (e.g., GRAFE et al. 2012, PREININGER et al. 2013). That interest has been focusing on acoustic and visual stimuli, while it remains poorly understood how tactile stimuli or sexual pheromones might be linked to, or complement, acoustic signals.

Hypsiboas punctatus is a common species that occurs in South America throughout the Amazon basin, south to the eastern Chaco region of Paraguay and along the banks of the Paraguay-Parana rivers, Argentina. This species has a prolonged reproductive season and congregates during the breeding season in the centres of large water bodies, generally associated with rivers, from lowlands to 1,400 m a.s.l. (DUELLMAN 1978, MARQUEZ et al. 1993, PRADO et al. 2005). Knowledge of the reproductive biology of *H. punctatus* is limited to the description of its advertisement call and some references to its variation (e.g., HOOGMOED 1979, MARQUEZ et al. 1993). It has two types of sexually dimorphic skin glands (SDSGs) that occur only in the mental and lateral regions of males (HOOGMOED 1979, BRUNETTI et al. 2012), suggesting some sort of chemical communication during courtship and/or male interactions. This scenario presents an exciting framework, since although SDSGs occur in several species of anurans, their function as a source of female-attracting pheromones has been demonstrated only for two of them (PEARL et al. 2000, POTH et al. 2012).

In this paper, we describe vocal interactions and territoriality in males of *H. punctatus*, female behaviour during mating, and the use of tactile stimuli during courtship and amplexus. Finally, we discuss our findings in terms of the significance of different signals (acoustic, and chemical and/or tactile) during courtship in anurans.

Materials and methods

Study areas

Two different populations of *Hypsiboas punctatus* were studied in the Parana River basin in Argentina. Population SF was located on the banks of a small river near Laguna Setúbal, in Alto Verde, near Santa Fe City, Santa Fe province. Population BV was located in a small lagoon connected to a floodplain, at Toropí, 8 km south of Bella Vista, Corrientes province. In both localities, *H. punctatus* was strongly associated with *Eichhornia crassipes* (water hyacinth), *Pistia stratiotes* (water lettuce), *Salvinia biloba* (giant salvinia), and *Azolla filiculoides* (water fern). Air and water temperatures were 26–32 and 29–31°C around noon, and 19–29 and 24–29°C at night, respectively.

Field observations

We made one survey in February 2011, and two surveys during February 2012 at locality SF, and one in February

2013 at locality BV, with three to five days of observation per survey. Observations were conducted from 20:30 to 4:00 h (approximately), beginning at sunset and finishing when anuran activity had decreased or ceased. To avoid disturbing animals, nocturnal observations were conducted with minimal lighting. Due to the inaccessibility to specimens, all observations were carried out from a small canoe. This allowed approaching individuals to distances as close as 30 cm. The field descriptions correspond to observations made on $n = 35$ males and $n = 12$ females at locality SF, and $n = 10$ males and $n = 4$ females at locality BV. Given that the purpose of this paper is to describe general aspects of the behaviour of *H. punctatus*, all field data are presented considering both populations and different years as a whole.

Observations under laboratory-controlled conditions

To complement field observations, we conducted behavioural observations and vocal recordings in the laboratory under temperature- and light-controlled conditions: The first took place during March of 2012 and involved specimens brought back from locality SF and placed in a single aquarium of 125 × 50 × 65 cm ($n = 5$ males and $n = 4$ females). The second was conducted during February of 2013 and used two groups of specimens brought back from locality BV. Each group was placed in a separate aquarium of 80 × 30 × 40 cm ($n = 3$ males and $n = 2$ females per group). Frogs were kept at 23–28°C, with a 14:10 light:dark period, and fed with small crickets. Each aquarium was outfitted with natural vegetation and 5 cm deep dechlorinated tap water. Once in the laboratory, the animals were allowed to acclimatise for two days after which we recorded the individuals' behaviour during five consecutive nights at minimum lighting with a SONY DCR-SR85 camera in night shot mode.

Data collection and analysis

Calling sites were categorized according to: (a) height above the water surface: water level or above water level, and (b) vegetation, considering the petioles or leaves of the most prominent species, *E. crassipes* (water hyacinth). Air and water temperatures were measured with an alcohol thermometer to the nearest 0.5°C.

Vocalizations of specimens from localities SF and BV were audio-recorded with a SONY (WM-D6C) equipped with an external microphone (SONY ECM-909A) and a Marantz PMD-430 with an Audiotechnika At-835B microphone, respectively. In all cases, microphones were positioned 40–70 cm from the caller. Calls were recorded on chrome cassette tapes at 4.75 cm/sec, and analysed using the software Raven pro 1.3 (16 bit resolution, 22 kHz of frequency sampling, FFT and frame length of 256 samples). The terminology of different call types follows that of WELLS (2007), except for fighting calls, which was defined

by MARTINS et al. (1998). We employ the term “territorial call” as it was defined by KLUGE (1981), rather than the “aggressive call given in long-range interactions” suggested by WELLS (2007). The main reason for this is that in our observations, this type of call did not seem to be emitted in response to another male’s call, which is required to qualify as aggressive call.

A detailed analysis of the different call types and their parameters are described by BRUNETTI et al. (in press), but briefly, males of *Hypsiboas punctatus* were heard emitting seven different types of call. Three of these were multi-note calls, whereas four of them were frequency-modulated whistles. Our behavioural observations allowed to assign them to specific functions: (1)–(2) advertisement calls (short and long duration), (3) territorial signalling, (4) courtship interactions, and (5)–(7) aggressive interactions (aggressive, fighting, and release calls). All calls consist of a single note and possess harmonic structures. In the present paper, we limited our descriptions to vocal interactions.

Behavioural observations in the field and under lab-controlled conditions were obtained using focal animal, sequence, and all occurrence methods (MARTIN & BATESON 1986). The number of times different individuals exhibited a particular behaviour is indicated in parentheses. Clutch size was quantified from three clutches from locality BV, and from three clutches in the laboratory. Egg sizes were measured with digital callipers to the nearest 0.01 mm from a randomly chosen sample ($n = 10$) of one of the clutches from the field, and two clutches in the laboratory, at developmental stages 9–13 (GOSNER 1960). Voucher specimens (adults, tadpoles, and egg clutches) of *H. punctatus* are deposited in the herpetological collection of the Museo Argentino de Ciencias Naturales “Bernardino Rivadavia” (MACN), Buenos Aires, Argentina. Results are presented in four sections: (1) behaviour and mating tactics of males, (2) mating tactics of females, (3) male and female interactions and description of the amplexus, and (4) description of the clutches.

