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ORIGINAL PAPER

Relationships between vocalization characteristics and sperm traits in a neotropical treefrog

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Abstract

Sexual selection can drive the evolution of any trait that enhances mating success and, when females mate with multiple males, competitive fertilization success. Sperm competition theory predicts a fundamental trade-off between traits under pre- and postmating sexual selection when resources are limited. However, variation in male condition and so resource availability for all fitness traits could lead to a positive association between the same traits across males. Such a link could be used by females to infer male fertility based on the expression of male traits under premating sexual selection (phenotype-linked fertility hypothesis). Most studies examining these types of covariation have focused on ornaments and armaments, but another widespread and costly trait category is acoustic signaling. In frogs, studies have examined sexual selection on either vocalization or sperm characteristics, but the link between them has been largely overlooked. Here, we examined the phenotypic covariation between multiple acoustic characteristics and testicular or sperm traits in the Brazilian treefrog *Scinax crospedospilus*. In multivariate analyses, we found the size of sperm heads and tails to covary positively or negatively with vocal traits, whilst sperm number increased with the call rate. By exploring beyond physical traits under premating or testis size under postmating sexual selection, our results revealed how males invest in different multivariate traits across episodes of selection to maximize their fitness. Including further traits is important considering the widespread use of other modes of costly sexual signaling and that multiple ejaculate traits may affect competitive fertilization success.

Keywords Amphibians · Bioacoustics · External fertilization · Hylidae · Sexual selection · Sperm morphology

Introduction

Sexual selection was proposed by Darwin (1871) as a selective force driving the evolution of exaggerated ornaments and armaments that mediate mate acquisition (i.e., premating sexual selection). In most species, however, females mate with multiple males and incite

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Extended author information available on the last page of the article competition among their sperm for fertilization of the ova (i.e., postmating sexual selection; Parker 1970; Birkhead and Møller 1998). Consequently, sexual selection will also target traits that enhance competitive fertilization success (Parker 1998; Simmons and Fitzpatrick 2012; Fitzpatrick and Lüpold 2014). Whilst there is little debate about the fact that a male's reproductive success ultimately depends on his fitness gains under both pre- and postmating sexual selection (Andersson and Simmons 2006; Evans and Garcia-Gonzalez 2016), predicting optimal male investments in traits targeted by different episodes of selection or the direction of coevolution between these traits is not necessarily straightforward (Parker 1998; Mautz et al. 2013; Parker et al. 2013; Evans and Garcia-Gonzalez 2016; Simmons et al. 2017).

Traits under pre- and postmating sexual selection can covary negatively or positively, depending on the context. Besides the influence of the underlying genetic variance and covariance of traits (e.g., Simmons and Kotiaho 2002; Hosken et al. 2008; Evans 2010), a key factor determining the direction of phenotypic relationships is the relative variation in resource acquisition and allocation between individuals (van Noordwijk and de Jong 1986; Reznick et al. 2000). Given that each male has limited resources to allocate to life-history traits, including reproduction (Roff 2002), sperm competition theory predicts a fundamental trade-off between traits under pre- and postmating sexual selection (Parker 1998). Indeed, negative associations between such traits have been widely reported (e.g., Blanco et al. 2002; Simmons and Emlen 2006; Fry 2006; Simmons et al. 2010; Somjee et al. 2018). However, when males vary in their resources to allocate to reproduction (i.e., condition *sensu* Rowe and Houle 1996; Hill 2011), some males may be able to invest more than others in both types of traits, which may result in a positive relationship across males (e.g., Sheldon 1994; Malo et al. 2005; Mautz et al. 2013). When pre- and postmating sexual traits are positively associated and linked to male condition, secondary sexual traits have the potential to advertise the male's fertility, providing useful information for female mate choice (phenotype-linked fertility hypothesis; Sheldon 1994). However, in populations with widespread multiple mating, there is also a higher risk of sperm depletion (Weir and Grant 2010), particularly for males with a high mating rate (e.g., Preston et al. 2001). To the extent that females can avoid sperm-depleted males, selection would be predicted to favor males that honestly advertise their fertility through exaggerated phenotypes (Sheldon 1994; Malo et al. 2005; Weir and Grant 2010; Foo et al. 2017).

