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Original Article

Show me you care: female mate choice based on egg attendance rather than male or territorial traits

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Female mate choice is often based on male traits, including signals or behaviors, and/or the quality of a male's territory. In species with obligate paternal care, where care directly affects offspring survival, females may also base their mate choices on the quality of a sire's care. Here, we quantified male reproductive success in a natural population of the glass frog *Hyalinobatrachium cappellei*, a species with male parental care, to determine the influence of territory quality, male traits, and paternal care behaviors on female mate choice. We found that attending males have a higher chance of gaining new clutches than nonattending males. Our results indicate that females do not select males based only on body condition, calling persistence, or territory traits. Instead, our findings support the hypothesis that females choose males based on care status. Indeed, males already attending a clutch were 70% more likely to obtain another clutch, and the time to acquire an additional clutch was significantly shorter. We also found that males adjust their parental care effort in response to genetic relatedness by caring only for their own offspring; however, remaining close to unrelated clutches serves as a strategy to attract females and increase chances of successful mating. Thus, males that establish territories that already contain clutches benefit from the signal eggs provide to females.

Key words: **female choice, male-offspring relatedness, mating strategy, parental care, paternity.**

INTRODUCTION

Classical sexual selection theory posits that males compete to gain access to choosy females who mate nonrandomly, selecting high-quality males as mating partners (Andersson 1994). Female mate choice is often based on resources, phenotypic traits, or behavioral displays, which signal male quality (Price et al. 1993; Andersson 1994; Kokko et al. 2006). By mating with a male with preferred attributes, a female obtains direct (increased offspring survival and quality; Price et al. 1993; Wagner 2011) or indirect benefits (increasing offspring attractiveness; Zahavi 1975; Kotiaho and Puurtinen 2007). Hence, selection will favor males that invest in advertising these benefits to females (Zahavi 1975; Andersson 1994). Body size or condition of males (Downhower and Brown 1980; Ryan 1980; Price 1984; Howard 1988; Bose et al. 2018), calling intensity (Smith and Roberts 2003; Wogel et al. 2005; Nemeth et al. 2012; Charlton et al. 2018), and quality of reproductive territories (Wells 1977; Roithmair 1992, 1994; Jones et al. 2014) are well-known indicators of male quality in vertebrate species.

In species with paternal care, females may base their mate choices not only on traits that indicate the genetic quality of potential sires but also the quality of the sire's care because that care directly affects offspring survival (Clutton-Brock 1991; Burrowes 2000; Farmer and Alonzo 2008; Klug et al. 2012). We expect that,

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when offspring have lowered or no chance of survival in the absence of a parent, any indicator for quality of parental care will be highly selectively advantageous. Indeed, male investment in the care of offspring plays an important role in female mate choice in fishes (Kraak and Videler 1991; Kraak and Groothuis 1994; Forsgren et al. 1996; Fagundes et al. 2007; Matsumoto et al. 2011) and arthropods (Tallamy 2001; Thomas and Manica 2005; Nazareth and Machado 2010; Requena and Machado 2015), where the presence, number, and developmental stage of embryos under male care influence female mate choice (Kraak and Weissing 1995; Goulet 1998; Manica 2002; Fagundes et al. 2007; Requena and Machado 2015). Mating cues are expected to reflect the qualities of males, favoring the evolution of specific morphological, physiological, and behavioral traits that maximize their chances of reproduction (Taborsky 1997). For example, males of the striped darter *Etheostoma virgatum* use egg-mimicking spots on their fins in a nesting display, enticing females to more frequently spawn in their territories (Porter et al. 2002). If the number of clutches attended by a male is an honest signal of male fitness, by laying eggs with attending males, females increase the survival rate of their own eggs because the risk of predation is reduced by the dilution effect (Sargent 1988) or the quality of care (Lindström et al. 2006).

A common mating strategy among frogs is to have territorial males, where a high certainty of paternity favors the evolution of paternal care (Sutton and Wilson 2019). Males of some anuran species remain in their territories attending multiple egg clutches (Summers and Tumulty 2014; Delia et al. 2017), offsetting the costs of care by increasing their fitness (Lehtinen and Nussbaum 2003). For example, although males of *Eleutherodactylus coqui* lose additional mating opportunities, they improve their reproductive success by providing care to their eggs until hatching (Townsend et al. 1984; Townsend 1986). In addition, because territory defense is energetically expensive, male territoriality associated with parental care likely serves as an honest signal of male quality (Nazareth and Machado 2010; Matsumoto et al. 2011; Mangold et al. 2015). Although paternal care is widespread in anurans (Vági et al. 2019), the signal value of clutches for female choice has been poorly explored in this group (but see Burrowes 2000; Martin et al. 2010).

Here, we investigated the drivers of female mate choice in the glass frog *Hyalinobatrachium cappellei*, a species that lays egg clutches on the undersides of leaves overhanging streams and in which males attend the eggs in their territories by cleaning, hydrating, and protecting them from predators (Noronha and Rodrigues 2018). As is typical for many frogs, sex ratio at breeding sites is highly male biased and we predict that females potentially choose partners based on body condition and oviposition site as reliable signal of male quality (Taylor et al. 2007; Mangold et al. 2015). In addition, egg attendance is essential for offspring survival in most centrolenids, including our focal species (Vockenhuber et al. 2008; Lehtinen et al. 2014; Valencia-Aguilar et al. in review); thus, we expect that females will make mating decisions based not only on male traits and territory quality but also on paternal effort. To test the relative importance of potential sexually selected traits, we evaluated the influence of male body size, calling persistence, oviposition site quality, and the presence of paternal care on female mate choice. If the presence of eggs is being used by females as a signal of quality of males or their paternal care, then attending males should achieve higher mating success than nonattending ones (Burrowes 2000; Lindström et al. 2006; Requena and Machado 2015).