Results

Behaviour and mating tactics of males

In the field, advertisement calls are emitted by males perched with a fully extended vocal sac on floating vegetation (*P. stratiotes*, *S. biloba*, *A. filiculoides*, and leaves of *E. crassipes*) at water surface level (Fig. 1A; $n = 40$). Calling males were observed to fight when a calling intruder approached. These fights took place within 10 cm from the calling site and were accompanied by fighting and release calls. Both males were then be positioned face to face and held tightly, usually with the hands on the contender’s back (Fig. 1B; $n = 5$). In this position, they used their pre-pollical spines to try and inflict injuries on the opponent. At locality BV, after having watched a fight for 30 minutes, we saw a juvenile *Caiman yacare* leap at the contenders and devour one of them.

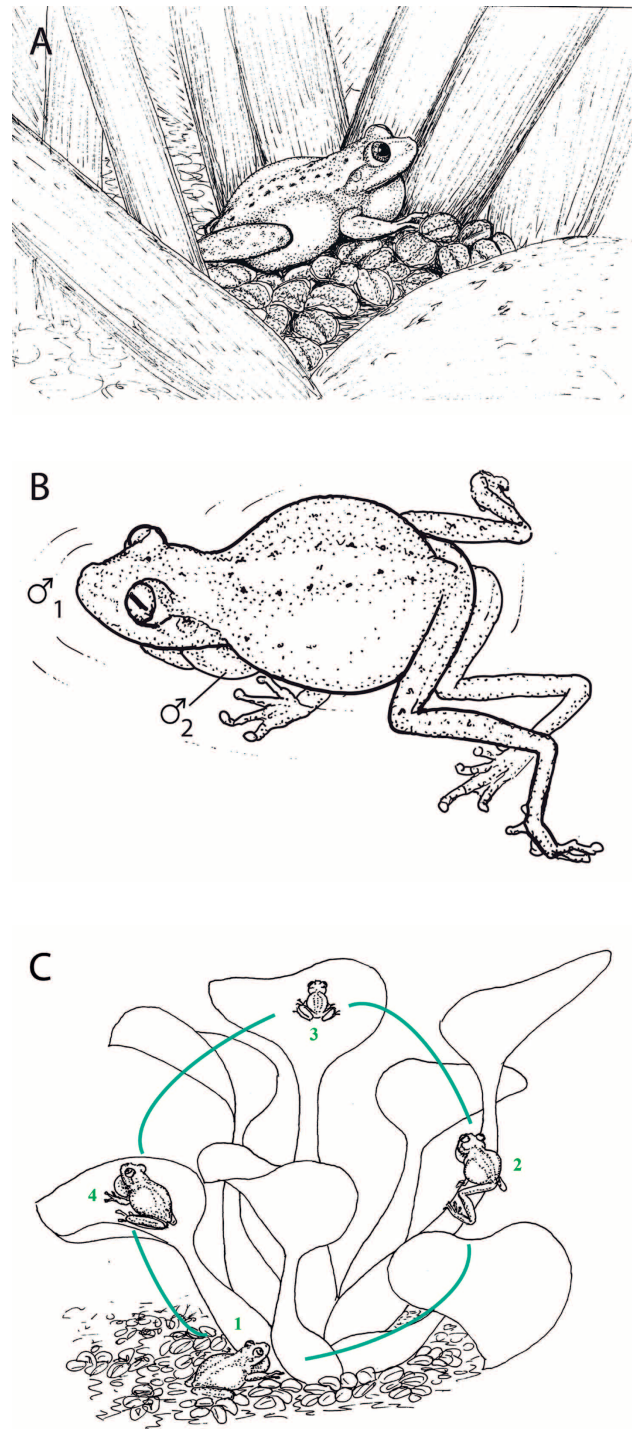


Figure 1. Reproductive behaviour of males of *Hypsiboas punctatus*: A) male emitting advertisement calls from his calling site at water surface level; B) male–male physical interactions. ♂1 pushes ♂2 under water. A fight ensues from both males facing each other, with ♂2 in an upside-down position; C) territorial signalling of males. A male giving off advertisement calls in position 1, stops calling and begins to climb the petioles of a water hyacinth. He then begins to emit sporadic territorial calls while moving to positions 3 and 4. After 30–50 minutes, he returns to position 1 and resumes advertising.

We observed a striking behaviour performed by some males that were emitting advertisement calls from their calling sites ($n = 4$). The sequence of this behaviour is as follows: the male begins to climb from the level of the water surface to different heights (50–100 cm) and distances (30–100 cm) on a circular path around his calling site while emitting territorial calls, which differ from the advertisement call mainly by the number of pulses per note and fundamental frequency (see BRUNETTI et al. in press, for detailed comparisons of call types). Later, he will return to his initial position and resumes calling (Fig. 1C). This behaviour lasts 30–50 minutes. Males vocalizing at their calling sites also abandoned it with fast movements, jumping on silent nearby males ($n = 3$), only to return soon to their original positions.

Under lab-controlled conditions, one or two males from each aquarium were observed moving about and sporadically emitting territorial calls during the first night. The second night, these males were emitting advertisement calls from secluded spots at water surface level. During the third night, these males were at the same sites, emitting advertisement calls, while at the opposite side, other males began to vocalize from water surface level as well. The fourth and fifth nights, all individuals maintained their locations and continued emitting advertisement calls. We observed fighting in two situations: in the first, two males had abandoned their calling sites while they were following a female (see description under Amplexus or rejection) and encountered each other at distances > 20 cm from their calling sites ($n = 2$). In the second, a fight ensued at the calling site of one of the males, in the manner observed in the field ($n = 2$). In one instance, one of the contestants was found dead at the site of the fight the next morning.

Both in the field and in the laboratory, neighbouring males were heard emitting advertisement calls in at least two different manners. In the first, a male would give off short advertisement calls at regular intervals and the likewise short advertisement calls of a second male alternated with those of the first (Fig. 2A; $n = 10$). The timing of alternation of calls among the males could vary, but both individuals gave approximately the same number of calls per minute (Fig. 2A; 6–18 calls/min). Most often, the call of the second male began immediately after the first male stopped vocalizing. Sometimes, a third male vocalized between those from the interacting duet (Fig. 2A). In the second, one of the males produced long advertisement calls while the second male continued giving short advertisement calls, resulting in their calls overlapping. In general, longer calls completely masked shorter calls (Fig. 2B; $n = 8$). Although long and short advertisement calls are multinote calls of similar appearance, they differ in some temporal parameters and are considered different (BRUNETTI et al. in press).