Over the past few decades, studies on the covariation between traits under pre- and postmating sexual selection have accumulated across a diversity of taxa (Mautz et al. 2013; Evans and Garcia-Gonzalez 2016; Simmons et al. 2017), from intraspecific relationships across individuals (Malo et al. 2005; Somjee et al. 2018) or populations (Parker et al. 2013) to broad interspecific associations (Ferrandiz-Rovira et al. 2014; Dines et al. 2015; Lüpold et al. 2014, 2017). However, only few studies have considered vocalization as the premating sexual trait in such comparisons (Simmons et al. 2010, 2011; Doyle 2011; Dines et al. 2015; Dunn et al. 2015), despite the pivotal role of acoustic signals in mate acquisition for many taxa, including frogs (Anura; Toledo et al. 2015).

In frogs, premating sexual selection is predominantly mediated by vocalization, including aggressive and reproductive calls (Wells 2007; Toledo et al. 2015). Aggressive calls are emitted sporadically and so are not heard very commonly (Toledo et al. 2015). By contrast, advertisement calls, the most common type of reproductive call, are emitted continually, and their role in attracting females and repelling other males indicates that they are under both selection by male-male competition and female choice (Toledo et al. 2015). Since males of many frog species can emit long bouts of calls throughout the reproductive season (Wells 2007), the number of calls or notes per minute and the duration of each note both are associated with the metabolic investment (Taigen and Wells 1985; Ryan 1988). Notes are often subdivided into pulses (Köhler et al. 2017), which themselves are costly (Wells 2001). Thus, variation in such acoustic properties can reflect the motivation and performance of males and has been linked to mating success (Wagner Jr 1992; Welch et al. 1998; Gerhardt and Huber 2002; Köhler et al. 2017). Further, the ability to produce low-pitch calls is typically correlated with a larger body (Gingras et al. 2013; Turin et al. 2018), and larger males have been shown to achieve higher mating success by being better at establishing and defending territories suitable for reproduction and attracting females to them (Ryan et al. 1992). Consequently, low-pitch calls are likely to be associated with a reproductive advantage and so favored by sexual selection.

In polyandrous species, males can also gain fitness by investing more in gametes to enhance competitive fertilization success (Parker 1998). In frogs, associations between proxies of sperm competition risk and intensity (e.g. relative testis size, sperm number or sperm size) have been reported both across (Kusano et al. 1991; Byrne et al. 2002, 2003; Zeng et al. 2014; Liao et al. 2018) and within species (Edwards et al. 2004; Dziminski et al. 2010; Buzatto et al. 2017). Additionally, the morphology of sperm themselves could contribute to variation in fertilization success (e.g., Oppliger et al. 2003; García-González and Simmons 2007; Lüpold et al. 2012; Bennison et al. 2015), although the evidence for such an effect is more mixed (Simmons and Fitzpatrick 2012; Lüpold and Pitnick 2018). For example, longer flagella have been demonstrated to enhance sperm swimming speed and so putatively competitive fertilization success in several species (Malo et al. 2006; Mossman et al. 2009; Fitzpatrick et al. 2010; Simpson et al. 2014; Bennison et al. 2015), but not so in others (Humphries et al. 2008; Lüpold et al. 2009; Firman and Simmons 2010; Simpson et al. 2014). Sperm head size, too, is positively, negatively, or not associated with swimming speed in the same studies. For frogs, we are aware of only one species to have been studied so far in this regard, the Australian quacking frog *Crinia georgiana* (Tschudi 1838), and no relationship has been found between sperm morphology and velocity (Hettyey and Roberts 2006; Dziminski et al. 2009; Simpson et al. 2014). This negative result leaves unresolved if and how sperm morphology mediates competitive fertilization success and is selected in other species. At least the comparative evidence across anurans and that the link between sperm form and function varies greatly among different avian or mammalian species (see above) suggest that positive selection on sperm dimensions is plausible in other anuran species.