Signals for mate choice should not have costs that reduce parenting ability itself (Kelly and Alonzo 2009); however, care for clutches is costly; thus, males could benefit by adopting strategies to manipulate a female's behavior to their own advantage (Zahavi 1975; Taborsky et al. 2008). If males with at least one clutch of eggs are more attractive to females, then males could adopt or take over already existing clutches of other males to enhance their mating success while bypassing mating costs associated with care of those foreign clutches (Taborsky et al. 1994; Thomas and Manica 2005; Zamudio and Chan 2008). For males, the time and energy invested in offspring survival can reduce mating opportunities (Townsend 1986), decrease food intake (Simon 1983), and increase chances of predation (Poo et al. 2016). Thus, it is expected that attending males will be the genetic fathers of all the offspring they care for (Alonzo and Klug 2012). We used molecular parentage analysis of adults and larvae collected in the field to test hypotheses about the fitness consequences of parental care and the drivers of female mate choice. Specifically, we asked 1) whether male body condition or characteristics of their territories are drivers of female choice, 2) whether parental care is being used by females as a signal for mate choice, and 3) whether males adopt or take over clutches that are not their own as a strategy to increase fitness while, at the same time, releasing them from costs of paternal care.

MATERIALS AND METHODS

Study population

Hyalinobatrachium cappellei is a small arboreal frog that cares for offspring during embryonic development (Noronha and Rodrigues 2018). Parental care takes place in the male's territory, where males care for eggs of multiple females either simultaneously or in sequence (Noronha and Rodrigues 2018). We monitored three adjacent stream (Alagado, Bosque, and Buritizal) populations of *H. cappellei* on a daily basis over two breeding seasons at the Fazenda São Nicolau (9°49'10.1"S and 58°15'29.9"W), municipality of Cotriguaçu, Mato Grosso state, Brazil. Vegetation in the area is classified as dense humid forest (Blaser et al. 2011), with monsoon climate, including a hot rainy summer (September to March) and a dry winter (June to August; Alvares et al. 2013). Temperature does not differ strongly between seasons (24 to 36 °C in summer and 32 to 38 °C in winter), but rainfall and humidity are greater in the summer (mean of 1300–1700 mm and 80% humidity) than in the winter (mean of 20–50 mm and 40% humidity) (Bittencourt-Rosa et al. 2013).

Field surveys and behavioral observations

During the months of January and February 2017 and 2018, we conducted two daily surveys, one during the day (8 AM to 1 PM) and one at night (8 PM to 2 AM). During each survey, we searched for attending and nonattending males and marked the male's territory with flagging tape to monitor male activity and development of clutches until hatching (Table 1). Here, we used the term "attending" to refer to males caring for one or more clutches and "nonattending" for males that did not have a clutch when sampling started. In some cases, we used a ladder to reach males and their clutches up to a maximum height of 7 m above the ground. We revisited each male's territory to monitor male activity, egg attendance, and embryonic development once during each daytime visit (for 20–30 min) and twice or three times (for 30–40 min) each night.

During each visit, we recorded male activity and the condition of any clutch(es). We considered egg attendance when the male hydrated eggs and remained close to the clutch (no further than

Table 1

Results of parentage analyses in *Hyalinobatrachium cappelei*. ID, male identity; *N* mates, number of mating partners; size, male size (mm); mass, male mass (g); *N*, number of clutches per male; mean eggs, mean number of eggs per clutch; min/max, minimum and maximum number of eggs per clutch; egg number, total number of eggs attended by male; %survival, percentage of hatched larvae

					Clutches						
Place	ID	*N* mates	Size	Mass	*N*	Mean eggs	SD	Min	Max	Egg number	% survival
Alagado 2016	MALE16_20	*,1	21.86	0.59	3	21.66	3.05	19	25	65	84.62
Alagado 2016	MALE16_15	*	20.68	0.48	3	20.33	5.03	15	25	61	100
Alagado 2016	MALE16_12	1	20.49	0.51	1	25				25	92
Alagado 2016	MALE16_14	1	22.78	0.64	1	17				17	100
Alagado 2017	MALE17_72	1	19.5	0.45	1	18				18	100
Bosque 2016	MALE16_22	2	21.6	0.65	2	19.5	0.70	19	20	39	87.18
Bosque 2016	MALE16_11	1	22.71	0.67	1	23				24	95.83
Bosque 2016	MALE16_18	2	22.86	0.66	2	20	1.41	19	21	40	97.5
Bosque 2016	MALE16_16	2	20.83	0.45	2	22.5	0.70	22	23	45	95.56
Bosque 2016	MALE16_23	1	22.4	0.58	1	16				16	75
Bosque 2016	MALE16_25	1	21.21	0.58	1	20				20	80
Bosque 2016	MALE16_26	1	20.77	0.57	1	18				18	94.44
Bosque 2016	MALE16_28	1	21.83	0.63	1	17				17	100
Bosque 2016	MALE16_29	1	21.53	0.58	1	17				17	88.24
Bosque 2017	MALE17_50	2	19.45	0.54	2	23.5	10.60	16	31	47	53.19
Bosque 2017	MALE17_52	2	20.4	0.61	2	26.5	4.94	23	30	53	86.79
Bosque 2017	MALE17_74	2	20.2	0.62	2	20.5	0.70	20	21	41	100
Bosque 2017	MALE17_67	*	20.35	0.5	3	21.33	6.11	16	28	64	93.75
Bosque 2017	MALE17_77	2	20.4	0.6	2	18.5	2.12	17	20	37	48.65
Bosque 2017	MALE17_81	4	21.05	0.53	4	15.66	1.41	15	16	60	96.67
Bosque 2017	MALE17_82	5	20.6	0.54	5	16	3.08	14	17	80	95
Buritizal 2017	MALE17_86	1	20.6	0.54	1	18				18	100
Buritizal 2017	MALE17_70	2	20.6	0.57	2	21	2.82	19	23	42	59.52
Buritizal 2017	MALE17_83	4	21.7	0.68	4	18	2.21	16	20	69	94.2
Buritizal 2017	MALE17_84	2	20.1	0.49	2	21.50	4.94	18	25	43	100
Buritizal 2017	MALE17_85	2	20.35	0.58	2	22	0	22	22	44	97.73

*Males were not the genetic father of the clutches close to them.