Mating tactics of females

In the field, soon after sunset (20.30 h), females were typically perched on *E. crassipes* petioles and leaves at different

heights (20–180 cm above the water) near males emitting advertisement calls. From there, they engaged in a stereotyped behaviour that we refer to as “Initial Exploration” and is summarized as follows: A female approaches the calling male, leaping from petioles and leaves of *E. crassipes* until reaching a position above him (at a vertical distance of between 30 and 80 cm from the male) and remains motionless with her head held horizontal for the next 5 to 20 min. Eventually, she would incline her head towards the male and observe him from above (Fig. 3A). She may then, (1) leave and continue to move exploring other males, or (2) continue descending while repeating the sequence of her approach and observation until she will have reached a distance of 5–15 cm from the male. She will then remain motionless for 10–20 min. once more. This behaviour was observed in five females. Two of them repeated the Initial Exploration phase twice during the same night focusing on the same male that each of them had explored in the first place (these were different males).

After the Initial Exploration phase, the female may hang from the *E. crassipes* leaves and contact the male’s flanks (or back), and soon after, jump into the water ($n = 3$). In this manner, she will move closer to the male, thereby reducing the distance to 3–5 cm. In general, the two individuals will now be orientated at different angles. Then, the female will turn her head towards the male and begin to move around him, contacting her snout to the flanks and gular region of the male (Fig 3B; $n = 3$). These contacts may be repeated once or twice while the female moves around the male’s calling site ($n = 2$). The male will remain motionless all the while, possibly emitting sporadic advertisement calls. We have named this behaviour “Proximate Exploration”, and two females repeated this behaviour twice with the male they had focused on first.

In the laboratory, we observed females exhibiting similar behaviours to those recorded in the field. Four females conducted an Initial Exploration phase and all repeated this behaviour twice with the same focus male. Also, two of these females explored two different males during the same night. The sequence of Proximate Exploration was observed in two females, with one of them also passing her dorsum under the gular region of the male.

Amplexus or rejection

We observed in the field and in the laboratory that females performing Proximate Explorations subsequently either left the male’s calling site or chose the resident male. In the first scenario, three different behaviours were observed in the males: (1) attempting to amplex the female with her freeing herself immediately ($n = 2$ in the field, $n = 1$ in the laboratory), (2) remaining motionless ($n = 2$ in the field, $n = 2$ in the laboratory), or (3) following the female while emitting courtship calls (Fig. 3C, $n = 2$ in the field, $n = 1$ in the laboratory). In the laboratory, we also observed that some males would follow females they had detected at distances < 20 cm ($n = 6$); however, the latter did not conduct Proxi-

mate Explorations, nor did the situation lead to amplexus, as these males eventually returned to their calling sites.

The complete sequence of events when a female chose a male was observed twice, once in the field and once in the laboratory, and it was similar in both situations. The female positioned herself close to the male, initially at an angle. She then turned to face the male at a distance of 3–5 cm for approximately 2 minutes, during which the male emitted advertisement calls. Subsequently, the female bumped the male, who used his hands to position her below him at an angle. The pair remained in this position for approximately two minutes. The male then aligned himself and mounted the female while pressing his gular region on her snout. During amplexus, the male's hands held on to the angles of

the female's jaw (Fig. 3D). Usually, the pair drifted around the male's calling site, while he arched his body forward and pressed his gular region on the female's snout and even on her eyes. In both instances, amplexus occurred after the female had conducted at least two Proximate Explorations. It is worth mentioning that during our observations in the field, amplexus was interrupted after five minutes by an unidentified fish that consumed the female.

Clutches

In the field, *H. punctatus* females deposited clutches of 173–342 eggs, split up in clusters of 7–15 individual eggs, which

