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# SEXUAL SELECTION IN THE NEOTROPICAL TREEFROG *SCINAX CROSPEDOSPILUS* (ANURA, HYLIDAE): INTEGRATING TESTES, CALLS AND SOCIAL INTERACTIONS

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# Sexual selection in the Neotropical treefrog *Scinax crospedospilus* (Anura, Hylidae): integrating testes, calls and social interactions

Dissertação apresentada ao Instituto de Ciências Biológicas, Programa de Pós-Graduação em Ciências Biológicas: Biodiversidade e Conservação da Natureza, da Universidade Federal de Juiz de Fora, como requisito para obtenção do grau de Mestre.

Orientador: Prof. Dr. Renato Christensen Nali

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# RESUMO

A seleção sexual é classicamente conhecida por moldar características pré-copulatórias, como ornamentos e armamentos, para aumentar as chances de acasalamento. Entretanto, nas últimas décadas, passou a haver consenso de que, quando as fêmeas acasalam com vários machos, os espermatozoides podem competir para fertilizar os ovócitos, e dessa forma, as características espermáticas vantajosas também devem estar sob seleção (seleção sexual pós-copulatória). As características pré- e pós-copulatórias podem covariar em duas direções diferentes. A teoria da competição espermática prevê um *trade-off* fundamental (correlação negativa) entre as características pré- e pós-copulatórias quando os recursos são limitados. Já a hipótese de fertilidade ligada ao fenótipo prevê uma correlação positiva, em que as fêmeas podem acessar o *status* de fertilidade dos machos através de características fenotípicas exageradas. Os anfíbios anuros são bem conhecidos por seu comportamento de vocalização, embora ainda faltem detalhes sobre o contexto social da emissão dos cantos, e novos estudos tem sido realizados no que diz respeito a características pós-copulatórias, como características de ejaculação. Neste trabalho, usamos a espécie de anuro *Scinax crospedospilus* (Anura: Hylidae) como modelo para testar, pela primeira vez em anfíbios, possíveis inter-relações entre características de vocalização e características de espermatozoides e testículos. Foram analisados cantos gravados em campo (Jardim Botânico da Universidade Federal de Juiz de Fora, Brasil) e o número de espermatozoides, a morfologia espermática e a massa dos testículos de machos coletados foram medidos em laboratório. Além disso, fizemos gravações em vídeo e áudio, e observações diretas em campo, para estudar as interações sociais dos indivíduos, bem como a fidelidade do sítio às bromélias em uma abordagem de marcação-recaptura. De acordo com a hipótese de *trade-off*, descobrimos que o comprimento da cabeça e da cauda do espermatozoide correlacionam-se negativamente com a duração da nota e a frequência dominante da segunda banda, respectivamente. Corroborando a hipótese da fertilidade ligada ao fenótipo, o comprimento da cauda do espermatozoide, o comprimento total do espermatozoide e o número de espermatozoides correlacionaram-se positivamente com a taxa de repetição do canto e o comprimento da cabeça do espermatozoide correlacionou-se negativamente com a frequência dominante da primeira banda; ou seja, machos investiram igualmente em dois tipos de características que são vantajosas ao mesmo tempo. Descrevemos, pela primeira vez, o canto de encontro da espécie, e também as interações entre machos, que incluem perseguição e quatro tipos de sinais visuais. Os indivíduos mostraram algum grau de fidelidade a bromélias, utilizadas como refúgio e sítio de vocalização. Este é o primeiro estudo demonstrando covariações entre vocalização e características de ejaculação em anuros, e destacamos a importância de incorporar o tamanho de partes do espermatozoide no contexto da seleção sexual pós-copulatória, a fim de entender a oportunidade total de seleção sexual nos animais. Em relação ao comportamento reprodutivo, nossos resultados ampliam o repertório de comunicação bimodal para *Scinax crospedospilus* muito tempo após sua descrição, o que irá contribuir para futuros estudos experimentais sobre seleção sexual em anuros, especialmente dentro do gênero *Scinax*, em que estudos de comportamento social são escassos.

Palavras-chave: Anuros; Hylidae, Neotrópico; comunicação acústica; características espermáticas

# ABSTRACT

Sexual selection is classically known to act on premating traits such as ornaments and armaments to increase mating success. In the last decades, however, the consensus is that, when females mate with multiple males, sperm can compete to fertilize the ova, so much so that advantageous characteristics of ejaculates should be also under selection (postmating sexual selection). Pre- and postmating traits should covary in two different directions. The theory of sperm competition predicts a fundamental trade-off (negative correlation) between pre- and postmating traits when resources are limited. In another direction, the phenotype-linked fertility hypothesis predicts a positive correlation in which females may access male fertility status through exaggerated phenotypic traits. Frogs are well known for their calling behaviour, although details on the social context of emission are still lacking, and new studies have investigated postmating traits such as ejaculate characteristics. We used the treefrog *Scinax crospedospilus* (Anura: Hylidae) as a model to test possible relationships between call traits vs. sperm and testes traits for the first time in amphibians. We analyzed calls recorded in the field (Botanical Garden of the Universidade Federal de Juiz de Fora, Brazil) and sperm number, morphology and testes mass of collected males in the lab. In addition, we made video and audio recordings and direct observations in the field to study the social interactions of individuals, as well as site fidelity to bromeliads in a mark-recapture approach. According to the trade-off hypothesis, we found that sperm head length and sperm tail length traded off against note duration and second band dominant frequency, respectively. According to the phenotype-linked fertility hypothesis, we found that sperm tail length, sperm total length and sperm number correlated positively with call rate and that sperm head length correlated negatively with first band dominant frequency, i.e., males invested equally in two types of traits that are advantageous at the same time. We described for the first time the species' encounter call, and male-male interactions that include chasing and four types of visual signals. Individuals showed some degree of site fidelity in bromeliads, which they might use as a refuge and calling site. This is the first study to show covariations between vocalization and ejaculate characteristics in frogs, and we highlight the importance to incorporate sperm size in the context of postmating sexual selection to understand the total opportunity for sexual selection in animals. Regarding the reproductive behaviour of *Scinax crospedospilus*, our results expand the communication repertoire for the species long after its description and should contribute for future experimental studies on sexual selection in frogs, especially within the *Scinax* genus, in which studies of social behaviour are sorely needed.

**Keywords**: Anurans; Hylidae, Neotropics; acoustic communication; ejaculate traits

# SUMMARY

# GENERAL INTRODUCTION

Sexual selection is known to arise when selective pressures act in given exaggerated and conspicuous characteristics such as weapons and ornaments, usually in males, in order to gain mates (Darwin, 1871; Andersson, 1994). Males are under stronger sexual selective pressures when compared to females since variance in male reproductive success is usually much larger (Bateman, 1948). This type of sexual selection is now known as premating sexual selection (Ryan, 1985; Catchpole, 1987; Tobias et al., 2012; Cholewiak et al., 2018) and it may decrease the chances of male survival while increasing male reproductive success (Darwin, 1971). There are two main mechanisms of premating sexual selection (Hosken and House, 2011): mate choice (intersexual selection), which is usually female preference by certain morphological or behavioural characteristics of males (Fisher, 1958; Catchpole, 1987), and competition for mates (intrasexual selection) which is usually in the context of male disputes for female preference (Miller, 2013).

Almost 100 years after Darwin's original ideas of sexual selection, Parker (1970), when studying fertilization of insects, postulated that sexual selection continues after mating (postmating sexual selection), mainly in the form of sperm competition. In this time, sperm and ejaculate characteristics should be under selective pressures when more than one sperm by different males compete for a given ova. Sperm competition happens when females benefit from mating with multiple males, a behaviour known as polyandry (Hosken and Stockley, 2003). In sequential polyandry, females mate with males not simultaneously, which is typical for internal fertilizers (Parker, 1970; Jenni, 1974), while simultaneous polyandry normally occurs in external fertilizers, such as frogs (Byrne and Whiting, 2008; Roberts and Byrne, 2011).

Covariations between pre- and postmating traits can occur in two different directions. The sperm competition theory predicts a fundamental trade-off (negative correlation; Simmons and Emlen, 2006; Fry, 2006; Simmons et al., 2010; Ferrandiz-Rovira et al., 2014) when resources are limited towards a total reproductive expenditure (Parker, 1998). In an opposite direction, the phenotype–linked fertility hypothesis (Sheldon, 1994) posits that males may signal fertility status through exaggerated phenotypes (Malo et al., 2005). The study of trade-offs between pre- and postmating sexual selection traits has been under attention in many different taxa such as insects (Simmons and Emlen, 2006; Durrant et al., 2016), fishes (Preston et al., 2011), birds

(Blanco and de la Puente, 2002), mammals (Dunn et al., 2015; Foo et al., 2018) and, more recently, amphibians (Parker et al., 2013). In counterpart, some studies have found that those types of traits should correlate positively (Malo, 2005, Cotton et al., 2010; Supryia et al., 2018; Durrant et al., 2020), which indicates that this relationship is more complex than the theory since many other variables may be predicted (Fry, 2006; Devigili et al., 2013), and that a more extensive work should be conducted across taxa.

In frogs, the patterns and mechanisms of sexual selection have been very well explored in the last century, but they were highly focused on how males obtain mates under the context of acoustic communication (Wells, 1977; Ryan, 1985; Gerhardt and Huber, 2002; Nali and Prado, 2014). Different types of calls are emitted under a myriad of social contexts (Toledo et al., 2015). For instance, advertisement calls, which are frequently used during the breeding season, are emitted by males to attract conspecific females (Wells, 1977), whereas aggressive calls are a group of different call types, e.g., males may emit encounter or territorial calls when other males invade their territories (Wells, 1978; Toledo et al., 2015). In this scenario, males usually call very close or just jump on and chases the invader (Abrunhosa and Wogel, 2004; Aguiar et al., 2022, personal observations). These observations are much rarer in the literature and still lack attention (Wells, 2007).

Elaborate studies of social behaviour and sexual selection in frogs are greatly concentrated on temperate species (Wells, 1978; Gerhardt and Huber, 2002), despite the fact that the Neotropics harbor the largest number of anuran species in the world, especially Brazil (Frost, 2022). Even so, most researchers focus on call descriptions without the investigation of social context (Bastos et al., 2011; Magrini et al., 2011; Manzano et al., 2022). Therefore, studies on anuran behaviour in the Neotropics are sorely needed.

On the other hand, studies of postmating sexual selection in frogs are scarce all over the world. Postmating sexual selection studies in frogs are concentrated in the last three decades only (Kusano et al., 1991; Byrne et al., 2002; 2003; Doyle 2011; Buzatto et al., 2015), and mainly within the families Myobatrachidae (Byrne, 2002; Parker et al., 2013; Buzatto et al., 2015) and Rhacophoridae (Fukuyama, 1991; Kusano et al., 1991; Jennions and Passmore, 1993), since polyandric events, and by consequence episodes of sperm competition are common in species of these families. Neither of these families have representatives in Brazil. Nevertheless, polyandry might happen more frequently in other families than thought (Roberts and Byrne, 2011), including the speciose Hylidae, a family of true treefrogs highly concentrated in the Neotropics in which most studies in sexual selection and reproductive behaviour have been conducted in Brazil (Bastos and Haddad, 2002; Toledo and Haddad, 2005; Nali and Prado, 2012; Zamudio et al., 2016; Foratto et al., 2021; Aguiar et al., 2022).

The genus *Scinax* is a diverse hylid genus of treefrogs that occur from southern Mexico to eastern Argentina with 129 species (Faivovich et al., 2002; 2005; Frost, 2022). This genus is mostly composed by species of the *Scinax rubra* group (Faivovich 2002). *Scinax crospedospilus* (Lutz, 1925) is a small-sized tree-frog with broad distribution in southeastern Brazil, specifically in the states of Rio de Janeiro, São Paulo and Minas Gerais (Silveira et al., 2020). The males usually call perched on trees and other vegetations in the edges of water bodies (Silveira et al., 2020; chapter 2), although calls can also be emitted from bromeliads where pairs have been found (Chapter 2). Very little is known about its calling repertoire, breeding biology and its social behaviour, and only its advertisement call has been described from a few individuals (Heyer et al., 1990; Bevier et al., 2008; Magrini et al., 2011). During the breeding season, males of *Scinax crospedospilus* aggregate in high density choruses to attract mates and amplexus is exposed (chapter 2), which could enhance chances of multimale spawning (Zamudio et al., 2016). In addition, a polyandric behaviour has been observed in the population under study (F.M.R.S. Pedro, personal communication).

