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MATING STRATEGIES AND PARENTAL CARE IN GLASSFROGS (AMPHIBIA ANURA CENTROLENIDAE)

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Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de doutora em Ciências Biológicas (Área de concentração: Zoologia).

Março-2020

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ABSTRACT

Many species in the Centrolenidae family, know as glassfrogs, exhibit paternal care of eggs. Although parental care promotes offspring survival, it may impose survival and mating cost; hence, males should intensify or weaken resource allocation between its fitness' components. Furthermore, anuran males compete to attract females, but since it is an energetically demanding activity, in some cases individuals use some tactics to offset these costs and gain access to mates. In this thesis, I studied different aspects of the paternal care and mating strategies in glassfrogs species, which were separated in four chapters. In chapter 1, we made field observations and conducted behavioral experiments in attending and non-attending males of the glassfrog *Hyalinobatrachium cappellei* to examine the importance of attending activities on offspring survival, as well as the effects of predation risk on male's behavior. We also assessed how parental care influences time allocation in males' activities. We found that care activities do not affect male's mating success and that paternal care is crucial to avoid embryo dehydration. Besides, our results show that although attending males are more risk tolerant, they will prioritize their own survival, even with negative effects on offspring, when the threat reaches a certain threshold. In chapter 2, we examined female mate preference in *H. cappellei*. Our results indicate that females of *H. cappellei* do not select males based on body condition, call persistence, or territory characteristics. Instead, females choose males based on care status; attending males had higher chance of attracting new females and gain new clutches. Additionally, we also found that males adjust their parental care effort in response to genetic relatedness, by caring only for their own

offspring; however, they may remain close to unrelated clutches as a strategy to attract females and increase chances of successful mating. In chapter 3, we experimentally investigated the existence of allopaternal care in two centrolenid species, *Hyalinobatrachium chirripoi* and *Centrolene peristicta,* as well as the possible costs and benefits of this behavior. Attending males of both species males adopted unrelated clutches, whereas non-attending males abandoned their territories when unrelated clutches were placed close to them. Contrary to our expectations, once males adopted the unrelated clutches, they cared for all the eggs in their territories with a similar frequency. Our finding suggest that alloparenting in glassfrogs only occurs in males that already have clutches of their own and, most likely, cannot distinguish unrelated clutches. Finally, in chapter 4, we tested whether glassfrog males that care for their offspring have smaller relative testes volume compared with species that do not exhibit paternal care. We collected data on testis size of breeding frogs using information from specimens we have collected, as well as from museum specimens, and literature. For the 26 species of glassfrogs included in our data set, we found that attending males tend to have smaller testes compared with non-attending males in glassfrogs. Besides, no significant effects of clutch size on testes size were detected. These results suggest that testes size may also vary in response to a pots-fertilization investment, such as parental care.

Keywords: Parental care, offspring, female choice, aloparental care, testes size

RESUMO

Em muitas espécies da família Centrolenidae, conhecidas como rãs-de-vidro, os machos cuidam de seus ovos. No entanto, apesar do cuidado paternal aumentar a sobrevivência da prole, pode impor custos de sobrevivência e acasalamento, portanto, os machos devem aumentar ou diminuir a alocação de recursos entre os componentes do seu *fitness*. Além disso, os machos competem para atrair fêmeas, mas, como é uma atividade exigente em termos energéticos, em alguns casos os indivíduos usam algumas táticas alternativas para compensar esses custos e obter acesso às parceiras. Nesta tese, eu estudei diferentes aspectos do cuidado paternal e estratégias de acasalamento nas rãs-de-vidro, separados em quatro capítulos. No capítulo 1, fizemos observações de campo e realizamos experimentos comportamentais com machos e desovas de *Hyalinobatrachium cappellei* para examinar a importância do cuidado na sobrevivência da prole, bem como avaliamos os efeitos do risco de predação no comportamento dos machos. Também avaliamos como o cuidado paternal influencia a alocação de tempo nas atividades dos machos. Descobrimos que as atividades de cuidado não afetam o sucesso de acasalamento nos machos e que o cuidado paterno é crucial para evitar a desidratação dos embriões. Além disso, nossos resultados mostraram que, embora os machos que cuidam desovas sejam mais tolerantes ao risco, eles priorizam sua própria sobrevivência, mesmo com efeitos negativos para a prole, quando a ameaça atinge um certo limiar. No capítulo 2, examinamos a preferência por parceiros nas fêmeas de *H. cappellei*. Nossos resultados indicam que as fêmeas não escolhem machos com base na condição corporal, persistência do canto

ou características do território. Em vez disso, as fêmeas escolhem machos com base no *status* de cuidado; machos com desovas tiveram maior chance de atrair novas fêmeas e ganhar novas desovas. Além disso, os machos ajustam seu esforço de cuidado em resposta ao grau de parentesco genético, cuidando apenas de suas próprias desovas. No entanto, eles podem permanecer próximos de desovas não relacionadas como uma estratégia para atrair fêmeas e aumentar as chances de acasalamento. No capítulo 3, investigamos, experimentalmente, a existência de cuidado aloparental em duas espécies de centrolídeos, *Hyalinobatrachium chirripoi* e *Centrolene peristicta*, bem como os possíveis custos e benefícios desse comportamento. Machos com desovas de ambas as espécies adotaram desovas não relacionadas, enquanto machos sem desovas abandonaram seus territórios quando as desovas não relacionadas foram colocadas em seus territórios. Contrariamente às nossas expectativas, uma vez que os machos adotaram as desovas não relacionadas, eles cuidaram de todos os ovos em seus territórios da mesma forma e constância. Nossos resultados sugerem que o cuidado aloparental nas rãs-de-vidro ocorre apenas em machos que já possuem desovas próprias e, muito provavelmente, não conseguem distinguir as desovas não relacionadas. Finalmente, no capítulo 4, testamos se os machos das espécies de rãs-de-vidro que cuidam das desovas têm volumes testiculares menores em comparação com as espécies que não exibem cuidados paternos. Coletamos dados sobre o tamanho dos testículos de machos reprodutivos a partir de espécimes em coletados em campo, bem como de espécimes de museus e a partir de literatura. Para as 26 espécies de rãs-de-vidro incluídas em nosso estudo, descobrimos que os machos que cuidam da sua prole têm testículos menores em comparação com os machos que não

cuidam. Além disso, não foram detectados efeitos significativos do tamanho da desova no tamanho dos testículos. Esses resultados sugerem que o tamanho dos testículos também pode variar em resposta a um investimento pós-fertilização, como o cuidado paternal.

Palavras-chaves: Cuidado parental, prole, escolha pela fêmea, cuiado aloparental, tamanho testicular

INTRODUCTION

Mating system is shaped by the reproductive strategies of males and females (Arnold and Duvall 1994), affecting their respective potential rates of reproduction (Clutton-Brock and Vincent 1991). For example, by mating with many females, males can typically increase the number of offspring that they produce. Thus, males are limited in their reproductive success by their access and monopolization of receptive females (Trivers 1972, Arnold and Duvall 1994). Usually males compete to attract females, through male-male competition (intrasexual selection) or display traits that signal their quality as mates (intersexual selection) (Trivers 1972, Andersson 1994). In species where males provide benefits that directly affect female's and offspring fitness, such as nest sites, territories and parental care, there should be strong selection on females to choose males that reliably reflect their ability to provide these direct benefits (Zahavi 1975, Price et al. 1993).

Particularly, in species with paternal care, parents provide care for their offspring and try to increase offspring survival exhibiting simple or elaborate forms of care (Trivers 1972, Ridley 1978, Smiseth et al. 2012). Parental care is broadly defined as any investment in offspring following fertilization of the female gamete (Trivers 1972, Smiseth et al. 2012). Trivers (1972) defined parental care in terms of the reproductive investment of the parent. That is, parental investment is any energy spent to increase the quality or future fitness of an offspring, at the cost of future reproductive success of the breeder, either in the form of reduced condition of the parent, or loss of mating opportunity (Trivers 1972). This inequality between the resource demands of the offspring and the

fitness demands of the parent results in conflict (Klug et al. 2012), leading to a considerable variation in the level and duration of care that parents provide to their offspring (Klug et al. 2012). For example, in some taxa, females invest not only in producing eggs or live offspring but also in care, while in others only the males invest in offspring care (Clutton-Brock 1991, Gross 2005). However, in some cases, parental investment can be equally shared and both parents invest substantial energy and time in parental care (Clutton-Brock 1991). Among vertebrate groups, amphibians show elaborate and conspicuous social and reproductive behaviors and many species with parental care have a terrestrial or semi-terrestrial mode of reproduction (McDiarmind 1978, Crump 1996, Lehtinen and Nussbaum 2003, Gomez-Mestre et al. 2012), making them a natural model system to investigate the relationship between parental investment and mating strategies (McDiarmind 1978, Ursprung et al. 2011, Gomez-Mestre et al. 2012, Summers and Tumulty 2014).

Parental care in amphibians is diverse and know in about 62% of anuran species (35 families) (Furness and Capellini 2019). Care behaviors probably evolved as a response to harsh or unpredictable environmental conditions, high levels of predation, parasitism or intense inter or intraspecific competition on land (Clutton-Brock 1991, Crump 1996, Wells 2007). Therefore, males, females or both display a broad set of parental behaviors, from egg attendance to froglet transport and viviparity to increase offspring survival (Furness and Capellini 2019, Vági et al. 2019). Egg attendance by the male is the commonest form of care in anurans (Vági et al. 2019) and is related with egg protection from pathogens and predators, desiccation (maybe the most important), and handling to prevent developmental abnormalities (McDiarmid 1978, Townsend et al.

1984, Crump 1996, Lehtinen and Nussbaum 2003, Wells 2007). Egg attendance is associated to the permanence of the parent with one or multiple clutches after oviposition, usually at male's territories (McDiarmind 1978, Crump 1996, Lehtinen and Nussbaum 2003, Wells 2007). Some authors have argued that paternal care probably evolve in circumstances where females lay eggs directly in a territory defended by a male (Ridley 1978, Townsend 1986, Werren et al. 1980). Indeed, if a male defends a territory before fertilization, then that sex can be selected as caregiver by association with their offspring and territoriality (territoriality hypothesis; Ridley 1978, Gross and Shine 1981). Consequently, if a male remains in his territory, it is possible that further females will lay their eggs there, reducing the care cost for males (Trivers 1972, Crump 1996). This hypothesis has shown to be plausible in males of numerous species of centrolenids (Greer and Wells 1980, Vockenhuber et al. 2008, Delia et al 2010, Valencia-Aguilar et al. 2012, Delia et al. 2017) and dendrobatids (Weygoldt 1987, Roithmair 1994, Summers and Tumulty 2014), which attend multiple clutches in their territories. In such cases, it is possible that males can offset the care costs by increasing their parental fitness (Lehtinen and Nussbaum 2003). For example, although in *Eleutherodactylus coqui* males lose additional mating opportunities, they improve their reproductive success by providing care to their eggs until hatching (Townsend et al. 1984, Townsend 1986). Besides, as territory defense is energetically expensive, male territoriality associated with parental care likely serve as an honest signal of male quality to females (McDiarmid 1978, Nazareth and Machado 2010, Mangold et al. 2015).

Other important aspects regarding paternal care costs may include mode of fertilization and genetic relatedness between male and offspring (Werren et al. 1980,

Klug et al. 2012). As mentioned above, in species with external fertilization and territorial males, females will lay eggs on the male's territory (Ridley 1978, Crump 1996, Lehtinen and Nussbaum 2003). As a consequence, external fertilization in territorial species could favor the evolution of male care (Ridley 1978, Gross and Shine 1981) because the low sperm competition will increase the level of genetic relatedness between male and offspring, and hence the certainty of paternity (paternity hypothesis; Werren et al. 1980, Gross and Shine 1981, Alonzo and Klug 2012). In this sense, it has been pointed out that parents should invest more in the care of an offspring with a higher degree of relatedness (Alonzo and Klug 2012), since paternal care represents a cost for males (Simon 1983, Townsend 1986). Indeed, models and experimental studies in fishes (Neff and Gross 2001, Neff 2003) and amphibians (Werren et al. 1980, Chen et al. 2011, 2013) have evidenced that males adjust their parental effort in response to variation in parentage degree. However, besides the “certainty of paternity”, other factors as environmental conditions (quality and availability of resources), costs-benefits or mating strategies can lead to variation in parental care behavior (Zahavi 1975, Lion and van Baalen 2007, Alonzo and Klug 2012, Chen et al. 2011).

A parental care strategy employed by some groups is named alloparental care (Riedman 1982, Brown 1998, Ramos et al. 2012), which is defined as the parental investment given by a conspecific individual that is not the genetic father (Wisenden 1999). Although, alloparental behavior can be very beneficial by increasing brood fitness and the reproductive output of the breeder (Wisenden 1999), it entails potential costs to the fecundity and fitness of the alloparent. In fact, individuals may lose all future reproductive potential by taking on the role of alloparent (Roldán and Soler 2011).

However, when the indirect reproductive benefits of adoption outweigh the direct fitness costs, care for unrelated offspring can be used as a strategy (Kalmbach 2006). For example, in Tessellated darters (*Etheostoma olmstedi*, Osteichthyes), females prefer males already associated with eggs, thus males can increase their attractiveness and mating success by caring for unrelated offspring (Stiver and Alonzo 2011). Likewise, males can adopt or include unrelated eggs/youngs into their nest to benefit from decreased offspring predation due to dilution effect, as observed in the cichlid *Cichlasoma nigrofasciatum* (Wisenden and Keenleyside 1994). On the other hand, in the absence of reliable cues to paternity (i.e. by clutch piracy or cuckoldry), selection may favor alloparental behavior in males to avoid potential costs of abandoning their own offspring (Trivers 1972, Clutton-Brock 1991).

As time and energy are limited, any investment in current reproduction may preclude investment in future reproduction or other components of fitness (Gadgil and Bossert 1970, Stearns 1992). For instance, if a male expends energy on current matings by means of sexual ornamentation or gonadal expenditure will have less energy available to offspring assistance (Gadgil and Bossert 1970, Stearns 1992, Requena and Alonzo 2017). Theoretical and experimental studies have considered that energetic limitations can become apparent in male sexual traits (Stiver and Alonzo 2009, Beltran et al. 2010). Indeed, specific sexual morphological features are known to be associated with the mating system in several groups (Pircher et al. 2005, Awata et al. 2006, Soulsbury 2010, Liao et al. 2011, Mascaro et al. 2013), in which it has been established a positive correlation between gonadal expenditure and alternative mating tactics, such as polyandry and clutch piracy, because of sperm competition (Byrne et al. 2002, Vieites

et al. 2004, Pircher et al. 2005). Furthermore, in species with paternal care, as larger testes provide an advantage in sexual competition because they can produce more sperm (Pitnick 1996), males may have evolved large testes to secure paternity and ensure that their efforts benefit their own offspring (Pitnick 1996).

In the glassfrogs (Centrolenidae family), all species are nocturnal and females lay clutches on the vegetation or rocks out of water, along lotic systems (streams and waterfalls), and, following hatching, larvae finish development in the water (McDiarmid 1978, Cisneros-Heredia and McDiarmid 2007, Guayasamin et al. 2009). Parental care of eggs is the main form of parental behavior in this family, with males protecting eggs from predators and desiccation (Jacobson 1985, Hawley 2006, Vockenhuber 2009, Delia et al. 2013, Lehtinen et al. 2014). However, maternal egg attendance has recently been described for one species of glassfrog (Bravo and Delia 2015). Parental care in this family has evolved in species of Centroleninae and Hyalinobatrachinae subfamilies (McDiarmid 1978; Wells 2007, Vargas-Salinas et al. 2014), where high reproductive success has been associated with egg and embryo attendance at male's territory (McDiarmind 1978, Vockenhuber et al. 2008, 2009; Delia et al. 2010, 2013, Valencia-Aguliar et al. 2012, Lehtinen et al. 2014, Delia et al. 2017). Here, we used glassfrog species as models to study mating strategies and parental behavior. In Chapter 1, we assessed how parental care influences time allocation in males' activities, tested the importance of attending activities on offspring survival in *Hyalinobatrachium cappellei*, as well as the effects of predation risk on male's behavior. In Chapter 2, using behavioral and molecular data, we tested hypotheses about the fitness consequences of parental care and the drivers of female mate choice in *H. cappellei*. In Chapter 3, we assessed in

the field the existence of allopaternal care in males of *Hyalinobatrachium chirripoi* and *Cenrolene peristicta* and discussed possible costs and benefits. Finally, in Chapter 4, we investigated whether testes size is related with paternal care in glassfrog males.

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CHAPTER I

MALE CARE STATUS INFLUENCES THE RISK-TAKING DECISIONS IN A GLASSFROG

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Male care status influences the risk-taking decisions in a glassfrog

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Abstract

Parental care increases offspring survival but also increases predation risk of parents. Hence, risk decisions are expected to vary according to the care status and the threat faced by the parents. Here, we tested the importance of egg attendance performed by males of *Hyalinobatrachium cappellei* on offspring survival, as well as the effects of predation risk on male's care behavior. For this purpose, we examined the risk-taking decisions of glassfrog males towards two different dummy predators in different contexts: attending and non-attending males. We found that paternal care is crucial for offspring survival and increase male's mating success. Besides, our results show that although attending males are more risk tolerant, they will prioritize their own survival, even with negative effects on offspring when the threat reaches a certain threshold. Overall, the findings of this study highlight the importance of evaluating the effect of predation risk level on parental risk-taking decisions.

Keywords: Anura, *Hyalinobatrachium cappellei,* male activities, paternal care, predation risk, offspring survival.

Significance Statement

In species with parental care, parents face a trade-off between investment in current offspring survival and chances of future reproduction. Hence, choosing the right strategy to preserve energy and decrease their risk of mortality is an important decision, which may have significant effects on offspring, consequently on parents' fitness. Using field observations and experiments, we found that attending males of the glassfrog *Hyalinobatrachium cappellei* increased not only the larvae hatching success, but also their own mating success. Besides, we also found that males were able to adjust their behavior according to the faced threat. Our results show that differences in reproductive status may play a role in determining the antipredator behavior and mating success in glassfrogs males.

Introduction

Parental care increases offspring survival (Klug et al. 2012), however, may affect parents' fitness by changing resource allocation patterns and increasing predation and injury risks (Clutton-Brock 1991; Klug and Bonsall 2014). Activities such as guarding and defending are energetically costly and as energy and time devoted to care behaviors are limited resources (Trivers 1972, Clutton-Brock 1991; Klug and Bonsall 201), any investment in care will come at the expense of another important activity (Klug et al. 2012, Klug and Bonsall 2014). As a result, parents may face a trade-off between the amount of resources (time and energy) they need to invest to ensure their offspring or their own survival (Trivers 1974; Klug et al. 2012). It has been shown that parents are able to assess and adjust their behavior accordingly to the faced threat (Lima and Dill 1990; Sih et al. 2004). However, any variation in parental care effort can affect the fitness of both offspring and parents (Clutton-Brock 1991; Klug and Bonsall 2014). For example, an increase in offspring defense or provisioning rate can lead to higher offspring survival (Mutzel et al. 2013; Poo et al. 2016; Bebbington and Hatchwell 2016) but with a high cost for the parents (Cook et al. 2001). In contrast, a decrease in parental investment when facing a high risk ensures parents' survival but increases offspring mortality (Ghalambor et al. 2013; Mahr et al. 2015).

Parent's decisions are expected to vary according to the threat that both the offspring and the parents face (Andersson et al. 1980). On one hand, if only the offspring faces a threat, parents can display diverse behavioral strategies as a risk-avoidance response to defend its current offspring against predators or stressful abiotic conditions (Toledo et al. 2011; Consolmagno et al. 2016). For example, a parent can choose

attacking, eating or avoiding potential predators (Simon 1983; Townsend et al. 1984; Bickford 2004; Vockenhuber et al. 2009; Rodrigues et al. 2011), or adjust their care frequency in response to warmest periods (Delia et al. 2013; Lehtinen et al. 2014; Consolmagno et al. 2016) to increase offspring's survival. On the other hand, when the threat is too high to both offspring and adults, parents have to decide between offspring and their own survival and future parental investment (Trivers 1972; Lima and Dill 1990; Coleman and Gross 1991). In other words, based on the type and threat intensity parents will probably prioritize their own survival, taking fewer risks to protect their offspring (Andersson et al. 1980; Consolmagno et al. 2016). Besides, variation in defense response can be determined by predators' traits (Lima and Dill 1990). Hence, based on the predator's size and density, for example, attending males can attack more often adult predators instead of younger ones (Poo et al. 2016) or escape when predator size exceeds their own size (Chuang et al. 2017).