5 cm). For attending males, we recorded the time that the male spent hydrating or touching egg clutches during the embryonic development period and photographed clutches to determine development and survivorship of embryos. Observations were recorded (for 30–40 min) using a digital video camera recorder with an infrared night-shot mode (Sony Handycam AVCHD) to minimize disturbance. When a new egg clutch was found, we recorded the number of eggs and embryonic development stage (Gosner 1960) and later classified them into two developmental categories: early-stage embryos (stages 1–19) and late-stage embryos (stages 20–25; following McDiarmid and Altig 1999). The time required for eggs to hatch ranges from 15 to 20 days, so, when embryos were close to hatching, we positioned a plastic bag below the clutches to collect them. We euthanized adults and larvae after the experiment and collected tissues from 13 females, 11 nonattending males, and 25 attending males with their clutches, which were fixed in 100% ethanol for molecular parentage analyses. Adults (males and females) and tissue samples are accessioned at the Coleção de Anfibios Célio F.B. Haddad, Departamento de Biodiversidade, Universidade Estadual Paulista "Júlio de Mesquita Filho," Rio Claro, São Paulo, Brazil.

DNA extraction and microsatellite genotyping

For DNA extraction, we used a variation of the HotShot (hot sodium hydroxide) method (Truett et al. 2000). Small pieces of the tail for larvae and liver or toe clip for adults (males and females) were individually placed into each well of a 96-well PCR plate with 50 µL of 25 mM NaOH and two drops of mineral oil. The samples were placed at 95 °C for 1 h and cooled to room temperature. Fifty microliters of a neutralization buffer (40 mM Tris-HCl) was added to each well and mixed. We performed three multiplexes with three 22-locus multiplex mixes (66 polymorphic microsatellite loci) (https://doi.org/10.5061/dryad.0rc447b) using an optimized protocol for high-throughput amplicons. First, we performed a multiplex PCR in a 10 µL volume (5 µL 2× master mix, 2.5 µL water, 1 µL primer mix, and 1.5 µL DNA template) for each sample on an ABI Geneamp 9700 using the following parameters: 95 °C–15 min, 30 cycles of 94 °C–30 s, 56 °C–1 min 30 s, 72 °C–1 min 30 s, and final hold at 15 °C. Two microliters of each PCR product were pooled with an equal volume of water in a new 384-well plate. The diluted PCR products were used as template for a second PCR to add Illumina Nextera dual barcode sequences with an initial denaturation at 92 °C (2 min) followed by six cycles of 94 °C–30 s, 62 °C–1 min, 68 °C–1 min, and a final extension at 68 °C–10 min. Two microliters from each barcoded sample were pooled, cleaned with 0.7× sample volume Ampure XP and resuspended in 0.5× AE buffer. Libraries were quantified using a Qubit DNA broad range assay kit and a Qubit 2.0 Fluorometer and submitted for sequencing on an Illumina MiSeq (v2 kit, 2 × 250 bp) at the Biotechnology Resource Center at Cornell University. Sequences were demultiplexed using bcl2fastq Conversion Software 296 v2.18 (Illumina, Inc.). All bioinformatic analyses were conducted using a custom amplicon.pl script (Supplementary Appendix S1) in a PuTTy terminal for Linux software.

Male mating success

We measured male traits (intrinsic variables) and territory characteristics (extrinsic variables) potentially under sexual selection in anurans (Liao et al. 2013; Correia et al. 2018) as predictors of male mating success. Intrinsic variables included snout-vent-length (mm, $N = 36$ males), body mass (g, $N = 36$ males), and calling persistence (defined as the percentage of nights in which each individual was recorded vocalizing during observations) of all attending and nonattending males. All size measurements were recorded to the nearest 0.5 mm using digital calipers and to the nearest 0.5 g using a precision analytical balance. Extrinsic variables included the length and width of the leaf (in centimeters) where we found males and their clutches, as well as the vertical distance (height) of the leaf from the water (in centimeters). The number of clutches that each male was caring for was considered as the measure of mating success. We compared the body size, body mass, calling persistence, and territory characteristics of attending and nonattending males using a *t*-test. We used the Shapiro–Wilk and Levene's test to check for normality and homogeneity of variances, respectively. In addition, we used a multiple linear regression model to evaluate possible associations among body condition (body mass over body length) of the attending males, the total number of clutches, and number of eggs within each clutch. We also plotted the residuals to test for normality and homoscedasticity to check the validity of the models.

To test whether male traits and territory characteristics were potential determinants of mating success, we used a generalized linear model with male mating success as the response variable and male status (attending and nonattending), body condition, calling persistence, and territory characteristics (leaf length, width, and height) as predictor variables. We also built models considering the additive effect and interaction between variables. For each analysis, we compared the fit of all alternative generalized linear mixed models using the Akaike information criterion (AICc) to rank and calculate the relative weight of all models fitted to the data. To test whether females of *H. cappellei* prefer to spawn in the territory of males already associated with egg clutches, we estimated the probability of a male gaining a first clutch (in the case of nonattending males) or a new clutch (in the case of attending males) using a logit model with all the observed males ($N = 36$). Finally, we also examined if the number of clutches accumulated by successful males was related with caring for clutches at the same stage (early- or late-stage embryos) versus caring for clutches at different stages using a Kruskal–Wallis test with a Dunn test as a post hoc analysis. We implemented all the models using the packages lme4, glmm, and AICcmodavg in R 3.1.0 (R Core Team 2014).

Mating system and mate selection

We created a genotype matrix with 1063 samples (13 females, 36 males, and 1014 tadpoles) for 66 microsatellite loci and discarded loci and samples that had >20% missing data. Observed/expected heterozygosity, polymorphism information content, the frequency of null alleles, and the average nonexclusion probability of each locus were estimated based on the allele frequencies of 49 adults (13 females and 36 males) and 49 loci using the computer program Cervus 3.0.7 (Marshall et al. 1998; Kalinowski et al. 2007). Hardy–Weinberg expectations and genetic linkage disequilibrium between pairs of loci were also calculated using Cervus 3.0.7.