This master's dissertation is divided in two chapters redacted as manuscripts. On the first chapter, we used *Scinax crospedospilus* as a model species to study possible covariations between vocalizations traits that are used to attract females and repeal other males (premating sexual selection) and sperm traits that are advantageous for fertilization (postmating sexual selection). We recorded males in the field and measured testes and sperm traits in the lab. We found, for instance, that sperm head length and sperm tail length correlated negatively with note duration, which corroborates the trade-off hypothesis. We also found, for example, that sperm tail length, sperm total length and sperm number correlated positively with call rate, which corroborates the phenotype-linked fertility hypothesis. On the second chapter, we studied its social behaviour focusing on aggressive calls, which had not been described yet. This call showed to be very distinguishable from its advertisement call. We also observed different social behaviours, including visual signals, mainly in the context of male-male competition, as well as some degree of side fidelity of males in bromeliads. Taken together, our results will contribute to researchers in the areas of behavioural ecology, specifically the total opportunity for sexual selection of frogs with a major contribution for the poorly studied treefrog *Scinax crospedospilus*. We hope that our results will foment the scarce research on the relationships between postmating sexual selection and animal vocalizations.

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# CHAPTER 1

## Relationships between vocalization characteristics and sperm traits in the Brazilian treefrog *Scinax crospedospilus*

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Running title: Trade-offs between calls and sperm morphology

## ABSTRACT

Sexual selection can act in premating traits, i.e., those that are advantageous to obtain mates and in postmating traits, i.e., those that enhance chances of fertilization, especially when sperm competition occurs. The theory of sperm competition predicts a fundamental trade-off between pre vs. postmating sexual traits when resources are limited. On the other hand, when males mate with multiple females, sperm depletion may occur, and females might choose males whose phenotypes indicate good fertility (the phenotype-linked fertility hypothesis). Despite many studies on sexual selection related to frog vocalizations, and the growing interest in sperm characteristics within the group, the interrelationship between these two components is poorly known. We tested the phenotype-linked fertility hypothesis and the trade-off hypothesis regarding covariation in acoustic traits and testicular/sperm traits in the Brazilian treefrog *Scinax crospedospilus*. We recorded and collected males to measure acoustic variables and testicular/sperm characteristics expected to be advantageous in reproduction and ran multiple regression analyses between these traits. We found covariations in both directions between sperm traits vs. acoustic variables. Supporting the trade-off hypothesis, males appeared to trade off sperm head and tail lengths against note duration and second band dominant frequency. We also found a positive association between sperm length/number and call rate and first band dominant frequency, which corroborates the phenotype-linked fertility hypothesis. This is the first study to find covariations in sperm characteristics and vocalizations in frogs, and we highlight the importance of sperm morphology variation regarding postmating sexual selection. Taken together, our results will aid in future studies regarding the evolution of pre and postmating sexual selection in acoustically oriented taxa.

**Keywords:** amphibians, bioacoustics, external fertilization, Hylidae, sexual selection, sperm morphology

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# INTRODUCTION

Sexual selection was proposed by Darwin (1871) as a selective force that drives the evolution of traits advantageous to obtain mates (premating sexual selection), such as ornaments, signals and armaments. Nevertheless, especially when females mate with multiple males (polyandry), sexual selection should continue in certain traits that enhance chances of fertilization (post-mating sexual selection), in the form of sperm competition – when sperm cells of different males compete to fertilize a given set of ova (Parker 1998; Parker et al 2013). How both types of sexual selection (pre- and postmating) covary to determine total male reproductive fitness has been under attention over the last few decades (Parker 1998; Mautz et al 2013; Evans and Garcia-Gonzalez 2016).

Covariations between traits related with pre and postmating sexual selection can occur in two different directions. If males allocate their limited resources towards a total reproductive expenditure (Parker 1998), sperm competition theory predicts a fundamental trade-off between pre- and postmating traits. In this case, pre- and postmating traits would show a negative relationship (Simmons and Emlen 2006; Fry 2006; Simmons et al 2010; Ferrandiz-Rovira et al 2014). On the other hand, the phenotype–linked fertility hypothesis (Sheldon 1994) posits that females might choose males whose phenotypes indicate good fertility. Thus, in populations in which males mate with multiple females, there is a higher risk of sperm depletion (Weir and Grant 2010) and while females avoid sperm-depleted males, one could expect a selection in males that honestly advertise fertility through exaggerated phenotypes (Malo et al 2005; Weir and Grant 2010; Foo et al 2017). In the last few decades, covariations regarding pre- and postmating traits have been observed in many taxa, across individuals (Somjee et al 2018), across populations of the same species (Parker et al 2013) and across species (Ferrandiz-Rovira et al 2014; Lüpold et al 2014). However, not much attention has been given to studies on the correlation of acoustic and sperm traits in animals (but see Simmons et al 2010; Doyle 2011; Simmons et al 2011; Dines et al 2014; Dunn et al 2015), even though many taxa depend on or use acoustic signals for reproduction, such as anuran amphibians (Toledo et al 2015).

In anurans, premating sexual selection is predominantly mediated by reproductive vocalizations including advertisement and aggressive calls (Wells 2007; Toledo et al 2015). Yet, aggressive calls are less common to observe and are emitted sporadically (Toledo et al 2015), while advertisement calls are emitted continually and functions both to attract females and repel other males (inter and intrasexual selection; Toledo et al

2015), suffering strong sexual selective pressures. In many species of frogs, during the reproductive season, males can emit long bouts of calls (Wells 2007). Because of that, the note or call rate (number of notes or calls per minute) and note duration usually increase with the energy cost due to aerobic metabolism (Taigen and Wells 1985; Ryan 1988). Notes are often subdivided in pulses (Köhler et al 2017), which are also costly (Wells 2001). Thus, in the context of sexual selection, such acoustic properties are known to indicate high performance due to motivation by males, increasing its mating success (Wagner Jr 1992; Welch et al 1998; Gerhardt and Huber 2002, Köhler et al 2017). Also, lower call frequencies, which usually infer larger body size across frogs (Gingras et al 2013; Turin et al. 2018), may repel conspecific males, due to a high probability of losing a fight, and attract females, due to this ability to win fights and keep territories that are advantageous for reproduction (Ryan et al 1992).

A greater reproductive advantage can also be obtained by male frogs that invest more in gametes (postmating sexual selection), and this could be assessed specifically in traits that enhance the probability of fertilization, such as sperm number and a correlated trait, testes size (Edward et al 2004; Buzatto et al 2017). It is also known across species (Gomendio and Roldan 1991; Fitzpatrick 2009), across populations (Malo et al 2006) and across individuals (Mossman et al 2009; Fitzpatrick et al 2010; Bennison et al 2015) that sperm with longer heads and tails swim faster, enhancing the probability of fertilizing an ovum. These selective pressures should be greater especially for simultaneous polyandrous species (Fukuyama 1991; Kusano et al, 1991; Byrne et al., 2002; Buzatto et al 2015; Zamudio et al 2016) such as the model Australian frog species *Crinia georgiana* (Tschudi 1838). Across populations, males of this species are known to trade off on testes against arm width, a sexually selected weapon (Parker et al 2013; Buzatto et al 2015, 2017).

Acoustic traits strongly depend on social condition in frogs (Wells 2007). Besides that, recent studies show that postmating traits such as sperm number may vary depending on the intraspecific sperm competition risk (Rahman et al 2013; Wylde et al 2020), and total sperm size and its constituent parts may show phenotypic plasticity (Hellriegel and Blackenhorn 2002; Crean and Marshall 2008; Morrow et al 2008; Immler et al 2010; Rahman et al 2013; Haeussler et al 2014). Therefore, both episodes of sexual selection may vary depending on resources availability and allocation strategies (Wylde et al 2020) and the traits related with these two episodes could potentially covary with each other (Mautz et al 2013; Ng et al 2018). However, the phenotype-linked fertility hypothesis was tested only once regarding vocalization in anurans (Doyle 2011), and the fact that males could trade off acoustic properties (premating) against testes characteristics (postmating) was never tested for the highly diverse anuran group. Therefore, this knowledge gap prevents a full comprehension of the dynamics of sexual selection in anurans (Evans and Garcia-Gonzalez 2016).

The treefrog family Hylidae is the most speciose amphibian family in the world, with 1035 species highly concentrated in the Neotropical region (Frost 2022), some of which are polyandric (d'Orgeix and Turner 1995; Zamudio et al 2016). *Scinax crospedospilus* is a small-sized treefrog endemic to the Atlantic Forest, southeastern Brazil (Heyer et al 1990; Frost 2022). Males are prolonged breeders (Wells 2007) that usually mate with many females throughout the rainy season (Heyer et al 1990) enhancing chances of sperm depletion (Weir and Grant 2010). In counterpart, this species shows exposed amplexus and a high density of males during the rainy months (author’s personal observation), which increases the probability of multiple male encounters and by consequence polyandrous mating events (Buzatto et al 2015; Zamudio et al 2016). In fact, this mating system has been observed in the species (F. M. S. R. Pedro, pers. comm.).

Here we aimed to test the relationships between testes/sperm traits and vocalizations, using *S. crospedospilus* as a model species. Specifically, we tested whether (1) males will invest more in traits that are advantageous to attract females (e.g., longer calls and lower frequencies), and less in sperm traits that are advantageous in fertilization (e.g., larger and more numerous sperm cells) – the trade-off hypothesis, or (2) males will invest equally in both traits, showing a positive covariation – the phenotype-linked fertility hypothesis. Our results will help elucidate the nature of both episodes of sexual selection across animal taxa with acoustic communication, especially in frogs, in which these complex covariations have been strongly underappreciated.

## MATERIALS AND METHODS

### Fieldwork

We conducted weekly expeditions during the breeding (rainy) season (October 2021 to March 2022) in an urban fragment of Atlantic Forest (Botanical Garden of the Universidade Federal de Juiz de Fora, Juiz de Fora municipality, state of Minas Gerais, southeastern Brazil). The vegetation consists of seasonal semideciduous trees, bushes and grassland (Brito and Carvalho 2014). We sampled males were at night (6 PM to 11 PM) near two lakes (GPS coordinates = lake 1: 21°44'06.8"S, 43°22'11.8"W; lake 2: 21°43'56.6"S, 43°22'12.6"W) and a permanent stream (21°43'49.7"S 43°22'13.7"W). We found males through visual and auditory searches and used red-light headlamps to minimize disturbance on behaviour (Nali and Prado, 2012). We then recorded each male for at least 2 minutes using a Marantz PMD 660 audio recorder with a unidirectional Sennheiser ME66 microphone at a standard distance of approximately 1 m. After the recording, we captured each male and placed it individually inside a plastic bag with some water, and measured air temperature with a thermo-hygrometer.

### Laboratory analyses and testes/sperm measurements

We took each captured male alive to the laboratory after each collection night. We euthanized them according to Heyer et al (1994), weighed them with an analytical scale (accuracy = 0.00001 g) and measured their snout–vent lengths (SVL) with a digital caliper (accuracy = 0.01 mm). We dissected males and weighed both testes together on the same analytical scale. We then calculated the relative testes mass using the residuals of the allometric relationship between soma mass (body mass – testes mass) and total testes mass, an approach that has been used in studies with anuran testes to control for body mass (Buzatto et al 2017; Chen et al 2023).