Some species in the Centrolenidae family, known as glassfrogs, exhibit parental care and studies have shown that embryo survival is positively correlated with parents' guard behavior (Vockenhuber et al. 2009; Delia et al. 2013; Delia et al. 2017). Main causes of embryo mortality include fungal infection, dehydration, predation and development failure (McDiarmind 1978). Alterations in both parental behavior and offspring survival have been related to predator-parent interaction (Delia et al. 2017). Although clutch predation has been reported for many glassfrog species (Valencia-Aguilar et al. 2012; Delia et al. 2017; Noronha and Rodrigues 2018), antipredator behavior or male-predator interactions remain poorly studied in this family (Delia et al. 2017; Ospina-L et al. 2019). The glassfrog *Hyalinobatrachium cappellei* occurs in the

Amazon basin (Frost 2020) and individuals reproduce during the rainy season (October to May) in central-north Brazil, along streams or flooded areas (Noronha and Rodrigues 2018). Males vocalize from leaves to attract females, clutches are deposited on the underside of leaves above the water and males care for the clutches, actively hydrating the eggs to prevent dehydration (Noronha and Rodrigues 2018).

As males of *H. cappellei* occupy a fixed territory before and after oviposition (A. Valencia-Aguilar, pers.obs.) and may attend multiple clutches for a couple of weeks (Noronha and Rodrigues 2018), this species is an excellent model to evaluate parents' effort and how threat level posed by different predators modify males' behavior. Thus, here we investigated the species paternal behavior trying to answer the following questions: (1) how do males distribute their time among different activities (e.g., calling, mating, resting, and guarding)? (2) Does egg attendance by males increase embryo's survival and, consequently, larvae hatching? (3) Are attending males more risk-tolerant than non-attending males? We predict that attending males will devote more time on care activities, while non-attending males will spend more time calling to attract females, and that larvae survival and hatching will be higher in those clutches cared for their parents. Although attending males might be more risk-tolerant because of the benefits associated to embryo protection, we predict that they will abandon the clutches and prioritize their own survival when facing a high risk of predation.

Methods

Study area

Field observations of *H. cappellei* were carried out at the São Nicolau farm (9º51'16.9" S; 58º14'57.7" W), from January to February in two consecutive years, 2016 and 2017, in the municipality of Cotriguaçu, north-western of Mato Grosso state, Brazil. The locality is close to the Juruena River with 7000 hectares of Amazon Forest (Rodrigues et al. 2010). The vegetation is classified as dense humid forest, inserted in the Brazilian Meridional Amazonia (Blaser et al. 2011). According to Köppen classification, monsoon climate predominates in the region (Rodrigues et al. 2010), with rainy season (September to March) and a dry season (June to August). Temperature does not differ strongly among seasons (24 to 36°C in the summer and 32 to 38°C in the winter), while rainfall and humidity is greater in the summer (1300-1700 mm, 80%), decreasing in the winter (20-50 mm, 40%) (Bittencourt-Rosa et al. 2013).

Attending and non-attending males' activities

We monitored 65 individuals (*N* = 20 attending and *N* = 5 non-attending males in 2016; *N* = 25 attending and *N* = 15 non-attending males in 2017) and 85 clutches (N = 31 in 2016 and N = 54 in 2017) by diurnal and nocturnal sampling with headlamps through visual and acoustic searches. Individuals and clutches were monitored from 15 to 20 days, which varied according to time spent by males in their territories and embryos' developmental rate. Each male and their clutches were constantly observed and recorded from one-meter distance for 20 - 40 minutes (once in the morning and twice or three times at night) until larvae hatching. We used a digital video camera recorder with an infrared night-shot mode (Sony Handycam AVCHD) to perform nocturnal observations and behavioral experiments to minimize male disturbance.

Individuals were identified using digital photographs of their dorsum spots patterns (Van Lidth de Jeude 1904, Kenyon et al. 2010) and sexed by morphological features; while males were identified by their calls and vocal sacs, females were identified after oviposition or by the presence of oocytes visible through their transparent skin. During focal observations, we recorded male activity (calling, mating, foraging, aggression or defense behaviors against potential predators or competitors), male position relative to eggs (next, touching, and sitting on), egg attendance frequency (proportion of time that male devoted to clutch maintenance during the day, between 08:00-13:00, and at night, between 20:00-02:00), and egg/embryo clutch condition (parasitism, predation, and embryonic stage of development). To determine time devoted to different activities and compare between attending and non-attending males, we calculated the proportion of time that each male spent to each activity (calling, foraging, mating, and attending) relative to the total daily hours of observation (08:00-13:00, and 20:00-02:00). Then, we estimated time budget for each male by summing occurrences of an activity across days and divided by the total days of observation. It was not possible to record data blind because our study involved focal animals in the field.

Benefits of egg attendance: embryo development and survivorship

To characterize and quantify egg care duration in *H. cappellei* and assess its benefits, we compared larvae hatching success between non-attended egg clutches (experimental group; N = 15 attending males removed, being 5 males in 2016 and 10 in 2017) with attended egg clutches (control group; N = 15 attending males not removed, being 10 males in 2016 and 5 in 2017). For non-attending clutches, we removed the

male-caregiver to prevent them from providing care for the eggs, while attended egg clutches received paternal care during embryonic development. Removed males were released ~10 m upstream or downstream from their clutches and none returned to their territories. We examined and photographed each clutch daily (once in the morning and twice or three times at night) to monitor embryo development and to quantify and categorize mortality. We registered embryo mortality by predation, dehydration, or developmental failure. The time required for embryos to hatch since they are laid is about 15-20 days (A. Valencia-Aguilar, pers.obs.), so when embryo were close to hatch we positioned plastic bags below clutches to determine embryo survival. Larvae hatching success was defined as the proportion of hatchlings relative to the number of eggs per clutch.

Predation risk and behavioral response of attending and non-attending males

To understand if male's responses to threat varied as a function of care status, we simulated different levels of risk, using rubber models of a cricket (25 mm) and a spider (50 mm), which were presented to attending and non-attending males. Crickets are common predators of both adults and clutches of the glassfrog *Cochranella granulosa* (Delia et al. 2017). However, based on previous observations (Noronha and Rodrigues 2018), as well as on our own observations between 2015 - 2017, crickets represent a threat for the clutches rather than for *H. cappellei* males. Moreover, we observed a spider preying on clutches of *H. cappellei* and a male leaving his territory after noticing the proximity of a spider, a common defense response to avoid predation (Toledo et al. 2011; Ortega-Andrade et al. 2013). For these reasons, we choose the

cricket and the spider as predators in our threat experiments, representing different levels of threat. We tested our rubber models using a dark resin ball as a control model, with males of *H. cappellei* (*N* = 10) and three sympatric anuran species: *Allophryne ruthveni* (*N* = 5), *Boana* cf. *fasciata* (*N* = 10), and *Dendropsophus brevifrons* (*N* = 10). All individuals used in the validation tests responded similarly to the three models; fled from predators' proximity and ignored resin ball models. After validation, we exposed two groups of attending (*N* = 5 in 2016 and *N* = 5 in 2017) and non-attending males (*N* = 5 in 2016 and *N* = 5 in 2017) to the following threats: a) physical disturbance to simulate a medium-risk predator attack; we used a cricket dummy that represented threat only for the clutches, and b) physical disturbance to simulate a high-risk predator attack; we used a spider dummy that represented threat for both males and clutches. In each treatment, we approached the dummy predators to the males and clutches using a wood stick. Then, we used the dummies to touch and squish the males and clutches (in the case of the attending males), and observed if males remained in their initial position, faced the predator or left the territory. We also registered the types of males' response (raising body, stop calling, remaining alert, fleeing, and cloacal discharge) (Toledo et al. 2011).

Besides, to test the effect of the stimuli variation in males' response, we exposed each group of males to the following test: 1) a random stimuli; for the first group of attending (N = 5 in 2016) and non-attending males (N = 5 in 2016), we presented each dummy during one minute, in a random sequence in consecutive nights. 2) Non-random stimuli; for the second group of attending (*N* = 5 in 2017) and non-attending males (*N* = 5 in 2017), we presented each dummy during one minute in the same sequence (cricket,

spider) in the same night, with an interval of 5 min between each stimulus. When a non-attending male abandoned his territory, we monitored the site for five consecutive nights to determine if the male would return to the same place or to a nearby leaf. Likewise, when an attending male abandoned his territory with clutches, we monitored the clutches until all embryos hatched or died.

Statistical Analyses

Proportion of time that attending and non-attending males spent in each activity was compared using a Kruskal-Wallis non-parametric test, because these behavioral variables were not normally distributed. We compared embryo survival between attended and non-attended clutches using a generalized linear model (GLM), with a quasibinomial distribution. In this model, we included larvae hatching success as the response variable, and mortality causes (predation, dehydration, and developmental failure) and male presence (present or removed) as explanatory variables. We also built models considering the additive effect and interaction between the explanatory variables. Besides, we used a Linear Regression to examine the influence of male care duration on larvae hatching success. Specifically, we evaluated whether an increase in the time of care led to an increase in the survival and hatch rate of the larvae. We used a Fisher's exact test for small sample sizes, to determine whether males' responses differed between treatments (a: medium risk; b: high risk). To determine whether care status (attending and non-attending) influenced males' behavior when facing a medium (cricket) and high (spider) risk situation, we used logistic regression models, with males' responses (raising body, stop calling, remaining alert, fleeing, and cloacal discharge) as

the response variable. As there were no differences in the males' responses between stimuli a and b (P = 0.99, Fisher's exact test medium risk; P = 0.99, Fisher's exact test high risk), data were combined for the logistic model. First, we tested whether isolated threat type (medium or high risk) influenced males' behavior change. Second, we included males' SVL, development stage of embryos (eggs or larvae) and the interaction between these variables as predictors, to test whether each of the five types of males' responses (raising body, stop calling, remaining alert, fleeing, and cloacal discharge) could be attributed to the size and care status of the males. For visual representation of the results, bar-plots were used. All analyses were conducted using R software version 3.4.3 (R Core Team., 2017).

Results

Attending and non-attending male's activities

Attending and non-attending males exhibited calling and care site fidelity, but time allocation for activities differed among them (Fig. 1a). Non-attending males spend much more time foraging (P < 0.001) and calling (P = 0.002) than attending males. However, attending males were more frequently observed in amplexus than non-attending males (P = 0.001). Indeed, non-attending males obtained their first clutch after a week (X ± SD = 7.16 ± 1.72 days, N = 6 males) while attending males obtained more clutches after a couple of days (second clutch: X ± SD = 3.05 ± 1.07 days, N = 19 males; third clutch: X ± SD = 2.77 ± 1.20 days, N = 9 males; fourth clutch: X ± SD = 2 ± 0.89 days, N = 6 males; fifth clutch: X ± SD = 2 ± 1.41 days, N = 2 males; sixth clutch: 1 day, N = 1 male). Attending males remained close to the egg clutches (between 1-6 clutches,

simultaneously) during both day and night. At day, males hydrated clutches only on dark, cloudy and rainy days for one hour (08:00-09:00h) and, during the night, they distributed their remaining time close to the clutches calling or alert (75% of the time), foraging (13%), hydrating or handling the eggs (7%), and in amplexus (5%; Fig. 1b). Non-attending males were inactive at day and remained in resting position on the same leaf used as calling site. During the night, non-attending males called from the same area but from different leaves, where they also foraged.

Benefits of egg attendance: embryo development and survivorship

Generally, males hydrated their eggs after heavy rains or during drizzling rain covering the clutches (X ± SD = 19.91±4.19 eggs per clutch, N = 85 clutches) with their bodies going up and down quickly and their hind limbs over the eggs, changing their position and starting over again until hydrating the entire clutch. Furthermore, we also observed the males using their hands and feet to rotate or clean the eggs probably from parasite. When males stopped hydrating the eggs, jumped and sat on wet areas of the upper side of leaves near to the clutches, probably to hydrate their bodies, storaging water in the bladder, and then re-hydrating the clutches. Embryo survival in attended clutches (control group) was higher when compared to that of non-attended eggs (ANOVA: $F_{2,23}$ = 11.28, P = 0.0003; Fig. 2a). Moreover, male's presence reduced predation (P = 0.0008) and desiccation (P = 0.019) in 98% of the cases (Figure 2b). Although developmental failure (Fig. 2b) was observed in clutches of both treatments, we did not detect this failure as a major cause of mortality (P = 0.99).

Regarding care duration, we found that the longer a male remains with his offspring, the higher larvae hatching success ($F_{2,30}$ = 64.94, R^2 = 0.79, P < 0.001, N = 30 males [15 males in 2016 and 15 males in 2017], 63 clutches [26 in 2016 and 37 in 2017]). On average, males spent 13.5 days (Range 10-19 days, N = 30 males) taking care of a single clutch, and when taking care for multiple clutches (more than one), hatching success of the offspring increased 3.54 times for each extra day that males spent with them. Spiders, crickets, ants, a cockroach, and a snake (juvenile *Leptodeira annulata*) were observed eating embryos of attended and unattended clutches during the night and day. In the case of the attended clutches, predation apparently occurred when males were absent from oviposition sites. On the other hand, we observed an attending male caring for two clutches on separate leaves, one in front of the other, exhibiting passive defensive behavior to prevent the predation of one of his clutches. When a cockroach began to eat the eggs, the male from a different leaf started to raise his body until the cockroach stopped and left the leaf. Although, in this case the male was successful in deterring the cockroach, probably males would prefer to flee to protect themselves when confronting larger predators as snakes. Indeed, although we observed the snake *L. annulata* preying on some egg clutches, we could not observe if the male fled from the snake or if he had already left the territory when the snake found the clutches. In any case, the male would not have been able to defend the embryos from the snake.

Predation risk and behavioral response of attending and non-attending males

Our results showed that males' responses to threat varied as a function of care status. During the medium-risk experiment (cricket dummy) attending and non-attending males remained in their territories, without changes in their behaviors (Table 1). While attending males raised their bodies a couple of times (*N* = 2 males in 2016, *N* = 1 male in 2017, Fig. 3a) or remained in the same position (*N* = 3 males in 2016, *N* = 4 males in 2017), non-attending males stopped calling and remained in the same position (*N* = 5 males in 2016, *N* = 5 males in 2017, Fig. 3b). On the other hand, during the high-risk experiment (spider dummy) the behavior of attending and non-attending males changed considerably (Table 1). Attending males crouched down and remained motionless (Fig. 3c). However, unlike non-attending males, attending males only fled after touching or squishing their dorsal region with the spider (*N* = 5 males in 2016, *N* = 5 males in 2017), releasing a colourless liquid through its cloaca (*N* = 2 males in 2016, *N* = 3 males in 2017) while escaping (Fig. 3d). Besides, attending males remained on the same leaf or around and returned close to the eggs few minutes after perturbation (*N* = 5 males in 2016, *N* = 5 males in 2017). By contrast, non-attending males fled when we started approaching the spider and never returned to the same leaf, but remained in the same area (*N* = 4 males in 2016, *N* = 5 males in 2017, Fig. 3e,f). Although attending and non-attending males did not exhibit aggressive reactions (calls or fighting behaviors) towards the dummies, differences in males' responses were related to the threat type (Table 1) instead of males' size or the development stage of the embryos (Table 1).

Discussion

We found that males of *H. cappellei* spent less time calling and foraging once they started to care for their clutches. However, although non-attending males spent more time calling, attending males continued mating and obtained more clutches from females. Calling and caring activities are energy-demanding, thus it is expected that males allocate certain amount of time or energy in one activity at a time (Lissåker and Kvarnemo 2006; Blue et al. 2016). However, contrary to some species where attending males tend to use more energy than non-attending males, reducing food intake, increasing mortality risk or losing new matings (Simon 1983; Steinhart et al. 2002; Requena et al. 2012), attending males of *H. cappellei* seem to increase the chance of acquiring both new mates and more clutches. Similar results have been reported for other species of anurans (Chen et al. 2011; Mangold et al. 2015), fishes (Coleman et al.1985; Matsumoto et al. 2011), and arthropods (Requena and Machado 2015), where males continue to mate while caring for clutches in their territories. Our results suggest that in *H. cappellei* both care investment and mating opportunities are non-mutually exclusive activities. Hence our ongoing study will investigate the role of parental investment for female mate choice in this glassfrog species.

Anurans exhibit diverse and complex parental care behaviors (Vági et al. 2019; Furness and Capellini 2019). Some species, for example, are active caregivers that constantly hydrate their clutches (e.g., *Kurixalus eiffingeri,* Chen et al. 2007) or cover long distances looking for suitable water bodies for larval deposition (e.g., *Allobates femoralis*, Ringler et al. 2013), whereas in glassfrogs (genus *Hyalinobatrachium*) males are mainly passive caregivers (Vockenhuber et al. 2008; Valencia-Aguilar et al. 2012; Bravo and Delia 2015; this study). In *H. cappellei*, caring males attend their clutches

during the embryonic development (approximately 10-19 days), using 42% of their time in caring activities, mostly remaining close to the clutches, but also hydrating or handling the eggs. Moreover, males hydrated the clutches few times and only during or after rains, using the water accumulated in the upper side of the leaves in their territories. Time devoted to caring activities in *H. cappellei* are similar to those found in *K. eiffingeri*, where males spent 34.3% of the time attending clutches, leaving their territories to find water to hydrate the eggs (Chen et al. 2007).

As we predicted, embryos were almost totally dependent on care for survival. Only, 20% of the embryos of the non-attended clutches survived, compared to 82% of survivorship in the attended clutches. High rates of embryo survival were also found in other glassfrogs (*H. aureoguttatum* 96.5%, *H. fleischmanni* 82%*, H. orientale* 79.8%*, H. valerioi* 50%*, Ikakogi tayrona* 78.2%, and *Centrolene peristicta* 78.89%), as well as in some terrestrial (*Eleutherodactylus cooki* 85%, *Cophixalus parkeri* 95%, and *Hylophorbus rufescens* 78.2%) and arboreal frogs (*Chiromantis hansenae* 75%, and *Oreophryne* sp. 100%) when parents (male or female) remained attending clutches (Simon 1983; Burrowes 2000; Bickford 2004; Vockenhuber et al. 2009; Valencia-Aguilar et al. 2012; Delia et al. 2013; Poo and Bickford 2013; Lehtinen et al. 2014; Bravo and Delia 2015; Salgado and Guayasamin 2018). It has been demonstrated that desiccation and predation are the main causes of mortality of eggs and embryos in animals (Andersson et al. 1980; Alonzo-Alvarez and Velando 2012). However, in the glassfrogs, parental care seems to be mainly associated with reduction in embryo dehydration rather than active defense against predation (Valencia-Aguilar et al. 2012; Delia et al. 2013). Thus, our observations also suggest that, although the parental investment in *H.*

cappellei can be influenced by the predation risk, avoidance of desiccation is more likely to be the primary driver of parental care in this species. We also found that males of *H. cappellei* caring for multiple clutches increased hatching success for each additional day they spent providing care. By extending the duration of care, parents may incur in energetic and survival costs, as well as reduce the time available for new breeding opportunities (Clutton-Brock 1991; Klug et al. 2012). However, our results suggest that males of *H. cappellei* do not pay high energetic and mating costs by prolonging parental care.

Our last prediction was that the level of threat posed by different predators would modify males' behavior. Indeed, we found that males of *H. cappellei* were able to discriminate between a medium and high-risk predation adjusting their responses accordingly. Similar to what have been described for other anurans (Poo et al. 2016; Chuang et al. 2017), fishes (Magnhagen 1992), and birds (Mahr et al. 2015), our results suggest that males of *H. cappellei* use visual cues to detect the presence of predators. Males of *H. cappellei* did not actively or aggressively defend their clutches from predators, contrary to what was reported for other glassfrog species (*H. valerioi, H. colymbiphyllum,* and *H. fleischmanni*), in which males defend their offspring by kicking the intruders wasps away (Drake and Ranvestel 2005; Vockenhuber et al. 2008; Delia et al. 2010). Wasps can be considered as a low threat for the males, which can drive off or consume them (McDiarmid 1978). However, crickets can be a threat not only for the clutches but also for males by preying on them (e.g., *Feihyla hansenae*, Poo et al. 2016; *Cochranella granulosa,* Delia et al. 2017) or causing injuries. Hence, to minimize energy expenditure and mortality risks, males of *H. cappellei* could be exhibiting passive

defensive behaviors to protect their clutches against medium-sized predators like cockroaches or our cricket dummy.