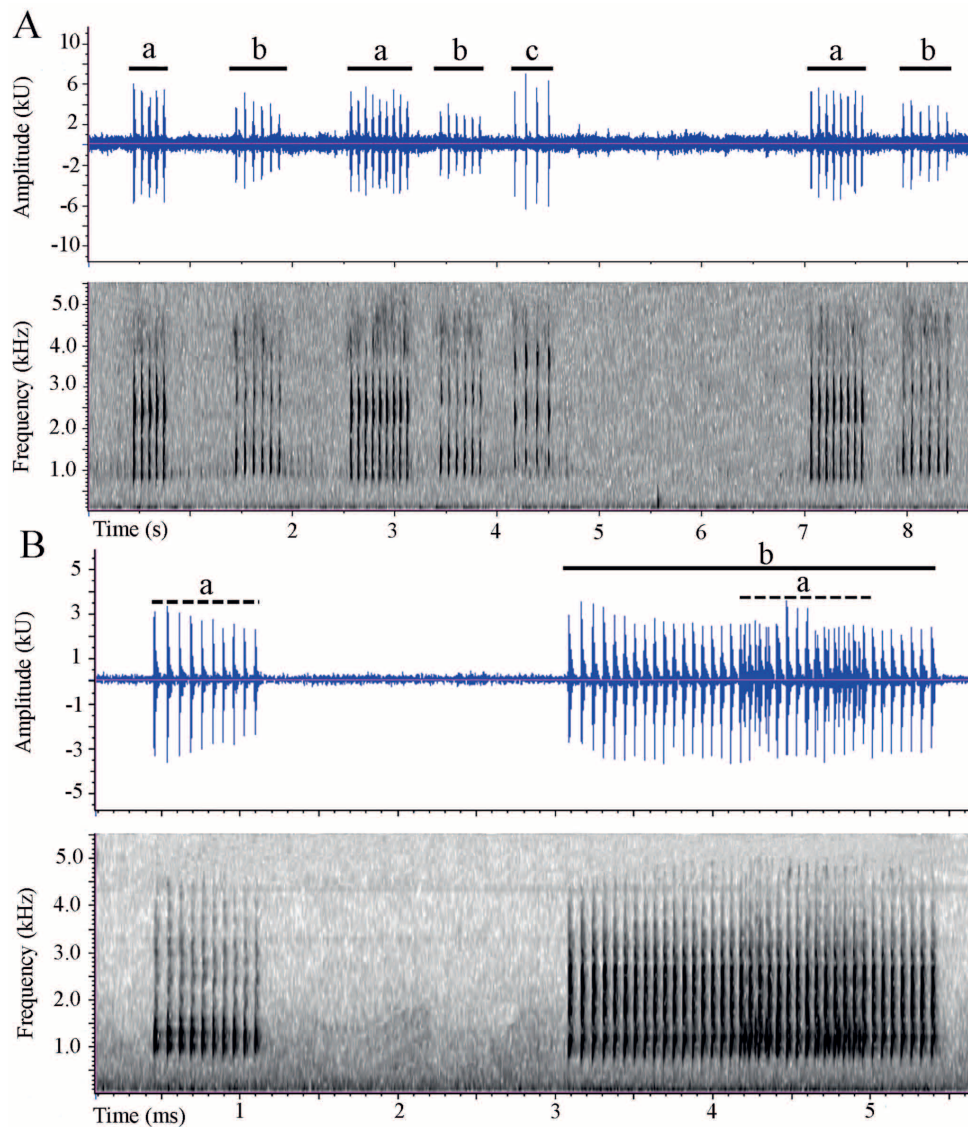


Figure 2. Waveform and sound spectrogram of male *Hypsiboas punctatus* recorded in the field at Santa Fe City, Santa Fe, Argentina. Air temperature = 22°C: A) alternating calls of male a and male b. Compare the differences in response time of male b at the beginning of the vocalizations with those emitted at the end. Between them are sporadic calls of male c; B) males a and b 10 min later: Male b emits a long advertisement call that completely masks the call of male a.

adhered to each other and formed a single-layer film on the water surface. The floating egg clutch adhered to surrounding vegetation (Fig. 4). In the laboratory, clutches contained 167–210 eggs and were deposited at sites from which males were emitting advertisement calls. The animal pole is black and the vegetal pole unpigmented. Eggs have a diameter of 1.4–1.7 mm (mean = 1.5 mm, SE = 0.1 mm, n = 30) and the jelly capsules have a diameter of 1.7–2.4 mm (mean = 2.0 mm, SE = 0.2 mm, n = 30). Eggs remained adhered together by their outer jelly. It was difficult to dislodge them from the surrounding vegetation. In the aquarium, eggs that sank to the bottom failed to develop.

Discussion

Male behaviour

Field and laboratory observations allow us to conclude that males of *H. punctatus* stake a territorial claim on their calling site and its immediate surroundings. This is supported by the following considerations: (1) males show site fidelity; (2) they defend their territory against other males by emitting calls and fending them off if necessary; (3) amplexus occurs and eggs are deposited at the calling site; and (4) males emit territorial calls within a circular area around their calling sites. Male behaviour of defending resource-based territories is almost always associated to the monopolisation of oviposition sites (see WELLS 2007 for a review),

and is more evident in species in which males build nests and defend them against other males, as is the case in the *H. faber* group or species of the genus *Limnonectes* (KLUGE 1981, MARTINS & HADDAD 1988, EMERSON 1992). However, as described here, it may also occur in other species that produce aquatic eggs and do not build nests (e.g., HOWARD 1978, KOKUBUM & GIARETTA 2005).

The most frequent calling pattern of two neighbouring males of *H. punctatus* was alternating short advertisement calls. However, this pattern could be altered by the emission of long advertisement calls that overlap at least part of the short call from the other male. Call alternation results in approximately the same number of calls per minute for either male, but also in reduced call overlaps. Call overlapping frequently obscures the short advertisement call from one of the males. It has been demonstrated that the males of some anuran species have the capacity to alter their calling strategies depending on the social context of communication (GRAFE 1996, SCHWARTZ et al. 2002, REICHERT & GERHARDT 2013). Avoiding acoustic interference would be desirable because it may facilitate females to locate and discriminate amongst males, as well as raising the males' chances of assessing the intensity of a neighbour's call and maintain adequate inter-male spacing (DYSON & PASSMORE 1988, GRAFE 1996). In turn, a high level of call overlap or overlay may function as a socially mediated response to escalated competition in which males shift their communication efforts away from attracting females and

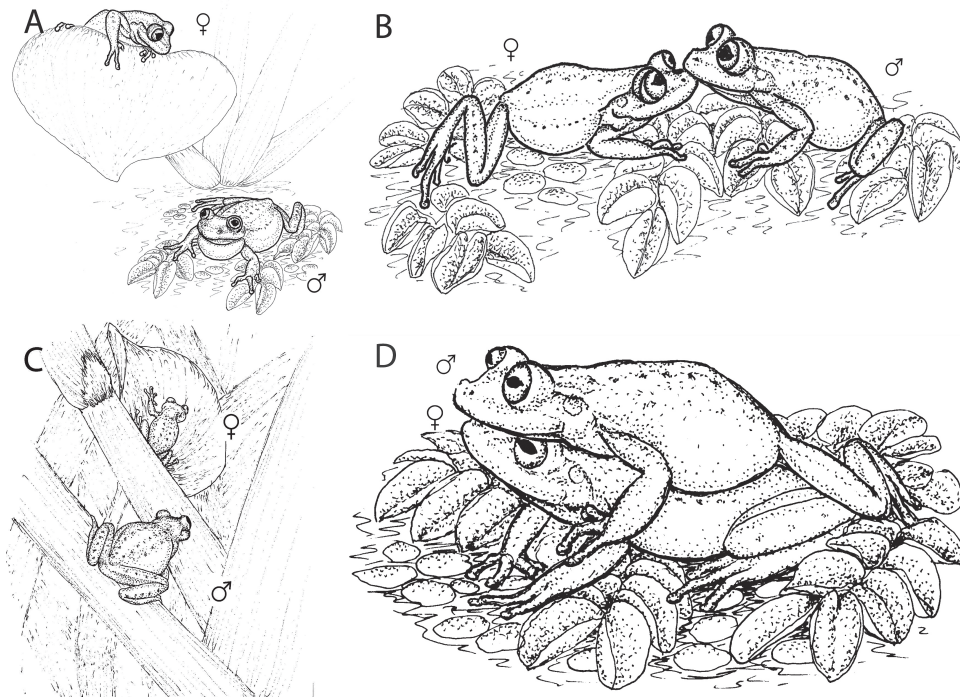


Figure 3. Female–male interactions in *Hypsiboas punctatus*: A) ♂ emits advertisement calls while ♀ observes him from above; B) ♀ contacting her snout to the mental gland of the ♂ at his calling site; C) ♂ pursuing ♀ after she has left his calling site; D) Amplexant pair. Note the position of the mental gland of the ♂ on the ♀ and the placement of his hands. Figures follow the sequence of occurrence. See text for definitions of different behaviours.

toward repelling rival males (SCHWARTZ et al. 2002, REICHERT & GERHARDT 2013). Call overlap of *H. punctatus* might represent an early phase of escalating competition behaviour, although detailed studies considering male and female density are needed to better understand the context in which males alter their calls.