We used one of the testes to determine sperm characteristics. Using a glass rod (diameter = 5.3 mm) it was macerated on a concave clock glass (diameter = 35 mm; depth = 5 mm) containing 10 µl distilled water. Additional 10 µl distilled water was added to the tip of the glass rod to remove any residuals. Then we took 6 µl of the sperm solution + 54µl 4% formaldehyde. We homogenized this final solution with the pipette and applied in each side of a Neubauer chamber until all coverslips were filled. After that we allowed sperm cells to settle for approximately 5 minutes. Under a phase-contrast microscope (Olympus Bx51) with a camera attached to a computer, we counted sperm cells from five quadrants at 40x magnification on both sides of the chamber, and an average was taken from the total value of the sides (measurement of sperm number; Absher 1973). To calculate the number of sperm cells/µl, we considered the total volume of all 5 squares ($0.02 \text{ mm}^3 = 0.02\ \mu\text{l}$) used for sperm count and then we were able to determine the number of sperm cells per 1 µl.

To analyze the morphometry of sperm cells, we placed 6 µl of the same sperm solution on a slide. We photographed sperm cells at 100x magnification using the same microscope above and measure them using the software ImageJ (N = ca. 10 sperm cells per individual; Byrne et al 2003). We did not measure the mid piece of each cell because it was indistinguishable from the head (e.g., Byrne et al 2003). For each sperm cell, we measured head length from the apex of the acrosome to the junction of the head with the tail (Figure S1). Sperm cells of *S. crospedospilus* present two filaments of tail (Fouquette and Delahoussaye 1977), so to measure tail length we took an average of both filaments for each of the 10 cells per individual. We measured each filament of the tail from the junction of the head with the filament to the apex of the filament (Figure S1). Because the filaments from the same sperm cell in this species are similar in size (Fouquette and Delahoussaye, 1977), we only measured sperm cells with similar filaments, thus avoiding measuring cells that were possibly harmed during preparation. In summary, we used the following variables as potential postmating traits under selective pressure for fertilization increase: relative testes mass (residuals, as explained above), sperm head length, sperm tail length, total sperm length, and we also included the variable ejaculate expenditure (sperm number multiplied by sperm total length; Ball and Parker 1996). Because we used the first collected males to standardize our sperm cell preparations, we obtained a slightly lower sample size for some sperm traits, at a minimum of 31. We did not control sperm variables for body size (male SVL) due to a lack of correlation between these variables (Table S1).

All specimens were deposited in the Coleção de Anfíbios da Universidade Federal de Juiz de Fora, municipality of Juiz de Fora, state of Minas Gerais, Brazil, under the vouchers CAUFJF 2112 – 2155.

## Acoustic analyses

We measured advertisement calls (Figure S2) in Raven Pro 1.6 software (Yang 2022) using the following configurations: Fast Fourier Transformation = 128; overlap = 85%; brightness = 70; contrast = 60. We averaged per individual 10 measurements of the acoustic variables peak frequency (automatic measurement for dominant frequency), note duration and number of pulses (number of pulses repeated within a note) (Köhler et al 2017). We averaged per individual 60 measurements of pulse duration across different notes. We calculated the call rate for each individual (number of calls emitted in one minute; in the case of *Scinax crospedospilus,* calls consist of only a single note). We also calculated two more acoustic variables for each individual (Köhler et al 2017): call effort (ratio between average inter-note interval and average note duration, expressed in percentage) and pulse rate (ratio between average number of pulses and average note duration per individual, expressed in pulses/s). Because the call of *S. crospedospilus* is composed of two stronger frequency bands (Magrini et al 2011; Figure S2), we measured the dominant frequency of both bands separately (first band dominant frequency and second band dominant frequency), since each one may show biological relevance (Foratto et al 2021). We also analyzed in each measured note which frequency band contained the dominant frequency and stated the proportion in percentage.

Acoustic variables in frogs may be influenced by air temperature and body size (Lingnau and Bastos 2007; Turin et al 2018). To remove the temperature influence, we first ran linear regression analyses between each acoustic variable vs. air temperature. The variables that showed significant correlations were then adjusted to the average air temperature measured across all recordings (21.4°C) using the formula: acoustic variable value – (regression estimate × measured temperature) + (regression estimate × average air temperature) (see Pröhl et al 2007). We repeated the procedure above to remove the body size influence, adjusting to the average SVL of 31.22 mm across all collected males (Pröhl et al 2007). We used this adjusted acoustic dataset in our analyses.

### Statistical analyses

Because the investigation of a trade-off between two types of traits do not specify which is the dependent and which is the independent variable, we ran all regression analyses arbitrarily considering sperm/testes traits as dependent variables and acoustic traits as independent variables. To make sure that this procedure did not bias our results, we chose some analyses and ran the models the other way around, i.e., using acoustic traits as dependent variables and sperm/testes traits as independent variables, and values were the same.

We used the stepwise selection method (Pierna et al 2009), using the function *step* in R, which creates sequential multiple linear models containing all predictor variables (full model) and excludes the least significant variables (highest p-values), one at a time, of the initial full model. The models are compared and ranked according to the Akaike Information Criteria (AIC; Bozdogan 1987), and the model with lowest AIC value is chosen. We log-transformed our dataset and ran all our analyses in R version 4.2.2 (R Core Team 2022). We used the function *report* within the package *report* (Makowski and Lüdecke 2019) to obtain effect sizes (standardized β) for each variable within the models with 95% confidence intervals that were computed using a Wald t-distribution approximation. We also evaluated every chosen model regarding posterior predictive check, normality of residuals, influential observations, linearity of residuals, homogeneity of variances and multicollinearity using the function *check_model* within the package *perfomance* (Lüdecke et al 2021).

## RESULTS

We sampled 40 males in the field with a SVL ranging from 29.81 to 33.19 mm, body mass ranging from 1.38 to 2.12 g and that were recorded across air temperatures that ranged from 17.4 to 23.5 °C.

The sperm cell of *Scinax crospedospilus* was similar to the diagram of Fouquette and Delahoussaye (1977) (Figure S1). Also, our results for sperm length measurements (table 1) agree with the 70 µm mean length of sperm cells within the *Scinax ruber* clade (Fouquette and Delahoussaye 1977; Faivovich et al 2005). All means, standard deviations and ranges for all sperm, testes and acoustic variables are shown in table 1. The dominant frequency of the note fell within the first frequency band in 75% of the cases, and within the second band in 25% of the cases.

Although most of our models were non-significant, we did find some significant relationships (table 2), and our models followed the expected assumptions (Figures S3-S8). Among these, we found two directions regarding covariances between testes/sperm traits vs. acoustic traits. According to the trade-off hypothesis, sperm head length correlated negatively with note duration and sperm tail length correlated positively with second band dominant frequency (Table 2; Figure 1). According to the phenotype-linked fertility hypothesis, sperm head length correlated negatively with first band dominant frequency, while sperm tail length, sperm total length and sperm number correlated positively with call rate (Table 2; Figure 1). Relative testes mass and ejaculate expenditure yielded non-significant whole-models, with no effect from any of the call variables.

## DISCUSSION

Covariations between pre- and postmating traits in anurans are still poorly known (Parker et al 2013), even more so considering calls and testes. Despite the single test for the phenotype-linked fertility hypothesis with calls in one frog species, no positive covariation was ever found (Doyle 2011). Regarding the trade-off hypothesis that stem from the sperm competition theory (Parker 1998), a negative covariation was found across populations of *Crinia georgiana*, between arm width and testes size (Parker et al 2013) but calls were never considered. By testing relationships between acoustic and sperm traits regarding fundamental trade-offs and phenotype-linked fertility hypothesis in the treefrog *Scinax cropsedospilus*, our results showed covariations in both directions for the first time in anurans.

We found that sperm size (tail and total length) and sperm number correlated positively with call rate and that sperm head length correlated negatively with first band dominant frequency. These associations are consistent with the phenotype-linked fertility hypothesis (Sheldon 1994), that is, females of *S. crospedospilus* may access male fertility through faster call rates and lower first band dominant frequencies. Lower dominant frequencies are usually correlated with larger males across many frog species (Gingras et al 2013) and therefore lower frequencies are usually preferred by females (Ryan 1991). Across all measured notes, dominant frequencies were indeed present within the first band in most cases. Also, some male frogs dishonestly signal larger body sizes to other males by lowering their frequencies (Nali and Prado 2014), and a similar strategy might also happen if males try to advertise their competitiveness of the ejaculate to other conspecific males (Malo et al 2005). We highlight that hormones may mediate these positive covariations. For instance, androgen levels regulate vocal activity and the development of vocal structures such as the larynx in frogs (Emerson and Hess 2001; Moore et al 2005). Since those hormones are responsible to control spermatogenesis (Dufau 1985; Wingfield et al 1990), they could mediate the direct relationships that we found. Given our significant correlations, the influence of hormone levels appears to be an interesting avenue of investigation (e.g. Leary et al 2008; de Assis et al 2012).

On the opposite direction, males of *S. crospedospilus* appear to trade off the length of sperm head and sperm tail against note duration and second band dominant frequency respectively (i.e., the trade-off hypothesis). Interestingly, the few studies that focused on the correlation of acoustic and postmating traits across animals only addressed this relationship with sperm number, sperm viability and testes size (Simmons et al 2010; Simmons 2011; Dines et al 2015; Dunn et al 2015; Ng et al. 2018), and ours is the first to address sperm morphology. Although costs in spermatogenesis are mostly related with sperm production (Parker and Pizzari 2010; Bunning et al 2015), Godwin et al (2017) demonstrated that the elongation of sperm can be costly at the risk of sperm competition. In addition, higher note durations in frogs can be extremely costly since it is related with aerobic metabolism (Ryan 1988; Wells 2001). One could wonder why the second band dominant frequency did not correlate in the same direction as the first band. According to our results, the dominant frequency of the note was only present in the second band in 25% of the cases, which indicates that females should focus less on this band. Consequently, we speculate that it is more advantageous for males to invest more in sperm size than in producing a second band with lower dominant frequencies Our results corroborate the importance of analyzing different frequency bands in studies of frog communication and sexual selection (Foratto et al 2021) In general, these findings agree with the fundamental trade-off hypothesis (Parker 1998), although further studies testing the availability of resources in *S. crospedospilus* are necessary.

Comparing temporal acoustic traits, our results showed that call rate in *S. crospedospilus* correlated positively with sperm length (indicating the phenotype-linked fertility hypothesis) and note duration correlated negatively with sperm head length (indicating a trade-off). Although both traits usually infer male quality (Gerhardt and Huber 2002), call rate is a variable that does not account for the duration of the note (Köhler et al. 2017), so much so that a higher call rate but with shorter notes would not be very costly (Prestwich 1994). We suggest that males might invest in call rate which can increase the changes of acquiring a female, but it would not incur as much higher energy expenditure as emitting longer notes. Thus, this may explain why sperm length correlated in opposite directions regarding call rate and note duration in *S. crospedospilus.*

Many studies over the last decades have corroborated that sperm size/morphology is fundamental in the evolution of postmating sexual selection (Gomendio and Roldan 1991; Byrne 2003; Snook 2005; Fitzpatrick et al 2009; Lüpold et al 2020). Still, constituent parts of the same premating trait may present different directions of correlations with a variety of postmating traits (Pitcher et al 2007; Evans et al 2015), which is consistent with our results that sperm head and tail may correlate differently with call traits. In addition, external fertilization takes place in environments usually disturbed by many factors, so both sperm size and number may suffer environmental pressures besides sexual selection (Levitan and Petersen 1995; Emerson 1997; Simmons et al 2009; Liao et al 2018), which makes the study of covariances in pre- and postmating traits challenging (Moore et al 2005). Another complicating factor is that *S. crospedospilus* displays different modes of communication, such as aggressive calls and visual signalization (author's unpublished data), which could also correlate with sperm traits. The lack of control for social context and number of reproductive events for every given male are caveats, but the presence of significant relationships indicate that these hypotheses should be carefully studied. For instance, we detected covariations with call variables by measuring sperm size and number, while relative testes mass showed no significance. Testes mass has been and continues to be one of the most used variables in studies of postmating sexual selection (Kusano et al 1991; Byrne et al 2002; Dunn et al 2015; Lüpold et al 2020). Byrne et al (2002) did not find a definitive relationship between testes mass and risk for sperm competition in Hylidae (family of *S. crospedospilus*), and suggested incapacity of increasing sperm production. Our results show that, at least for covariations with acoustic variables in frogs, testes mass should be replaced with more refined operational variables that are consistently linked with fertilization success (Gomendio and Roldan 1991; Edwards et al 2004; Fitzpatrick et al 2009).