Theoretical models assume that by acting and behaving more cautiously, caregivers will be able to lower predation costs (Andersson et al. 1980). Likewise, experimental studies have shown that parental risk decisions varied in relation to their own, but not offspring risk, i.e., when the threat is too high, parents will abandon their current offspring to avoid being preyed and increase their chances of future reproduction (Shaffer and Formanowicz 1996; Mahr et al. 2015; Chuang et al. 2017). However, in the glassfrog species, studies have evidenced an opposite pattern. Attending males of *Centrolene savagei* (Ospina-L et al. 2019), *H. cappellei* (this study) and attending females of *Ikakogy tayrona* (Bravo and Delia 2015) are more risk-tolerant and required higher levels of threat stimuli to abandon their territories and clutches, than non-attending individuals. Attending males of *H. cappellei* did not actively protect their clutches during spider experiment. However, although spider dummy was twice males' size, males did no abandoned their territories, despite the higher threat to their own survival. In *Hyalinobatrachium aureoguttatum* (Valencia-Aguilar et al. 2012), an attending male was observed trying to attract the attention of a harvestmen that was preying on some of his clutches by moving close to the predator. Probably, males of *H. cappellei* remain in their territories in an attempt to attract the attention of larger predators like our spider dummy, and moving away after the predator approach. However, although we squeezed the males with the spider a couple of times before they moved away, in a natural situation this response would have cost male's life (Ortega-Andrade et al. 2013). Hence, although the increased tolerance of males to a high-threat

may increase their mortality risk, could be an alternatively parental defense exhibited only under certain circumstances (e.g., when larvae are ready to hatch), to increase offspring survival. In fact, in the glassfrogs, embryos are capable of hatching prematurely after paternal abandonment or to escape from predation (Delia et al. 2014; 2018). However, this hatching plasticity is possible only after a period of obligate care (approximately of five days in *Hyalinobatrachium fleischmanni*) (Delia et al. 2014). Hence, by tolerating more risk, attending males of *H. cappellei* could be decreasing their survivorship to ensure their offspring "escape' from predation. However, since defensive behaviors performed by parents are poorly studied in anurans, further studies would be necessary to elucidate this hypothesis.

In conclusion, we found a strong offspring dependency on parental care related more to dehydration avoidance than predator defense, since males of *H. cappellei* did not aggressively defend their clutches. However, although we did not observe active defense, males were able to identify, assess and respond accordingly to the threat level imposed by the predator (e.g., predator type and behavior). Besides, we found that care activities did not affect male's mating opportunities in this species, since attending males continued obtaining clutches. Hence, parental care in *H. cappellei* could be considered as energy conservative, with attending males exhibiting high mating success while avoiding extra predation risks. We suggest that differences in reproductive status may play a significant role in the antipredator behavior pattern exhibited by glassfrogs, which needs further investigation, and that future studies are also needed to evaluate the role of male reproductive status for female choice.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

Field experiments were approved by the Ethics Committee on Animal Use of the Universidade Estadual Paulista (UNESP), Rio Claro, São Paulo state, Brazil (protocol number 9457) and, the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, license number 51479-4).

Data availability

All data generated or analyzed during this study are included in this published article.

Author Contributions

AVA and CPAP designed the original research questions. AVA collected field data, performed all statistical analyses and drafted the manuscript. All authors substantially edited and approved the final manuscript.

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Table 1 Logistic regression for factors associated with changes in the responses of attending and non-attending males of *Hyalinobatrachium cappellei* submitted to two levels of predation risk (medium and high) during a fieldwork experiment conducted in the municipality of Cotriguaçu, Mato Grosso state, north-western Brazil.

	df	t value	*p* value	Coefficient
Threat type				
Medium risk	18	-0.007	0.99	-18.72
High risk	18	-108.50	<0.001	-51.132
Attending males				
Threat	15	-99.02	<0.001	<0.01
Embryo development	15	<0.001	0.00	1.00
SVL	15	<0.001	<0.001	1.00
Clutches development*SVL	15	<0.001	<0.001	1.00
Non-attending males				
Threat	16	<0.01	0.003	-3.38
SVL	16	<0.001	1.00	0.00
Threat*SVL	16	<0.001	1.00	0.00

Figure captions

Fig 1. Percentage of time spent by males in different activities during two breeding seasons (January - February 2016 and January - February 2017) at São Nicolau Farm, municipality of Cotriguaçu, Mato Grosso state, north-western Brazil. (a) Time spent in each activity by attending and non-attending males of *Hyalinobatrachium cappellei*. (b) Time that attending males spent in different caring activities. * indicates significant differences between bars.

Fig 2. (a) Larvae hatching success of attended (control group) and non-attended egg clutches (experimental group) of the glassfrog *Hyalinobatrachium cappellei*. (b) Causes of embryo mortality identified as predation, dehydration, and development failure for both attended (N = 30) and non-attended clutches (N = 20) monitored during our study in north-western Mato Grosso state, Brazil. * indicates significant differences between bars.

Fig 3. Behavioral responses of attending males of *Hyalinobatrachium cappellei* to two levels of disturbance. (a) Attending and (b) non-attending males' responses to medium-risk predation (cricket dummy). (c,d) Attending and (e,f) non-attending males' responses to high-risk predation (spider dummy). The arrow indicates male cloacal discharge after being touched by the spider.

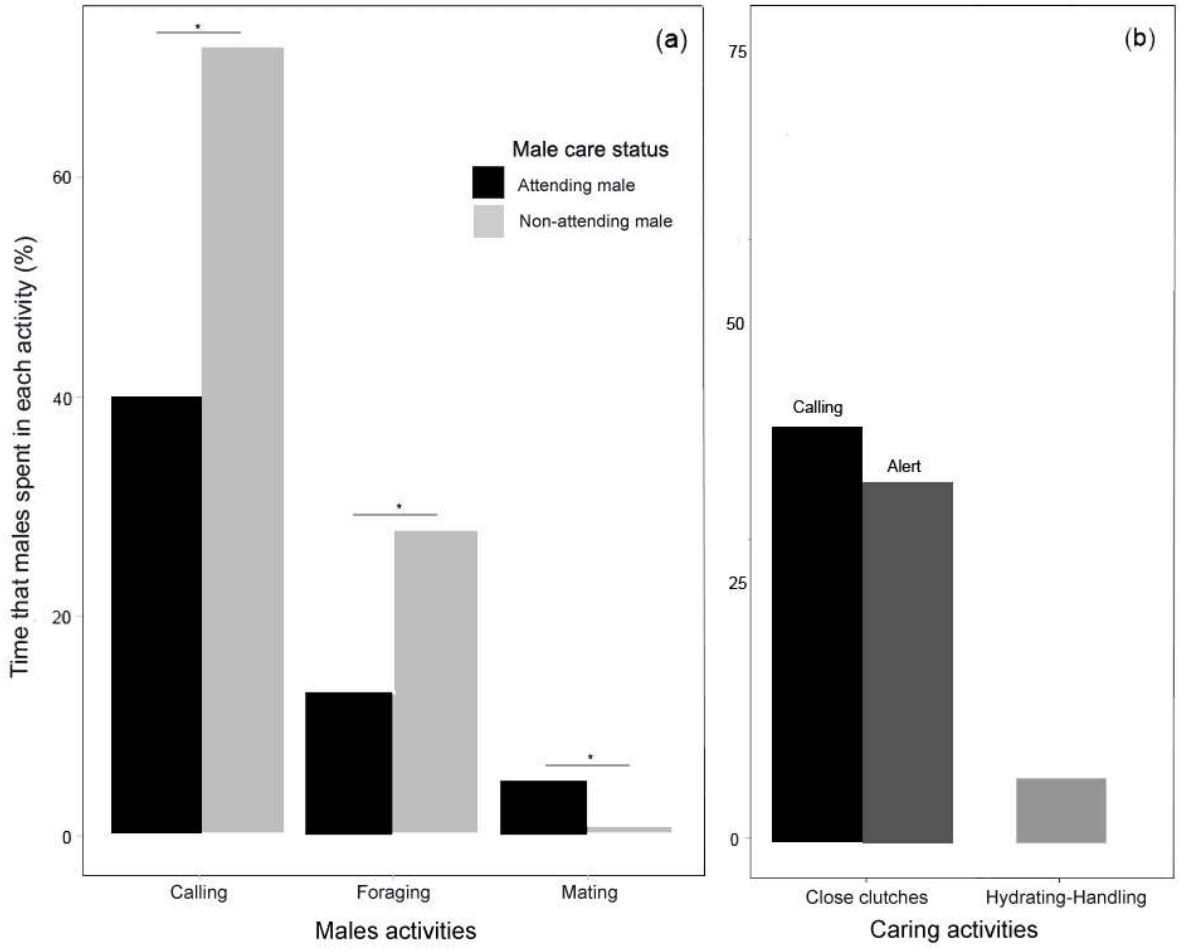

(a)
(b)
Male care status
Attending male
Non-attending male
Time that males spent in each activity (%)
0
20
40
60
0
25
50
75
*
*
*
Calling
Foraging
Mating
Males activities
Calling
Alert
Close clutches
Hydrating-Handling
Caring activities

Fig 1.

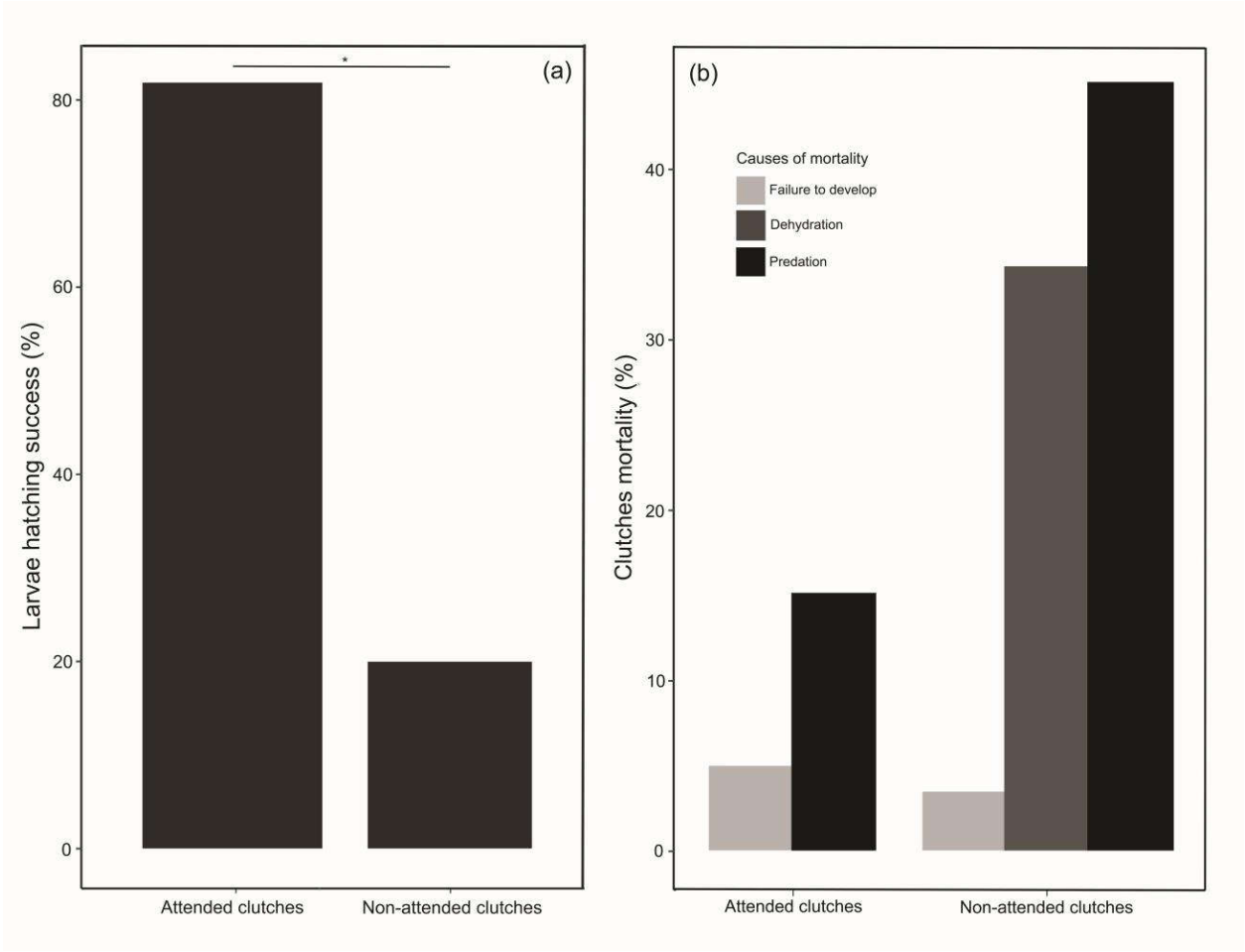

(a)
*
Larvae hatching success (%)
0
20
40
60
80
Attended clutches
Non-attended clutches
(b)
Causes of mortality
Failure to develop
Dehydration
Predation
Clutches mortality (%)
0
10
20
30
40
Attended clutches
Non-attended clutches

Fig 2.

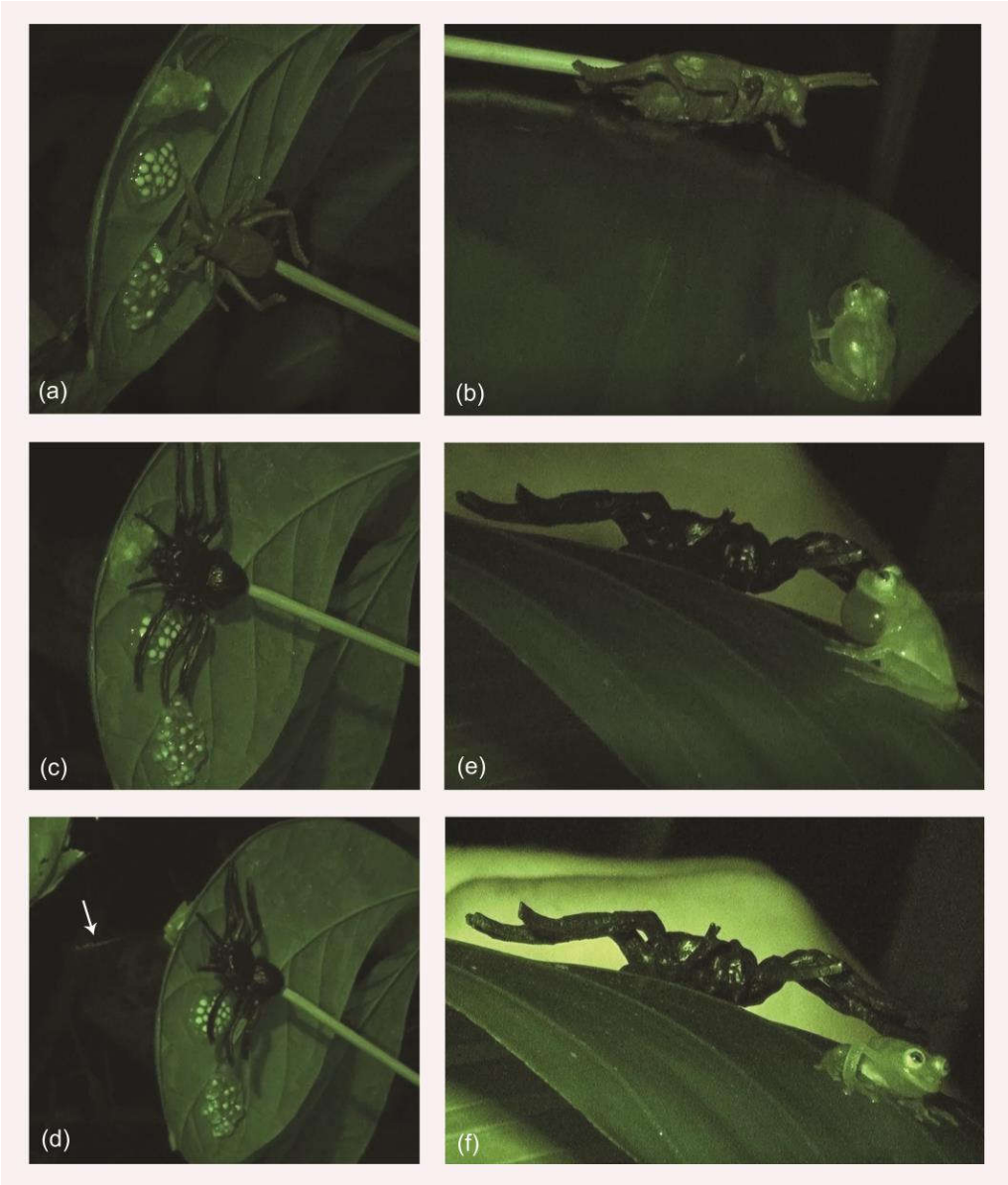


Fig 3.

CHAPTER II

SHOW ME YOU CARE: FEMALE MATE CHOICE BASED ON EGG ATTENDANCE RATHER THAN MALE OR TERRITORIAL TRAITS

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

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Running title: Female mate choice in a frog with paternal care

Abstract

Female mate choice is often based on male traits or the quality of territories they hold. However, male mating success can also be enhanced by male signals or behaviors that are part of male mating strategies. In species with obligate paternal care, where the care of eggs directly affects offspring survival, females may instead base their mate choices on the quality of a sire's care. Here, we quantified male reproductive success in a natural population of the glassfrog *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 non-attending 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. 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 that egg clutches provide to females.

Keywords: Parental care, paternity, male-offspring relatedness, female choice, mating strategy

Lay summary

Being a good dad matters, but pretending to be one is also useful. Using behavioral and molecular data, we show that females of a Neotropical glassfrog prefer to mate with males already caring for clutches. In this species, care is critical for offspring survival, hence by remaining close to unrelated clutches males increase their chances to attract females by pretending to be “good caretakers”.

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 fitness (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; Nemeth et al. 2012; Wogel et al. 2013; Charlton et al. 2018), and quality of reproductive territories (Wells 1977; Roithmair 1992; 1994; Jones et al. 2014) are well-known indicators of male fitness 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 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 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; Nazareth and Machado 2010;

Requena and Machado 2015), where the presence, number, and developmental stage of clutches 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 glassfrog *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 evidence 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 non-attending 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; 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 changes 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 two adjacent stream populations of *H. cappellei* on a daily basis over two breeding seasons at the Fazenda São Nicolau (9°49'10.1" S, 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 rainy (September to March) and a dry season (June to August) (Alvares *et al*. 2013). Temperature does not differ strongly between seasons (24 to 36°C in rainy and 32 to 38°C in dry), but rainfall and humidity are greater in the rainy (mean of 1300-1700 mm and 80% humidity) than in the dry season (mean of 20-50 mm and 40% humidity) (Bittencourt-Rosa *et al*. 2013).

Field surveys and behavioral observations

We conducted two surveys, one during the day (08:00-13:00) and one at night (20:00-02:00) during the months of January-February 2017 and 2018. During each survey, we searched for attending and non-attending males, and marked the male's territory with flagging tape to monitor male activity and development of clutches until hatching (Table 1). 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 visited 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). For attending males, we recorded the time that the male spent hydrating or touching egg clutches and photographed clutches to determine development and survivorship of embryos. We considered egg attendance when the male hydrated eggs and remained close to the clutch (no further than 5 cm). 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 its

size (number of eggs) and development stage (Gosner 1960) and later classified the eggs into two developmental categories: embryos (stages 1-19) and hatchlings (stages 20-25) (following McDiarmid and Altig, 1999). The time required for eggs to hatch ranges from 15-20 days, so when larvae were close to hatching we positioned a plastic bag below the clutches to collect them. We collected tissues of 13 females, 11 non-attending males, and 25 attending males with their clutches in 100% ethanol for later use in molecular parentage analyses.

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 2x master mix, 2.5 µl water, 1 µl primer mix and 1.5µL 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 0.7x sample volume Ampure XP and resuspended in 0.5 x 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 x 250 bp) at the Biotechnology Resource Center (BRC) 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 (Appendix S1, Supporting information) 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 (SVL 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 non-attending males. All size measurements were recorded to the nearest 0.5 mm using digital calipers and to the nearest 0.005 g using a precision analytical balance. Extrinsic variables included the length and width of the leaf (in cm) where we found males and their clutches, as well as the vertical distance (height) of the leaf from the water (in cm). 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 non-attending males using a t-test. In addition, we used a multiple linear regression model (MLR) to evaluate possible associations between body condition (index between body size and body mass) of the attending males, the total number of clutches, and number of eggs within each clutch.

To test whether male traits and territory characteristics were potential determinants of mating success, we used a generalized linear mixed model (GLMM) with male mating success as the response variable and male status (attending, non-attending), 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 GLMMs 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 non-attending males) or a new clutch (in the case of attending males) using a logit model with all the observed males (n = 36). Finally, we used a Kruskal-Wallis test with a Dunn test as a post-hoc analysis to determine if the number of clutches accumulated by successful males was related with caring for clutches at the same stage (embryos or hatchlings) vs. caring for clutches at different stages. We implemented all the models using the packages lme4, glmm and AICcmodavg in R 3.1.0 (R Development 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 (PIC), the frequency of null alleles, and the average non-exclusion probability of each locus were estimated based on the allele frequencies of 987 individuals (49 adults and 938 tadpoles) 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 pair-wise 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 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 multi-locus 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), 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. In some cases, Cervus and Colony could not assign a genotype from the sampled parents to certain clutches, and in those cases, we used a simulated parental genotype provided by Colony. When multiple females or males were assigned to a single clutch in Cervus or Colony, we compared genotypes of each larva within the clutch with all the candidate parents to select the most likely parent pair for the clutch. We assigned a parent to the entire clutch if there were ≤ 2 allele mismatches between the candidate parent (mother or father) and each larva within the clutch. 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 (Figure S2, Supporting information).