Whilst traits under either pre- or postmating sexual selection are well studied in many frog species (reviewed in Ryan 1991; Kelley 2004; Roberts and Byrne 2011), the relative investments between them have rarely been explored. Among the few studies available, males have been shown to trade off testes size against arm width as a sexual weapon across populations of *C. georgiana* (Parker et al. 2013; Buzatto et al. 2015, 2017). The relationship between these two traits has further been shown to shift from positive to negative across species with an increasing probability of multimale amplexus, resulting in stronger post- compared to premating sexual selection (Lüpold et al. 2017). However, besides such links between testes and weapons, only one study has explored how male vocalization relates to ejaculate investments, finding no significant association between call duration or rate and sperm number or viability (Doyle 2011). Beyond this study of calling intensity, how acoustic properties themselves (i.e., call quality) covary with testis or sperm characteristics in the context of allocation trade-offs has never been explored in frogs despite their taxonomically widespread advertisement calls. This knowledge gap prevents a comprehensive understanding of the dynamics of sexual selection in this highly diverse taxon.

The treefrog family Hylidae is the most speciose amphibian family in the world, with 1,047 species highly concentrated in the Neotropical region (Frost 2023), some of which are polyandric (d'Orgeix and Turner 1995; Zamudio et al. 2016). *Scinax crospedospilus* is a small treefrog endemic to the Atlantic Forest, southeastern Brazil (Heyer et al. 1990; Frost 2023). During prolonged breeding throughout the rainy season (Heyer et al. 1990; Wells 2007), males are found at high densities and show exposed amplexus (P. L. Aguiar, pers. obs.), increasing the probability of multimale mating (F. M. S. R. Pedro, pers. comm.; also see Buzatto et al. 2015; Zamudio et al. 2016; Lüpold et al. 2017). Hence, males are predicted to invest in large ejaculates (Liao et al. 2018), which, combined with frequent mating itself, increases the risk of sperm depletion (Weir and Grant 2010).

Here, we examined the relationships between traits under pre- and postmating sexual selection in *S. crospedospilus* to test the predictions from sperm competition theory (Parker 1998) against the phenotype-linked fertility hypothesis (Sheldon 1994). Specifically, we compared vocalization parameters normally related to mate acquisition (e.g., longer and lower-pitch calls) with testicular and ejaculate traits that might be favored by postmating sexual selection (e.g., larger and more numerous sperm). These traits should covary negatively based on sperm competition theory (Parker 1998) but be positively related if male vocalization were to signal high fertility (Sheldon 1994). Further, a negative association between pre- and postmating sexual traits would indicate greater variation in resource allocation strategies than in resource availability across males, whereas a positive relationship would suggest the opposite (Simmons et al. 2017). Such joint exploration of sexually selected traits is necessary to elucidate the dynamics and relative importance of different episodes of selection, a context rarely studied in species that rely primarily on acoustic communication for mate acquisition.

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 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 behavior (e.g., Nali and Prado 2012). We then recorded each male for at least 2 min using a Marantz PMD 660 audio recorder connected to 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 to the nearest 0.1 °C with a digital termohygrometer (J-prolab).

Laboratory analyses and testes/sperm measurements

After each collection night, we took all captured males to the laboratory. We euthanized each male individually by spraying a solution of lidocaine 5% in the gular region to allow cutaneous absorption (McDiarmid 1994; CONCEA 2018). The male was then placed in a plastic bag full of air until they ceased all movement and heart beating (about 5 min), the latter of which is possible to see in ventral position. We then weighed them with an analytical balance (Shimadzu; accuracy = 0.00001 g) and measured their snout–vent length (SVL) using a digital caliper (accuracy = 0.01 mm). Subsequently, 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 frog testes to control for body mass (Buzatto et al. 2017; Chen et al. 2023).

To determine sperm characteristics, we macerated one of the testes per male using a glass rod (diameter = 5.3 mm) on a concave clock glass (diameter = 35 mm; depth = 5 mm) containing 10 µl distilled water, rinsing any residual sperm from the tip of the rod with another 10 µl distilled water. Then, we combined 6 µl of this sperm solution with 54 µl 4% formaldehyde and homogenized this solution using the pipette. For estimates of sperm quantity, we homogenized the 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 min. 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 each side of the chamber and averaged the total value of the two sides (measurement of sperm number; Absher 1973). When the sperm count of the two chambers differed by less than 10%, the sample was considered valid (Zhang et al. 2020). To calculate the sperm concentration as cells/µl, we considered the total volume of all 5 squares ($0.02\ mm^3 = 0.02\ \mu l$) used for sperm count and determined the number of sperm cells per 1 µl. To authenticate the validity of this methodology, we ran a linear regression between sperm count and the size of the testes, and expected a positive relationship.