Female behaviour and choice

During this study, we observed females of *H. punctatus* conducting two methods of investigating calling males. We have named these approaches Initial Exploration and Proximate Exploration, respectively. During Initial Exploration, females never descended to the water surface at the calling site of a male, while Proximate Exploration only occurred subsequent to Initial Exploration at the calling site of a male. Similar methods of sampling were suggested for *H. rosenbergi* by KLUGE (1981). According to this author, females make their choice, outside and within the nest of males, on the basis of different criteria. Following this idea, the behaviour of females of *H. punctatus* during Initial Exploration may be a means of assessing information on a male's call characteristics. Our interpretation agrees with the experimental results obtained for *Hyla gratiosa* and *Hyla versicolor* (SCHWARTZ & BUCHANAN 2001, MURPHY & GERHARDT 2002).

Proximate Exploration served females of *H. punctatus* to inspect a male's territory. During the inspection, the females were always observed moving around the calling site and contacting the dorsal, lateral, and/or gular regions of the resident male with their snout and hands. This behaviour is comparable to that observed in *H. faber* and *H. rosenbergi* during nest inspection, which are two species of the *H. faber* group within the Cophomantini for which well documented data exists on their reproductive biology (KLUGE 1981, MARTINS & HADDAD 1988). Some good examples of female choice based on territory quality were described in species of *Lithobates* (WELLS 1977b, HOWARD 1978) and *Ameerega trivittata* (ROITHMAIR



Figure 4. Egg clutch deposited in the field on 14 January 2013, at Toropi, Corrientes, Argentina.

1994). However, no correlations were found for *H. faber* or *H. rosenbergi* between nest characteristics and successful nests in terms of clutch deposition (KLUGE 1981, MARTINS 1993). Whether females of these species assess information on a male's territory and/or the quality of this male during their inspection is not entirely clear. However, according to KLUGE (1981), female choice in *H. rosenbergi* was likely based on a male's physical quality, as assessed through physical contact. The type and extent of information that is gathered by means of physical contact is yet unknown. A possible explanation, at least for *H. punctatus*, may take into account the existence of a secondary sexual character in males: the sexually dimorphic skin glands (SDSGs) that are located in the lateral and mental regions in this species (BRUNETTI et al. 2012). Histological studies in *H. punctatus* revealed that SDSGs have specialized mucous glands (SMGs) and specialized serous glands (SSGs) that are characterized by a proteinaceous content in their secretions (BRUNETTI et al. 2012). Even though SDSGs are known to be the source of pheromones secreted by male urodela during courtship (HOUCK & SEVER 1994), such information is scarce concerning anurans, and only three studies have provided experimental evidence on the existence of chemical signals involving SDSGs during reproduction (PEARL et al. 2000, POTH et al. 2012, WILLAERT et al. 2013).

The lack of information on the subject in anurans is at least partially explained by the poor knowledge existing on the reproductive biology of most species while histological studies are required in other cases. Within Cophomantini, other examples of physical contact have been described in species of *Aplastodiscus* and *B. ibitiguara* prior to the pair entering the nest (HARTMANN et al. 2004, HADDAD et al. 2005, ZINA & HADDAD 2007, NALI & PRADO 2012), while recently, CAMURUGI & JUNCÁ (2013) reported that under laboratory conditions another species of the *H. punctatus* group, *H. atlanticus*, females would use their snout to contact the mental gland of a male before facilitating amplexus ($n = 3$). It is worth noting that both *H. atlanticus* and *B. ibitiguara* have SDSGs (FAIVOVICH et al. 2009, J. FAIVOVICH pers. comm.).

Finally, current results do not allow concluding which features of a male make the females of *H. punctatus* choose or reject it. However, our observations should significantly contribute to elucidating the different levels that are involved in this choice-making process, and in particular how female makes use of SDSGs as chemical source of information used during courtship at close range sampling.

Amplexus

As has been reported for some species of *Aplastodiscus*, *B. ibitiguara*, *H. atlanticus*, *H. faber*, and *H. rosenbergi* (LUTZ 1960a, KLUGE 1981, HARTMANN et al. 2004, HADDAD et al. 2005, ZINA & HADDAD 2007, NALI & PRADO 2012, CAMURUGI & JUNCÁ 2013), amplexus in *H. punctatus* will only occur after the female has made pre-mating physical con-

tact with the male. Males clasping females with their hands at the angle of the female's jaw have been reported for *H. atlanticus* (CAMURUGI & JUNCÁ 2013), *H. cinerascens* (TELLES et al. 2013; Fig. 1A), *H. faber*, and *H. rosenbergi* (KLUGE 1981, MARTINS & HADDAD 1988). KLUGE (1981) also described that the male arched his back down while his head completely covered the female's snout, while LUTZ (1960a) mentioned that during mating, the male of *H. faber* would repeatedly press the female down. Similar movements were observed to occur several times in *H. atlanticus* during egg deposition (CAMURUGI & JUNCÁ 2013). MARTINS & HADDAD (1988) suggested that position adjustment from the traditional axillary amplexus in *H. faber* would prevent females from being injured by the males' prepollical spines, while CAMURUGI & JUNCÁ (2013) interpreted these movements as facilitating a proper alignment of male and female cloacae to maximize egg fertilization. An equally plausible and non-exclusive alternative, at least in *H. atlanticus*, *H. cinerascens*, and *H. punctatus*, would be that this modified amplexus allows the male to position his mental gland closer to the nostrils of the female, while the movements would facilitate maintaining pressure on the gland while the pair is moving around during egg deposition. In view of all these pieces of evidence, and taking into account the potential role of SDSGs as a source of sexual pheromones (PEARL et al. 2000, BRUNETTI et al. 2012, WILLAERT et al. 2013), it needs to be investigated whether the male delivers chemical signals to the female during amplexus.