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 (‘non-mates’) 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, hydrating) to the clutch during our timed behavioral observations. 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 procedures were approved by the Ethics Committee on Animal Use at Universidade Estadual Paulista (UNESP), 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 (ICMBio/IBAMA; license number 51479-4).

Results

We monitored and sampled 49 individuals of *H. cappellei* (36 males, 13 females) and 1407 larvae from 76 clutches. Males typically returned to the same territories to breed, calling to attract females. After mating, females abandon male territories, while 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 (SD = 1.43, range = 1–6 clutches, N = 76 clutches) with a total number of eggs per clutch from 15 to 53 ($\overline{X}$ = 30.31 eggs, N = 76 clutches). We found no differences between the size (number of eggs per clutch) of the clutches cared for by 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 touch or hydrate their clutches (Figure 1C) until the larvae hatch. Some embryos died due to predation, although on average 90% (SD = 14.63) of the larvae 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 while 33% did not acquire any clutches. We found no differences in body size (*t* test: *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 non-attending 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, non-attending) 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 non-attending male territories and calling persistence, non-attending males had a small chance of obtaining their first clutch (estimated probability = 0.27, 95% 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 found that clutch number also influenced male mating success. Males caring for multiple clutches received new clutches of eggs more frequently (on average after 3 days) compared to males caring for just one clutch (on average after 6 days). Moreover, although we found more males (68%) caring for clutches at the same stage (only embryos or only hatchlings) than males (32%) caring for clutches at different stages (embryos + hatchlings), males caring for embryos + hatchlings 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 of embryos and hatchlings and males caring for only clutches of hatchlings 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 57 clutches (938 larvae, Table 1). Because females in our focal species leave the mating territory shortly after amplexus, our capture rates for females is significantly lower than for males who remain at territories to call and attend clutches. For seven clutches (119 larvae), we were able to assign both parents from the study area, and for the other 50 clutches (819 larvae) 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 case 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 mate with multiple partners during each breeding season. However, although males often mate with different

females, 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 re-mating events occurred approximately two to three weeks apart ($\bar{X}$= 15-20 days). We estimated clutch interval by comparing oviposition 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%) out of 57 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 the 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 hydrating or touching care behaviors. After approximately five 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 the 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 non-related 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 glassfrog 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 non-attending 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 female glassfrog species with obligate parental care depend on signals

of male parental investment for female mate choice. However, glassfrog 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 glassfrogs with parental care have shown that larger males, calling continuously (*Centrolene savagei*, Vargas-Salinas et al. 2014; Ospina-L et al. 2017), from higher calling sites (*H. fleischmanni*; Greer and Wells 1980) obtained more mates. However, in other species females do not choose mates according to either male traits or relatedness (*Hyalinobatrachium valerioi* Mangold et al. 2015), but instead based on male care status (*H. cappellei* Noronha and Rodrigues 2018, this study). Given this variation, it will be interesting to further examine whether care status is used as a signal for female mate choice more frequently in species with higher dependence on parental care.

In *H. cappellei* offspring survival is highly dependent on male care; mortality in the attended clutches averages 14%, whereas in the non-attended 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 that reliably attend clutches will be more successful (Zahavi 1975; Price *et al*. 1993; Alonzo 2012). Two non-exclusive hypotheses explain why females would choose already mated males. The parental-investment hypothesis (Trivers 1972; Sargent 1988) posits that females of *H. cappellei* use this signal to directly increase the survival of their offspring during the period of paternal care. The female copying hypothesis (Gibson and Höglund 1992; Alonzo 2008) posits that they increase their fitness by using already existing clutches as an indicator trait of male quality. Not only did attending males receive more new clutches, our results

show that number of clutches, and diversity in egg developmental stage are 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. Thus, the longer a male holds a territory with eggs, the higher the chance he will acquire new clutches laid sequentially with a few days lag between them. A similar pattern has been observed in some fish and arthropod species with male 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 ellipes*) females (Sikkel 1989; Matsumoto et al. 2011) 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 non-attending males, it is not clear how (for example 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.

Males of most glassfrog species decrease parental care at later embryonic stages (*Hyalinobatrachium valerioi,* Vockenhuber *et al*. 2008; *H. aureoguttatum,* Valencia-Aguilar et al. 2012; *H. orientale,* Lehtinen et al. 2014; *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 *Hyalinobatrachium cappellei* (Author, pers. obs.) and *H. fleischmanni (*Delia et al. 2014), predators preferentially consume embryos and hatchlings 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 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 by natural selection because of offspring survival (Gross 2005), but also favored by sexual selection because of the evolution of female preferences (Alonzo 2012).

Our genetic analysis showed that our focal species is primarily polygynous, with most males and only a few 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 glassfrogs (*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 to females. In glassfrogs, mating success in males is limited by their ability to attract females and hold a territory, while 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, 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. 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 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*, fifteen-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), while males of the fifteen-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* always had full paternity of their clutches and were able to recognize and care only for their own offspring. 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 glassfrog 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 daidaleum, Rojas and Cabello 2011). Independent of whether usurped clutches are abandoned or are the result of territorial take-overs, 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. Behavioral reproductive tactics to maximize individual fitness are common in animals (Taborsky et al. 2008). Typically, adopting a particular 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 non-successful 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 otherwise poorer males that 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 non-paired 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 size traits, 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, egg attendance 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 through enhanced mating success as well as higher 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.

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Data accessibility

The genotypes from collected individuals (males, females, larvae) of *Hyalinobatrachium cappellei* are deposited in the Dryad Digital Repository: https://doi.org/10.5061/dryad.0rc447b.

Author Contributions

AVA, KRZ and CPA designed the original research questions, AVA collected field data and samples, performed DNA extractions and genotyping, SMB developed microsatellites and performed genotyping, AVA did all statistical analyses, AVA drafted the manuscript, and all authors substantially edited and approved the final manuscript.

Conflict of interest

The authors declare no conflict interests.

Table 1. Results of parentage analyses in *Hyalinobatrachum 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, SD = standard deviation, Min/Max = minimum and maximum number of eggs per clutch, Egg number = total number of eggs attended by male, %survival = percentage of hatched larvae.

Place	ID	N mates	Size	Mass	Clutches					Egg number	% survival
					N	Mean Eggs	SD	Min	Max		
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.

Figure legends

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)** Larvae 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 non-attending males. Probabilities were estimated using the coefficients of the selected model and the 95% CIs for each male group (attending, non-attending). **B)** Delay time for attending males to acquire a new clutch, and for non-attending males to acquire their first clutch.

Figure 3. The total number of clutches acquired by males caring for clutches they fathered 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.

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, and symbol sizes represent the number of mating partners per individual (1-4).

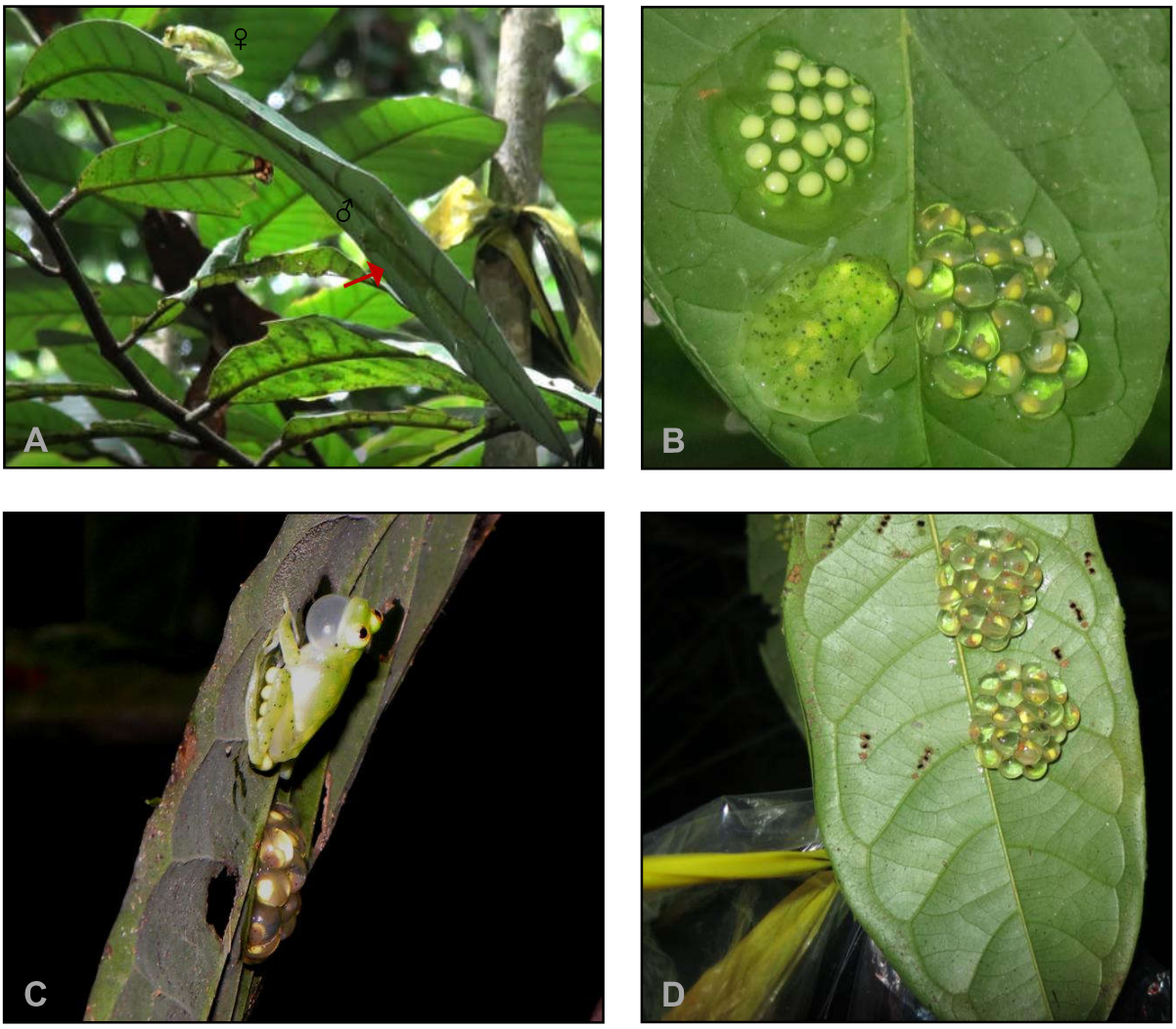


Figure 1

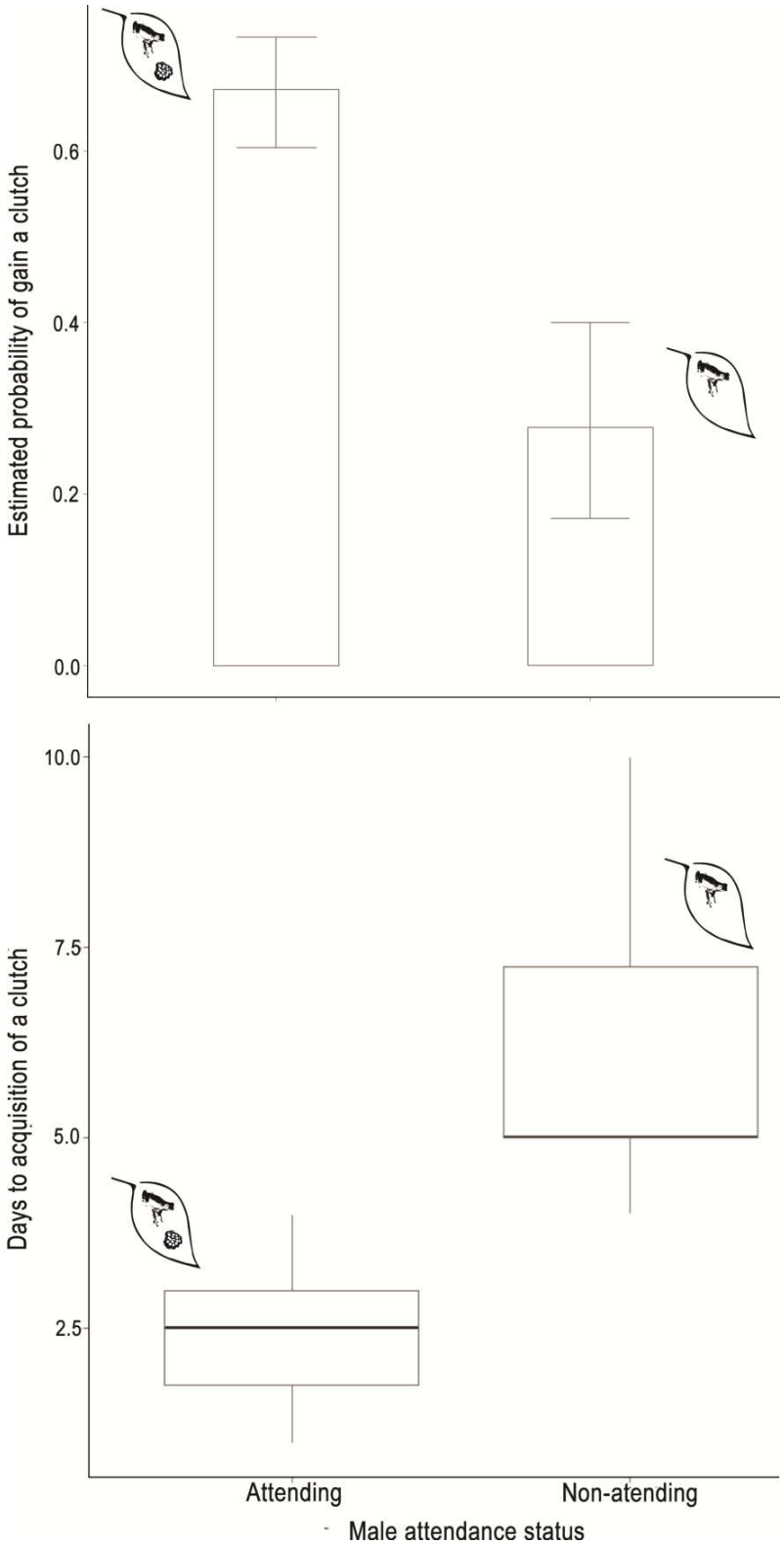

Estimated probability of gain a clutch
0.0
0.2
0.4
0.6
Days to acquisition of a clutch
2.5
5.0
7.5
10.0
Attending
Non-atending
Male attendance status

Figure 2

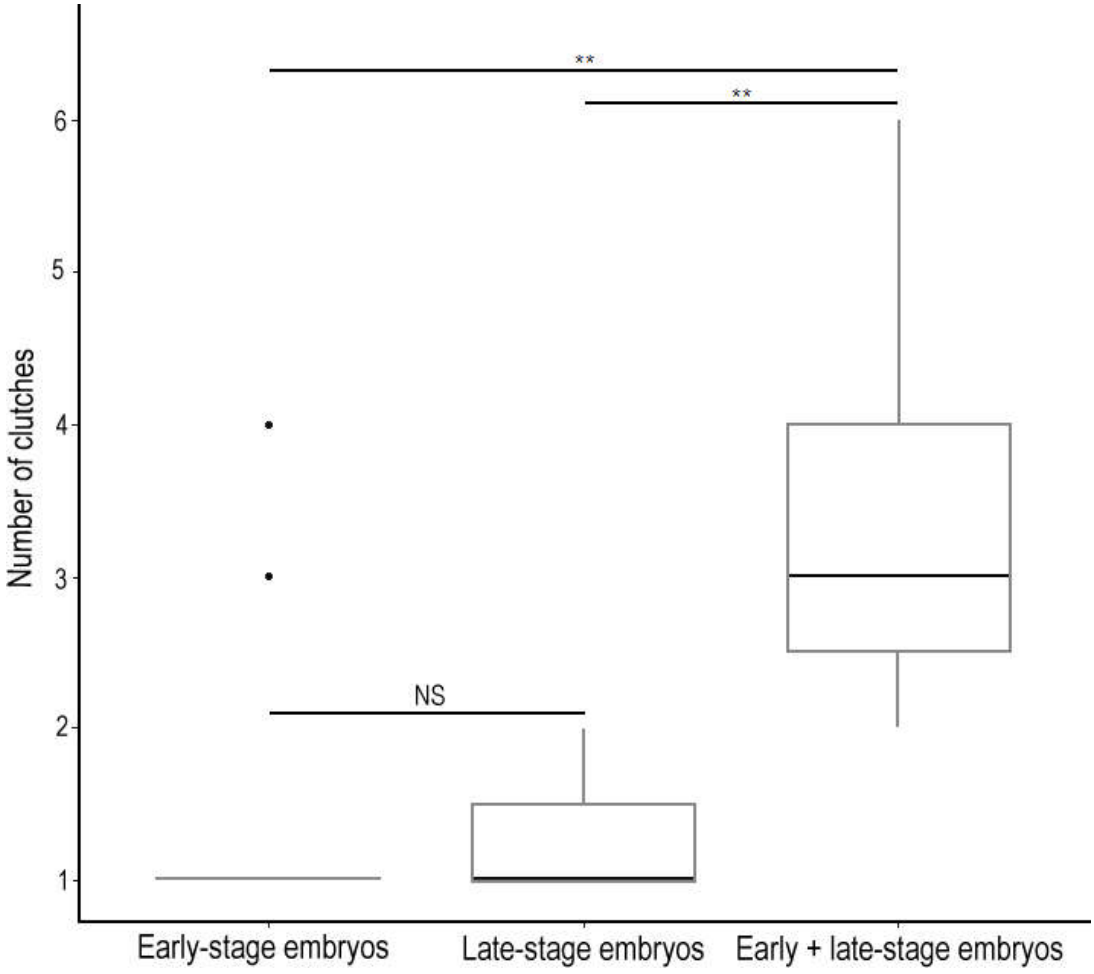

**
**
NS
Number of clutches
1
2
3
4
5
6
Early-stage embryos
Late-stage embryos
Early + late-stage embryos

Figure 3

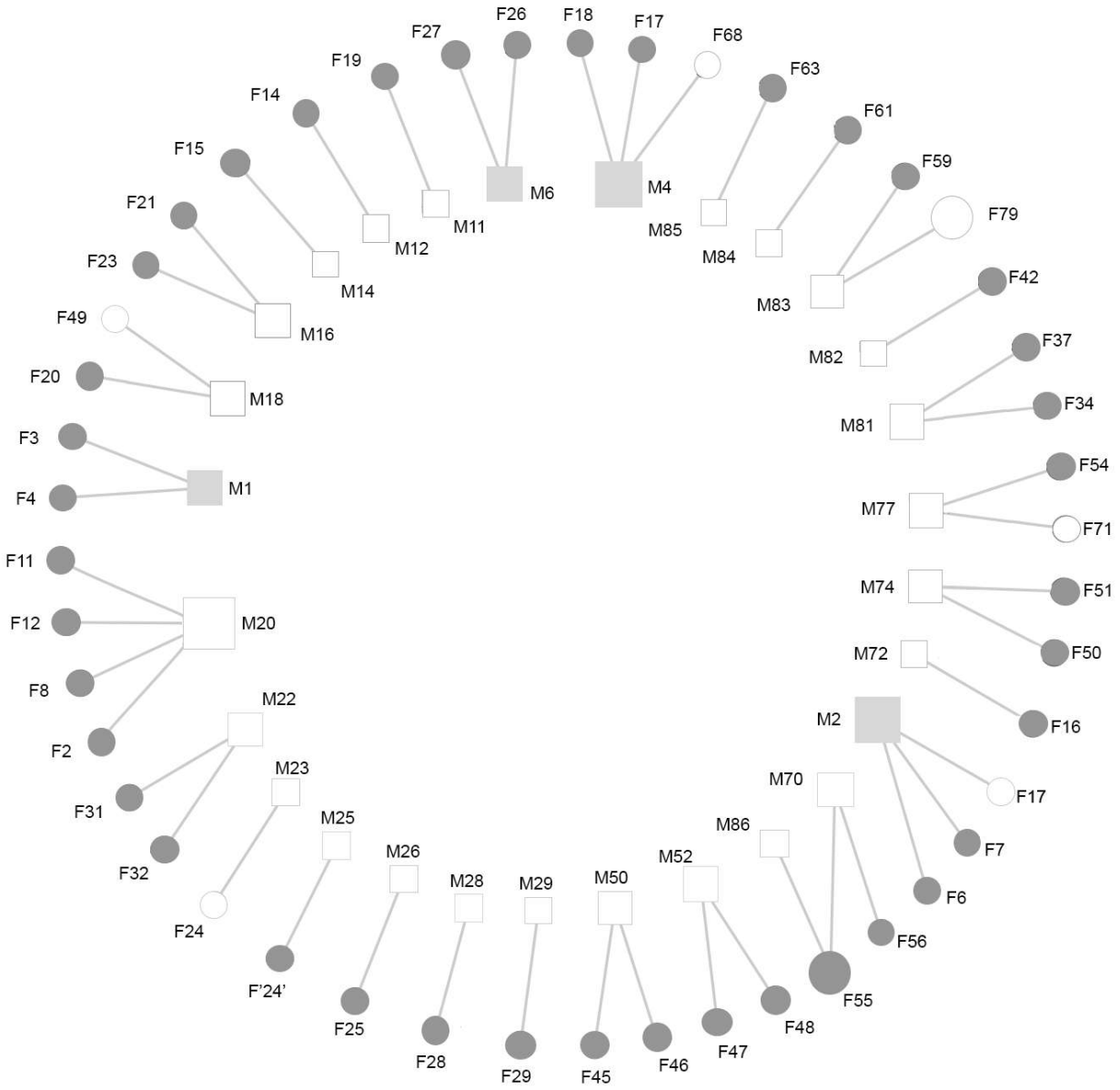

F26
F18
F17
F27
F68
F19
F63
F14
F61
F15
F59
M6
M4
F21
F79
M11
M85
F23
M12
M84
F42
M14
M83
F49
M16
F37
F20
M82
M18
F34
F3
M81
M1
F54
F4
M77
F71
F11
M74
F51
M20
F12
M72
F50
F8
M22
F16
M2
F2
M23
F17
M70
F31
M25
M86
F7
M26
M52
F32
M28
M29
M50
F6
F24
F56
F'24'
F55
F25
F48
F28
F47
F29
F45
F46

Figure 4

Supplemental Information for:

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

Anyelet Valencia-Aguilar, Kelly R. Zamudio, Célio F.B. Haddad, Steve M. Bogdanowicz, Cynthia P.A.Prado

Appendix S1. Analyzing multiplex amplicon data with amplicon.pl script

#Working directory (workdir):

-bash-4.2$ cd /workdir

#User-specific directory (smb31) in workdir:

-bash-4.2$ mkdir /workdir/smb31

#Copy .fastq.gz files to working directory:

-bash-4.2$ cp /home/smb31/Ldis_Oct2015/*.fastq.gz /workdir/smb31

Copy key, sample, ref files to working directory:

-bash-4.2$ cp /home/smb31/Ldis_Oct2015/Ldis_* /workdir/smb31

#Covert key, sample, ref files to unix format:

-bash-4.2$ mac2unix Ldis_key_5Oct15.txt

mac2unix: converting file Ldis_key_5Oct15.txt to Unix format ...