In summary, our study is the first to show the existence of covariations between vocalization and postmating traits in anurans, either in a trade-off fashion following the sperm competition theory or corroborating the phenotype-linked fertility hypothesis, i.e., that some acoustic traits may signal male fertility to females. In addition, we showed that measuring sperm morphology, which had not been considered before in covariation studies, as well as using sperm number rather than testes mass, contribute to the understanding of the relative variance in reproductive success in animals oriented acoustically. We expect this work to foment studies in the areas of behavioural and evolutionary ecology, specifically regarding pre- and postmating sexual selection in animals that communicate by sound.

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# Tables

**Table 1.** Means, standard deviations (SD), minimum and maximum values for sperm, testes and acoustic variables of *Scinax crospedospilus*, municipality of Juiz de Fora, southeastern Brazil. *N* = number of males.

| | **N** | **Mean ± SD** | **Min** | **Max** |
|---|---|---|---|---|
| **Sperm/Testes variables** | | | | |
| Sperm Head Length (µm) | 35 | 15.91 ±1.335 | 12.05 | 18.92 |
| Sperm Tail Length (µm) | 31 | 37.60 ± 2.59 | 33.17 | 41.78 |
| Total sperm length (µm) | 31 | 53.62 ± 3.53 | 47.76 | 60.50 |
| Sperm number (/µl) | 40 | 19,642 ± 13,218 | 2,050 | 59,950 |
| Ejaculate expenditure | 31 | 111,4 ± 76,18 | 9,79 | 323,93 |
| Testes mass (g) | 40 | 0.0039 ± 0.0014 | 0.0019 | 0.0071 |
| **Acoustic variables** | | | | |
| First band dominant freq. (Hz) | 40 | 1,447 ± 43.77 | 1,378 | 1,550 |
| Second band dominant freq. (Hz) | 40 | 3,368 ± 219.12 | 2,929 | 4,022 |
| Pulse duration (s) | 40 | 0.034 ± 0.0033 | 0.029 | 0.041 |
| Call rate (notes/min.) | 40 | 58.12 ± 24.19 | 10 | 93 |
| Pulse rate (pulses/note) | 40 | 28.79 ± 2.64 | 22.98 | 33.5 |
| Note duration (s) | 40 | 0.219 ± 0.0259 | 0.167 | 0.276 |
| Call effort (%) | 40 | 35 ± 8.3 | 18 | 54 |
| N° of pulses | 40 | 6.27 ± 0.55 | 5 | 7.8 |
| Internote interval (s) | 40 | 0.66 ± 0.193 | 0.41 | 1.18 |

**Table 2.** Results (t-tests) from multiple linear regression analyses between sperm and testes variables vs. temporal acoustic variables in *Scinax crospedospilus*, southeastern Brazil. We also report the standardized β (effect size) with confidence intervals for each variable within the model. The $r^2$ relates to the whole-model. The sign of the t-value depicts the type of correlation (negative or positive). The most accurate multiple linear regression model for each variable was selected based on the Akaike Information Criterion using stepwise regression.

| **Trait** | **$r^2$** | **t** | **p** | **β (95 % CI)** |
|---|---|---|---|---|
| **Sperm head length** | | | | |
| Fist band dominant freq. | 0.254 | -3.287 | **0.002** | -0.49 (-0.80, -0.19) |
| Note duration | | -2.159 | **0.038** | -0.32 (-0.63, -0.02) |
| **Sperm tail length** | | | | |
| Second band dominant freq. | 0.329 | 2.349 | **0.027** | 0.41 (0.05, 0.77) |
| Fist band dominant freq. | | -1.823 | 0.080 | -0.29 (-0.61, 0.04) |
| Call effort | | -1.825 | 0.087 | -0.52 (-1.11, 0.07) |
| Call rate | | 2.717 | **0.011** | 0.76 (0.18, 1.34) |
| Pulse Duration | | -1.774 | 0.098 | -0.28 (-0.60, 0.04) |
| **Sperm total length** | | | | |
| Second band dominant freq. | 0.316 | 2.039 | 0.052 | 0.34 (-0.00345, 0.68) |
| First band dominant freq. | | -1.916 | 0.076 | -0.30 (-0.63, 0.02) |
| Call effort | | -1.951 | 0.062 | -0.56 (-1.16, 0.03) |
| Call rate | | 2.731 | **0.011** | 0.77 (0.19, 1.35) |
| Pulse Duration | | -1.83 | 0.087 | -0.29 (-0.61, 0.04) |
| **Sperm number** | | | | |
| Call rate | 0.103 | 2.342 | **0.024** | 0.36 (0.05, 0.66) |
| **Ejaculate expenditure** | | | | |
| First band dominant freq. | 0.054 | 1.396 | 0.174 | 0.26 (-0.12, 0.64) |
| Call effort | | 1.671 | 0.106 | 0.31 (-0.07, 0.69) |
| **Testes mass (residuals)** | | | | |
| Call rate | 0.037 | -1.211 | 0.233 | -0.19 (-0.52, 0.13) |

# Figure

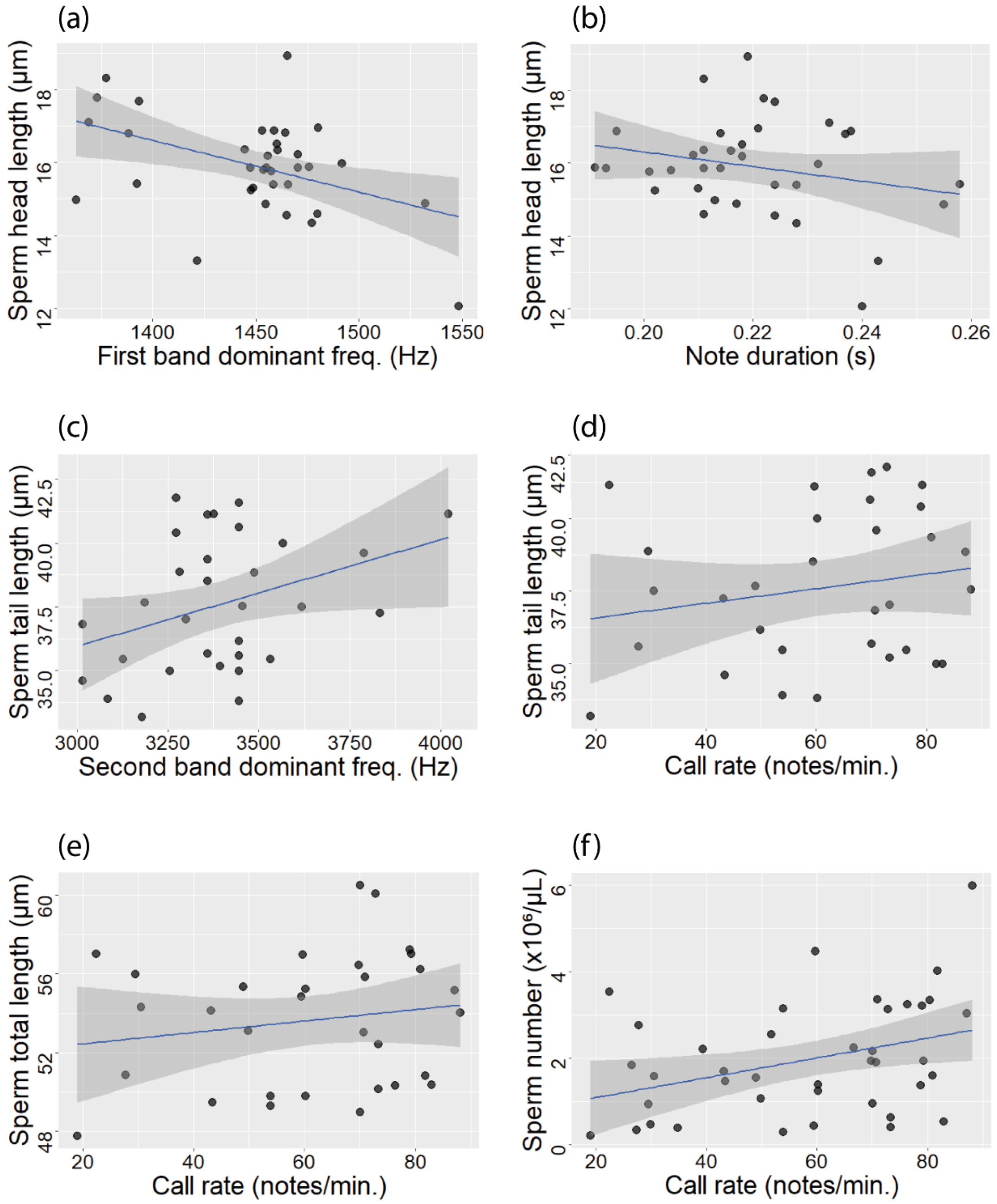

**Figure. 1.** Correlations between vocalization and sperm characteristics for *Scinax crospedospilus*, municipality of Juiz de Fora, southeastern Brazil. All plots show linear regression analyses representing significant correlations among sperm variables vs. acoustic variables (see table 2).

# CHAPTER 2

# Social interactions in the treefrog *Scinax crospedospilus* (Anura, Hylidae): encounter calls, visual signalizations and aspects of its reproductive behaviour

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## ABSTRACT

Little is known about the breeding behaviour of the treefrog *Scinax crospedospilus*, despite its wide distribution in southeastern Brazil. So far, only few aspects of its reproduction and advertisement call have been described. Using video and audio recordings and direct observations in the municipality of Juiz de Fora, Minas Gerais, Brazil, we described its encounter call, verified site fidelity, and discussed social contexts with highlights on male-male interactions with the display of visual signals. The encounter call of *Scinax crospedospilus* was clearly distinguished from the advertisement call, being a non-pulsed note formed by two parts, one of which shows frequency modulation. In counterpart, dominant frequency and note duration of both calls were overall similar. *Scinax crospedospilus* showed some degree of site fidelity to bromeliads. We observed four distinct visual signals (arm waving; leg kicking; head shaking and spasm-like movement) during male-male interactions, and a brief courtship behaviour. Our results provide the first description of several types of social interactions for the poorly known species *Scinax crospedospilus* and should contribute for future studies on the ecology, behaviour and sexual selection of frogs.

Keywords: acoustic communication, aggressive behaviour, Hylidae, sexual selection, social behaviour

## INTRODUCTION

Acoustic communication is the most conspicuous reproductive behaviour in anurans (Wells, 2007), being crucial in sexual selection as males usually can send information such as body size, spatial territory and reproductive status to conspecific females and other males (Wells, 1977a; Wells, 1978; Nali and Prado 2014). Thereby, anuran vocalizations are important on studies regarding evolution, ecology and behaviour (Foratto et al., 2021; Nali et al., 2022a). With a diverse repertoire, frog calls can be separated by social context (Toledo et al., 2015), although the study of social context is fairly underrepresented in anuran bioacoustics (Toledo et al., 2015; Guerra et al., 2018).

The advertisement call is the most described type of anuran vocalization in the literature (Wells, 1977a; Toledo et al., 2015) and it has the function to attract conspecific females and organize male choruses (Toledo et al., 2015). Another well-known type are the aggressive calls, which functions primarily to maintain inter-male spacing (Wells, 1978; Toledo et al., 2015; Köhler et al., 2017). This is true especially in prolonged-breeding species that can show site fidelity and territoriality (Borges et al., 2018). The aggressive calls can be divided in different types. For example, when a conspecific intruder male enters the resident's territory, the encounter call is usually emitted by males at close-range distance (Toledo et al., 2015). This call is usually associated with escalated behaviour because it alerts other intruder males and therefore may avoid physical combat (Pombal et al., 1998; Aguiar et al., 2022) that can cause unwanted injuries (Nali and Prado, 2012).