For sperm morphometry, we placed 6 µl of the same sperm solution on a slide under a coverslip and photographed sperm cells at 100× magnification under the same microscope as above. Using the software ImageJ v. 1.53t, we measured the lengths of the sperm head and the tail for ca. 10 sperm cells per male (Byrne et al. 2003). We did not measure midpiece length because it was indistinguishable from the head (e.g., Byrne et al. 2003). Head length was the distance between the apex of the acrosome and the junction of the head with the tail (Electronic supplementary material 2, Figure S1). Since sperm of *S. crospedospilus* have two tail filaments of equal size (Fouquette and Delahoussaye 1977), we averaged the lengths of both filaments, each measured from its junction with the head to its apex (Electronic supplementary material 2, Figure S1). We did not measure any sperm with visually unequal filament lengths to avoid measuring cells that may have been damaged during preparation. In summary, we used the following variables as potential traits under postmating selection: relative testes mass (residuals, as explained above), sperm head length, sperm tail length, total sperm length, and ejaculate expenditure (sperm number multiplied by sperm total length; Ball and Parker 1996). Because we used the first males collected to standardize our sperm cell preparations, we obtained a slightly lower sample size for some sperm traits, but a minimum of $N=31$. We did not control sperm variables for body size (male SVL) due to a lack of correlation between these variables (Electronic supplementary material 1, 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 (Electronic supplementary material, 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 of peak frequency (automatic measurement for dominant frequency), note duration, and number of pulses repeated within a note (Köhler et al. 2017). We further averaged per individual 60 measurements of pulse duration across different notes. We calculated the call rate for each male as the number of calls emitted in one minute, with each call consisting of a single note in *S. crospedospilus*. We also calculated two more acoustic variables for each male (Köhler et al. 2017): call effort (ratio between average note duration and average inter-note interval, 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; Electronic supplementary material, Figure S2), we measured the dominant frequency of both bands separately (first and second band dominant frequencies), since either one may be biologically relevant (Foratto et al. 2021). We also analyzed for each male which frequency band contained predominantly the dominant frequency.

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 effect, we first ran linear regression analyses between each acoustic variable and air temperature. Variables showing 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 effect, 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 covariation of two traits does not specify which is the dependent and independent variable, respectively, we ran all regression analyses arbitrarily considering sperm/testes traits as dependent variables and acoustic traits as independent variables. To ensure that this procedure did not bias our results, we re-ran a subset of analyses by reversing focal variables, i.e., using acoustic traits as dependent variables and sperm/testes traits as independent variables, and values were the same.

We log-transformed our dataset and ran all our analyses in R version 4.2.2 (R Core Team 2022). We used the stepwise selection method (Pierna et al. 2009) implemented in the *step* function of the *stats* package (R Core Team 2022). Starting from an initial linear model containing all predictors of interest (full model), this function sequentially excludes the variable with the highest p-value until the minimum adequate model is reached by stepwise model comparisons based on the Akaike Information Criterion (AIC; Bozdogan 1987). We used the function *report* within the package *report* (Makowski and Lüdecke 2019) to obtain effect sizes (standardized β) with 95% confidence intervals for all predictor variables based on a Wald t-distribution approximation. We also evaluated model performance based on posterior predictive check, normality and linearity of residuals, influential observations, homogeneity of variances and multicollinearity using the function *check_model* within the package *performance* (Lüdecke et al. 2021).

Results

We sampled 40 males of *S. crospedospilus* in the field, ranging in SVL from 29.81 to 33.19 mm and in body mass from 1.38 to 2.12 g. We recorded their calls across air temperatures between 17.4 and 23.5 °C. The means, standard deviations and ranges of all variables are shown in Table 1.

The general sperm phenotype resembled that in Fouquette and Delahoussaye (1977) for the *Scinax ruber* clade (Electronic supplementary material 2, Figure S1), but its total length (mean ± SD = 53.62 ± 3.53 µm; Table 1) was slightly below the clade mean of 70 µm (Fouquette and Delahoussaye 1977; Faivovich et al. 2005). The dominant frequency of the note fell predominantly within the first band in 75% of the males, and within the second band in 25% of the males.