-bash-4.2$ mac2unix Ldis_ref_5May15.fas

mac2unix: converting file Ldis_ref_5May15.fas to Unix format ...

-bash-4.2$ mac2unix Ldis_sample_Oct2015.txt

mac2unix: converting file Ldis_sample_Oct2015.txt to Unix format ...

#Run script:

```
-bash-4.2$ nohup analyze_amplicon.pl -s Ldis_sample_Oct2015.txt -k
Ldis_key_5Oct15.txt -r Ldis_ref_5May15.fas -x 40:90:90 -d 10:150 -i 8 -t 8 -m
clustalw:FASTA -o Ldis_out_6Oct15 &> Ldis_6Oct15 &

#Check last 100 lines of output:
-bash-4.2$ tail -100 Ldis_6Oc
```

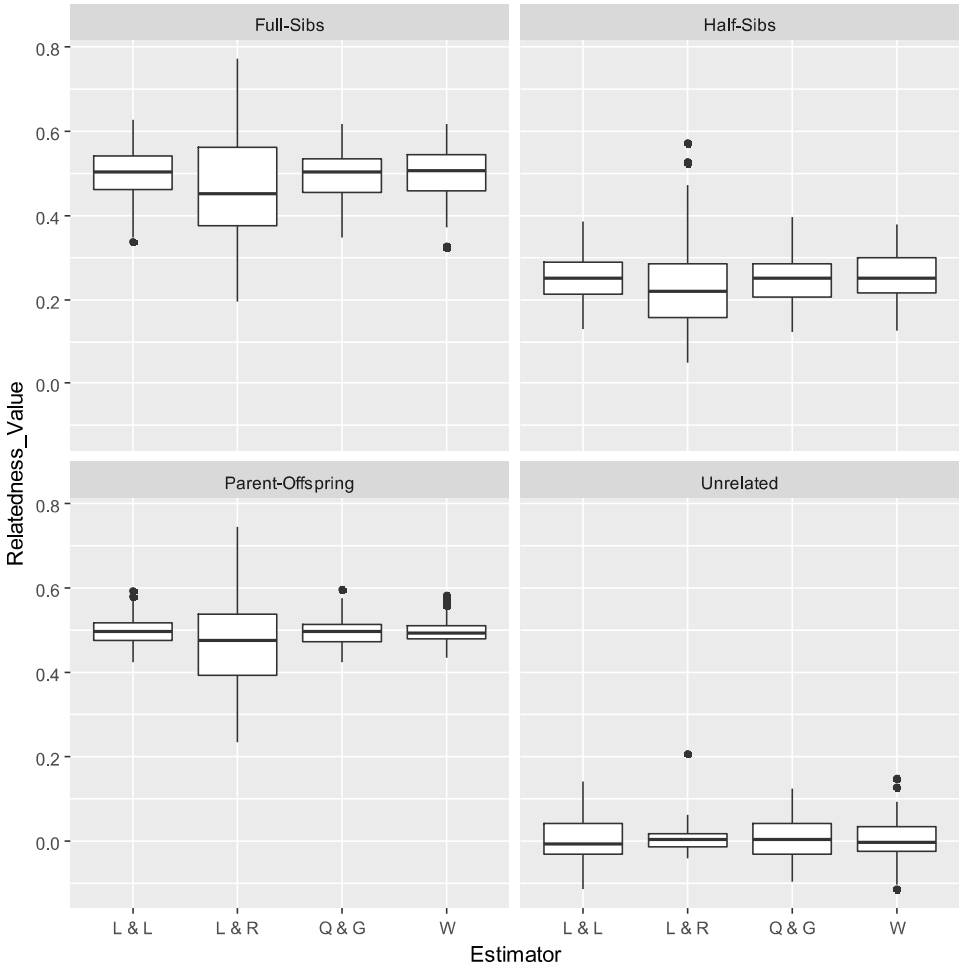


Figure S1. Plot comparing the relatedness estimates using four estimators, and simulated individuals of known relatedness. L & L=lynchli estimator, L & R=lynchrd estimator, Q & G=quellergt estimator, and W=wang estimator.

CHAPTER III

ALLOPARENTAL CARE IN GLASSFROGS: MALES CARE FOR UNRELATED CLUTCHES ONLY WHEN ASSOCIATED WITH THEIR OWN CLUTCHES

(Target Scientific Reports)

Alloparental care in glassfrogs: males care for unrelated clutches only when associated with their own clutches

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Parental care is costly, thus, parental investment theory predicts that parents should only care for their own offspring, avoiding care of unrelated offspring. Despite this, alloparenting – care for unrelated offspring – has been reported in many taxa, because it may increase survivorship of their own offspring or mating success. In this study, we experimentally investigated the existence of allopaternal care in two species of glassfrogs and discuss possible costs and benefits. Males of *Hyalinobatrachium chirripoi* and *Centrolene peristicta* mated with multiple females and cared for clutches in their territories, while continued to call. In our experiment, males were randomly assigned to two different treatments: (i) attending males: individuals caring for their own clutches that were experimentally given one additional unrelated clutch, (ii) non-attending males: individuals without clutches that were given one unrelated clutch. Attending males adopted unrelated clutches, whereas non-attending males abandoned their territories when unrelated clutches were placed close to them. Contrary to our expectations, once males adopted the unrelated clutches, they cared for all the eggs in their territories with a similar frequency. Our finding suggests that alloparenting in glassfrogs only occurs in males that already care for their own clutches and, most likely, cannot distinguish unrelated clutches, which, in natural conditions, may result from reproductive events when females are intercepted by satellite males. Our results suggest that alloparenting in glassfrogs may have been selected because of the benefits related to higher offspring survival and mating success.

Key words: Anura, clutch adoption, paternal care, offspring discrimination

Introduction

Parental care involves any parental behavior that increases both parents and assisted offspring fitness, with potential costs to the caregivers (1). Since the amount of parental care provided to the offspring could decrease parental survival and future reproduction (2), it is expected that parents should avoid care for unrelated offspring to save resources for present or future reproductive events (3). Indeed, care adjustment in response to certainty of paternity has been reported in some species of birds (4), fishes (5) and arthropods (6), where parents reduced care assistance when perceiving a reduction in paternity. Then, if parental care is beneficial only when directed toward genetic descendants (2), why do parents sometimes care for unrelated offspring? It has been proposed that alloparental care is the result of a misdirected parental care (7,8), which can result from intraspecific clutch piracy (9) or brood parasitism (10,11), such as sneak fertilization by younger or smaller males (7,12). However, in some cases alloparents care for unrelated offspring because of direct benefits, such as higher survivorship of their own offspring or higher mating success (7). There is a strong selection on females to choose males that provide direct benefits, such as nest sites, territories and parental care, to improve females' and offsprings' fitness (13,14). Particularly, in species with paternal care, it is expected that females take into account not only traits that indicate the genetic quality of potential partners, but also the quality of paternal activities associated with offspring survival (1,15,16). In fact, in some animal groups, females prefer to mate and spawn in the territory of males already

associated with clutches (17,18). Thus, males may use clutch adoption as part of a reproductive strategy to attract mates (19).

Among tetrapods, amphibians show elaborate and conspicuous social and reproductive behaviors, making them a natural model system to investigate mating and parenting behaviors (20,21). In the Neotropical family Centrolenidae, males of some species of the genera *Hyalinobatrachium* and *Centrolene* attend clutches deposited on the undersides of leaves above water (21). Based on paternity analysis and behavioral observations, we found that males of the Amazonian species *H. cappellei* use paternal care as a mating strategy, i. e., males' chances of mating increased with the number of attended clutches (Valencia-Aguilar et al. in review).

Furthermore, although males of *H. cappellei* used unrelated clutches to attract females, they did not care for them, abandoning the unrelated offspring after acquiring their own (Valencia-Aguilar et al. in review). In fact, in species where alloparental care has evolved, although both related and unrelated offspring may serve for female attraction, only related clutches would contribute directly to male's fitness, thus related offspring should be more valuable than unrelated ones for the males (7,19,22).

As a follow up, here we used behavioral observations and clutch translocation experiments to investigate allopaternal care in *Centrolene peristicta* and *Hyalinobatrachium chirripoi*, by comparing both caring frequency and condition of the clutches experimentally placed in the territories of attending (males already taking care of clutches) and non-attending males (without clutches). Under a scenario where males do not exhibit alloparental care, we predict that

attending males will care only for their own clutches, while non-attending males will remain close to the unrelated clutch or in the territory to attract females, but without caring for the clutches. In this regard, we tested if males invest more care in related than in unrelated clutches. If males adopt unrelated clutches, we expect that they will invest less time and energy to care for them, compared to the investment directed towards their own clutches. Parental care decisions can also be influenced by territorial signals (23,24) because males may use contextual cues (e.g., spatial location, quality of surrounding resources) to recognize their offspring (25,26), ignoring, abandoning or consuming their own clutches when detecting changes in their territories (23,24). On that basis, we experimentally modified the territories of attending males of *C. peristicta* and *H. chirripoi* to determine the importance of spatial and territorial cues in the recognition of clutches by males.

Results

Males of *Hyalinobatrachium chirripoi* and *Centrolene peristicta* mated with multiple females and cared for clutches in their territories, while continued calling to attract mates. Attending males of *C. peristicta* cared for up to two clutches, with a total number of eggs per clutch varying from 19 to 33 ($\dot{X}$ = 24.05, *sd* = ± 2.87 eggs, *N* = 36 clutches), and spent on average 27.8% (*sd* = ± 16.5, *N* = 10) of their time assisting the eggs. Likewise, males of *H. chirripoi* cared for up to three clutches, with a total number of eggs per clutch from 58 to 71 ($\dot{X}$ = 62, *sd* = ± 5.7 eggs, *N* = 20 clutches), and spent on average 21.9% (*sd* = ± 9.2, *N* = 10) of their time assisting the eggs.

All attending males of *C. peristicta* (*N* = 6) cared for the unrelated clutches experimentally placed in their territories, accepting them as their own clutches (Figure 1A, Table 1). In *H. chirripoi* half of the attending males (*N* = 3/6) cared (hydrating and handling) for the unrelated clutches placed in their territories (Figure 1B, Table 1). Of the remaining three clutches (each one placed in a different territory), the embryos of one died dehydrated, so we concluded that the male did not provided care. Although we did not observed males hydrating the other two clutches, we observed them touching the unrelated embryos, which developed into larvae and hatched as well as each male's own embryos. Once males of both species noticed the presence of the introduced clutches, they remained between 10–20 minutes close to the new clutch, only observing it; they repeated this behavior three to five times per night after hydrating their own clutches. It took between 2 to 3 days for the males to start attending the unrelated clutches (Figure 2A and B, see S1). However, contrary to our expectations, once males accepted the unrelated clutches, they cared for all of them in their territories (related and unrelated) in a similar frequency (*C. peristicta*: $\dot{X}$ = 28.50%, *sd* = 3.33 related, $\dot{X}$ = 27.83%, *sd* = 3.97 unrelated, *t* test: $t_{28.89}$ = 0.45, *p* = 0.60; *H. chirripoi*: $\dot{X}$ = 21.16%, *sd* = 2.13 related; $\dot{X}$ = 20.33%, *sd* = 1.75 unrelated, *t* test: $t_{20.98}$ = 0.57, *p* = 0.15), even after acquiring new clutches (Figure 2). Males of both glassfrog species that were not caring for their eggs (non-attending males) abandoned their territories when unrelated clutches were placed close to them. Once these males noticed the presence of the clutch, they slowly approached and touched (*N* = 2 males of *H. chirripoi*) or observed it (*N* = 4 males of *H. chirripoi*; *N* = 6 males of *C. peristicta*) for a few

minutes before moving away. Even more interesting, males moved away few meters from their original location (*C. peristicta*: 3–5 m, *N* = 6; *H. chirripoi*: 4–6 m, *N* = 6) and established a new territory, where some of them acquired their own clutches (*C. peristicta N* = 3 males; *H. chirripoi N* = 2 males).

Regarding the territory relocation experiment, as we expected, all males in the control group (*N* = 3 males of *H. chirripoi*; *N* = 3 males of *C. peristicta*) remained in their territories despite the changes, caring for their clutches until larvae hatching. In the relocation experiment, 50% of males (3/6) of *H. chirripoi* abandoned the clutches, while all males (6/6) of *C. peristicta* remained and cared for the clutches until larvae hatched. For *H. chirripoi* it is not clear whether males abandoned clutches due to perturbation during the relocation of the territory (too much shaking because of the slippery rocks in the stream) or because they could not recognize the new area. Despite this, 50% of the males of *H. chirripoi* remained in their territories after relocation and exhibited the same care behavior that we observed in the attending males in the control group. The time spent caring for the clutches after territory relocation was not different compared to assistance time provided by males of the control group (*C. peristicta t* test: $t_{20.98}$ = 01.42, p = 0.15; *H. chirripoi t* test: $t_{17.91}$ = 0.62, p = 0.32). All *C. peristicta* males whose territories were relocated cared for their clutches despite territory manipulation. In addition, we monitored some non-attending males until they acquired one clutch and then we removed it after four days of attendance, placing it again close to the corresponding father two days later. However, contrary to what we expected, males of both species (*H.*

chirripoi N = 1; *C. peristicta N* = 2) abandoned their territories, even when their own clutches were relocated on the same leaf, although in a different site within the territory.

Discussion

Overall, we found that attending males of *Centrolene peristicta* and *Hyalinobatrachium chirripoi* cared for unrelated clutches in their territories. However, the presence of eggs per se does not stimulate parental care behavior in the non-attending males (without clutches), since they abandoned their territories when noticing the presence of unrelated clutches. In contrast, for example, males of the poison frog *Allobates femoralis* transported unrelated tadpoles placed in their backs into natural and artificial pools, suggesting that tactile stimuli were enough to induce care behaviors in males of this species (27). Unlike non-attending males of *A. femoralis,* care behaviors were not triggered by external stimuli in non-attending males of *C. peristicta* and *H. chirripoi*, suggesting that other factors like steroid hormonal levels could be regulating care behaviors in these glassfrog species (28). It has been documented that hormonal changes can lead to or stimulate paternal care behaviors in species in which care involves attendance, carrying and provisioning of the offspring (28,29,30). For example, attending males of the tropical frog *Eleutherodactylus coqui* showed low androgen levels throughout the egg attendance period; however, once the eggs hatched and the males began to call and mate again, the plasma androgen levels increased rapidly (28). Likewise, the levels of testosterone decrease while cortisol increases in

both females and males of the poison frog *Dendrobates tinctorius* during parental care (31). It is assumed that a decrease in testosterone levels facilitates paternal care via reduction of mating investment and aggressive behavior (32,33). However, neither territorial nor aggressive behaviors were observed in males of *H. chirripoi* and we only observed one territorial aggression between males of *C. peristicta* during our fieldwork (2 months of daily observations). Besides, males of both species continued calling and acquiring clutches during parental care period (Valencia-Aguilar, pers. obs). Townsend et al. (28) found low androgen levels in males of *E. coqui* during parental care period; however, in a subsequent experimental study, they showed that paternal care was independent of testosterone levels, suggesting that changes in behavior were not dependent on plasma androgen levels (34). In this sense, since some minimal level of circulating androgens are necessary for males to call (33,34), we would expected that testosterone levels between attending and non-attending males of *C. peristicta* and *H. chirripoi* will have no differences.

Care activities in some species of fish (35), anurans (31), birds (36) and mammals (37), have been associated to an increase in cortisol levels, which play a central role in regulating reproductive behavior, mobilizing the energy reserves that maintain care behaviors (offspring attendance or defense) (33,38). In this sense, we suggest that alloparenting in *C. peristicta* and *H. chirripoi* is contextual-dependent because unrelated clutches were adopted only by males already associated with their own clutches. In other words, if the physical contact of the males with their own clutches induces a “turn on” hormonal

response (i.e. an increase in cortisol levels), we would expect that they will continue caring for all the clutches inside their territories regardless their parentage (24,27). As in *E. coqui* and *D. tinctorius,* hormonal shifts in males of *C. peristicta* and *H. chirripoi* may also contribute to promote paternal care behaviors; however, it is not currently possible to generalize across species. Hence, future experimental studies will help to understand the role of hormones in the paternal behavior expression in these glassfrogs. However, independent on what triggers this behavior, adoption of unrelated clutches may be advantageous for alloparents if the fitness benefit offset the costs (7). Indeed, caring for unrelated clutches could be costly for non-attending males, reducing their own fitness if they do not acquire their own clutches. Hence, the observed abandonment of foreign clutches by males of *C. peristicta* and *H. chirripoi* may serve as a saving-resource strategy (2,39). Besides, since males acquired new clutches after we placed the foreign clutch and they continued caring for all the eggs (related and unrelated), attending males seems to benefit from alloparenting. However, it is necessary to determine if males' allocare investment has no significant extra cost (40), and if would increase both offspring survival (7) and mating success (41).

Territory changes, as well as territory relocations did not affect care behavior of the attending males in *C. peristicta* and *H. chirripoi.* However, contrary to our expectations, males rejected their own clutches after changing their spatial location inside the male's territories, suggesting that parents did not recognize their own offspring, even after caring for them for several days before

relocation. Hence, in *C. peristicta* and *H. chirripoi* kin discrimination does not appear to be present (26, this study). Indeed, we could not test whether males were able to discriminate between related and unrelated clutches directly, but our results suggest that attending males of these species use clutch's location inside their territories rather than chemical, visual, or tactile cues for offspring discrimination. These results could explain why males abandoned their clutches when they were moved to an adjacent place on the leaf, as well as the fact that males chose to care for the unrelated clutches placed close to their own clutches regardless of offspring relatedness.

Attending males did not accept the unrelated clutches immediately; instead, they spent a couple of days observing the eggs without caring for them, suggesting that males were able to differentiate the presence of the new clutch in some way. Therefore, if males detected the presence of the foreign clutch, why they did not decrease care frequency after been cuckolded? Wisenden (7) pointed out that many parents do not discriminate between related and unrelated offspring when caring for them, either because the cost of attending is very low or the cost of eliminating or abandoning them is too high. Hence, a possible answer to the question raised above is that the benefits of allopaternal care are conditional to the fitness effects that a male would face by care desertion (45).