Another channel of communication used by frogs is visual signalization (Hödl and Amezquita, 2001), emitted in different reproductive contexts such as courtships and aggressive interactions between males (Hödl and Amezquita, 2001; Hartmann et al., 2004; 2005). When compared with the conspicuous anuran vocalizations, studies on visual signals are relatively scarce (Hödl and Amezquita, 2001) and are mostly concentrated in diurnal species (Haddad and Giaretta, 1999; Hödl and Amezquita, 2001; de Luna et al., 2010). However, over the past decades, visual signaling has been found in nocturnal species as well (Hartmann et al., 2005; Giasson and Haddad, 2006; Toledo et al., 2007; Augusto-Alves et al., 2018), showing that social interactions in frogs are much more complex than once thought. For instance, during courtships, communication between males and females may involve, besides courtship calls (e.g. Nali et al., 2022b), other sensorial modalities such as several types of visual signals (Hödl and Amezquita, 2001) and tactile stimuli (Haddad and Sawaya, 2000; Nali and Prado 2012).

Male frogs usually call from different types of microhabitats, especially in the Neotropics, where a great variety of microhabitats exist (Wells, 2007). Additionally, they may also use these sites as refuges. For instance, bromeliads are frequently used as calling sites and shelters by bromelicolous treefrogs, while bromeligenous frogs use bromeliads also for reproduction (e.g., oviposition; Silva et al., 2011; Moravec and Campos, 2020). In this context, studies regarding site fidelity are important to understand preferences for bromeliads and spatial organization of individuals during reproduction (Silva et al., 2011; McCracken and Forstner, 2014). This is especially important considering that mark-recapture studies are still rare for Neotropical frogs (Borges et al., 2018).

The treefrog genus *Scinax* is the most species-rich (129 spp.) in the subfamily Hylinae (Faivovich, 2005; Frost, 2022). *Scinax crospedospilus* is a small-sized treefrog endemic from the Atlantic Forest with a wide distribution in southeastern Brazil, in the states of Minas Gerais, Rio de Janeiro and São Paulo (Silveira et al., 2020). Individuals are usually found close to water bodies perched at different heights of vegetations such as low bushes, bromeliads and trees (Heyer, 1990; Pacheco et al., 2016; Silveira et al., 2020).

Little is known about the reproductive behaviour and site fidelity of *Scinax crospedospilus,* with only the description of its advertisement call and general environment of reproduction (Heyer et al., 1990; Bevier et al., 2008; Magrini et al., 2011). There is no description of its social behaviour in the literature, with only few studies describing complex social interactions for the entire *Scinax ruber* group, to which the species belong (Hartmann et al., 2005; de Carvalho et al., 2015). Herein we redescribe the advertisement call of *Scinax crospedospilus*, describe for the first time its encounter call, verify site fidelity of males in bromeliads and also discuss its social context with highlights on visual signals.

## MATERIALS AND METHODS

### Fieldwork

We conducted field expeditions during the breeding (rainy) season, specifically from September to November/2019, from November/2020 to February/2021 and from October/2021 to February/2022. The sampling locality was an urban fragment of Atlantic

Forest, the Botanical Garden of the Universidade Federal de Juiz de Fora, Juiz de Fora municipality, state of Minas Gerais, southeastern Brazil (GPS coordinates = 21°44’08.1”S; 43°22’11.8”W, elevation = 855 m). The vegetation consists mainly of seasonal semideciduous trees, bushes and grassland (Brito and Carvalho 2014). Individuals were located at night (6 PM to 11 PM) and observed preferably using red-light headlamps to minimize behavioural disturbance (e.g. Nali and Prado, 2012). Individuals were found (1) in the vegetation within and on the proximities of two lakes (lake 1: 21°44'06.8"S 43°22'11.8"W; lake 2: 21°43'56.6"S 43°22'12.6"W), (2) in bromeliads in different heights (0 to 5 meters) close to them and (3) in a permanent stream close to lake 2.

### Recordings, body measurements and behavioural observations

We recorded calling males for at least 2 minutes utilizing a Marantz PMD 660 recorder with a unidirectional Sennheiser ME66 microphone at a standardized distance of approximately 1 m. After the recording, each male was captured and placed individually inside a plastic bag with some water, and air temperature was measured with a thermo-hygrometer with an accuracy of 0.1°C.

We also observed the behaviour of all individuals using the methods of focal animal, all occurrences and samples by sequence (Altmann, 1974). Annotations and video recordings of social contexts were made including male-male and male-female interactions, as well as the presence of amplexus. We sampled a total of 40 males for advertisement call and we recorded a total of 5 males that emitted encounter calls. We measured the snout-vent length (SVL) and body mass of males and females with a portable digital scale (accuracy = 0.01 g), and their snout–vent lengths (SVL) were measured with a digital caliper (accuracy = 0.01 mm).

### Bioacoustic analyses

We measured advertisement and encounter calls in Raven Pro 1.6 software (Yang, 2022) using the following parameters: Fast Fourier Transformation = 128; overlap = 85%; brightness = 70; contrast = 60. The measured spectral variables were frequency 5%, frequency 95% and peak frequency; we measured the peak frequency of the two prominent bands of the advertisement call separately (Magrini et al., 2011). The temporal measured variables were note duration, inter-note interval, call rate (number of calls emitted in one minute), number of pulses per note and pulse duration. According to

Köhler et al. (2017), frequency 5%, frequency 95% and peak frequency correspond to measures for minimum frequency, maximum frequency and dominant frequency, respectively. For the encounter calls, we measured separately the duration of the non-modulated and modulated parts of the note (Figure 1). Also, at the modulated part, we measured both ascending and descending dominant frequency modulation within the band that contained the dominant frequency.

To test for differences in parameters between both types of calls, we compared the dominant frequency and durations of advertisement vs. encounter calls with a Mann–Whitney test (McKnight and Najab, 2010), since males that we used to measure advertisement calls were different from those used in encounter calls. Because we had much more advertisement calls measured, we included 15 random individuals and 5 measurements of advertisement calls per individual for the analysis, and all individuals and measurements of encounter calls.

### Site fidelity to bromeliads

We marked nine males found in bromeliads of the species *Portea petropolitana*, near the two lakes from 09 September to 05 November 2019 to analyze site fidelity to bromeliads. These individuals were marked by toe-clipping (Waichman, 1992). The cut was performed in the first capture and followed the code established by Waichman (1992). A single digit was cut per individual, and the digits were kept as tissue samples for future molecular studies. The manipulation, marking (toe-clipping) recording and/or collection of individuals was approved by CEUA/UFJF (Protocol 31/2019) and ICMBio (SISBIO license # 69659). Collected males and females were euthanized and fixed according to Heyer et al. (1994). Most specimens were deposited in the Coleção de Anfíbios da Universidade Federal de Juiz de Fora, Juiz de Fora, Brazil, under the vouchers CAUFJF 2112 – 2155, and the tissues were deposited at the same collection.

## RESULTS

We conducted 57 nightly field expeditions, totaling approximately 211 hours of observation. We collected and measured a total of 50 males (SVL = 31.12 ± 0.84 mm, range: 29.8 – 33.2 mm; body mass = 1.71 ± 0.21 g; range: 1.15 - 2.2 g) and a total of 10 females (SVL = 30.49 ± 0.98 mm, range: 28.95 – 31.97; body mass = 2.05 ± 0.2 g, range:

1.76 – 2.4 g). In addition, we observed differences in coloration (sexual dichromatism), as males usually presented a darker green color and females usually a brighter green color.

### Calling sites, activity and vocalization descriptions

We found individuals usually perched in many vegetations heights up to 5 meters, close to water bodies. Individuals seem to be bromelicolous, since most were found using bromeliad tanks as shelters or even calling sites, but no clutches or tadpoles were observed in them. Males also vocalized closer to the edge of the leaves and closer to the tanks. Males presented a typical lek behaviour, calling perched and spaced, attracting females. *Scinax crospedospilus* showed to be highly skittish; individuals normally moved backwards towards the base of the bromeliad tank to hide when we approached them. Also, in nights with many calling males, they showed to be highly restless and agitated, emitting many variations of their encounter call note (see below). Apparently, this call was not only emitted in close-range when males visualized each other, but also when nearby males started to emit advertisement calls.

The advertisement call of this population is similar to the original description: it was a simple call, composed by a complex pulsed note (average number of pulses = 6; Figure 1A) with two prominent frequency bands (Magrini et al., 2011; Table 1). Dominant frequency fell either within the first band (fundamental frequency; 75% of sampled individuals; N = 30) or the second band (25% of sampled individuals; N = 10). The encounter call (Table 2) was usually emitted in a context of many calling males at close proximity (ca. 30 cm), and we found at least four different variations (Figure 1B-E).

Males were restless and agitated in the presence of intruder males, and this call likely functions for inter-male spacing. Occasionally in days with few calling males, this call was also heard. It is clearly distinguished from the advertisement call, consisting of a non-pulsed note (tonal), similar to a whine. The note is formed by two different parts, herein called modulated and non-modulated, referring to the fact that they may or may not have ascending and descending frequency modulation (Fig. 1B-E). The first part of the note (non-modulated) is typically longer with greater variation of the duration (0.118 ± 0.095 s) and may be interrupted (Fig. 1). It resembles a hoarse and unfinished sound. In situations of more aggressive male-male interactions (e.g, during chases), this part tended to be longer (Fig. 1E). The second part of the note (modulated) typically presented an average ascending frequency modulation of 675 ± 126.758 Hz and average descending frequency modulation of 766 ± 84.188 Hz and varies little in duration (0.103 ± 0.006 s). The modulated part of the encounter call also presents band frequencies although they are not very well separated from each other.

Dominant frequency from advertisement and encounter calls were not different (W = 1250.5, p = 0.932), which was also the case for note duration (W = 1522, p = 0.058).

### Male-male interactions and visual signals

All interactions were observed at night and mostly at two main vegetations approximately 2 m from each other, a medium-sized tree and a set of bromeliads near lake 2, which typically contained many individuals, mostly males. Males of *Scinax crospesdospilus* interacted using encounter calls, visual signals and chasing behaviour. Physical combat was not observed.

We observed at least four different types of movements/postures associated with visual signaling (Table 3). Following the nomenclature of Hartmann et al. (2005), we observed (1) arm lifting, either in silence or during the emission of advertisement or encounter calls and (2) leg kicking, during the emission of advertisement or encounter calls. We also propose the following signals: (3) head shaking: rapid up and down movements with the head which we observed in silence or during the emission of advertisement calls, and (4) spasm-like movements: a very rapid tremble with the whole body, which we observed always during the emission of encounter calls. On 06 January 2020 on the trunk of a medium-sized tree, we observed two silent males facing each other and one above the other at a distance of ca. 3 centimeters. The male below had its body arched and soon after it slightly lowered his body. It started to emit encounter calls and after 16 seconds, it raised the posterior part of its body briefly as if in a spasm and continued emitting the encounter call, which lasted up to 25 seconds. While it emitted its last call, it advanced towards the upper male, staying less than 1 centimeter distant (still facing each other). After 2 seconds, the lower male advanced on the upper one, which dodged to the side of the trunk. Interestingly, the longest encounter call note (0.3243 s) measured during this interaction coincides with the moment when the spasm occurs. On 6 December 2021, on branches of this same tree, we heard a male emitting encounter calls and saw a static individual a little further up at a distance of ca. 30 cm. A silent male nearby displayed head shaking and soon after, arm lifting. After 3 seconds, it started to emit advertisement calls and after 14 seconds it displayed head shaking at least 4 times. The advertisement call lasted ca. 1 minute.

On 20 December 2021, we observed a chasing behaviour involving two males perched on a *Cecropia* tree ca. 2 m above the ground/water. We observed a male below on a branch and a male above on a leaf ca. 40 cm apart. The male on the leaf was emitting advertisement calls, while the male perched below was emitting shorter encounter calls (e.g., figure 1D), but after 26 seconds this male jumped and climbed the branch towards the above male, still emitting short encounter calls. This chasing male was very restless and inflated its body, and when reached one leaf ca. 30 cm distant from the above male, it started to emit encounter calls with a longer non-modulated part (e.g., figure 1E). It continued emitting encounter calls while doing spasm-like movements. As a result, the chased male stopped calling. Similar chasing behaviours were also observed in bromeliads on at least two other occasions, with restless males doing spasm-like movements and emitting longer encounter calls while likely chasing other nearby males (not visualized) that emitted advertisement calls.