As expected, sperm number and testes mass correlated positively ($t = 4.4$; $p < 0.001$; $r^2 = 0.34$). Regarding the phenotype-linked and trade-off hypotheses, although most results were non-

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	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 (mg)	40	3.9 ± 1.4	1.9	7.1
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

N = number of males

significant, we did find some significant relationships (Table 2), and our models followed the expected assumptions (Electronic supplementary material, Figures S3-S8). Among these, we found two directions regarding covariances between testes/sperm traits vs. acoustic traits. Sperm head length correlated negatively with note duration and the first band dominant frequency, whereas sperm tail length covaried positively with the second band dominant frequency and call rate (Table 2; Fig. 1). The call rate was also positively associated with sperm total length and sperm number (Table 2; Fig. 1). Relative testis mass and ejaculate expenditure, however, were not significantly associated with any of the call variables.

Table 2 Results (t-tests) from multiple linear regression analyses between sperm and testes variables vs. temporal acoustic variables in *Scinax crospedospilus*, southeastern Brazil

Trait	r^2	t	p	β (95% CI)
Sperm head length				
First 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)
First 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 (-0.003, 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)

The r^2 relates to the whole model. The sign of the t-value depicts the type of correlation (negative or positive). Significant relationships are highlighted in bold for p values. We also report the standardized β (effect size) with confidence intervals for each variable within the model. The most accurate multiple linear regression model for each variable was selected based on the Akaike Information Criterion using stepwise regression

Discussion

Our knowledge of covariations between traits under pre- and postmating sexual selection in frogs is still limited (Parker et al. 2013; Buzatto et al. 2017; Lüpold et al. 2017), particularly when considering that vocalizations are among the most common traits under premating sexual selection. So far, to the best of our knowledge only one study has compared call with ejaculate characteristics to test the phenotype-linked fertility hypothesis in frogs, finding no association between these traits in *Dryophytes versicolor* (Doyle 2011). Here, we documented both positive and negative covariation between call parameters and different ejaculate traits. To the extent that these traits are costly and affect fitness, these relationships suggest that both the fundamental trade-off and the phenotype-linked fertility hypotheses may apply to the treefrog *S. cropsedospilus*.

We found that sperm size (tail and total length) and sperm number correlated positively with call rate and that sperm head length covaried negatively with first band dominant frequency. These associations follow the predictions of the phenotype-linked fertility hypothesis (Sheldon 1994) insofar as these ejaculate traits indeed mediate fertilization success in frogs (e.g., sperm number: Dziminski et al. 2009). If so, male *S. crospedospilus* with faster call rates and lower first band dominant frequencies would have the potential of advertising superior fertility to females. Female frogs have been shown to prefer males with lower dominant frequencies (Ryan et al. 1992; Felton et al. 2006; Calsbeek et al. 2022), which generally are linked to larger body sizes (Gingras et al. 2013) and thus possibly more experience (Felton et al. 2006). Some male frogs, however, dishonestly signal larger body size to other males by lowering their call frequencies (Nali and Prado 2014). Hence, the link between call frequencies and ejaculate characteristics may allow for a similar strategy to advertise their sperm competitiveness to conspecific males (Malo et al. 2005).

These positive associations between call and sperm traits may also be mediated by hormones. In frogs, androgen levels regulate vocal activity and the development of vocal structures such as the larynx (Emerson and Hess 2001; Moore et al. 2005), and the same hormones also control spermatogenesis (Dufau 1984; Wingfield et al. 1990). This dual function of androgens opens interesting avenues for future research of the mechanisms underlying the phenotypic links we documented here (e.g. Leary et al. 2008; de Assis et al. 2012).

In contrast to these positive trait associations, the lengths of sperm head and tail tended to covary negatively and positively with note duration and second band dominant frequency, respectively. Since these traits (except second band dominant frequency) are expected to come with some energetic cost (Taigen and Wells 1985; Ryan 1988; Pitnick et al. 1995; Wells 2001; Lüpold et al. 2016; Godwin et al. 2017), the negative association between sperm head length and note duration would be consistent with the trade-off hypothesis (Parker 1998). The few studies to date on the correlation between acoustic and postmating sexual traits focused on sperm number, sperm viability and testis size (intraspecific: Simmons et al. 2010; Simmons 2011; Ng et al. 2018; interspecific: Dunn et al. 2015), but here we found a trade-off with sperm morphology. Longer sperm can be costly in terms of both energy and time taken for their production (Pitnick et al. 1995; Godwin et al. 2017), particularly combined with the well-established costs of producing vast numbers of sperm (Dewsbury 1982; Parker and Pizzari 2010; Bunning et al. 2015). In addition, longer note durations in frogs can be extremely costly through elevated aerobic metabolism (Ryan 1988; Wells 2001). It remains unclear why the first band dominant frequency correlated negatively with sperm