Glassfrog males do not actively defend their eggs from dangerous predators (e.g., skanes, spiders) instead, the main function of parental care in this group is related to prevention of embryos' dehydration (46,47,48,49,50). Thus, for *C. peristicta* and *H. chirripoi*, the cost of adopting clutches may actually be lower

than the cost of abandoning. Besides, males can also allocate time and energy not only in care activities but also in mate attraction and foraging (Valencia-Aguilar et al. in review). Field observations showed that unattended clutches of both species died by dehydration, parasitism and predation (51, Valencia-Aguilar, pers.obs.). In this sense, by adopting unrelated clutches, male's own clutches may benefit from the dilution effect in cases of a predator attack or fungal infection (52).

Moreover, by caring for both related and unrelated clutches, males avoid potential costs of abandoning their own offspring. If a male interrupts offspring assistance, he will waste time, resources and energy allocated in parental and mating effort (1,2,45). Finding a new mate can be difficult for males of both species due to temporal variation in population density and the female 'time out' mating. The 'time out' required to recover from a mating event was estimated to be around two weeks for *H. cappellei* females (Valencia-Aguilar et al. in review). Hence, by losing both, care and mating investment as a result of desertion (1, 8), a male may not find a new receptive female and loose the entire breeding season. Consequently, attending males may be tolerant to cuckoldry because alloparenting behavior would not reduce their present or even future reproductive success (8), as observed in *C. peristicta* and *H. chirripoi*, where males continued acquiring clutches after clutch adoption.

To conclude, here we showed that males of *Centrolene peristicta* and *Hyalinobatrachium chirripoi* provide alloparental care when we experimentally introduced an unrelated clutch. However, male's decision to provide allocare seems to be dependent mostly on his own care-status condition. We suggest

that parental decision making, involving a switch between adoption or desertion of clutches, may have evolved in the absence of direct offspring recognition cues (e.g., tactile, chemical). Thus, interesting questions related to kin recognition, costs and benefits of alloparental care in *C. peristicta* and *H. chirripoi* remains to be further investigated.

Methods

Study site and parental care behavior

Observations and behavioral experiments were carried out in the Reserves Itapoa (December /2018, 0°07'22.2"N, 79°16'16.2"W), Canandé (January /2019, 0°31'18.6"N, 79°08'09.8"W) and Las Gralarias (February-March /2019, 0°00’33" S, 78°44’15" W), situated in the Esmeraldas and Pichincha provinces of Ecuador, respectively. We monitored one population of *Centrolene peristicta* (*N* = 10 males; Las Gralarias reserve) and two of *Hyalinobatrachium chirripoi* (*N* = 10 males; Itapoa and Canandé reserves) for an entire breeding season to collect data on mating system, as well as paternal care activities. Males were localized and monitored during the day and night with headlamps (red-light) through visual and acoustic searches. Focal observations were made at one-meter of distance from each male during 15 minutes at day and 20-40 minutes at night, when individuals were active (twice or three times per night). During observations we recorded male activity (calling, mating, aggressive or defensive behaviors against potential predators or competitors), male position relative to eggs (next, touching, sitting on), egg attendance frequency (time that male expended in clutch maintenance during the day, between 09:00-13:00, and at

night, between 19:00-02:00), and egg/embryo clutch condition (parasitism, predation, embryonic development). These data allowed us to determine how often males care for their eggs (hydrate, touch or protect them), information that was used as a base line for behavioral experiments and comparison. Each male was used just once in the behavioral experiments (see below). A high-resolution digital video camera recorder with an infrared night-shot mode (Sony Handycam AVCHD) was used to visual monitor both behaviors and experiments. The video camera was held in the territory of each male (close enough for monitoring without disturbance) and activated to record in the night vision function to allow observations without flashlights.

Clutch adoption experiment

Males of *C. peristicta* (*N* = 12) and *H. chirripoi* (*N* = 12) were randomly assigned to two different treatments: (1) attending males (*N* = 6): individuals already caring for their own clutches, to which one unrelated clutch was experimentally added; (2) non-attending males (*N* = 6): individuals without clutches, to which one unrelated clutch was also experimentally added. All unrelated clutches were obtained from males located 50-80 m away and embryos were at the same age and size, in the case of the attending group. In order to move clutches, we cut a piece of the leaf where the clutch was placed and relocated it in another male's territory, carefully stitching the leaf with thread and needle during the day (Figure 1), when males were inactive, to minimize disturbance. To compare male's behavior relative to related versus unrelated clutches, we measured the care frequency of both clutch types (related vs. unrelated), which

was defined as the proportion of time that an individual spent hydrating, defending or in contact with the eggs (touching them with their body, hands or feet) relative to the length of the observation period. Care-frequency differences between related and unrelated clutches were compared using a Student's t-test. All statistical analyses were performed using the software R 3.1.0 (R Development Core Team 2014).

Territory changes and territory relocation experiments

Attending males were randomly assigned to the control (*N* = 3 males of each species, due to limited availability of attending males) and the relocated group (*N* = 6 males of each species). In the control group, males' territories were partially modified to control for handling effects. We cut some of the branches and leaves near the leaf where the male was caring for their clutches. In the relocated group, the entire branch with leaves, where the male established its territory, was removed from its original location and transferred to a new one. Males were relocated after providing 5 days of egg care (embryos at Gosner stages 19–20) to examine the importance of spatial location in the care behavior. To ensure that the new location was different from the original one, we moved males' territories with their clutches 60 m away, either up or down stream from their original position. Besides location, we also modified the original height of the territories (3–5 m above the stream), placing them between 2–3 m above the stream. All changes and relocations of the territories were made during the day, when males were inactive, to minimize disturbance. The time that males spent in their territories after the relocation was used as the

behavioral response. We also observed parental behavior performed by males in both groups. We compared care behavioral responses between males in the control group and males in the relocated territory using a Student's t-test. All statistical analyses were performed using the software R 3.1.0 (R Development Core Team 2014).

Ethical considerations

Field observations and behavioral experiments were carried out in accordance with the Association for the Study of Animal Behaviour guidelines and current Ecuadorian legislation. Fieldwork was approved by the Ministry of Environment of Ecuador (scientific research N°019-2018-IC-FAU-DNB/MAE).

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Author contributions statement

AVA and CPAP designed the original research questions, AVA performed the field experiments, analyzed the data and drafted the manuscript. All authors discussed, review, edited and approved the final manuscript.

Additional Information

Conflict interests

The authors declare no conflict interests

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Figure Legends

Figure 1. Glassfrog males caring for related and unrelated clutches in different developmental stages. Unrelated clutches were carefully stitched with thread and needle. **(A)** Male of *Centrolene peristicta* caring for an unrelated clutch (pointed by arrow) placed close to its own clutches. **(B)** Male of *Hyalinobatrachium chirripoi* caring for an unrelated clutch (pointed by arrow) placed in its territory. Note that a new clutch was laid partially on the stitched leaf, where the unrelated clutch was experimentally placed.

Figure 2. Care behavior response of attending males of two glassfrog species in the clutch adoption experiment. **(A)** Time length (days) and caring frequency of related and unrelated clutches by *Centrolene peristicta* males. **(B)** Time length (days) and caring frequency of related and unrelated clutches by *Hyalinobatrachium chirripoi* males.

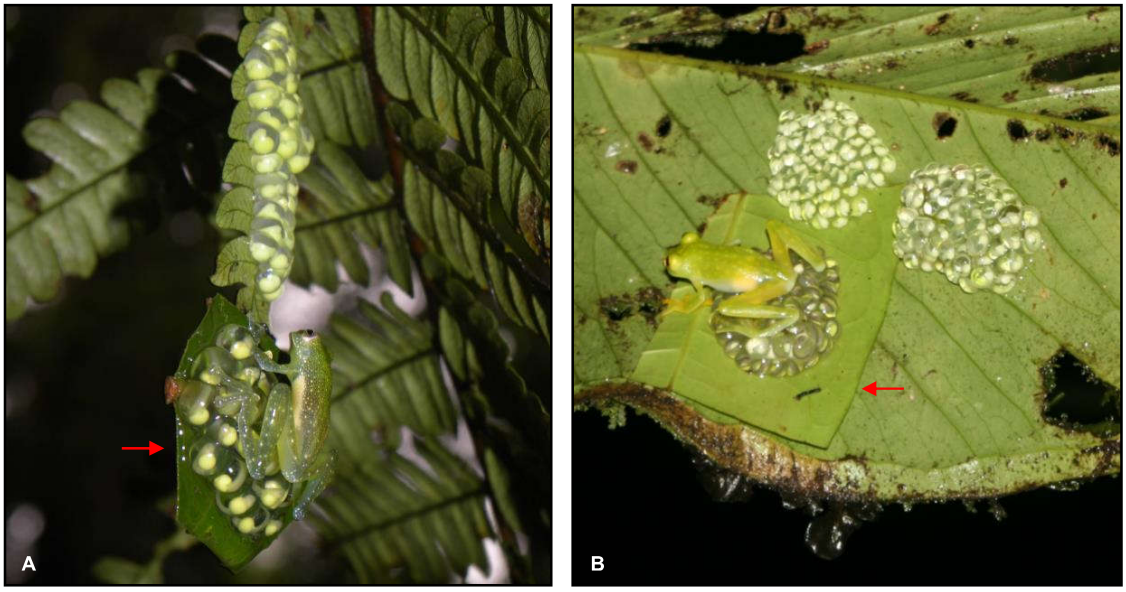


Figure 1

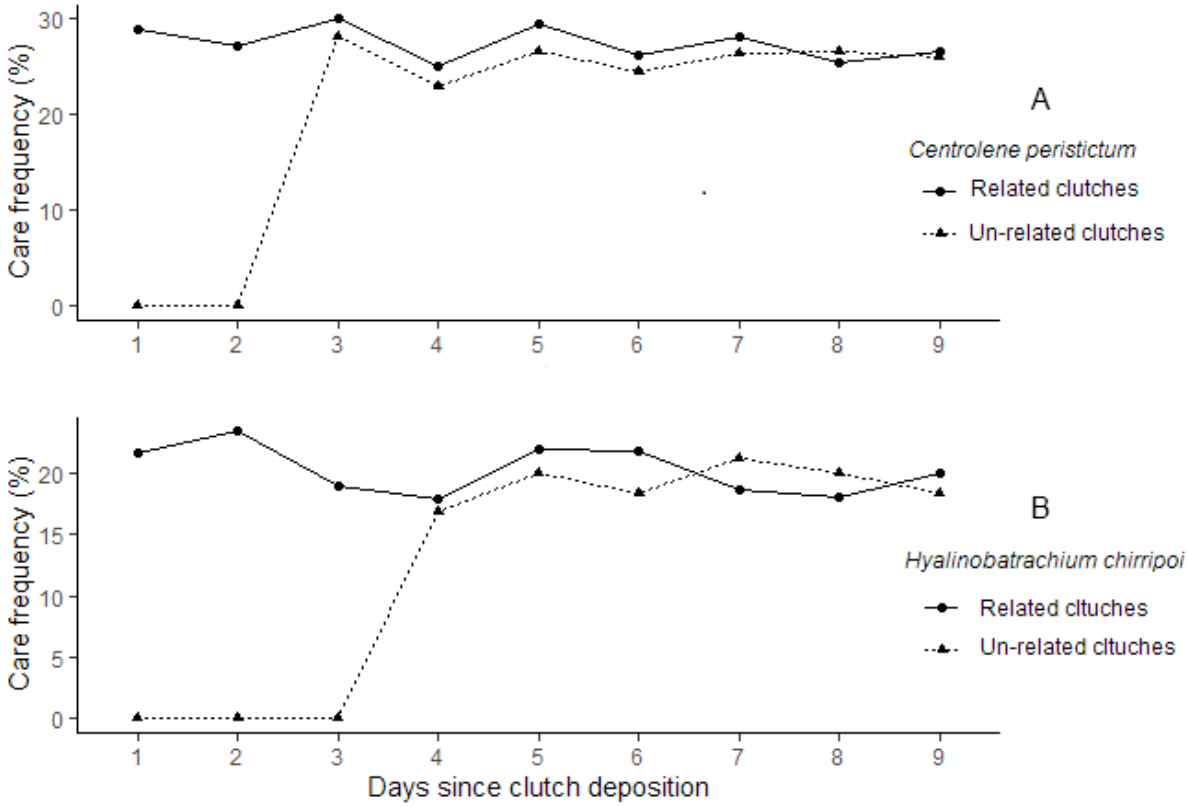

Care frequency (%)
30
20
10
0
1
2
3
4
5
6
7
8
9
A
Centrolene peristictum
Related clutches
Un-related clutches
Care frequency (%)
20
15
10
5
0
B
Hyalinobatrachium chirripoi
Related cltuches
Un-related cltuches
Days since clutch deposition

Figure 2

Table 1. Parental care and hatching success for unrelated and related clutches of *Centrolene peristicta* and *Hyalinobatrachium chirripoi*. Male = code for each male monitored in the field; Related clutch = number of clutches that each male was caring for before the clutch introduction experiment; Unrelated clutch = number of clutches placed in each male's territory; New clutch = number of clutches that each male acquired after unrelated clutch introduction; Hatching = number clutches that hatched after experiment.

Species	Male	Related clutch	Unrelated clutch	New clutch	Hatching
Centrolene peristicta	1	2	1	0	Both
	2	1	1	0	Both
	3	1	1	0	Both
	4	2	1	1	Both
	5	1	1	0	Both
	6	1	1	0	Both
Hyalinobatrachium chirripoi	1	1	1	0	Only related one
	2	1	1	0	Both
	3	1	1	0	Both
	4	1	1	1	Both
	5	1	1	1	Both
	6	2	1	0	Both

CHAPTER IV

TESTES SIZE AND PATERNAL CARE INVESTMENT IN GLASSFROG SPECIES (ANURA: CENTROLENIDAE)

(Target American Naturalist)

Testes size and paternal care investment in glassfrog species (Anura: Centrolenidae)

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Abstract

Empirical and theoretical studies have explored how sperm competition, clutch size, and variation in hormonal levels affect the relative testis size among frogs. However, the relationship between paternal investment and variation in testis size has been underexplored in anurans. Since paternal care might be energetic demanding, precluding investment in testis size, and testosterone levels may be negatively related with caregiving activities, here we tested the hypothesis that males of glassfrog species that care for their offspring have smaller relative testes volume compared with species that do not exhibit paternal care. Besides, we also examined whether females' fecundity influence testis size in the Centrolenidae family. To test these hypotheses we collected data on testis size of breeding frogs using information from specimens we have collected, as well as from museum specimens, and literature. We measured testes size, body size of males and females, and clutch size (number of eggs) of 26 species of glassfrogs. Controlling for body size and phylogenetic relationships, we found that attending males haved smaller testes compared with non-attending males in glassfrog species. Besides, no effects of clutch size on testes size were detected. These results suggest that testes size may also vary in response to a post-fertilization investment, such as parental care.

Keywords: Amphibian, clutch size, gonadal investment, external fertilization, parental investment.

Introduction

Life-history models predict that organisms face trade-offs between mating and parenting investment to maximize their reproductive success (fitness) because of the finite amounts of energy to expend in reproduction (Gadgil and Bossert 1970, Stearns 1992). As a consequence, we expect that certain traits (morphological, anatomical, and physiological) could be affected by the mating system, especially those that require large amounts of energy (Stearns 1992, Stiver and Alonzo 2009). For example, sexual morphological features are known to be associated with specific mating systems in many species of insects (Bangham et al. 2002), fishes (Awata et al. 2006), anurans (Byrne 2004, Liao et al. 2011), birds (Pircher et al. 2005), and mammals (Soulsbury 2010, Mascaro et al. 2013), in which there is a positive correlation between gonadal expenditure and alternative mating tactics, such as polyandry and clutch piracy (Byrne et al. 2002, Vieites et al. 2004, Pircher et al. 2005).

Polyandrous mating systems, where females frequently mate with multiple males during a single reproductive period (Parker 1970, Moller 1991), may lead to sperm competition, an extended form of male–male competition between sperm from rival males to fertilize females' eggs (Parker 1970). In particular, males of species with high levels of sperm competition may have evolved large testes to fertilize as many eggs as possible in a reproductive cycle (Moller 1991, Zamudio et al. 2016, Rowley et al. 2019), since larger testes provide an advantage in sexual competition, because they produce more sperm (Emerson 1997). However, larger testes also produce more testosterone (Mascaro et al. 2013), inhibiting some reproductive behaviors in males, such as

parental care (Mascaro et al. 2013, Pikus et al. 2018); therefore, large testes may be selected against in species with extensive paternal care.

To date, studies about the role of paternal care in the evolution of testis size show contradictory results. Comparative studies in fishes, birds and mammals, testing the factors associated with testis size (Pitcher et al. 2005, Mascaro et al. 2013), demonstrated that species with paternal care had smaller testes than species without care (Mascaro et al. 2013) because males that invest more in mating participate less in caregiving. However, this pattern was not observed in species with male incubation (birds, Moller 1991) or different brooding types (fishes, Kvarnemo and Simmons 2004), which is of particular interest considering that, although the studied species have parental care, they exhibit differences in their fertilization mode (internal vs. external, respectively). Alternatively, females' fecundity has also been pointed out as a driver of testes size (Emerson 1997); larger clutches (egg number) may require larger ejaculates, hence larger testes, to ensure fertilization of all eggs (Emerson 1997, Stockley et al. 1997).

Here, we investigated the relationship between paternal care, females' fecundity, and testis size in glassfrog species using phylogenetic comparative methods. Glassfrogs provide an excellent opportunity to test the effect of paternal care on testis size because of the differences in care investment in this family (Delia et al. 2017). Although species of the Centroleninae and Hyalinobatrachinae subfamilies present ecological similarities (Guayasamin et al. 2009), in the Hyalinobatrachinae subfamily almost 90% of the species exhibit an intense paternal care of eggs (McDiarmid 1978, Vockenhuber et al., 2008,

Valencia-Aguilar et al. 2012, Delia et al., 2013, Lehtinen et al. 2014), while few species in the Centroleninae subfamily exhibit some degree of care behavior (e.g., male guarding the clutches; Lynch et al. 1983, Dautel et al. 2011, Vargas-Salinas et al. 2014, Salgado and Guayasamin 2018). Specifically, we tested the hypothesis that males of glassfrogs that care for their eggs have smaller relative testes size compared with species that do not exhibit paternal care, a pattern that might have been driven by the inverse relationship between testosterone level and caregiving. In contrast, according to life-history models, we also predict that males of species that do not exhibit paternal care will have larger testes because they invest more in mating and might be under sperm competition risk. Moreover, we also examined whether species with larger clutches will present larger testes size. Although testes size is positively correlated with clutch size in some groups (Pircher et al. 2005, Soulsbury 2010), there is no evidence of large variation in clutch size among glassfrogs (Delia et al. 2017) hence, we predict that testes size will not be correlated with clutch size.

Methods

Data collection

We collected some data during fieldworks in Brazil (Fazenda São Nicolau 9°49’10.1” S, 58°15’29.9”W) and Ecuador (Itapoa 0°07'22.2"N, 79°16'16.2"W and Canandé 0°31'18.6"N, 79°08'09.8"W reserves), as well as from specimens deposited in the Zoology Museum at the Pontificia Universidad Católica del Ecuador (QCAZ), the Célio F.B. Haddad (CFBH) Amphibian Collection,

Universidade Estadual Paulista, Rio Claro, São Paulo, the Coleção de Anfíbios do Centro de Coleções Taxonômicas da Universidade Federal de Minas Gerais (UFMG), and material provided by Dr. Marco Rada from Colombia. We supplemented the dataset with data from primary research publications, online database (AmphibiaWeb 2019, Ron et al. 2019), and peer-reviewed books (Savage 2002, Kubicki 2007). Our final database (Table S1), contains information for 26 glassfrog species; eight from the Hyalinobatrachinae subfamily, all in the genus *Hyalinobatrachium* and with paternal care, and 16 species in six genera from the Centroleninae subfamily, including four species of *Centrolene* (two with paternal care), three of *Espadarana,* four of *Nymphargus,* two of *Sachatamia,* two of *Teratohyla* and three of *Vitreorana* all without paternal care. The Hyalinobatrachinae subfamily contains two genera and 34 species (Guayasamin et al. 2009, Frost 2020), where paternal care is widespread in *Hyalinobatrachium* (Delia et al. 2017). On the other hand, although the Centroleninae subfamily contains nine genera and 121 species (Guayasamin et al. 2009, Frost 2020), paternal care has been observed in only six species in the genus *Centrolene,* which holds 27 species (Delia et al. 2017).