## Amplexus and courtship behaviour

We observed a total of 16 amplexus during all field expeditions, all of which were axillary. Most occurred at the edges of water bodies and a few were found perched on trees, also close to water bodies. On 13 December 2021 we observed an amplexus on the tip of a bromeliad leaf. Further back on the same leaf, a male was emitting advertisement calls. On the same day, we observed four pairs in amplexus perched in a tree, approximately 30 cm distant from each other.

On 3 January 2022, we observed courtship interactions. In a jabuticaba-tree at approximately 2 meters high, we observed a female approximately 2 cm behind of a male with both legs extended. After 3s, the female walked in front of the male, which emitted six notes of a call similar to an advertisement call, likely a courtship call. The female then turned a little to the side and within less than 1s the male jumped on top of her in an inverted-amplexus position (male head to female cloaca). The female was seemingly reluctant and for about 4 seconds the male struggled to turn around while kicking once and tapping six times onto the female’s leg; finally, a lateral movement of the female helped the male stabilize in the right amplexus position. Right after that, the male tapped onto the side of the female, close to the axillary area.

## Site fidelity on bromeliads

All marked males were recaptured from one to four times at the same bromeliad in which they were collected (Table 4). One of the males (male A2 from Table 4) remained in the same bromeliad for at least eight weeks.

## DISCUSSION

No previous study reported the description or even the presence of any other different vocalizations than the advertisement call for *Scinax crospedospilus* (Heyer et al., 1990; Bevier et al., 2008; Magrini et al., 2011). We observed a different vocalization emitted in aggressive contexts (male-male interactions) when other males were close by or not, corroborating this call type as an encounter call (Toledo et al., 2015).

The encounter call structure is clearly distinguishable from the advertisement call since it is formed by a non-pulsed note. A similar note structure has been observed in other hylids such as *Scinax perereca*, *Boana raniceps*, *Dryophytes versicolor* and *Bokermannohyla ibitiguara* (Pombal et al., 1995; Bastos, 2003; Reichert and Gerhardt, 2013; Nali and Prado, 2014). Although the average duration of advertisement calls (0.219 s) and encounter calls (0.220 s) were very similar, note duration of the encounter call were much more variable depending on the social context. We clearly observed this here in a male-male chasing context in which one male emitted graded aggressive calls regarding duration (Pombal et al., 1998; Aguiar et al., 2022). Because note duration is usually a dynamic parameter (Gerhardt, 1991), this graded call is likely related to escalated aggressive behaviour under a male-male chasing context, in which increased vocal effort also indicates higher male aggressiveness to conspecific intruder males (Gerhardt and Huber, 2002, Owen and Gordon, 2005). Still, the dominant frequencies of both call types were similar, and this reflect the idea that spectral variables are usually static traits (Gerhardt 1991).

Many anuran vocalizations concentrate frequencies in different regions of the note (Köhler et al., 2017). Both dominant frequencies of the advertisement and encounter call showed variation of dominant frequencies in different bands, although the dominant frequencies of the advertisement call were much more prominent and distinguishable than those of the encounter call. Also, we found that in a quarter of the advertisement calls analyzed, the dominant frequency fell at the second frequency band, and in three quarters, in the other band, which indicates that this could be important in sexual selection contexts;

for instance, males could change their harmonic structure to attract females or access information through different harmonics of conspecific males (Reichert and Gerhardt, 2013; Foratto et al., 2021). Future playback experiments with frequency band manipulation (e.g. Foratto et al., 2021) should clarify how males respond to different bands and the full potential information transmission of such call traits in *Scinax crospedospilus*, especially in light of the male-male interactions (intrasexual selection) found.

Although visual signals in anurans are highly diverse (Hödl and Amezquita, 2001) they are still poorly described when compared to acoustic signals, especially for nocturnal frogs (Hartmann et al., 2005; Giasson and Haddad, 2006; Toledo et al., 2007). Visual cues in frogs are mostly known in diurnal species (Haddad and Giaretta, 1999; Summers et al., 1999; Grafe and Wanger, 2007; Preininger et al., 2009) and are used in the contexts of agonistic encounters or courtship behaviour (Hödl and Amezquita, 2001; Narins et al., 2003). Only in the 2000s did studies with nocturnal visual signaling start to emerge (Hartmann et al., 2005; Giasson and Haddad, 2006). We observed on many occasions visual displays that were more often performed simultaneously with calls (Table 3). Therefore, we can assume that *Scinax crospedospilus* presents bimodal communication, in which males could enhance their information transmission to conspecific males or females (Narins et al., 2002; Grave and Wanger, 2007). In addition, we observed movements in silent intervals between calls, which could be an energy saving strategy due to the energetic costs of calling (Wells, 2001). Most movements were displayed with no evident males in front of each other. Hartmann et al. (2005) observed in the noctunal hylids *Boana albomarginata* and *Scinax eurydice* that movements were displayed without a close rival in the vicinity most of the times. Still, they could be related to other contexts and used as visual signals secondarily. For instance, Zhao et al. (2022) suggested that defense movements against blood-sucking insects could enhance female preference for acoustic signals, i.e., evolve secondarily as visual signals for reproduction. A larger sample would be necessary to better understand the function of those movements within male and female interactions.

Complex courtship behaviours in frogs are usually performed at close-range distances and involve multiple sensory modalities (Wells, 1977b; Nali and Prado, 2012). Studies describing courtship behaviour in the genus *Scinax* are few compared to its large number of species, and they indicate that such behaviour is relatively simple in the group (Bourne, 1992; Toledo and Haddad, 2005), like we found for *S. crospedospilus* We recorded a male of *Scinax crospedospilus* with both legs extended, emitting a call similar as an advertisement call. In fact, courtship call resembles advertisement call, but with low intensity and a higher repetition rate (Owen and Tucker, 2006; Toledo et al., 2015). Because we were unable to analyze call parameters due to low audio quality (extracted from the video recording), we cannot confirm whether this was indeed a courtship call. Nevertheless, the social context strongly suggests that this is the case, which increases the complexity of social interactions in this species.

Studies regarding site fidelity seek to analyze the spatial structure and behavioural patterns of a given species in relation to a possible oviposition site and male territoriality (Ringler et al., 2009; Lima et al., 2010). Nevertheless, mark-recapture studies in the context of reproductive behaviour are still rare for Neotropical frogs (Borges et al., 2018). Five of the nine marked individuals were recaptured at least twice at the same bromeliad, which demonstrates some degree of site fidelity (Ringler et al., 2009; Borges et al., 2018). Since the bromeliads where males were collected (*Portea petropolitana*) presented many thorns along their leaves and their tanks were increasingly tapered, these environments could provide protection against predators, as well as humid shelters during the day (Silva et al., 2011; Moravec and Campos, 2020). Up to this point, *Scinax crospedospilus* might be considered a bromelicolous species (Peixoto, 1995) since no spawning or tadpoles were found inside the tanks, but further studies can help confirm that, since we found one amplexus in this location, as well as many calling males.

Reproductive behaviours of frogs, especially Neotropical ones, tend to be complex and multifaceted (Pombal and Haddad, 1998; Toledo et al., 2007; Nali and Prado, 2012; 2014; Valencia-Aguilar et al., 2020). After 30 years of the species description, and despite being widely distributed in southeastern Brazil (Silveira et al., 2020), we described for the first time, courtship interactions for the species, expanded its bimodal communication repertoire by describing aggressive calls and visual signals, and confirmed the occurrence of site fidelity in bromeliads. This study will contribute to future experimental studies that should focus on the context of sexual selection in this species.

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# Tables

**Table 1.** Means, standard deviations (SD), minimum and maximum values for acoustic variables of the advertisement call of 40 males of *Scinax crospedospilus*, municipality of Juiz de Fora, southeastern Brazil. A total of 10 measurements were taken for each male.

| | Mean ± SD | Min | Max |
|---|---|---|---|
| Max. Frequency (Hz) | 3,909 ± 370.53 | 3,273 | 4,660 |
| Min. Frequency (Hz) | 1,387 ± 33.89 | 1,292 | 1,464 |
| First Band Dominant Freq. (Hz) | 1,447 ± 43.77 | 1,378 | 1,550 |
| Second Band Dominant Freq. (Hz) | 3,368 ± 219.12 | 2,929 | 4,022 |
| Pulse duration (s) | 0.034 ± 0.0033 | 0.029 | 0.041 |
| Call rate (notes/min.) | 58.12 ± 24.19 | 10 | 93 |
| Pulse rate (pulses/note) | 28.79 ± 2.64 | 22.98 | 33.5 |
| Note duration (s) | 0.219 ± 0.026 | 0.167 | 0.276 |
| Call effort (%) | 35 ± 8.3 | 18 | 54 |
| Inter-note interval (s) | 0.66 ± 0.193 | 0.41 | 1.18 |
| N° of pulses | 6.27 ± 0.55 | 5 | 7.8 |

**Table 2.** Means, standard deviations (SD), minimum and maximum values for acoustic variables of the encounter call of *Scinax crospedospilus*, municipality of Juiz de Fora, southeastern Brazil. We measured 33 calls from five different males.

| | Mean ± SD | Min | Max |
|---|---|---|---|
| Min. Frequency (Hz) | 1,529 ± 218.54 | 1,356 | 1,894 |
| Max. Frequency (Hz) | 4184.291 ±403.77 | 3,617 | 4,616 |
| Dominant Frequency (Hz) | 2140.77 ± 586.26 | 1,636 | 3,076 |
| Ascending Frequency Modulation (Hz) | 675 ± 126.758 | 512 | 813 |
| Descending Frequency Modulation (Hz) | 766 ± 84.188 | 647 | 880 |
| Non-modulatory duration (s) | 0.118 ± 0.095 | 0.058 | 0.285 |
| Modulatory duration (s) | 0.103 ± 0.006 | 0.094 | 0.11 |
| Total note duration (s) | 0.220 ± 0.098 | 0.156 | 0.391 |

**Table 3.** Number of males of *Scinax crospedospilus* performing distinguishable movements that were captured on video, associated or not with advertisement and encounter calls. Arm lifting and leg kicking *sensu* Hartmann et al. (2005). We propose the nomenclature for the other two visual signals.

| | Arm lifting | Leg kicking | Head shaking | Spasm-like movement |
|---|---|---|---|---|
| Silent individuals | 1 | - | 1 | - |
| Advertisement call | 2 | 1 | 1 | - |
| Encounter call | 1 | 1 | - | 4 |

**Table 4.** Capture and recapture of males of *Scinax crospedospilus* at the same bromeliad in the year of 2019, municipality of Juiz de Fora, southeastern Brazil. Male code according to Waichman (1992); see text for details.

| Male Code | Inicial Capture | Recaptures | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 29/Sep | 2/Oct | 6/Oct | 13/Oct | 14/Oct | 22/Oct | 28/Oct | 5/Nov | 5/Nov |
| A1 | 9/Sep | | | | | X | | | | |
| A2 | 9/ Sep | | | X | | | X | | X | |
| A3 | 22/ Sep | X | | X | | | | | | |
| A4 | 23/ Sep | | | | | | | X | | |
| B1 | 23/ Sep | | | | X | | | | | |
| B2 | 23/ Sep | | | | | | X | | | X |
| B3 | 16/ Sep | | | X | | | | | X | |
| B4 | 16/ Sep | | X | | | | X | X | X | |
| C1 | 6/Oct | | | | | X | | | | |