Two types of analysis were performed: parentage assignment and sibship reconstruction (Jones et al. 2010). Parent–offspring assignment was estimated in Cervus 3.0.7, which assigns the most-likely parent (father) to each offspring based on a pairwise likelihood comparison approach. The program generates locus-by-locus likelihood scores for each candidate parent for each offspring and assigns parentage to a candidate parent with the highest logarithm of odds (LOD) score (Marshall et al. 1998; Kalinowski et al. 2007). The program Colony 2.0.6.4 was used to confirm the parent–offspring pairs assigned with Cervus, to identify siblings, and to reconstruct the genotype of unsampled parents. Colony infers sibships and parentage among individuals using multilocus genotypes and a full-pedigree likelihood method. The program considers the likelihoods of entire pedigree configurations rather than individual pairs of offspring and parents (Wang 2004; Jones and Wang 2010).

For all Cervus analyses, we specified all sampled males ($N = 36$) as "potential fathers" and females ($N = 13$) as "potential mothers," with default typing error of 1% and default levels of 80% (relaxed) and 95% (strict) confidence. In Colony, we selected the full likelihood model with medium precision and polygamous mating for both males and females without inbreeding. Based on our field observations, we excluded the possibility of multiple maternity for a clutch but did not exclude multiple paternity. We chose the strong sibship prior parameter, a paternal sibship size of 80 (because a male can attend up to six clutches; ~120 eggs) and a maternal sibship of 20 (each clutch has ~20 eggs), and provided a list of known maternal (for some offspring) and paternal sibships for improving Colony analysis. For both programs, all 49 genotyped adults (36 males and 13 females) were considered as candidate parents for each offspring in each analysis.

Parent–offspring assignments in Cervus agreed with 88% of assignments in Colony. Parental assignments were accepted if ≤2 allele were mismatched between the candidate parent (mother or father) and each larva within the clutch. In the cases where Cervus and Colony could not assign a sampled parent to certain clutches, we assumed that the parent was unsampled and assigned a parental genotype inferred by Colony to these clutches. We used the R package *related* (Pew et al. 2015) to estimate pairwise relatedness for four degrees of kinship: full siblings, half siblings, nonrelated, and parent–offspring. Moreover, we simulated 100 pairs of individuals of each degree of relatedness to obtain reference intervals (first to third quartile) for expected pairwise relatedness. Based on these simulations, we defined full siblings as all individual pairs with r values >0.45, half siblings with values between 0.2 and 0.45, and unrelated individuals with values below 0.2 (Supplementary Figure S1).

We also estimated the relatedness between each possible mated pair using the Wang relatedness estimator (Wang 2002, 2006) in Coancestry 1.0.1.8, using the genotypes from collected individuals, or reconstructed genotypes of males and females in Colony, to determine whether females are possibly choosing mates based on relatedness (e.g., by avoiding to mate with close relatives). We conducted an *F*-test between the Wang relatedness values of the observed and the estimated mated individuals from our study population to test for variance differences. Then we used a one-tailed *t*-test to determine whether the expected pairwise relatedness under the assumption of random mating differed from the observed pairwise relatedness from our study population. We also tested whether the estimated pairwise relatedness values of females and their mated males ("mates") differed from their relatedness to all other males ("nonmates") using a paired *t*-test. Finally, we used a Pearson correlation to test whether the attendance frequency was correlated with the relatedness degree of the male in each clutch. We measured attendance frequency as the time that a male spent close (touching and hydrating) to the clutch during our time of

behavioral observations as mentioned above. To generate a visual representation, we constructed a network graph showing all inferred mated pairs from Colony using the igraph package in R and the program Cytoscape 3.6.1.

Ethical considerations

Collections and field sampling procedures were approved by the Ethics Committee on Animal Use at Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil (Protocol # 9457) and the Instituto Chico Mendes de Conservação da Biodiversidade/Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (license number 51479-4).

RESULTS

We monitored and sampled 49 individuals of *H. cappellei* (36 males and 13 females) and 1407 larvae from 76 clutches. Males typically bred and cared for their clutches in their territories. After mating, females leave the male territories, whereas males remain with the eggs day and night during the developmental period (Figure 1a). Among attending males, 31.25% cared for one clutch in their territories and 68.75% cared simultaneously for multiple clutches at various stages of embryonic development (Figure 1b). On average, attending males cared for 2.4 clutches (standard deviation [SD] = 1.43, range = 1–6 clutches, $N = 76$ clutches) with a total number of eggs per clutch from 15 to 53 ($\bar{X} = 30.31$ eggs, $N = 76$ clutches). We found no differences in the size of clutches (number of eggs per clutch) cared for by individual males; indeed, the total number of eggs accumulated by each male was positively correlated with the total number of clutches ($F_{1,20} = 71.59$, $r^2 = 0.77$, $P = 4.85e^{-08}$). During embryonic development (approx. 15–20 days), males used their hands and feet to rotate and clean the eggs, which prevents fungal infections (Valencia-Aguilar et al. in review), and they used their ventral surface to hydrate their clutches (Figure 1c) until the embryos hatched. Some embryos died due to predation, although, on average, 90% (SD = 14.63) of the embryos attended by males survived to hatching (Figure 1d).

Male mating success

All males included in the study were observed calling during our nighttime surveys; however, not all males obtained mates; 67% of the males were successful, whereas 33% did not acquire any clutches. We found no differences in body size (*t*-test: degrees of freedom [df] = 29.98, $T = 0.52$, $P = 0.60$), body mass (*t*-test: df = 29.50, $T = -0.81$, $P = 0.42$), calling persistence (*t*-test: df = 17.87, $T = -0.41$, $P = 0.67$), or characteristics of the territories (perch height *t*-test: df = 29.92, $T = 1.46$, $P = 0.15$; leaf length *t*-test: df = 16.78, $T = 1.37$, $P = 0.18$; leaf width *t*-test: df = 27.18, $T = 0.07$, $P = 0.94$) between attending and nonattending males. We also found no correlation between male body condition and the total number of clutches ($r^2 = 0.03$, $P = 0.15$) or eggs per clutch ($r^2 = 0.08$, $P = 0.05$) cared for by males. Our analyses showed that male status (attending, nonattending) was the only significant predictor for the mating success of males ($P = 8.48e^{-09}$), with attending males having significantly higher probability of obtaining another clutch. Although we found no significant differences between attending and nonattending male territories and calling persistence, nonattending males had a small chance of obtaining their first clutch (estimated probability = 0.27, 95% confidence interval [CI] = 0.17–0.416; Figure 2). However, once males started caring for one clutch, the probability of obtaining more clutches more than doubled (estimated probability = 0.671, 95% CI = 0.604–0.732; Figure 2).