Life-history variables

For the 26 species of glassfrogs, we collected the following variables: (1) Male snout-vent length (SVL); (2) female SVL; (3) clutch size (number of eggs); (4) testes volume, and (5) paternal care treated as binary variable (present or absent). Snout-vent length (SVL in mm) was measured for males and females, and mean values separated by sexe were used in the analysis. Clutch size was

defined as the number of eggs laid during one spawn event. Testes volume was calculated as $4/3\pi*a^2*b$, where *a* and *b* are the width and length of each testis, respectively. The length and width of the right testis of each male were measured using an ocular micrometer (0.1 mm scale) fitted to a stereomicroscope (Zeiss Stemi SV11). In some anuran species, testes tend to decrease in size outside the breeding season (Lofts 1974) or to vary in size depending on environmental conditions (Sun et al. 2011) or male-male interactions (Awata et al. 2006, Liao et al. 2011). Hence, specimens were only used if they had been collected within their respective breeding seasons in the same locality and date. For this reason, we could only obtain measurements for three-to-eight males per species. Males and females' SVLs, clutch size, and testes volume were log transformed to ensure homoscedasticity.

Phylogenetic comparative analysis

Preliminary analyses showed significant positive correlations between testes volume and males SVL (R^2 = 0.51, P < 0.01; N = 26) (Figure S1A), and between clutch size and female SVL (R^2 = 0.179, P = 0.019; N = 25) (Figure S1B). To remove the effect of body size (Revell 2009), we performed regression analyses between testicular volume vs. male SVL, as well as between clutch size vs. female SVL, and used the regression residuals in subsequent analyses. To run these analyses and to test for associations between testicular volume, paternal care and clutch size, we used Phylogenetic Generalized Least Squares (PGLS) (Symonds and Blomberg 2014) and a pruned version of a glassfrogs phylogeny that contained all analyzed glassfrog species (Delia et al. 2017,

Figure S2). This approach controls for the non-independence among species by incorporating a variance–covariance matrix that represents their phylogenetic relationships. All analyses incorporated phylogenetic dependence by estimating Pagel's ʎ (Kamilar and Cooper 2013). We built separate PGLS models with testicular volume as the response variable and paternal care (present or absent) and clutch size as the predictors. We also built models considering the additive effect and interaction between variables. All analyses were carried out using R v. 3.6.0 (R Team 2019) with the 'caper' package (Orme et al. 2013).

Ethical Treatment of Animals

Specimens were collected in accordance with the Association for the Study of Animal Behaviour guidelines and current Brazilian and Ecuadorian legislation. Fieldwork in Ecuador was approved by the Ministry of Environment of Ecuador (scientific research N°019-2018-IC-FAU-DNB/MAE), and in Brazil by the Instituto Chico Mendes de Conservação da Biodiversidade/Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (ICMBio/IBAMA; license number 51479-4) and Ethics Committee on Animal Use of the Universidade Estadual Paulista (UNESP), Rio Claro, São Paulo state, Brazil (protocol number 9457). Specimens collected in Brazil and Ecuador were housed at the Célio F.B. Haddad (CFBH) Amphibian Collection, Universidade Estadual Paulista, Rio Claro, São Paulo and at Muzeo de Zoología of the Universidad San Francisco de Quito (ZSFQ), respectively.

Results

Overall, females (X = 24.80 ± 3.36, N = 25 species) of the examined glassfrog species were larger than males (X = 22.51 ± 3.09, N = 26 species; Table 1, t = 7.83, P < 0.001). Clutch size ranged from 14 to 26 eggs in *Centrolene* species (X = 19.91 ± 5.58, N = 4 species), 26 to 44 in *Espadarana* (X = 33.74 ± 9.31, N = 3 species), 19 to 69 in *Hyalinobatrachium* (X = 33.76 ± 19.58, N = 8 species), 24 to 50 in *Nymphargus* (X = 34.5 ± 11.03, N = 4 species), 22 to 48 in *Sachatamia* (X = 35.12 ± 18.20, N = 2 species), 18.55 to 20.75 in *Teratohyla* (X = 19.65 ± 1.55, N = 2 species), and 16.67 to 33 in *Vitreorana* (X = 25.89 ± 8.36, N = 3 species, Table 1). Testicular volume varied from 1.23 (*Hyalinobatrachium aureoguttatum*) to 24.04 mm^3 (*Centrolene buckleyi*, Table 1, Figure S3) and was positively correlated with male' SVL (R^2 = 0.510, $F_{1, 24}$ = 27.08, P < 0.001). We found that testicular volume was three times larger in species without paternal care (X = 8.23 mm^3 ± 7.42, N = 16 species; Table 1), when compared to species with paternal care (X = 2.36 mm^3 ± 0.65, N = 10 species; Table 1). Likewise, within *Centrolene* testicular volume in species without paternal care (*C. buckleyi* and *C. hybrida; X* = 13.73 mm^3 ± 14.58) was larger than in species with paternal care (*C. daidalea* and *C. peristicta; X* = 1.89 mm^3 ± 0.32, Table 1).

PGLS models showed that a significant amount of the variation in testicular volume was explained by the variables examined (full model, R^2 = 0.246, $F_{2, 22}$ = 4.928, P = 0.017). However, only parental care had a significant independent contributions to the model (PGLS, $F_{1,24}$ = 4.81, P = 0.04, Table 2). Species without paternal care had larger testes relative volume (median = 0.089; range = -0.21-0.617; N = 16) compared to those where males care for

their offspring (median = -0.188; range = -0.38-0.14; N = 10) (Figure 1). As expected, clutch size varied with female body size (SVL), where larger females produce more eggs per clutch than smaller ones (R^2 = 0.180, P = 0.02), even after controlling for phylogeny. However, we found no differences (t = 1.77, P = 0.30) in clutch size between species with (X = 32 ± 17.71, N = 10 species,) and without (X = 29 ± 11.13, N = 16 species) paternal care. Furthermore, variation in testicular volume was not related with variation in clutch size (PGLS, $F_{1,23}$ = 1.773, P = 0.2) (Figure 2).

Discussion

We found that, in glassfrogs, variation in testicular volume was not correlated with clutch size, but with paternal care investment. As we predicted, males of the species without paternal care have larger testicular volume than males of species with paternal care. Testes size has been associated with testosterone levels, which plays a central role in the expression of numerous sexual traits (Wingfield et al. 1990, Andersson 1994). However, although elevated levels of circulating testosterone will positively influence a male's testes size (Emerson 1997, Mascaro et al. 2013), elevated levels of testosterone will also decrease male contributions toward parental care (Townsend and Moger 1987, Lynn et al. 2005, Fischer and O'Connell 2019). In this sense, we suggest that the evolution of smaller testes in some glassfrog species could be the result of females choosing males that exhibit more parental care and, thus, have lower levels of testosterone (and smaller testes). To adequately test this hypothesis in glassfrogs, we still need to confirm that

testosterone levels are dependent on testes size in this family. Also, this hypothesis requires that females actually assess the degree of parental care provided by different males; visual or auditive cues, such as males calling close to egg clutches during consecutive nights or number of eggs/clutches at male´s territory, could provide such information to females (Valencia-Aguilar et al. in review, chapter 2).

An alternative explanation for our results could be that the large testes size of male glassfrogs without parental care is associated to a higher matting effort. It has been pointed out that differences in sperm production and, consequently, the relative testis size among males are related to variation in resource allocation for mating and parenting (Requena and Alonzo 2017). Paternal effort could lead to a decrease in gonadal investment, because if an individual invests more in parental care he will have less energy to invest in survival, growth, or gamete production (life history theory; Trivers 1972, Alonzo and Kindsvater 2008). In fact, sperm production can be costly (Olsson et al. 1997, Dowling and Simmons 2012; Fitzpatrick and Lüpold 2014) and since organisms have finite amounts of energy to expend on reproduction, it is expected that the allocation of resources toward either mating or parenting will be optimized to maximize males' fitness (Requena and Alonzo 2017). Hence, sexual selection will favor different allocation patterns depending on the mating system (Andersson 1994). For instance, a study with more than 1000 species of birds show that the testes size in males was smaller in monogamous species that breed solitarily and participate in feeding their offspring (Pitcher et al. 2005). Likewise, mating effort and paternal nurturing behavior in mammals are

negatively related and testes volume decrease with paternal investment (Mascaro et al. 2013). Similar studies also showed that morphological traits can be affected by mating system, with males of polygynandrous species investing more energy in gamete production, therefore larger testes, than monogamous males (Beltran et al. 2010, Soulsbury 2010).

Larger testes may also be favored in polygynandrous species with no paternal care because of higher risk of sneakers trying to fertilize recent clutches, leading to sperm competition (Parker 1970, 1982, 1998, Vieites et al. 2004). Previous comparative studies on a variety of taxa have shown that sperm competition is associated with increased testes size or larger ejaculates to fertilize a larger amount of eggs (Moller 1991; Byrne et al. 2002; Soulsbury 2010; Prado and Haddad 2003; Rowley et al. 2019). Thus, although there is no evidence of sperm competition among centrolenids, we hypothesize that males of glassfrog species that do not care for their offspring have larger testes because they invest more energy in mating and might have higher risk of sperm competition, contrasting to males of species with parental care that protect their eggs from opportunistic males that could try to fertilize some of the eggs recently deposited by the pair (Vieites et al. 2004). Matting effort measures and paternity analysis comparing species with paternal care versus those without this behavior will be useful to clarify whether these forces are driving testes size in this family.

Larger testes may be selected in species with large clutches, because larger clutches require relatively more sperm to be fertilized (Emerson 1997, Pitcher et al. 2005). A comparative study in the family Ranidae showed a

positive correlation between testes size and clutch size (Emerson 1997). However, subsequent studies in more than 180 Australian frogs, found that testes size was not related with clutch size, but instead with sperm competition risk, male' body mass, and site of oviposition (Byrne et al 2002, Byrne 2004). Our data also showed that teste size was not related with clutch size. In fact, clutch sizes of species with paternal care were not different from those species with no care. Females of the Myobatrachidae and Hylidae Australian frogs lay their eggs in aquatic or terrestrial habitats, embedded in jelly masses or foam nests. For these families, type of clutch (jelly or foam) seems to influence ejaculate expenditure, therefore testes size (Byrne et al. 2002). Females of glassfrogs lay eggs in a jelly mass on the vegetation or rocks out of water, along lotic habitats (McDiarmid 1978, Cisneros-Heredia and McDiarmid 2007, Guayasamin et al. 2009). In our study, oviposition sites across species were similar (e.g., vegetation close to streams) and all centrolenids deposit eggs embedded in jelly mass (Guayasamin et al. 2009). Hence, although we could not test these factors on testes size, we suggests that our results would not have changed because oviposition site and clutch type (jelly mass) are quite similar across glassfrog species.

Our results support previous studies in mammals and birds, which suggest that male participation in defense, incubation, or provisioning of offspring is driving teste size in males. Although variation in relative testes size in anuran species has been attributed mainly to sperm competition (Zamudio et al. 2016) and hormonal levels (Emerson 1997), we found that behavioral traits, such as paternal care, can also account for a significant portion of the variation

in testes size. Indeed, what we suggest is that there might be a complex relationship between paternal care, testes size, hormone levels (e.g., testosterone), and sperm competition in glassfrogs, which deserves further studies.

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Data availability

All data generated or analyzed during this study are included in this published article.

Author Contributions

AVA and CPAP designed the original research questions. AVA collected field data, performed all statistical analyses and drafted the manuscript. All authors substantially edited and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Body measurements (SVL and mass), testicular volume (volume of the right testis), and clutch size for 26 glassfrog species (Centrolenidae) of seven genera. Means and SD are shown, N in parenthesis.

Species	SVL (mm)		Body mass (g)		Gonads		Testes / SVL correction	Paternal care
	Females	Males	Females	Males	Testicular volume (mm^3)	Clutch size (number)		
Centrolene buckleyi	31.39 ± 0.43 (N = 2)	28.54 ± 0.32 (N = 4)	1.38 (N = 1)	1.58 ± 0.17 (N = 4)	24.04 ± 13.49 (N = 4)	16.5 ± 2.12 (N = 2)	0.408	Absent
C. daidalea	25.50 (N = 1)	20.68 ± 0.48 (N = 6)	-	0.53 ± 0.09 (N = 4)	2.12 ± 0.74 (N = 5)	25.90 ± 5.80 (N = 2)	-0.155	Present
C. hybrida	21.80 ± 1.31 (N = 4)	21.75 ± 1 (N = 6)	0.68 ± 0.02 (N = 2)	0.57 ± 0.03 (N = 6)	3.42 ± 1.30 (N = 6)	14 ± 2.65 (N = 3)	-0.024	Absent
C. peristicta	20.50 (N = 1)	19.73 ± 1.03 (N = 4)	-	0.34 ± 0.03 (N = 4)	1.66 ± 1.79 (N = 4)	23.24 ± 2.74 (N = 12)	-0.189	Present
E. andina	26.76 ± 1.86 (N = 5)	23.99 ± 1.15 (N = 7)	1.46 (N = 1)	0.87 ± 0.06 (N = 4)	4.38 ± 1.85 (N = 7)	44.08 ± 20.67 (N = 6)	-0.067	Absent
E. audax	25.85 ± 2.56 (N = 3)	22.05 ± 0.92 (N = 4)	1.09 (N = 1)	0.55 ± 0.11 (N = 3)	3.53 ± 1.43 (N = 4)	26 (N = 1)	-0.031	Absent
E. prosoblepon	26.75 ± 1.57 (N = 11)	24.05 ± 1.30 (N = 37)	1.09 ± 0.08 (N = 6)	0.74 ± 0.11 (N = 37)	4.53 ± 1.48 (N = 6)	31.16 ± 6.93 (N = 9)	-0.040	Absent
Hyalinobatrachium aureoguttatum	23.30 (N = 1)	19.94 ± 0.71 (N = 17)	-	0.31 ± 0.06 (N = 17)	1.23 ± 0.34 (N = 8)	30.86 ± 4.55 (N = 10)	-0.336	Present
H. cappellei	21.61 ± 1.03 (N = 17)	21.09 ± 1.19 (N = 4)	0.64 ± 0.11 (N = 15)	0.47 ± 0.05 (N = 4)	3.23 ± 0.47 (N = 5)	19 ± 4.75 (N = 11)	-0.002	Present
H. chirripoi	26.75 ± 0.81 (N = 4)	25.38 ± 1.01 (N = 27)	1.02 ± 0.1 (N = 3)	0.77 ± 0.11 (N = 27)	3.20 ± 0.89 (N = 6)	69 ± 9.64 (N = 3)	-0.270	Present
H. colymbiphyllum	26.75 ± 3.18 (N = 2)	25.80 ± 0.59 (N = 7)	-	0.71 ± 0.11 (N = 7)	2.91 ± 1.21 (N = 7)	60.44 ± 20.60 (N = 6)	-0.355	Present
H. fleischmanni	25.40 ± 4.42 (N = 4)	21.01 ± 0.88 (N = 9)	-	0.48 ± 0.15 (N = 9)	2.04 ± 0.75 (N = 4)	22.67 ± 6.04 (N = 9)	-0.174	Present
H. iaspidiense	22.20 ± 0.35 (N = 4)	19.17 ± 0.34 (N = 3)	0.37 ± 0.05 (N = 4)	0.29 ± 0.009 (N = 3)	2.59 ± 1.79 (N = 3)	22.50 ± 0.71 (N = 2)	0.047	Present
H. ibama	22.24 ± 0.83 (N = 3)	21.75 ± 0.49 (N = 4)	0.63 ± 0.04 (N = 2)	0.52 ± 0.01 (N = 4)	2.11 ± 1.08 (N = 4)	20 ± 7.07 (N = 2)	-0.234	Present
H. mondolfii	21.40 ± 1.55 (N = 2)	18.54 ± 1.94 (N = 3)	0.37 ± 0.009 (N = 2)	0.31 ± 0.08 (N = 3)	2.52 ± 1.16 (N = 3)	25.67 ± 5.13 (N = 6)	0.086	Present
N. grandisonae	31.22 ± 2 (N = 2)	27.51 ± 1.30 (N = 9)	-	0.96 ± 0.13 (N = 9)	11.70 ± 4.32 (N = 8)	50 ± 18.91 (N = 5)	0.152	Absent
N. griffithsi	24.23 ± 4.07 (N = 4)	24.42 ± 1.12 (N = 7)	-	0.54 ± 0.06 (N = 7)	21.07 ± 5.13 (N = 6)	33 ± 18.08 (N = 3)	0.589	Absent
N. lasgralarias	-	23.95 ± 0.26 (N = 2)	-	0.56 ± 0.05 (N = 2)	20.52 ± 8.59 (N = 2)	24 ± 16.97 (N = 2)	0.607	Absent
N. rosada	27.03 ± 0.80 (N = 2)	26.09 ± 0.62 (N = 3)	1.09 (N = 1)	0.98 ± 0.13 (N = 3)	12.85 ± 3.03 (N = 3)	31 ± 8.97 (N = 5)	0.273	Absent
Sachatamia albomaculata	27.35 ± 2.65 (N = 9)	23.63 ± 1.32 (N = 8)	1.23 ± 0.14 (N = 4)	0.75 ± 0.13 (N = 8)	3.43 ± 0.19 (N = 5)	48 ± 18 (N = 6)	-0.106	Absent
S. ilex	31.40 ± 2.75 (N = 10)	28.11 ± 0.98 (N = 26)	2.11 ± 0.22 (N = 12)	1.22 ± 0.09 (N = 26)	5.69 ± 1.64 (N = 6)	22.25 ± 4.99 (N = 4)	-0.191	Absent
Teratohyla midas	23.57 ± 2.13 (N = 3)	19 ± 0.44 (N = 7)	-	0.33 ± 0.04 (N = 7)	3.57 ± 0.46 (N = 7)	20.75 ± 8.42 (N = 4)	0.200	Absent
T. spinosa	20.47 ± 0.91 (N = 13)	19.38 ± 1.04 (N = 40)	0.60 ± 0.07 (N = 13)	0.51 ± 0.08 (N = 40)	1.67 ± 0.25 (N = 6)	18.55 ± 4.37 (N = 11)	-0.140	Absent
V. eurygnatha	21.57 ± 1.69 (N = 6)	18.27 ± 0.43 (N = 6)	0.47 ± 0.08 (N = 2)	0.43 ± 0.05 (N = 6)	3.69 ± 1.28 (N = 5)	28 ± 2.83 (N = 2)	0.267	Absent
V. ritae	20.41 ± 1.85 (N = 7)	18.90 ± 0.71 (N = 5)	0.69 ± 0.07 (N = 4)	0.35 ± 0.06 (N = 5)	3.69 ± 2.17 (N = 5)	16.67 ± 4.16 (N = 3)	0.223	Absent
V. uranoscopa	24.40 ± 1.54 (N = 4)	22.53 ± 2.32 (N = 6)	-	0.51 ± 0.15 (N = 6)	3.91 ± 0.64 (N = 6)	33 ± 6.98 (N = 4)	-0.020	Absent

Table 2. Testicular volume variation in relation to behavioral and life-history variables in glassfrog species (N = 26) using phylogenetically corrected generalized linear square (PGLS) models. Paternal care was used as a binary variable (present or absent). Clutch volume was log-transformed prior analyses. We provide parameter estimates with standard error (β ± S.E), the corresponding *t* and *P* and the adjusted R^2 for the model including F (d.f.) and *P*, respectively. (A: full model; B,C: reduced model).

	Estimated (S.E)	***t***	***P***
A			
Paternal care	-0.218 (0.083)	-2.620	0.015
Clutch size	-0.290 (0.260)	-1.116	0.276
model	0.246	4.928 *2,22*	0.017
B			
Paternal care	-0.313 (0.142)	-2.193	0.038
C			
Clutch size	-0.380 (0.285)	-1.331	0.196

Figure legend

Figure 1. Differences in testicular volume between glassfrog species with and without paternal care, using residuals of phylogenetic size-correction (GLS regression). Residual values from ten glassfrog species where males exhibit care activities (*Centrolene peristicta* in the figure) and 16 species where males do not care for their offspring (*Sachatamia ilex* in the figure). Boxplots show the median (thick line), the interquartile range (box), and whiskers extending to the most extreme values.

Figure 2. Relationship between the residual values of testicular volume and clutch size for all glassfrogs included in our dataset, (N = 25).