Clutches

The clutch structure of *H. punctatus* is that of an adhesive floating film as defined by ALTIG & MCDIARMID (2007) and has also been reported for its closely related species, *H. cinerascens* (TELLES et al. 2013). Within the Cophomantini, similar floating egg films have been observed in species of the *H. albopunctatus* group (*H. albopunctatus*, *H. calcaratus*, *H. fasciatus*, *H. lanciformis*; DUELLMAN 1978, 2005, MUNIZ et al. 2008), *H. faber* group (*H. albomarginatus*, *H. crepitans*, *H. faber*, *H. lundii*, *H. pardalis*, *H. rosenbergi*; BREDER 1946, LUTZ 1960a, b, JIM 1980, MARTINS & HADDAD 1988, CALDWELL 1992, GIASSON & HADDAD 2006), *H. pellucens* group (*H. rufitelus*; DUELLMAN 1970), *H. semilineatus* group (*H. boans*, *H. geographicus*, and *H. wavrini*; DUELLMAN 1978, MARTINS & MOREIRA 1991), and species of *Aplastodiscus* (HARTMANN et al. 2004, HADDAD et al. 2005). It has been suggested that floating egg films may cope better with the oxygen deprivation that is typically associated with small volumes of stagnant water (HADDAD et al. 2005). From our observations, we may infer that the eggs need to be at the surface to develop normally, which would be favoured by their forming a single-layer film. To the same end, the arched-downward position during amplexus would allow both mating partner to lift their cloacae and deposit the eggs on the water surface. As has been described for *H. rosenbergi* (KLUGE 1981), it is probable that the continuous monolayer of eggs observed

in *H. punctatus* is formed when groups of eggs cluster after having been distributed slowly over the water surface from different oviposition points.

Clutch sizes and egg diameters described here are consistent with previous reports for *H. punctatus* (KENNY 1969, DUELLMAN 1978). Compared to other Cophomantini that produce floating adhesive film clutches, the number of eggs of *H. punctatus* is similar to those reported for two species of *Aplastodiscus* (HADDAD & SAWAYA 2000, HADDAD et al. 2005: 219–227 eggs), *H. atlanticus* (CAMURUGI & JUNCÁ 2013: 160–307, n = 17), and *H. cinerascens* (DUELLMAN 2005: 310 eggs, n = 1 clutch; TELLES et al. 2013: 240 eggs, n=1 clutch), but much smaller than in species of the *H. albopunctatus* group (*H. albopunctatus*, MUNIZ et al. 2008: 572–979 eggs, n = 6 clutches; *H. calcaratus* and *H. lanciformis*, DUELLMAN 1978: 1,060–1,250 and 2,100–2,400 eggs, respectively, both n = 3 clutches; *H. fasciatus*, DUELLMAN 2005: 1,248–1,268 eggs, n = 2 clutches), the *H. faber* group (e.g. *H. rosenbergi*, KLUGE 1981: 1,780–3,057 eggs, n = 16 clutches; *H. faber* MARTINS & HADDAD 1988: 1,000–2,700 eggs, n = 7 clutches), and the *H. semilineatus* group (e.g., *H. boans*, DUELLMAN 1970: 2,722 eggs, n = 1 clutch; *H. geographicus*, DUELLMAN 1978: 2,434 eggs, n = 1 clutch). The variation in the number of eggs between the species may be, in part, related to differences in the size of the frogs (KLUGE 1981), but this deserves further study.

Multimodal communication during female choice?

The idea that mate choice is based on several cues, rather than one, has resulted in an increase of studies on complex signal function (see CANDOLIN 2003, and HEBETS & PAPAJ 2005 for a review). In anurans, those studies are focused on the interaction among acoustic and visual components (e.g., TAYLOR et al. 2011, GRAFE et al. 2012, PREININGER et al. 2013), while two studies have suggested the existence of a dual system based on acoustic and chemical signals (PEARL et al. 2000, STARNBERGER et al., 2013). The results presented here concerning female choice behaviour allow us to propose that in *H. punctatus* its mate-attraction system relies on at least two signal components that are conducted at different levels; i.e., Initial Exploration and Proximate Exploration. The former addresses acoustic signals, while it needs to be investigated whether chemical signals secreted by SDSGs are involved in the latter, or if physical contact only provides tactile stimuli.

It is worth noting that males of all anuran species for which sexual pheromones (e.g., splendipherin: *Litoria splendida*, WABNITZ et al. 2000; macrolides: mantellids, POTH et al. 2012), or SDSGs (e.g. *H. punctatus*, BRUNETTI et al. 2012; 16 *Bokermannohyla* spp., FAIVOVICH et al. 2009; several species of different taxonomic groups, BRIZZI et al. 2003) have been described, vocalize. Therefore, the study of possible multimodal components in *H. punctatus* in particular, but also in other species of anurans producing sexual pheromones, SDSGs, and/or tactile stimuli, creates

an exciting framework for learning how these different signals interact during courtship.