We also found that clutch number had a marginal influence on male mating success (*t*-test: df = 4, $T = 2.09$, $P = 0.05$). Males caring for multiple clutches received new clutches of eggs more

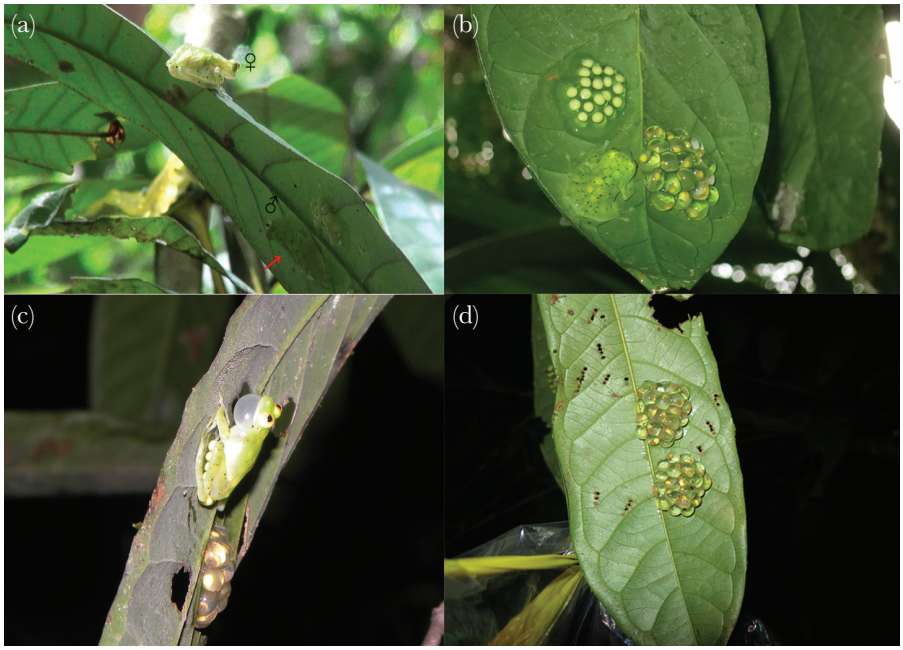

Figure 1
Mating and parental care behaviors in *Hyalinobatrachium cappellei*. (a) Female leaving the male's territory after spawning; male indicated by the red arrow. (b) An attending male resting during the day close to his clutches. (c) A male hydrating his newest clutch. (d) Embryo ready to hatch (Gosner stage 25); plastic bags were positioned below the leaves to collect emerging tadpoles.

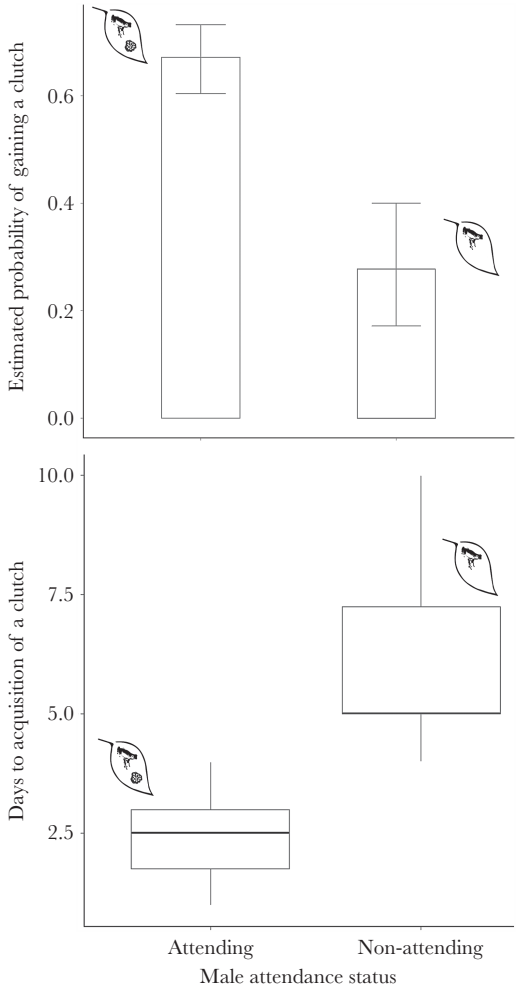

Figure 2
Estimated effect of care status on male reproductive success in the glass frog *Hyalinobatrachium cappellei*. (a) Attending males have a better chance of attracting females to deposit a new clutch compared to nonattending males. Probabilities were estimated using the coefficients of the selected model and the 95% CIs for each male group (attending and nonattending). (b) Delay time for attending males to acquire a new clutch and for nonattending males to acquire their first clutch.

