

## Breeding and parental behaviour in the glass frog *Centrolene savagei* (Anura: Centrolenidae)

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We examine factors that influence male mating success in the glass frog *Centrolene savagei*; in addition, we describe courtship behaviour, oviposition and early parental behaviour. Fieldwork was conducted in the Colombian Andes during the rainy season from April 2006 to January 2011. Larger males had more chances of mating than smaller males; neither the height of calling site nor number of nights calling influenced the male mating success. Apparently, females choose males on the basis of call frequency attributes. Ours is the first study in glass frogs to report mating success skewed toward larger males. Courtship behaviour includes acoustic signals and visual displays. Additionally, we recorded an unusual routine interruption of amplexus by the male, and a synchronized behaviour between the female and male that indicates the beginning of male parental behaviour.

**Keywords:** amplexus interruption; Centrolenidae; Colombia; courtship call; female choice; visual display

### Introduction

Anuran reproductive biology has attracted the attention of researchers for decades. Detailed research has focused on reproductive endocrinology (e.g. Fernández and Ramos 2003; Rastogi et al. 2005), mating systems (Halliday and Tejedo 1995; Sullivan et al. 1995), communication (Ryan 2001; Gerhardt and Huber 2002),

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parental care (Crump 1995; Lehtinen and Nussbaum 2003), reproductive modes (Crump 1974; Gomez-Mestre et al. 2012), and larval biology (McDiarmid and Altig 1999). Yet our understanding of the ecology and evolution of anuran reproduction is far from complete, as suggested by observations of peculiar behaviour and natural history in some species (Wells 2007). Detailed field research of frog behaviour may even change current thoughts on the evolutionary biology of anurans. For instance, recent evidence has revealed a cryptic mechanism of mate choice (Bruning et al. 2010), an ultrasonic communication system (Feng et al. 2006; Arch et al. 2008), seismic communication in arboreal and aquatic species (Caldwell et al. 2010; Forti and Encarna o 2012), and plasticity in reproductive modes and embryo development (Touchon and Warkentin 2008; Warkentin 2011). Also, diurnal species have been recorded during elaborate courtships involving auditory, visual or tactile signals (e.g. Silverstone 1973; Limerick 1980), and recent research indicates that multimodal (visual and acoustic) communication occurs even in nocturnal species (e.g. Am ezquita and H ohl 2004; Zina and Haddad 2007). Altogether, these findings suggest that breeding behaviour is still poorly understood in many anurans even within relatively well-studied lineages.

The family Centrolenidae (glass frogs) includes about 152 species (AmphibiaWeb 2013) of nocturnal neotropical anurans that reproduce along streams. Egg laying typically occurs on vegetation or rocks overhanging streams, where larval development occurs (Guayasamin et al. 2009). Males attend their eggs in several species of glass frogs, particularly within the genus *Centrolene* (Vargas-Salinas et al. 2007; Dautel et al. 2011; Cardozo-Urdaneta and Se ariz 2012) and *Hyalinobatrachium* (Jacobson 1985; Hayes 1991; Vockenhuber et al. 2008, 2009). Several studies have examined activity patterns, calling behaviour, territoriality, parental care and mortality in egg clutches of glass frogs (e.g. Greer and Wells 1980; Wells and Schwartz 1982; Guayasamin and Barrios-Amor s 2005; Vockenhuber et al. 2008, 2009; Delia et al. 2010). However, the natural history of the glass frogs and their evolutionary biology remain poorly documented.

The behavioural interactions during courtship and the factors explaining differential mating success among males inform our understanding of the interactions between sexual selection, mating systems and parental care in the evolution of glass frogs (Andersson 1994; Sullivan et al. 1995). In glass frogs, it is common to observe a calling male attending one or more egg clutches laid by different females (Jacobson 1985; Vockenhuber et al. 2008; Dautel et al. 2011), but only two studies have attempted to examine the correlates of male mating success (Greer and Wells 1980; Jacobson 1985). These studies found that the number of days for which a male *Hyalinobatrachium fleischmanni* occupied a calling site and the height of the calling perch were positively correlated with male mating success. Also, higher calling perches are better suited for auditory signal propagation than are lower perches (Wells and Schwartz 1982). The positive relationship between calling time and call site height and mating success has been found in other anuran taxa (Townsend 1989; Pr ohl and H ohl 1999; Wells 2007). Lastly, there is detailed information about courtship and oviposition behaviour in diverse anuran taxa (e.g. Rabb and Rabb 1963; Silverstone 1973), but similar information on glass frogs is uncommon (but see Greer and Wells 1980; Jacobson 1985; Vockenhuber et al. 2008).

The glass frog *Centrolene savagei* is a species of relatively small frogs (mean snout–vent length, SVL, females = 23.6 mm, males = 21.1 mm), known from

mid-elevation montane forests 1600–2410 m elevation, in the western and central Andes of Colombia (Ruiz-Carranza et al. 1991a; Rojas-Morales et al. 2011). Males have been observed calling on leaves typically within 0.8–3.0 m above streams; females lay 15–27 cream-coloured eggs, and males are known to guard clutches by frequently sitting on them day and night (Vargas-Salinas et al. 2007; Rojas-Morales et al. 2011). The tadpole and the advertisement call of *C. savagei* have been recently described (Diaz et al. 2013), but other aspects of its natural history and ecology remain almost unknown. Here we examine the ecological correlates of male mating success in *C. savagei*, and provide detailed descriptions of courtship, oviposition and parental behaviours in this species.