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References

- ALTIG, R. & R. W. McDIARMID (2007): Morphological diversity and evolution of eggs and clutch structure in amphibians. – *Herpetological Monographs*, **21**: 1–32.
- BOURNE, G. R. (1992): Lekking behavior in the Neotropical frog *Oolygon rubra*. – *Behavioral Ecology and Sociobiology*, **31**: 173–180.
- BREDER, C. M. (1946): Amphibians and reptiles of the Rio Chucunaque drainage, Darien, Panama, with notes on their life histories and habits. – *Bulletin of the American Museum of Natural History*, **86**: 375–436.
- BRIZZI, R., G. DELFINO & S. JANTRA (2003): An overview of breeding glands. – pp. 253–317 in: JAMIESON, B. G. M. (Ed.): *Reproductive biology and phylogeny of Anura*. – Science Publishers Inc., Enfield, New Hampshire.
- BRUNETTI, A. E., G. N. HERMIDA & J. FAIVOVICH (2012): New insights into sexually dimorphic skin glands of anurans: the structure and ultrastructure of the mental and lateral glands in *Hypsiboas punctatus* (Amphibia: Anura: Hylidae). – *Journal of Morphology*, **273**: 1257–1271.
- BRUNETTI, A. E., C. TABOADA & J. FAIVOVICH (in press): Extended vocal repertoire in *Hypsiboas punctatus* (Anura: Hylidae). – *Journal of Herpetology*.
- CALDWELL, J. P. (1992): Diversity of reproductive modes in anurans: facultative nest construction in gladiator frogs. – pp. 85–97 in: HAMLETT, W. C. (Ed.): *Reproductive biology of South American vertebrates*. – Springer-Verlag, New York.
- CANDOLIN, U. (2003): The use of multiple cues in mate choice. – *Biological Reviews*, **78**: 575–595.
- CAMURUGI, F. & F. JUNCÁ (2013): Reproductive biology of *Hypsiboas atlanticus* (Anura: Hylidae). – *Herpetological Notes*, **6**: 489–495.
- DARWIN, C. (1871): *The descent of man and selection in relation to sex*. D. Appleton and Company, New York.
- DUELLMAN, W. E. (1970): *Hylid frogs of Middle America*. – *Monograph of the Museum of Natural History, University of Kansas*, 1–2, 1–753.
- DUELLMAN, W. E. (1978): *The biology of an Equatorial herpetofauna in Amazonian Ecuador*. – *Miscellaneous Publication, Museum of Natural History, University of Kansas*, **65**: 158–159.
- DUELLMAN, W. E. (2005): *Cusco Amazonico. The lives of amphibians and reptiles in an Amazonian rainforest*. – Cornell University Press, Ithaca.
- DYSON, M. L. & N. I. PASSMORE (1988): Two-choice phonotaxis in *Hyperolius marmoratus* (Anura: Hyperoliidae): the effect of temporal variation in presented stimuli. – *Animal Behaviour*, **36**: 648–652.
- EMERSON, S. B. (1992): Courtship and nest-building behavior of a Bornean frog, *Rana blythi*. – *Copeia*, **4**: 1123–1127.
- EMLÉN, S. T. & L. W. ORING (1977): Ecology, sexual selection, and the evolution of mating systems. *Science*, **197**: 215–223.
- FAIVOVICH, J., L. LUGLI, A. C. C. LOURENÇO & C. F. B. HADDAD (2009): A new species of the *Bokermannohyla martinsi* group from central Bahia, Brazil with comments on *Bokermannohyla* (Anura: Hylidae). *Herpetologica* **65**, 303–310.
- GERHARDT, H. C. & F. HUBER (2002): *Acoustic communication in insects and anurans*, University of Chicago press, Chicago and London.
- GIASSON, L. O. M. & C. F. B. HADDAD (2006): Social interactions in *Hypsiboas albomarginatus* (Anura: Hylidae) and the significance of acoustic and visual signals. – *Journal of Herpetology*, **40**: 171–180.
- GOSNER, K. L. (1960): A simplified table for staging anuran embryos and larvae with notes on identification. – *Herpetologica*, **16**: 183–190.
- GRAFE, T. U. (1996): The function of call alternation in the African reed frog (*Hyperolius marmoratus*): precise call timing prevents auditory masking. – *Behavioral Ecology and Sociobiology*, **38**: 149–158.
- GRAFE, T. U., D. PREININGER, M. SZTATECSNY, R. KASAH, J. M. DEHLING, S. PROKSCH & W. HÖDL (2012): Multimodal communication in a noisy environment: a case study of the Bornean rock frog *Staurois parvus*. – *PLoS ONE* **7**, e37965.
- HADDAD, C. F. B. & A. A. GIARETTA (1999): Visual and acoustic communication in the Brazilian torrent frog *Hylodes asper* (Anura: Leptodactylidae). – *Herpetologica*, **55**: 324–333.
- HADDAD, C. F. B., J. FAIVOVICH & P. C. A. GARCIA (2005): The specialized reproductive mode of the treefrog *Aplastodiscus perviridis* (Anura: Hylidae). – *Amphibia-Reptilia*, **26**: 87–92.
- HADDAD, C. F. B. & R. J. SAWAYA (2000): Reproductive modes of Atlantic Forest hylid frogs: a general overview and the description of a new mode. – *Biotropica*, **32**: 862–871.
- HEBETS, E. A. & D. R. PAPAJ (2005): Complex signal function: developing a framework of testable hypotheses. – *Behavioral Ecology and Sociobiology*, **57**: 197–214.
- HARTMANN, M. T., P. A. HARTMANN & C. F. B. HADDAD (2004): Visual signaling and reproductive biology in a nocturnal treefrog, genus *Hyla* (Anura: Hylidae). – *Amphibia-Reptilia*, **25**: 395–406.
- HÖDL, W. & A. AMÉZQUITA (2001): Visual signaling in anuran amphibians. – pp. 121–141 in: RYAN, M. J. (ed.): *Anuran communication*. – Smithsonian Institution Press, Washington.
- HOOGMOED, M. S. (1979): Resurrection of *Hyla ornatissima* Noble (Amphibia, Hylidae) and remarks on related species of green tree frogs from the Guiana area. – *Notes on the herpetofauna of Surinam VI. Zoologische Verhandelingen*, **172**: 1–46.
- HOUCK, L. D. & D. M. SEVER (1994): Role of the skin in reproduction and behaviour. – pp. 351–381 in: HEATWOLE, H. & G. T. BARTHALMUS (Eds): *Amphibian Biology*. Vol. 1, The Integument. – Surrey Beatty & Sons, Chipping Norton.
- HOWARD, R. D. (1978): The evolution of mating strategies in bullfrogs, *Rana catesbiana*. – *Evolution*, **32**: 850–871.