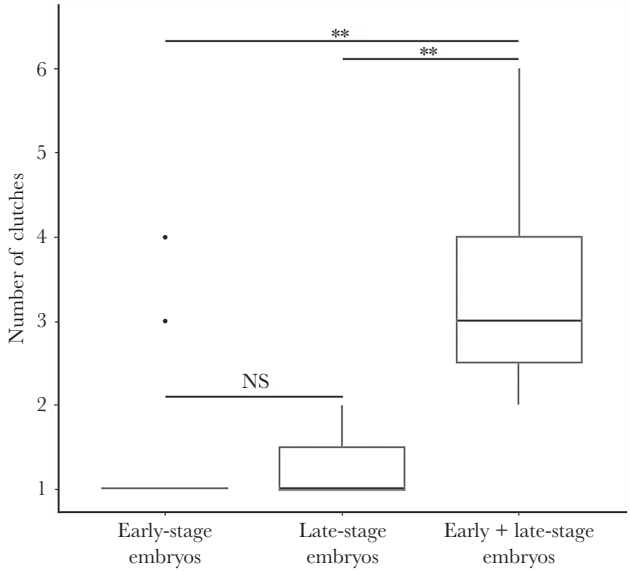

Figure 3
The total number of clutches acquired by male *Hyalinobatrachium cappellei* caring for clutches at similar and different developmental stages. The line in each boxplot indicates the median number of clutches, the bottom and top edges of the box mark the first and third quartiles, and the boxplot whiskers define the range. Circles indicate outlier values. Males attending more than one clutch have clutches at different developmental stages, indicating that females oviposited sequentially, over a period of time, during the time period each male held his territory. Asterisks indicate significant differences between groups.

frequently (on average, after 2.13 days) compared to males caring for only one clutch (on average, after 3.46 days). Moreover, although we found more males (68%) caring for clutches at the same stage (only early-stage or only late-stage embryos) than males (32%) caring for clutches at different stages (early-stage embryos + late-stage embryos), males caring for embryos at different stages obtained more new clutches ($P = 0.003$) than males caring for clutches at only one developmental stage ($P = 0.89$; Figure 3). Both males caring for clutches at different embryonic stages and males caring for embryos at the same stage spent, on average, 82% of their time close to the clutches; however, males caring for clutches at different stages acquired additional clutches faster; thus, it is possible that egg developmental stage could be another factor influencing female mate choice.

Mating system and female choice

Estimates of mating system and mate choice were performed using genetic parentage analysis for 36 males, 13 females, and 938 larvae (from 57 clutches; Table 1). Because females in our focal species leave the mating territory shortly after amplexus, our capture rates for females were lower than for males. For 119 larvae (from seven clutches), we were able to assign both parents from the study area and, for the remaining 819 larvae (from 50 clutches), a known male was assigned and the female's genotype was inferred but not assigned to a sampled female. We found no evidence of multiple paternity or maternity within clutches; all clutches were sired by one parent pair. In all cases of males with multiple clutches, we found that a different female spawned each clutch (Figure 4). Eight of the 13 females sampled in the field were the parent of at least one clutch. In the four cases when a female was observed in amplexus with a male or close to a recent clutch, our data confirmed their parentage of the eggs in that clutch.

Our data also revealed that both females and males mated with multiple partners during each breeding season. However, although males often mate with different females, we found that females sometimes mate again with the same or a different male (FEMALEAVA79 mated twice with MALE17AVA83, FEMALE 55 mated with MALE17AVA86 and MALE17AVA70; Figure 4). In both cases, these female remating events occurred approximately 2–3 weeks apart ($\bar{X}$ = 15–20 days). We estimated these clutch intervals by comparing ovipositon date between clutches after having maternity of all the sampled clutches.

Males were not always the genetic fathers of the clutches close to them. For 49 (85.96%) of the 57 sampled clutches, the attending male observed in the field was assigned as the genetic father in our analyses. However, in eight cases (14.03%), males were not genetically related to the clutches in their territories. Specifically, males AVA15 and AVA67 each had three clutches and AVA20 had two that were not fathered by them. Although these males remained close to the clutches during the day and night while calling, they did not attend the eggs and we observed none of the typical