# Figure

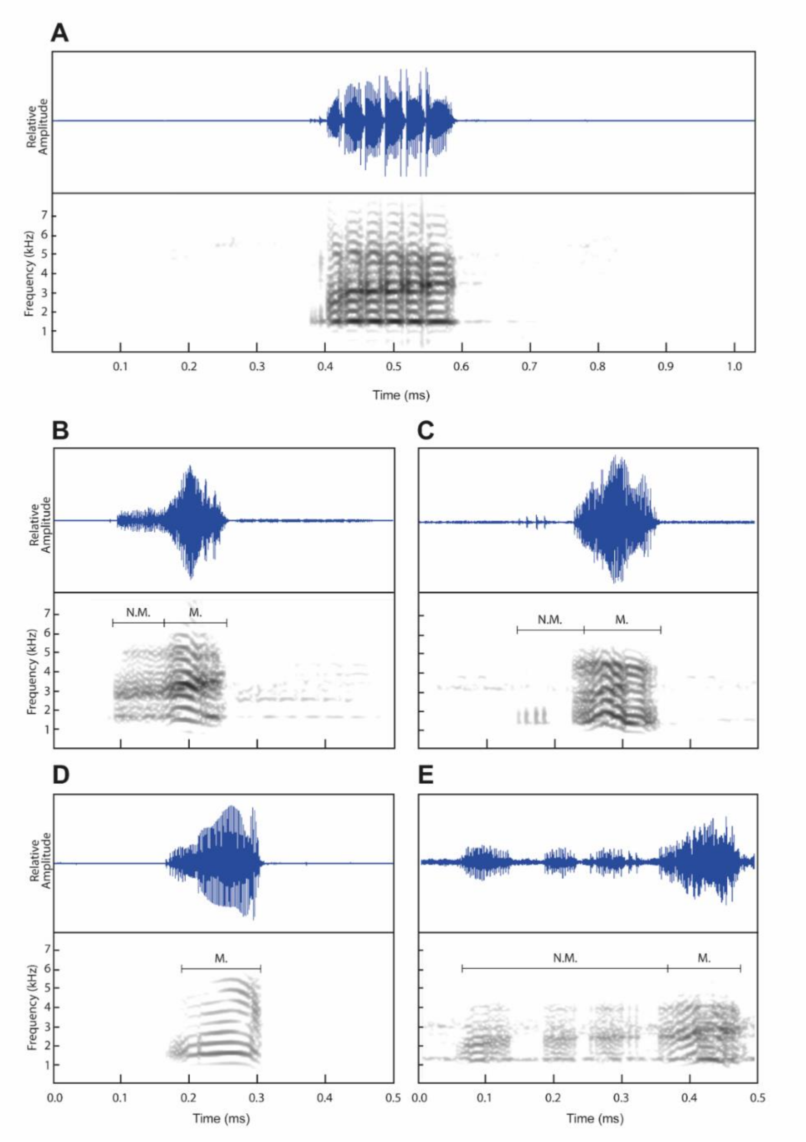

Figure 1. The advertisement call note (A) and four distinct variations of the encounter call (B-E) of *Scinax crospedospilus* observed in six uncollected males. The encounter call is formed by a note with generally two distinct parts: non-modulated (N.M.) and modulated (M.), relating to frequency modulation. (A) The advertisement call is a simple call composed by a note of an average of six complex pulses with two prominent frequency bands. (B) The non-modulated part of the encounter call is not long and not interrupted (recorded on 2 November at 20,1 °C). This type of note was observed in two individuals. (C) The non-modulated part of this note starts with a brief pulsed-like structure with an interruption (recorded on 2 November at 20 °C), observed in one individual. (D) A short note composed basically by the modulated portion. This note was observed in one individual intercalating with a nearby male emitting an advertisement call. (E) This note presents a long non-modulated part with interruptions. It was observed in one individual.

# FINAL CONSIDERATIONS

Using the treefrog *Scinax crospedospilus* as a model, we evidenced for the first time in frogs the existence of covariations between vocalizations and sperm characteristics, a relationship poorly explored in animals. Specifically, according to the phenotype-linked fertility hypothesis, sperm size (tail and total length) and sperm number correlated positively with call rate and sperm head length correlated negatively with first band dominant frequency. On the opposite direction, according to the trade-off hypothesis, sperm head length correlated negatively with note duration and sperm tail length correlated positively with second band dominant frequency.

We showed that sperm size, besides characteristics normally used in these types of analyses (sperm number and testes size), might explain covariations with acoustic traits in animals.

The male-male interactions of *Scinax crospedospilus* involve aggressive calling and chasing behavior, but physical combats were not observed. The courtship interaction observed was brief and may include courtship calls.

The communication in the species is complex. We described the encounter call with at least four variations, as well as four types of visual signals used in male-male interactions. Because visual signals and calls could be emitted simultaneously, we corroborate that the individuals display at least bimodal communication.

We found some degree of site fidelity of individuals in bromeliads, which indicates that this might be a good environment for protection, and, in the case of males, for calling sites.

Taken together, our results should contribute for future studies to understand the total opportunity for sexual selection in the treefrog *Scinax crospedospilus.* In addition, we contribute in the areas of behavioural and evolutionary ecology, specifically regarding both pre- and postmating episodes of sexual selection in acoustically oriented animals, a relationship still poorly investigated.

# SUPPLEMENTARY MATERIAL

**Table S1.** Linear regressions between male SVL (snout-vent length) and all sperm and testes traits.

| **Traits** | **n** | **$r^2$** | **t** | **p** |
|---|---|---|---|---|
| **Male SVL** | | | | |
| Sperm head length | 35 | 0.003 | -0.356 | 0.724 |
| Sperm tail length | 31 | 0.079 | -1.578 | 0.126 |
| Sperm total length | 31 | 0.058 | -1.338 | 0.191 |
| Sperm number | 40 | 0.013 | -0.698 | 0.489 |
| Ejaculate expenditure | 31 | 0.006 | -0.413 | 0.683 |
| Testes mass | 40 | 0.014 | -0.739 | 0.464 |

Figure S1. Sperm cell of *Scinax crospedospilus* in phase contrast microscope, municipality of Juiz de Fora, southeastern Brazil.

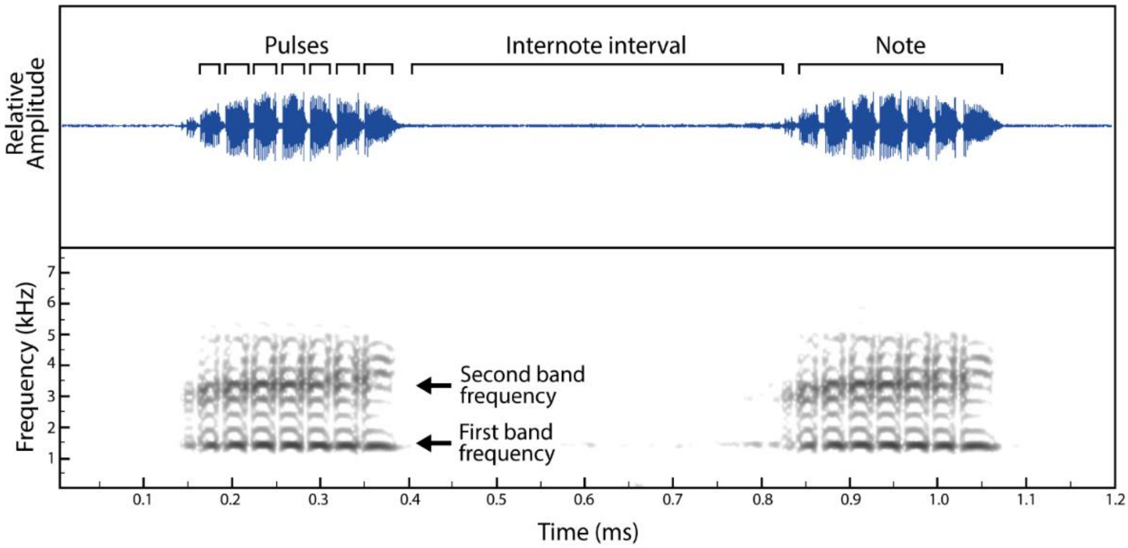

Figure S2. Representation of two calls (= notes in this species) of the treefrog *Scinax crospedospilus*, municipality of Juiz de Fora, state of Minas Gerais, Brazil.

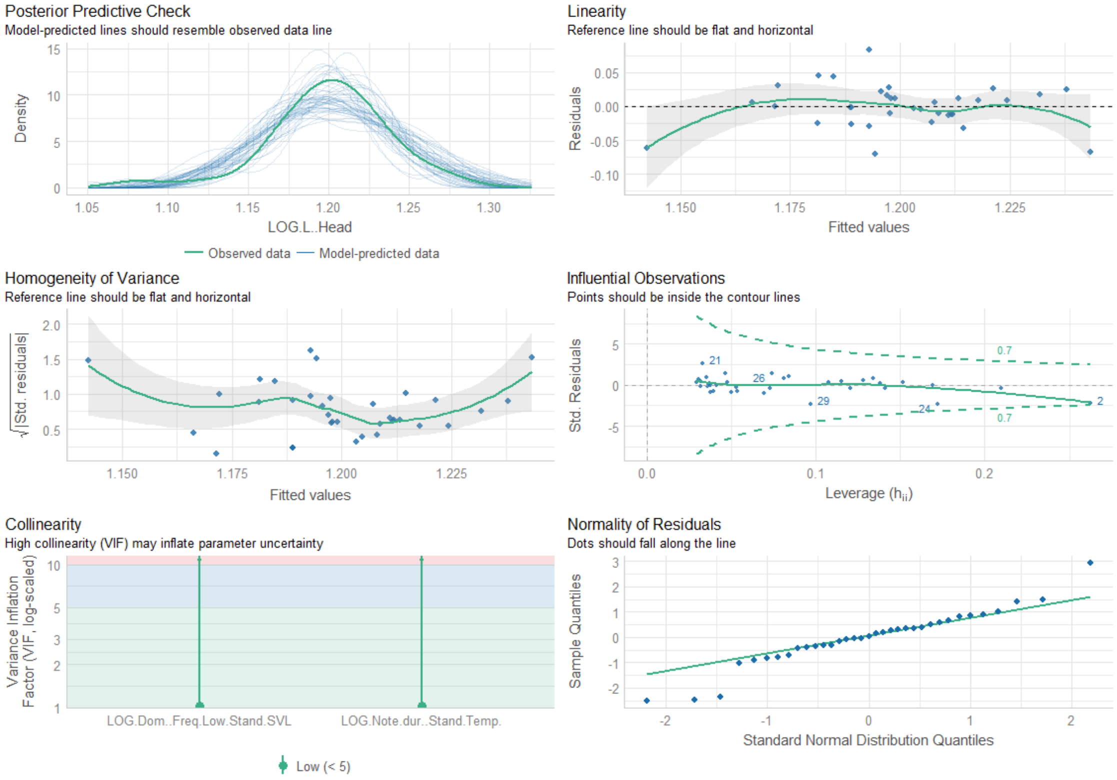

Figure S3. Diagnostics of the multiple regression model for the dependent variable sperm head length.

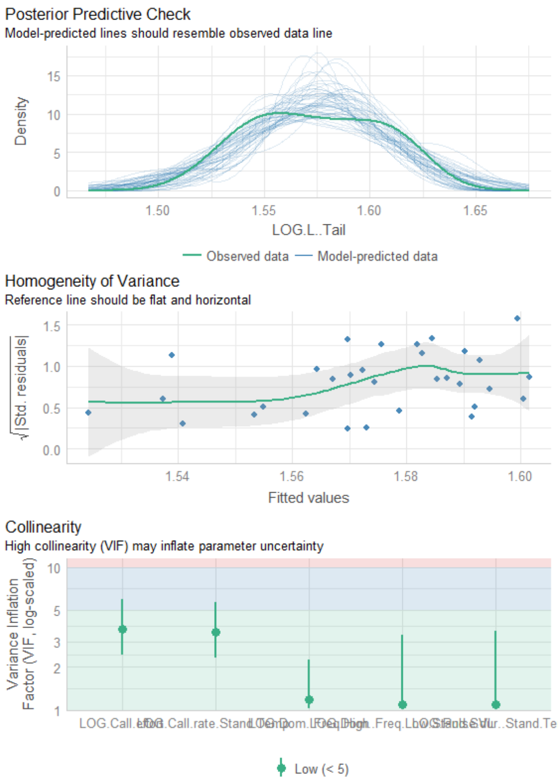

Figure S4. Diagnostics of the multiple regression model for the dependent variable sperm tail length.