- JIM, J. (1980): Aspectos ecológicos dos anfíbios registrados na região de Botucatu, São Paulo (Amphibia, Anura). – Unpubl. Ph.D. Thesis.
- KENNY, J. S. (1969): The Amphibia of Trinidad. – Studies on the fauna of Curaçao and other Caribbean islands, **108**: 1–78.
- KLUGE, A. G. (1981): The life history, social organization, and parental behavior of *Hyla rosenbergi* Boulenger, a nest-building gladiator frog. Miscellaneous Publication, Museum of Zoology, University of Michigan, **160**: 1–170.
- KOKUBUM, M. N. D. C. & A. A. GIARETTA (2005): Reproductive ecology and behaviour of a species of *Adenomera* (Anura, Leptodactylidae) with endotrophic tadpoles: Systematic implications. – Journal of Natural History, **39**: 1745–1758.
- LUTZ, B. (1960a): Noção de território em anfíbios anuros *Hyla faber* Wied. – Anais da Academia Brasileira de Ciências, **32**: 143–145.
- LUTZ, B. (1960b): The clay nests of *Hyla pardalis* Spix. – Copeia, **1960**: 364–366.
- MARQUEZ, R., I. DE LA RIVA & J. BOSCH (1993): Advertisement calls of Bolivian species of *Hyla* (Amphibia, Anura, Hylidae). – Biotropica, **25**: 426–443.
- MARTIN, P. & P. BATESON (1986): Measuring behaviour: Introductory guide. 2nd edn. Cambridge University Press, Cambridge.
- MARTINS, M. (1993): Observations on nest dynamics and embryonic and larval development in the nest building gladiator frog, *Hyla faber*. – Amphibia-Reptilia, **14**: 411–421.
- MARTINS, M. & C. F. B. HADDAD (1988): Vocalizations and reproductive behaviour in the smith frog, *Hyla faber* Wied (Amphibia: Hylidae). – Amphibia-Reptilia, **9**: 49–60.
- MARTINS, M. & G. MOREIRA (1991): The nest and the tadpole of *Hyla wavrini*, Parker (Amphibia, Anura). – Memórias do Instituto Butantan (São Paulo), **53**: 197–204.
- MARTINS, M., J. P. POMBAL JR. & C. F. B. HADDAD (1998): Escalated aggressive behaviour and facultative parental care in the nest building gladiator frog, *Hyla faber*. – Amphibia-Reptilia, **19**: 65–73.
- MUNIZ, K. P. R., A. A. GIARETTA, W. R. SILVA & K. G. FACURE (2008): Auto-ecologia de *Hypsiboas albopunctatus* (Anura, Hylidae) em área de Cerrado no sudeste do Brasil. – Iheringia, Série Zoologia, **98**: 254–259.
- MURPHY, C. G. & H. C. GERHARDT (2002): Mate sampling by female barking treefrogs (*Hyla gratiosa*). – Behavioral Ecology, **13**: 472–480.
- NALI, R. C. & C. P. A. PRADO (2012): Habitat use, reproductive traits and social interactions in a stream-dweller treefrog endemic to the Brazilian Cerrado. – Amphibia-Reptilia, **33**: 337–347.
- PEARL, C. A., M. CERVANTES, M. CHAN, U. HO, R. SHOJI & E. O. THOMAS (2000): Evidence for a mate-attracting chemosignal in the Dwarf African Clawed Frog *Hymenochirus*. – Hormones and Behavior, **38**: 67–74.
- POTH, D., K. C. WOLLENBERG, M. VENCES & S. SCHULZ (2012): Volatiles amphibians pheromones: macrolides from mantellid frogs from Madagascar. – Angewandte Chemie International Edition, **51**: 2187–2190.
- PRADO, C. P. A., M. UETANABARO & C. F. B. HADDAD (2005): Breeding activity patterns, reproductive modes, and habitat use by anurans (Amphibia) in a seasonal environment in the Pantanal, Brazil. – Amphibia-Reptilia, **26**: 211–221.
- PREININGER, D., M. BOECKLE, A. FREUDMANN, I. STARNBERGER, M. SZTATECSNY & W. HÖDL (2013): Multimodal signaling in the Small Torrent Frog (*Micrixalus saxicola*) in a complex acoustic environment. – Behavioral Ecology and Sociobiology, **2013**: 1–8.
- REICHERT, M. S. & H. C. GERHARDT (2013): Socially mediated plasticity in call timing in the gray tree frog, *Hyla versicolor*. – Behavioral Ecology, **24**: 393–401.
- ROITHMAIR, M. E. (1994): Male territoriality and female mate selection in the dart-poison frog *Epidobates trivittatus* (Dendrobatidae, Anura). – Copeia, **1994**: 107–115.
- SCHWARTZ, J. J., B. W. BUCHANAN & H. C. GERHARDT (2001): Female mate choice in the gray treefrog (*Hyla versicolor*) in three experimental environments. – Behavioral Ecology and Sociobiology, **49**: 443–455.
- SCHWARTZ, J. J., B. W. BUCHANAN & H. C. GERHARDT (2002): Acoustic interactions among male gray treefrogs, *Hyla versicolor*, in a chorus setting. – Behavioral Ecology and Sociobiology, **53**: 9–19.
- STARNBERGER, I., D. POTH, P. S. PERAM, S. SCHULZ, M. VENCES, J. KNUDSEN, M. F. BAREJ, M.-O. RÖDEL, M. WALZL & W. HÖDL (2013): Take time to smell the frogs: vocal sac glands of reed frogs (Anura: Hyperoliidae) contain species-specific chemical cocktails. – Biological Journal of the Linnean Society, **110**: 828–838.
- SULLIVAN, B. K., M. J. RYAN & P. A. VERREL (1995): Female choice and mating system structure. – pp. 469–517 in: HEATWOLE, H. & B.K. SULLIVAN (Eds): Amphibian Biology. Vol. 2, Social Behaviour. – Surrey Beatty & Sons, Chipping Norton.
- TAYLOR, R. C., B. A. KLEIN, J. STEIN & M. J. RYAN (2011): Multimodal signal variation in space and time: how important is matching a signal with its signaler? – Journal of Experimental Biology, **214**: 815–820.
- TELLES, D. O. C., S. A. F. VAZ & M. MENIN (2013): Reproductive ecology, size and diet of *Hypsiboas cinerascens* (Anura: Hylidae) in two urban forest fragments in Central Amazonia, Brazil. – Phyllomedusa, **12**: 69–76.
- WABNITZ, P. A., J. H. BOWIE, M. J. TYLER, J. C. WALLACE & B. P. SMITH (2000): Differences in the skin peptides of the male and female Australian tree frog *Litoria splendida*. – European Journal of Biochemistry, **267**: 269–275.
- WELLS, K. D. (1977a): The social behaviour of anuran amphibians. – Animal Behaviour, **25**: 666–693.
- WELLS, K. D. (1977b): Territoriality and male mating success in the green frog (*Rana clamitans*). – Ecology, **58**: 750–762.
- WELLS, K. D. (2007): The ecology and behavior of amphibians, University of Chicago Press, Chicago.
- WELLS, K. D. & J. J. SCHWARTZ (2006): The behavioral ecology of anuran communication. – pp. 44–86 in: NARINS, P. M., A. S. FENG, R. R. FAY & A. N. POPPER (Eds): Hearing and sound communication in amphibians. – Springer, New York.
- WILLAERT, B., F. BOSSUYT, S. JANSSENSWILLEN, D. ADRIAENS, G. BAGGERMAN, S. MATTHIJS, E. PAUWELS, P. PROOST, A. RAEPSAET, L. SCHOofs, G. STEGEN, D. TREER, L. VAN HOOREBEKE, W. VANDEBERGH & V. BOCXLAER (2013): Frog nuptial pads secrete mating season-specific proteins related to salamanders pheromones. – The Journal of Experimental Biology, **216**: 4139–4143.
- ZINA, J. & C. F. B. HADDAD (2007): Courtship behavior of two treefrog species, *Aplastodiscus arildae* and *A. leucopygius* (Anura: Hylidae), from the Atlantic rainforest, southeastern Brazil. – Herpetological Review, **38**: 282–285.