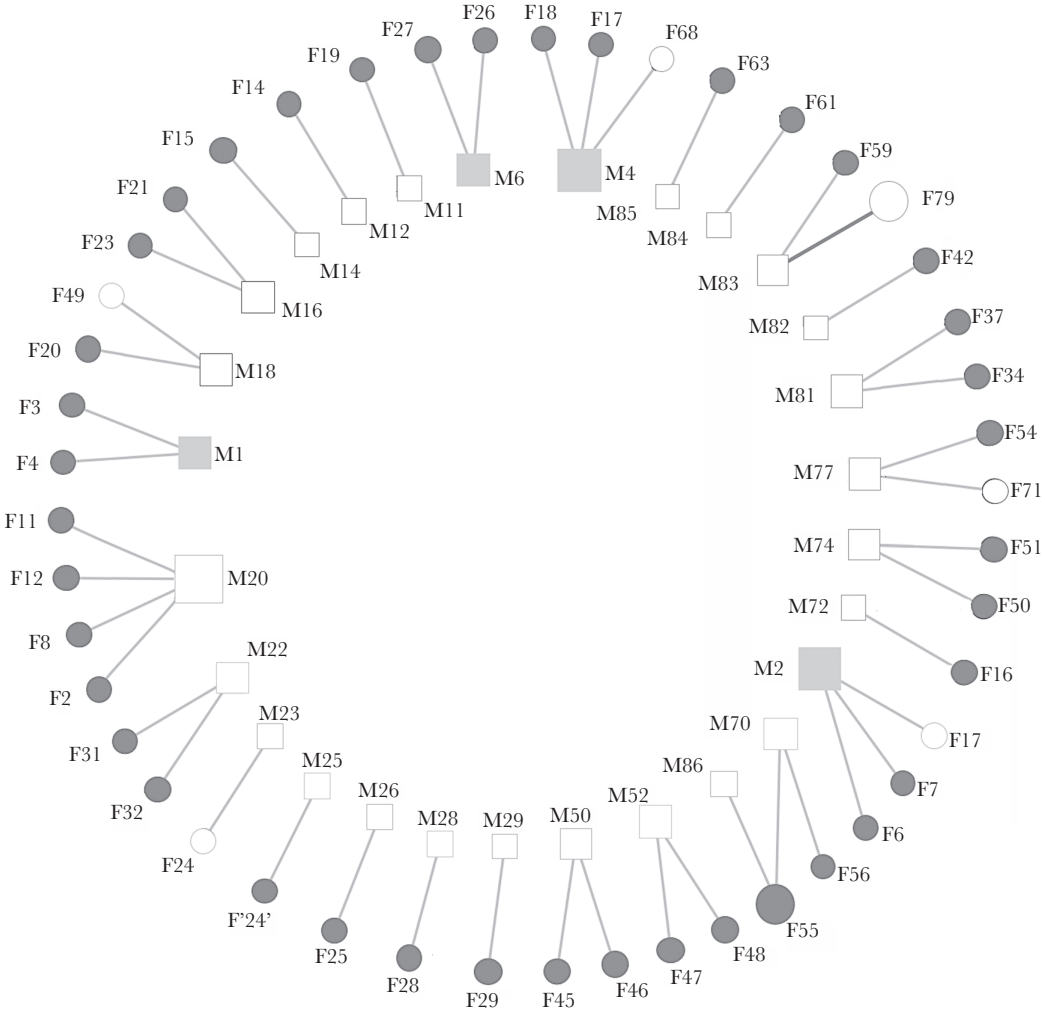

Figure 4

Mating network for *Hyalinobatrachium cappellei* during two breading periods at Cotriguaçu, Mato Grosso, Brazil. Males and females are represented as squares and circles, respectively. Simulated female and male genotypes are shaded, symbol sizes represent the number of mating partners per individual (1–4), and line width represents multiple matings by the same pair (1–2).

hydrating or touching care behaviors. After approximately 5 days, males AVA15, AVA20, and AVA67 were observed caring for new clutches (AVA15 one, AVA20 two, and AVA67 six clutches) on a new leaf within the same territory, and the original clutches, not fathered by them, were abandoned.

We were only able to analyze the degree of relatedness between males AVA15 and AVA67 and their original clutches, not the subsequent new clutches, because we lost the new ones in a heavy rainstorm. However, we analyzed one of the new clutches that AVA20 cared for and we found that he was the genetic father of the entire clutch. In fact, analyzing behavioral and genetic data together, we found that attendance frequency was associated with the degree of relatedness between males and offspring (r_p = 0.97, P = 2.2e^{-16}). When a male was the genetic father of a clutch, he spent one-third of his time touching or hydrating the eggs (after or during rains) until hatching. However, males AVA15, AVA20, and AVA67, the males associated with clutches that were not their own, called close to nonrelated clutches and performed no parental care until they successfully obtained their own clutches.

We estimated relatedness among all 2419 possible male–female paired dyads from the inferred and collected parental genotypes and found two full-sibling pairs (0.08%), seven half-sibling pairs (0.28%), and 2410 unrelated pairs (99.62%). In our observed mated sample, we found that only 2.22% of all matings occurred between half siblings and 97.77% between unrelated individuals. The observed and expected matings in each relatedness category did not differ significantly (*t*-test: df = 3.99, T = 0.004, P = 0.99), indicating that males and females are mating randomly. Likewise, the relatedness of the female and their mates did not differ from that of females and other potential partners in the population (*t*-test: df = 8.75, T = 1.57, P = 0.151), indicating that kin-biased mate selection (either positive or negative) is not occurring in this population.

DISCUSSION

We found that male mating success in our focal glass frog species is not related to male condition, calling persistence, or territorial quality but instead to male care status; attending males have a greater chance of gaining new clutches compared to nonattending males. This indicates that female choice and, consequently, male reproductive success in *H. cappellei* is directly related to parental

effort. Our results support the hypothesis that glass frog species with obligate parental care depend on signals of previous male mating success for female mate choice. However, glass frog species vary in whether they have parental care or not and, in the species with care, the degree of parental investment also varies widely (Delia et al. 2017). Previous studies in some glass frogs with paternal care have shown that larger males, calling continuously (*Centrolene savagei*; Vargas-Salinas et al. 2014; Ospina-L et al. 2017), from higher calling sites (*Hyalinobatrachium fleischmanni*; Greer and Wells 1980) obtained more mates. However, in *Hyalinobatrachium aureoguttatum*, *H. cappellei*, and *Hyalinobatrachium valerioi*, the mating success of males was not associated with male traits, territory quality (the height, distance to water, water depth, leaf area and temperature, canopy coverage, stream velocity, pH, and dissolved oxygen) or relatedness (Valencia-Aguilar et al. 2012; Mangold et al. 2015; Noronha and Rodrigues 2018). Given this interspecific variation, it will be interesting to further examine whether care status is more frequently used as a signal for female mate choice in species with higher dependence on paternal care.

In *H. cappellei*, offspring survival is highly dependent on male care; mortality in attended clutches averages 14%, whereas, in nonattended clutches, mean mortality is 93% (Valencia-Aguilar et al. in review). Hence, as we predicted, females use male attendance as an indicator of male quality and commitment to paternal care (Kokko et al. 2006; Kelly and Alonzo 2009) and, therefore, males in association with clutches will be more successful (Zahavi 1975; Price et al. 1993; Alonzo 2012). Two nonexclusive hypotheses could explain why females would choose already mated males. According to the parental investment hypothesis (Trivers 1972; Sargent 1988), females of *H. cappellei* should use this signal to directly increase the survival of their offspring during the period of paternal care. On the other hand, by choosing already mated males, females could increase their fitness by using already existing clutches as an indicator of male quality (female copying; Gibson and Höglund 1992; Alonzo 2008). Not only did attending males gain more new clutches but our results also showed that the number of clutches and diversity in egg developmental stage were also positively correlated with male mating success. Males caring for multiple clutches at different ages acquired new clutches faster than males caring for just one clutch or clutches at the same developmental stage. These results indicate that females potentially use information from the egg clutches themselves in their decision-making. Because males usually leave their territories a few days before embryos hatch (Valencia-Aguilar et al. in review), females should prefer to spawn in territories containing early-stage embryos, ensuring that males will remain longer caring for embryos. Similar patterns have been observed in some fish and arthropod species with paternal care, where female mate-choice is based on egg presence (Kraak and Groothuis 1994; Requena and Machado 2015), number of eggs (Kraak and Weissing 1995; Fagundes et al. 2007), and egg developmental stage (Sikkel 1989, 1994; Matsumoto et al. 2011). For example, garibaldi fish (*Hypsypops rubicundus*) and barred-chin blenny (*Rhabdoblennius nitidus*) females (Sikkel 1989; Matsumoto et al. 2011 as *Rhabdoblennius ellipes*) prefer not only to deposit eggs among other clutches to reduce the risk of predation per egg but also preferentially deposit eggs close to clutches at early developmental stages, thus extending the time of developmental overlap during which eggs would remain together. Although we found that females prefer attending over nonattending males, it is not clear how (e.g., by chemical, tactile, or visual signals) females discriminate specific characteristics of eggs, such as number or age, and the mechanisms of female signal reception still need further investigation.