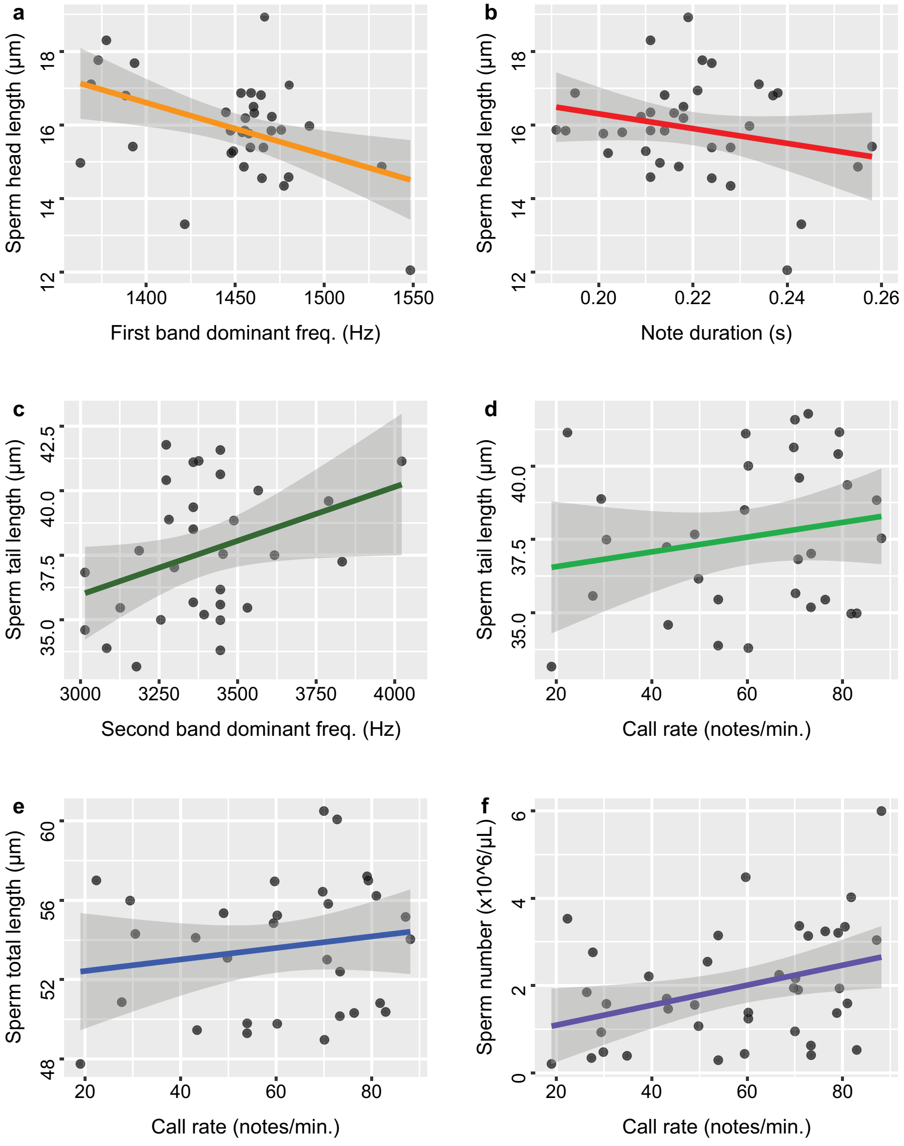

Fig. 1 Correlations between vocalization and sperm characteristics for *Scinax crospedospilus*, municipality of Juiz de Fora, southeastern Brazil. All plots show linear regression analyses representing significant correlations among sperm variables vs. acoustic variables (see Table 2)

head length, and the second band dominant frequency correlated positively with sperm tail length. According to our results, the dominant frequency of the note was predominant in the second band in only 25% of the males, which indicates that this band should be less relevant for female mate assessment compared to the first band. Consequently, we speculate that in *S. crospedospilus* it is more advantageous for males to increase their investment in sperm size to enhance sperm competitiveness than to produce a second band with lower dominant frequencies with limited fitness gains under premating sexual selection. Our results corroborate the importance of analyzing different frequency bands in studies of frog communication and sexual selection (Foratto et al. 2021). Further studies testing the availability of resources and the condition-dependence of acoustic and ejaculate traits in *S. crospedospilus* could provide deeper insight into allocation patterns between these traits across contexts.