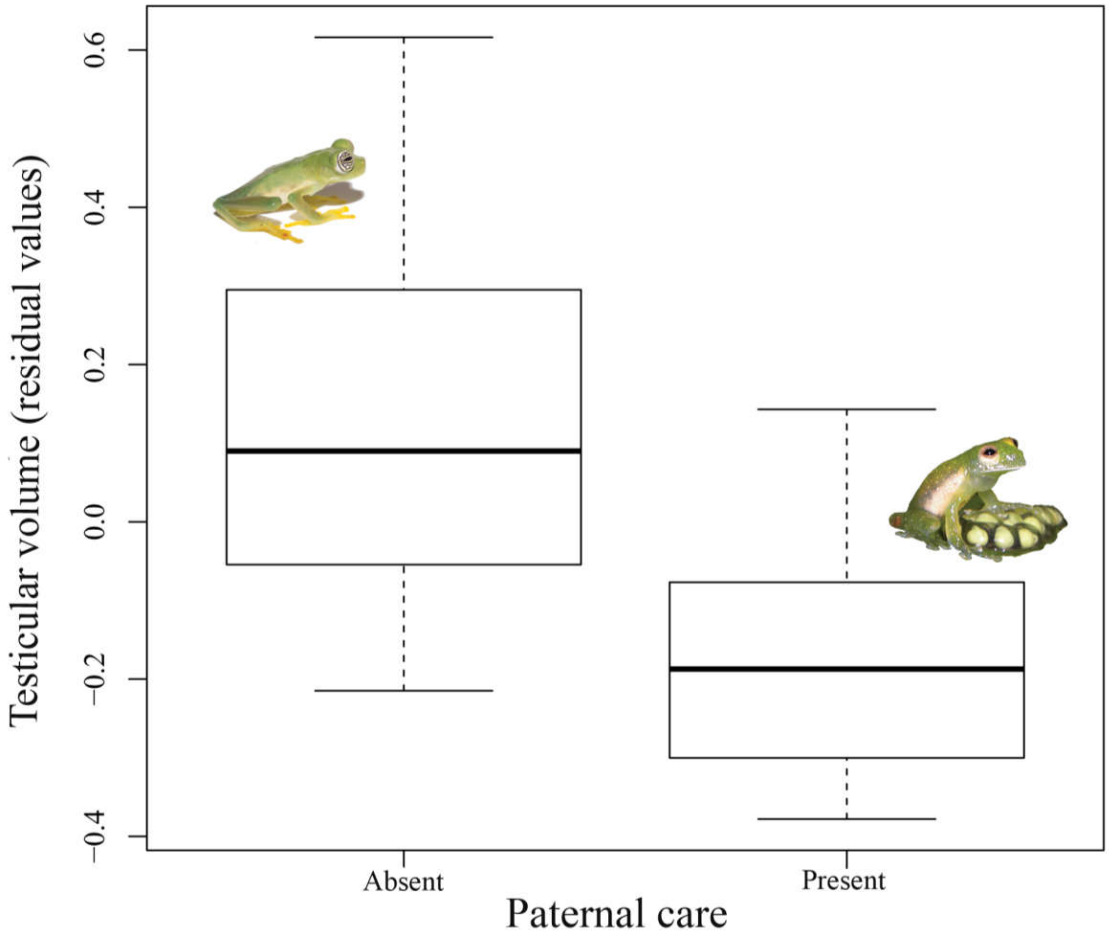

Testicular volume (residual values)
0.6
0.4
0.2
0.0
−0.2
−0.4
Absent
Present
Paternal care

Figure 1.

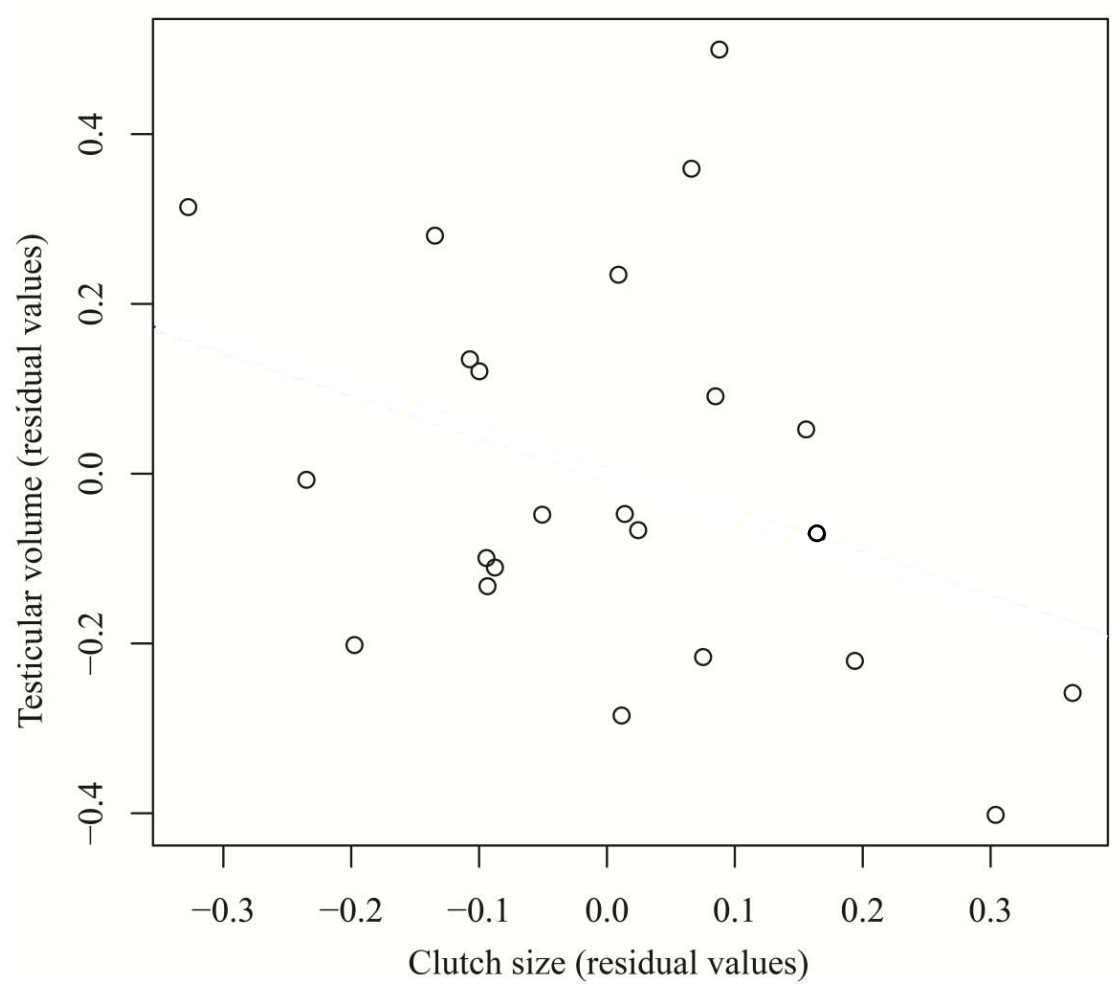

Testicular volume (residual values)
0.4
0.2
0.0
−0.2
−0.4
−0.3
−0.2
−0.1
0.0
0.1
0.2
0.3
Clutch size (residual values)

Figure 2

Supplemental Information for:

Testes size and paternal care investment in glassfrog species (Anura: Centrolenidae)

Anyelet Valencia-Aguilar[1,*], Juan M. Guayasamin[2,4], Cynthia P.A. Prado[1,3]

Table S1. Species and summary of data used in comparative analysis for the family Centrolenidae. SVL= snout-vent length, TL = testes length, TW = testes width, TV = testicular volume, PC = parental care, CS = clutch size. ND = no data.

Species	MALE					FEMALE		References
	SVL	TL	TW	TV	PC	CS	SVL	
Centrolene buckleyi	28.55	3.14	1.31	22.68	N	16.5	31.4	Guayasamin et al. 2006, 2018. Anfibios del Ecuador; this study
Centrolene daidalea	20.68	1.27	0.66	2.32	Y	25.9	25.5	Ruiz and Lynch 1991 (III); Rada et al. 2007; Cardozo and Señaris 2012
Centrolene hybrida	21.75	1.42	0.74	3.28	N	14	21.8	Ruiz and Lynch 1991; Rada et al. 2017; this study
Centrolene peristicta	19.73	1.03	0.51	1.14	Y	23.24	20.5	Lynch and Duellman 1973; Salgado and Guayasamin 2018; this study
Espadarana andina	24	1.8	0.76	4.35	N	44.08	26.76	Señaris and Ayarzaguena 2005; Cabanzo et al. 2013; Cabanzo and Ortega 2018; this study
Espadarana audax	22.05	1.38	0.74	3.17	N	26	25.85	Guayasamin not publis.; this study
Espadarana prosoblepon	23.81	1.66	0.8	4.46	N	31.16	26.75	Taylor 1949; Lynch and Duellman 1973; Jacobson 1985; Kubicki 2007; Hoffmann 2010; Bastos-Riascos et al. 2017; this study
Hyalinobatrachium aureoguttatum	19.94	1.13	0.54	1.4	Y	30.86	23.3	Barrera and Ruiz 1989; Valencia-Aguilar et al. 2012; this study
Hyalinobatrachium cappellei	21.09	1.73	0.74	3.99	Y	19	21.88	Señaris and Ayarzaguena 2005; Castroviejo et al. 2011; this study
Hyalinobatrachium chirripoi	25.07	1.45	0.75	3.43	Y	69	26.75	Taylor 1958; Kubicki 2007; this study
Hyalinobatrachium colymbiphyllum	25.81	1.38	0.7	2.81	Y	60.44	26.75	McDiarmid 1978; Jaramillo et al. 1997; Savage 2002; Kubicki 2007; Hughey et al. 2017
Hyalinobatrachium fleischmanni	20.71	1.52	0.56	1.99	Y	22.67	25.4	Taylor 1958; Starrett 1960; McDiarmid and Alder 1974; Greer and Wells 1980; Jacobson 1985; Kubicki 2007; Salazar and Pino 2015
Hyalinobatrachium iaspidiense	19.18	1.29	0.65	2.3	Y	22.5	22.2	Ayarzaguena 1992; Señaris and Ayarzaguena 2005; Castroviejo et al. 2011; Guayasamin et al. 2018a
Hyalinobatrachium ibama	21.75	1.75	0.6	2.68	Y	20	22.24	Ruiz and Lynch 1998; Rada et al. 2007
Hyalinobatrachium mondolfii	18.55	1.18	0.7	2.42	Y	25.67	21.4	Señaris and Ayarzaguena 2005; Castroviejo-Fisher et al. 2011
Nymphargus grandisonae	27.51	2.36	1.03	10.46	N	50	31.22	Hutter et al. 2013; Guevara and Vargas 2014; Vanegas-Guerrero et al. 2014
Nymphargus griffithsi	24.42	2.54	1.29	17.57	N	33	24.23	Goin 1961; Lynch and Duellman 1973; Hutter and Guayasamin 2012; Arcila-Perez et al. 2017
Nymphargus lasgralarias	23.95	2.67	1.35	20.46	N	24	N.D	Hutter and Guayasamin 2012
Nymphargus rosada	26.09	2.47	1.11	12.78	N	31	27.03	Rada et al. 2017
Sachatamia albomaculata	22.98	1.58	0.62	2.55	N	48	27.35	Savage 2002; Guayasamin et al. 2006; Kubicki 2007; Hoffmann 2010; Rada et al. 2017; this study
Sachatamia ilex	28.05	1.96	0.88	6.39	N	22.25	31.4	Savage 1967; Savage 2002; Kubicki 2007; Hoffmann 2010; Rada et al. 2017; Guayasamin et al. 2018b; this study
Teratohyla midas	19.01	1.4	0.65	2.46	N	20.75	23.57	Lynch and Duellman 1973; Duellman 1978; Diaz et al. 2016; Rojas-Padilla et al. 2019

Teratohyla spinosa	19.13	1.14	0.56	1.49	N	18.55	20.47	Starrett 1960; Kubicki 2007; this study
Vitreorana eurygnatha	18.37	1.65	0.73	3.66	N	28	21.57	Lutz 1947; Taylor and Cochran 1953; Heyer et al. 1985; Heyer et al. 1990
Vitreorana uranoscopa	22.53	1.85	0.79	4.83	N	33	24.4	Heyer et al. 1985; Heyer et al. 1990; Haga et al. 2014; Zaracho 2014
Vitreorana ritae	18.9	1.3	0.8	3.48	N	16.67	20.41	Señaris and Ayarzaguena 2005; Guayasamin et al. 2006; Guayasamin et al. 2008; Menin et al. 2009; Lima et al. 2006

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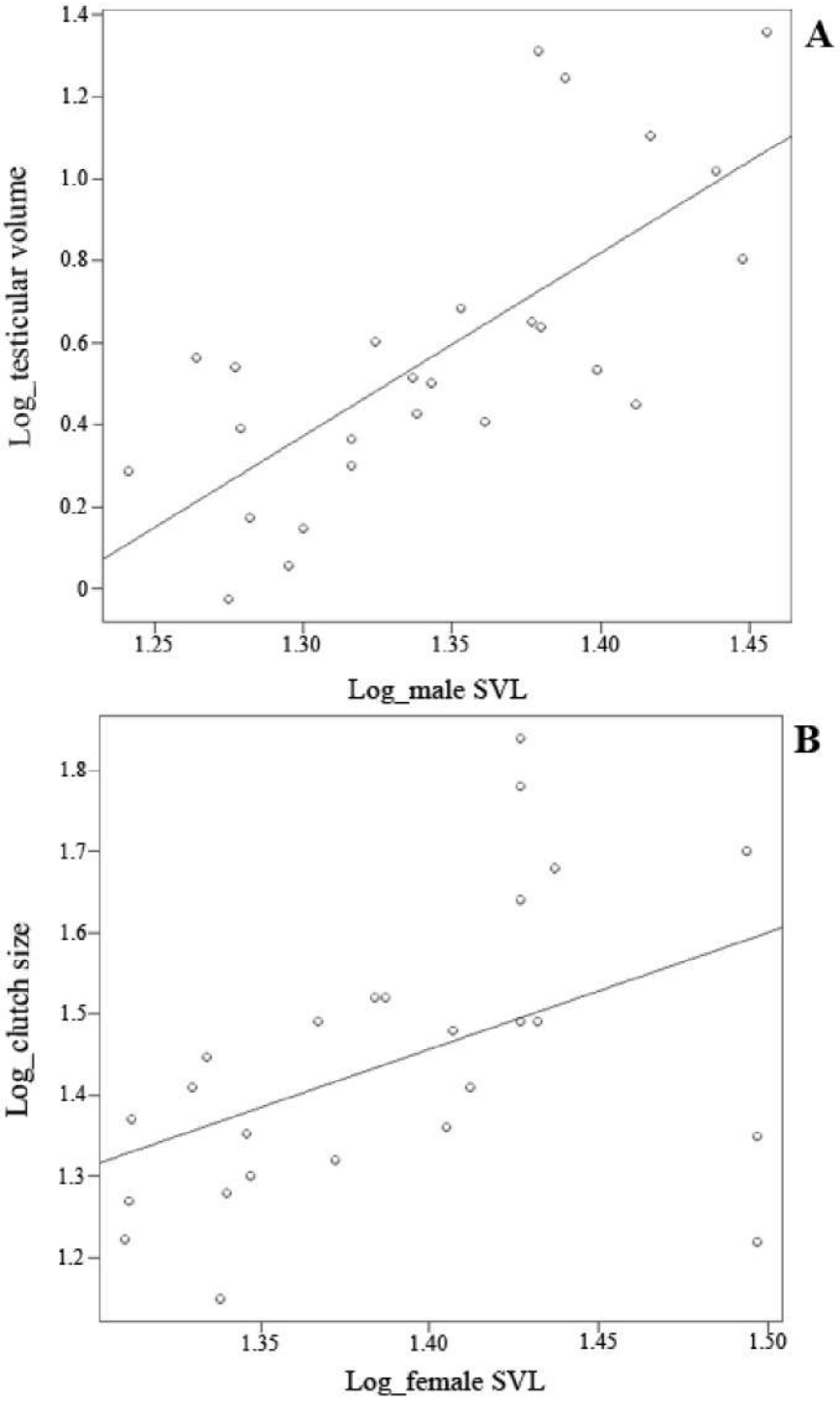


Figure S1. (**A**) Relationship between testicular volume and snout vent length (SVL) in glassfrog males (N = 26 species) and (**B**) clutch size and snout vent length (SVL) in glassfrog females (N = 25 species).

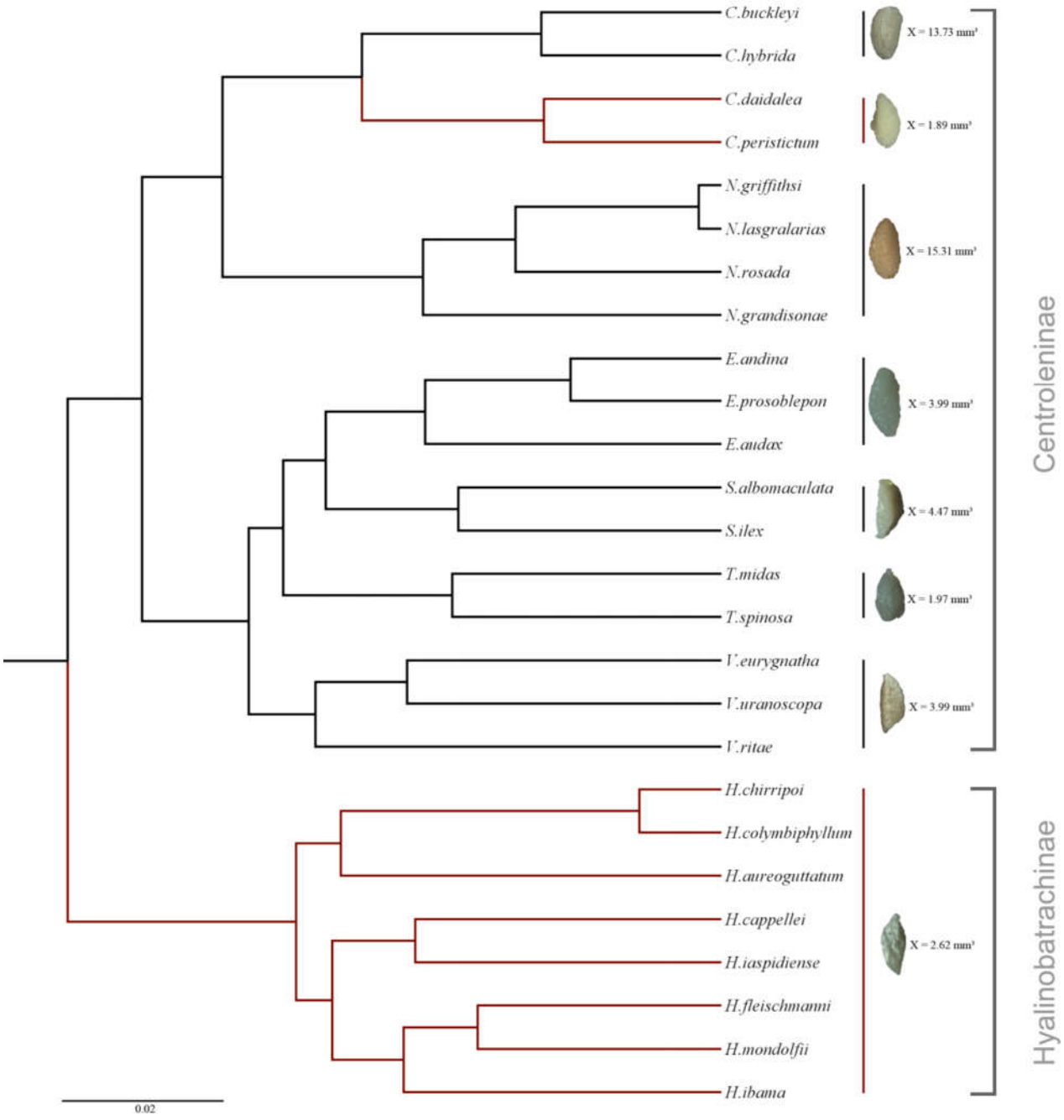


Figure S2. Phylogenetic relationships among glassfrog species (N = 26) used in our analyses for associations between testicular volume and paternal care. Parental care is indicated in different colors (black: species without paternal care, red: species with paternal care). Prune phylogenetic hypothesis for the group from Delia et al. (2017).

GENERAL CONCLUSION

Prevailing knowledge poses that parental care can be time consuming, energetically demanding, expose parents to predation or disease and decrease their mating success. However, recent discussions suggest that the trade-off between current and future mating success might be more complex than previously assumed. In fact, although parental care can decrease mating success in some species (i.e. fishes, birds, and mammals), where caregiver will not accept new mates. We found that in the glassfrog *Hyalinobatrachium cappelli* females prefer males that provide parental care, thus the evolution of paternal care in this family might be favored also by sexual selection. In this case, if care was favored by sexual selection, are costs likely substantially reduced for parents? Although we do not have enough evidence to answer this question our results suggest that care could be in fact energetically less costly than calling. This because, care can also be used as a strategy to increase males' mating success, decreasing males' costs associate to matting effort. Besides, in glassfrog males do not have cost associated with nest building, feeding, or actively defending of their offspring.

Besides, we found that paternal care, as a mating strategy, can increase males´ mating success but also constrains males' gonadal investment driven likely by the inverse relationship between testosterone level and caregiving. Further studies are needed to elucidate other important questions raised in this study, such as the underlying mechanisms driving alloparental care behavior in glassfrogs. Finally, our results highlight the importance of natural history to uncover trends and generate testable hypotheses about mating strategies and behavior evolution in frogs.