Parents of most glass frog species decrease parental care at later embryonic stages (*H. valerioi* [Vockenhuber et al. 2008]; *H. aureoguttatum* [Valencia-Aguilar et al. 2012]; *Hyalinobatrachium orientale* [Lehtinen et al. 2014]; and *Ikakogi tayrona* [Bravo and Delia 2015]). However, when males acquire new clutches, they continue caring for all clutches, thus increasing overall larval hatching success (Delia et al. 2017). In *H. cappellei* (Valencia-Aguilar, personal observations) and *H. fleischmanni* (Delia et al. 2014), predators preferentially consume embryos with thinner jelly layers, which is caused by low hydration levels due to the lack of male care. Thus, by extending care for their offspring and attending to both early- and late-stage embryos with the same frequency, males not only increase their mating success but also reduce the probability of predation on their offspring. These results are consistent with previous models of sexual selection applied to the evolution of parental care (Kirkpatrick 1985; Alonzo 2012), where paternal care is favored not only by natural selection because of offspring survival (Gross 2005) but also by sexual selection because of the evolution of female preferences (Alonzo 2012).

Our genetic analysis showed that our focal species is polygamous, with most males and some females of *H. cappellei* mating more than once. Among sampled individuals in our study population, we found no differences in mating success between males (67%) and females (61%), and those rates were similar to those observed in other glass frogs (*H. valerioi*; Mangold et al. 2015) and poison frogs (*Allobates femoralis*; Ursprung et al. 2011); nonetheless, we found a slightly higher mating frequency in males compared with females. In glass frogs, mating success in males is limited by their ability to attract females and hold a territory, whereas females are limited by their energetic investment in egg production (Wells 2007). So, the low female mating frequency in *H. cappellei* is likely due to differences in the cost of gamete production between females and males (Bateman 1948) because it takes approximately 15–20 days for females to mate again once they spawn.

Our results also support the hypothesis that males of *H. cappellei* adopt an alternative strategy to improve their mating success while avoiding the costs of parental care. The majority of attending males were the genetic father of the egg clutches that they cared for; however, we also found that some males (11%) remained close to unrelated clutches without caring for them. Studies with other externally fertilizing species have shown that males either adjust their paternal effort in relation to the degree of paternity (Chen et al. 2011) or care for related and unrelated offspring (Östlund-Nilsson 2002). For instance, in the arboreal frog *Kurixalus eiffingeri*, 15-spined sticklebacks (*Spinachia spinachia*), and bluegill sunfishes (*Lepomis macrochirus*), males either shared paternity or were not even fathers of the clutches in their nests (Neff and Gross 2001; Östlund-Nilsson 2002; Neff 2003; Chen et al. 2011). Field experiments showed that males of the arboreal frog and the bluegill sunfish were able to assess their paternity, adjusting parental effort (Neff and Gross 2001; Neff 2003; Chen et al. 2011), whereas males of the 15-spined stickleback did not (Östlund-Nilsson 2002). In contrast, we never observed male–male interference during amplexus or any evidence of sneaker males and, in fact, attending males of *H. cappellei* almost always had full paternity of their clutches and were able to recognize and care only for their own offspring. In the few cases where males were not the genetic fathers of the clutch, they did not care for them but remained close. Because caring activities are costly, we infer that males of *H. cappellei* are using

abandoned clutches or they have successfully usurped territories of other males to obtain partners at the expenses of the mating investment of another male (Clutton-Brock 1991; Klug et al. 2012). We never observed territorial fighting in *H. cappellei*; however, this behavior has been documented in other glass frog species with parental care (*Hyalinobatrachium colymbiphyllum*; Savage 2002; *H. fleischmanni*; Delia et al. 2010; *H. valerioi*; McDiarmid and Adler 1974; *Cochranella granulosa*; Kubicki 2007; *Centrolene daidalea*; Rojas-Runjaic and Cabello 2011). Independent of whether usurped clutches are abandoned or are the result of territorial takeovers, the strategy of using foreign clutches as a signal to females clearly works. The males we observed associated with unrelated clutches obtained their own clutches only a few days later, underscoring our findings that egg attendance is a successful mating strategy and increases male attractiveness. Alternative reproductive tactics to maximize individual fitness are common in animals (Taborsky et al. 2008). Typically, adopting a particular alternative strategy depends on some aspect of the status or condition of an individual relative to others in the population (Andersson 1994). In *H. cappellei*, we found no differences in body size or territory quality between successful and nonsuccessful males, and the males found in association with unrelated clutches were not smaller or in poorer condition; thus, we have no evidence that this strategy is adopted by poorer males that otherwise would not have the opportunity to mate. However, the advantage of having a clutch for male mating success is significant; our data show that, by occupying the territories of previously successful males, the mating success of nonpaired males could double. Therefore, we propose that this alternative strategy of usurping signals of reproductive success might play a key role in maximizing male fitness in this species.

In summary, our results show that females of *H. cappellei* mate randomly relative to male body size, calling persistence, characteristics of their territories, and their kinship with males in the population. However, females have a notable preference for males already associated with clutches, highlighting the importance of male parental care for female choice. Likewise, association with egg clutches likely increases male attractiveness because some males use unrelated clutches to attract females while avoiding parental care costs. Male *H. cappellei*, thus, obtain higher fitness via paternal investment, increasing mating success, as well as survival of offspring. Finally, it seems that parents are able to recognize their own eggs, although the mechanism used to do so remains to be investigated. The close association between mates during amplexus and the high degree of paternity certainty likely contribute to male strategies and female choice in this species.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Data availability: Analyses reported in this article can be reproduced using the data provided by Valencia-Aguilar et al. (2019).

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