Comparing temporal acoustic traits, our results showed that call rate in *S. crospedospilus* correlated positively with sperm length and that note duration declined with increasing sperm head length. These results again suggest potential support of the phenotype-linked fertility and fundamental trade-off hypotheses, respectively. Although both vocal traits tend to reflect male quality (Gerhardt and Huber 2002), the call rate itself does not account for the duration of notes (Köhler et al. 2017), potentially resulting in relatively low costs for a high call rate if each note is short (Prestwich 1994). We suggest that males might invest in call rate to enhance mate acquisition, but emitting shorter notes instead might keep the energetic costs down. If so, this might explain why in *S. crospedospilus* sperm head and tail length covaried in opposite directions with note duration and call rate, respectively.

While our study has provided valuable insights, it is important to consider some limitations that could affect our interpretations. For instance, the environment where external fertilization takes place can change rapidly in response to many factors, and by extension also the selection on both sperm size and number, in addition to sexual selection (Levitan and Petersen 1995; Emerson 1997; Simmons et al. 2009; Liao et al. 2018). Such a varying selective environment can complicate the study of ejaculate traits and their covariance with premating sexual traits (Moore et al. 2005). Another complication is that *S. crospedospilus* employs different modes of communication, including aggressive calls and likely visual signaling (P. L. Aguiar, unpublished data). Since male frogs use aggressive calls (e.g., encounter calls; Toledo et al. 2015) in intrasexual selection and visual signaling for both intra- and intersexual selection (Hödl and Amezquita et al. 2001), males of *S. crospedospilus* might advertise their ejaculate quality through a combination of acoustic and visual traits rather than single vocal parameters.

The lack of control for the social context and male mating history is a further caveat in our study. Yet, the patterns that we did find at least provide partial support of our main hypotheses and suggest that further detailed studies are warranted. For instance, we detected covariation of call parameters with sperm size and number, but not with relative testis mass, which is one of the most widely studied traits under postmating sexual selection (reviewed in Simmons and Fitzpatrick 2012; Zamudio et al. 2016; Lüpold et al. 2020; Baker et al. 2020). The reason for this negative result remains unclear, but a comparative study across hylid frogs (family of *S. crospedospilus*) might provide some insight. Byrne et al. (2002) found no clear relationship between testes mass and the level of sperm competition across species and hypothesized that this could be linked to potential constraints to increasing sperm production. We do not know if such constraints might explain why testes mass differed from gamete size and number in the covariation with vocal traits. However, further insight could be gained by substituting relative testes mass 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 revealed contrasting covariation between different vocalization and ejaculate traits for the first time in a frog species, including sperm morphology that has not previously been considered in such context for any animal species to the best of our knowledge. We hope that this work can contribute to a broader investigation of pre- versus postmating sexual selection, moving beyond the traditional focus on ornaments and armaments. Considering the prevalent use of acoustic signaling in mate acquisition, our study opens up new avenues for understanding the dynamics of sexual selection.

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Author contributions PLA collected and analyzed field and laboratory data. PLA led the writing with important contributions from SL and RCN. RCN idealized the project and supervised the activities. All authors approved the final version of the manuscript.

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Data Availability Some of the data is available as supplementary material. Further data can be made available upon reasonable request. Collected specimens were deposited in the Coleção de Anfíbios da Universidade Federal de Juiz de Fora, Juiz de Fora municipality, Minas Gerais State, Brazil (CAUFJF 2112–2155).

Code Availability The code can be made available upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare that there is no conflict of interest.

Ethics approval Capture and recordings of individuals in the field were authorized by the Chico Mendes Institute for Biodiversity Conservation (SISBIO/ICMBio), under the license # 78878-1, as well as by the Ethics Committee of Animal Use (CEUA/UFJF) under the protocol # 18/2021.

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Consent for publication PLA, SL and RCN give their consent for the publication of all data and conclusions in this journal.

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