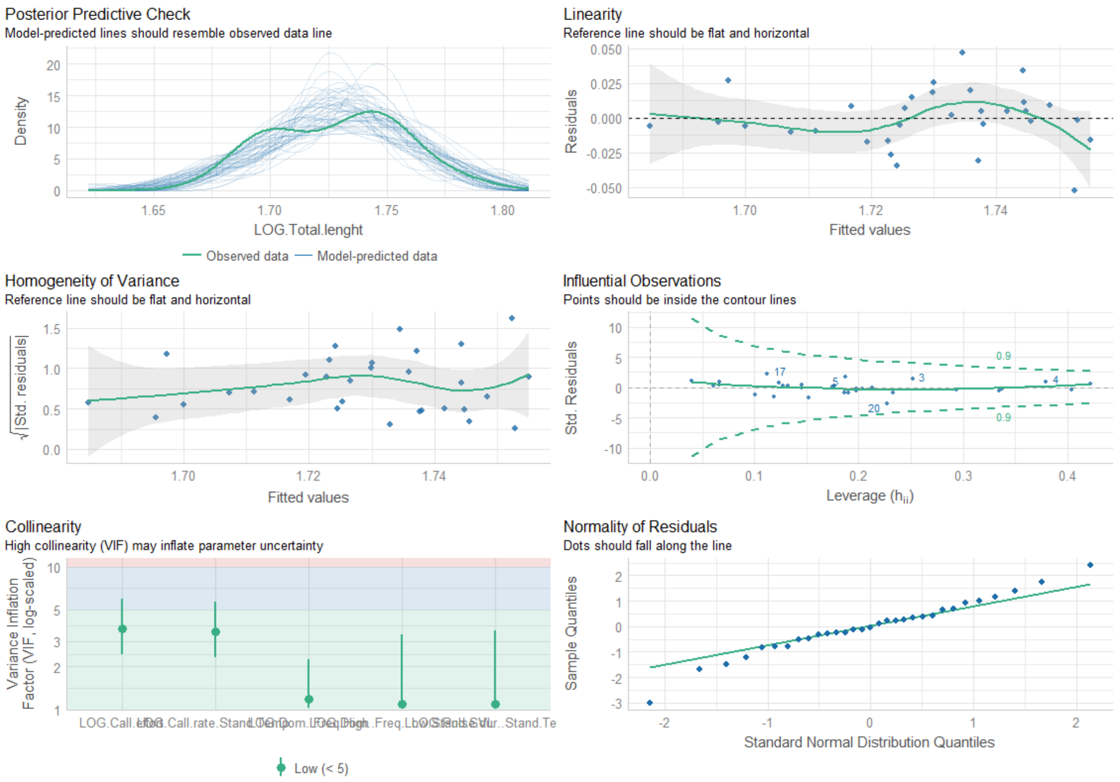

Figure S5. Diagnostics of the multiple regression model for the dependent variable sperm total length.

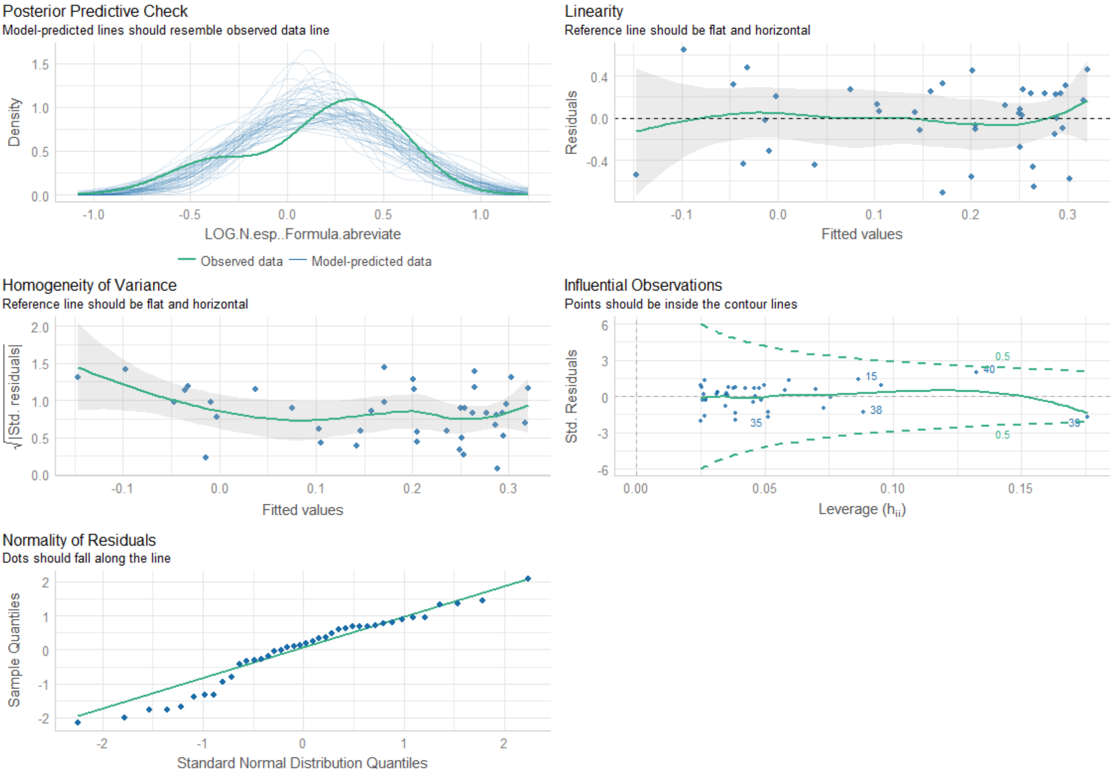

Figure S6. Diagnostics of the multiple regression model for the dependent variable sperm number.

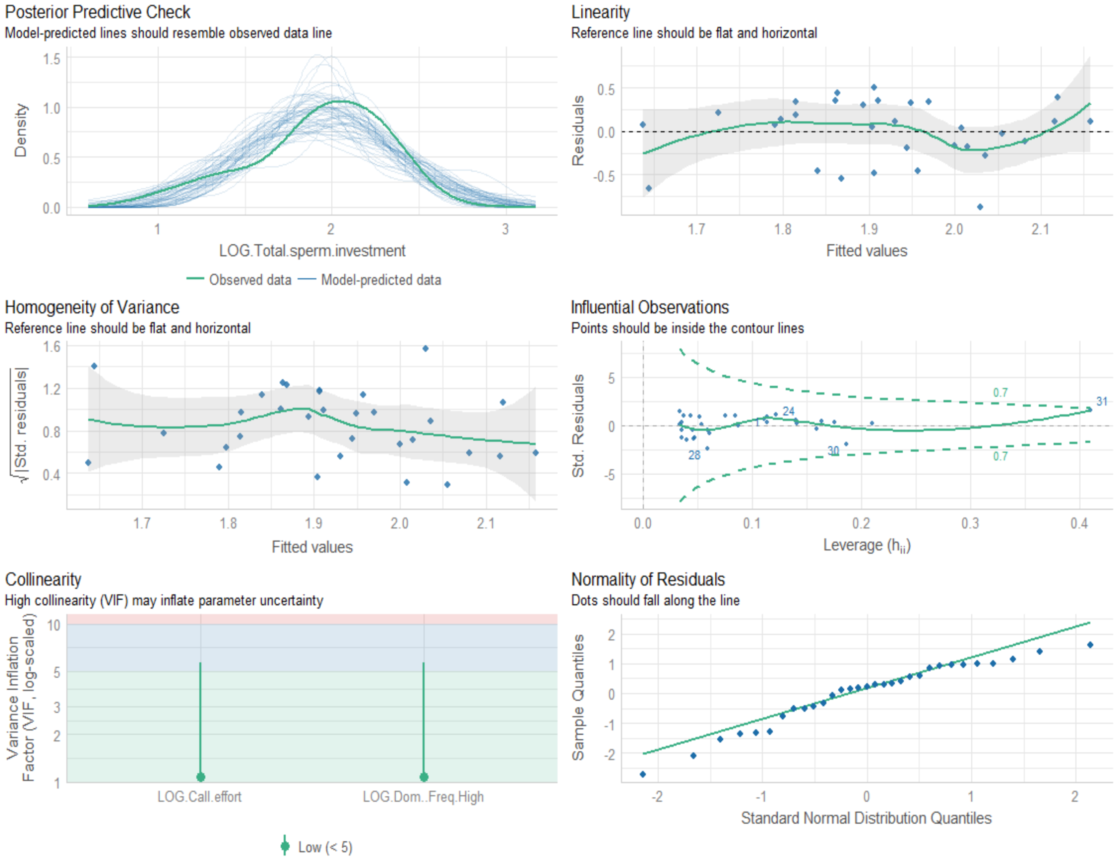

Figure S7. Diagnostics of the multiple regression model for the dependent variable ejaculate expenditure.

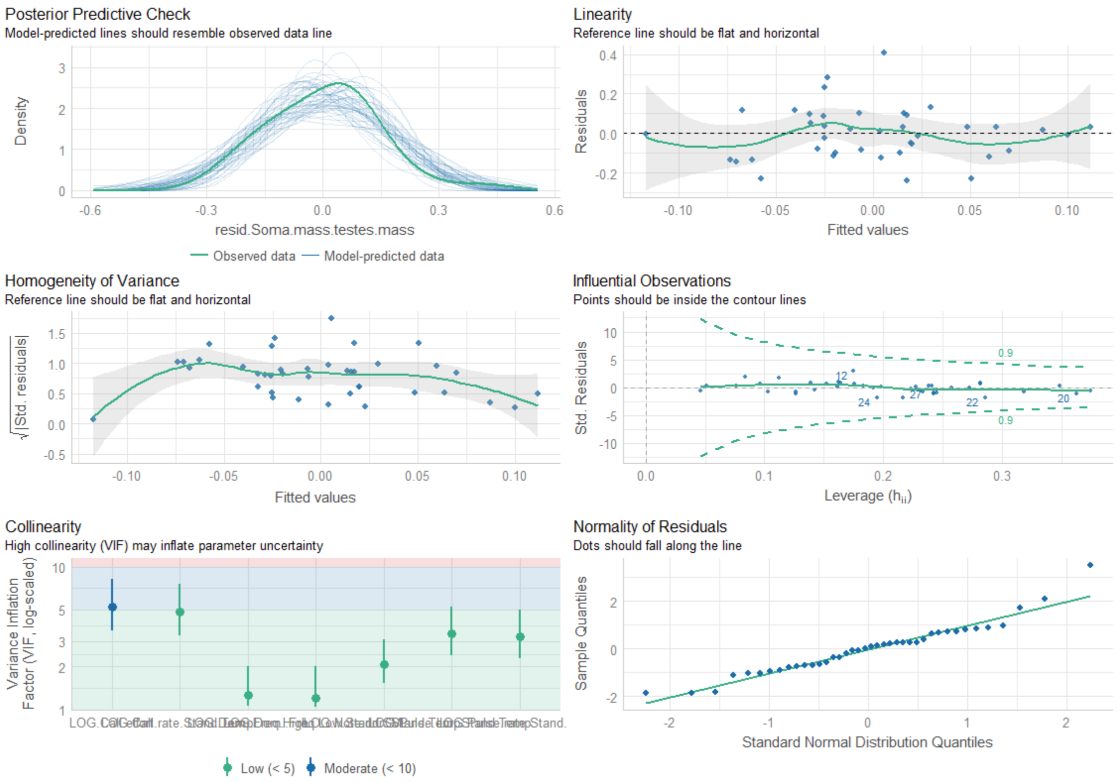

Figure S8. Diagnostics of the multiple regression model for the dependent variable relative testes mass.