### Material and methods

Fieldwork was conducted at four localities in Colombia between April 2006 and January 2011 and always during the rainy season (Table 1). Two localities were in Cordillera Central, Departamento del Quindío: Filandia (Cañón del Río Barbas and Reserva Forestal Bremen), and Departamento de Caldas: Alto Bonito and El Águila villages. The third and fourth localities were in Cordillera Occidental, Departamento del Valle del Cauca: Piedramesa (Finca Piedramesa) and Yotoco (Reserva Forestal Bosque de Yotoco).

To examine the relationship between male attributes and male mating success, male advertisement calls were recorded from September 2010 to January 2011 only at Alto Bonito-Águila. At this locality we recorded calls with a Sennheiser ME64 microphone connected to a Marantz PMD620 digital recorder. At the time of recordings, dorsal surface temperatures of the calling males were measured with an infrared hygro-thermometer (Extech EA25). Calls were digitised at 16 bits resolution and 44.1 kHz sampling rate. Audiospectrograms and oscillograms were analysed with a fast Fourier transformation (window size = 256; window type = Blackman) in Raven Pro 1.4 (Bioacoustics Research Program 2011). We measured the body size (SVL) of all males with a digital calliper (to the nearest 0.1 mm), and also recorded the microhabitat type (e.g. leaves, rocks, trunks) of the calling site used by each male, and measured the perch height (in cm) above water surface with a measuring tape. To allow repeated measurements of the same individuals, frogs were individually marked by toe clipping (Donnelly et al. 1994). Body measurements were recorded each time a frog was recaptured and the final mean values were used for statistical analysis. We used a logistic regression analysis to test whether mating success (a binary variable, males either bred or did not breed during the observation time) was influenced by male body size, and the height of calling perch. Observations of an amplexus with the subsequent egg clutch fertilization or male egg attendance (i.e. egg brooding) were considered as evidence of male mating success. Because male body size may influence the probability of mating through its pleiotropic effect on male call traits, we examined the relationship between body size and call features (call frequency and duration) using linear regression analysis.

We also made observations on courtship behaviour during nightly (19.00 h to midnight) walks along the stream transect in all other localities (Filandia, Yotoco and Piedramesa; from April 2006). When we found an amplexant pair or a female approaching a calling male, we observed and photographed the individuals until oviposition occurred or until sunrise (06.00 h). We also recorded vocalizations during

Table 1. Summary of localities visited in the western Andes (WA) and central Andes (CA) of Colombia to study breeding behaviour of the glass frog *Centrolene savagei*.

	Locality			
	Piedramesa (WA) <sup>a</sup>	Yotoco (WA) <sup>a</sup>	Filandia (CA) <sup>b</sup>	Alto Bonito-Águila (CA) <sup>c</sup>
Coordinates (datum = WGS84)	3°11'12" N, 76° 41'21" W	3°53'18" N, 76° 20'5" W	4°42' N, 75° 38' W	5°07'27" N, 75° 29'57" W
Elevation (m)	1580	1200–1600	1700–2100	1950–2050
Precipitation (mm)	1200	1100	2500	2600
Temperature (°C)	21	22	17	18

Notes: <sup>a</sup>CVC, Corporación del Valle del Cauca, Colombia; <sup>b</sup>CAR, Corporación Autónoma de Risaralda, <sup>c</sup>Corpocaldas (2002).

Data shown for annual precipitation and temperature are mean values.

courtship with the same equipment as described above. The dorsal surface temperatures of the calling males were measured with an infrared thermometer (Oakton 35629). Males were not captured to avoid disrupting their breeding behaviour; nevertheless, male body size (SVL) was estimated in the field with a calliper (nearest 0.1 mm) placed close to the male. In the locality of Alto Bonito-Aguila we used red lights to reduce disruption of the frogs' behaviour.

## Results

### *Correlates of mating success*

We monitored mating success in 30 males at Alto Bonito-Águila. Two males mated three times (6.7%), one male mated twice (3.3%), 13 males mated once (43.3%), and 14 males were not observed mating during the study (46.6%). Larger males had higher probability of mating than smaller males (logistic regression, Wald's statistic = 5.74,  $\beta = 1.436$ ,  $p = 0.017$ ; Figure 1A). The height of calling perches (always the upper surface of leaves in our study) did not affect the probability of attracting a mate (Wald's statistic = 0.415,  $\beta = -0.006$ ,  $p = 0.52$ ). Since there was an inverse relationship between call frequency and male body size ( $r^2 = 0.358$ ,  $F = 8.37$ ,  $p = 0.011$ ;  $n = 17$ ; Figure 1B), mated males tended to have lower pitched calls than unmated males ( $t = 2.09$ ,  $df = 15$ ,  $p = 0.054$ ). Call duration was unrelated to body size ( $r^2 = 0.042$ ,  $F = 0.659$ ,  $n = 17$ ;  $p = 0.43$ ).

### *Courtship behaviour*

We observed one pre-amplexus courtship interaction in Yotoco and two in Alto Bonito-Águila. In Yotoco, the female was observed on a leaf located ~20 cm above a male, which was calling from a leaf about 270 cm above the stream edge. The female approached the male by hopping from leaf to leaf. When she arrived on the calling perch, he temporarily ceased advertising, turned his body towards her and then resumed calling. Seconds later, the male displayed body push-ups and rapid movements of his arms and legs (like limb shaking *sensu* Hödl and Amézquita, 2001).

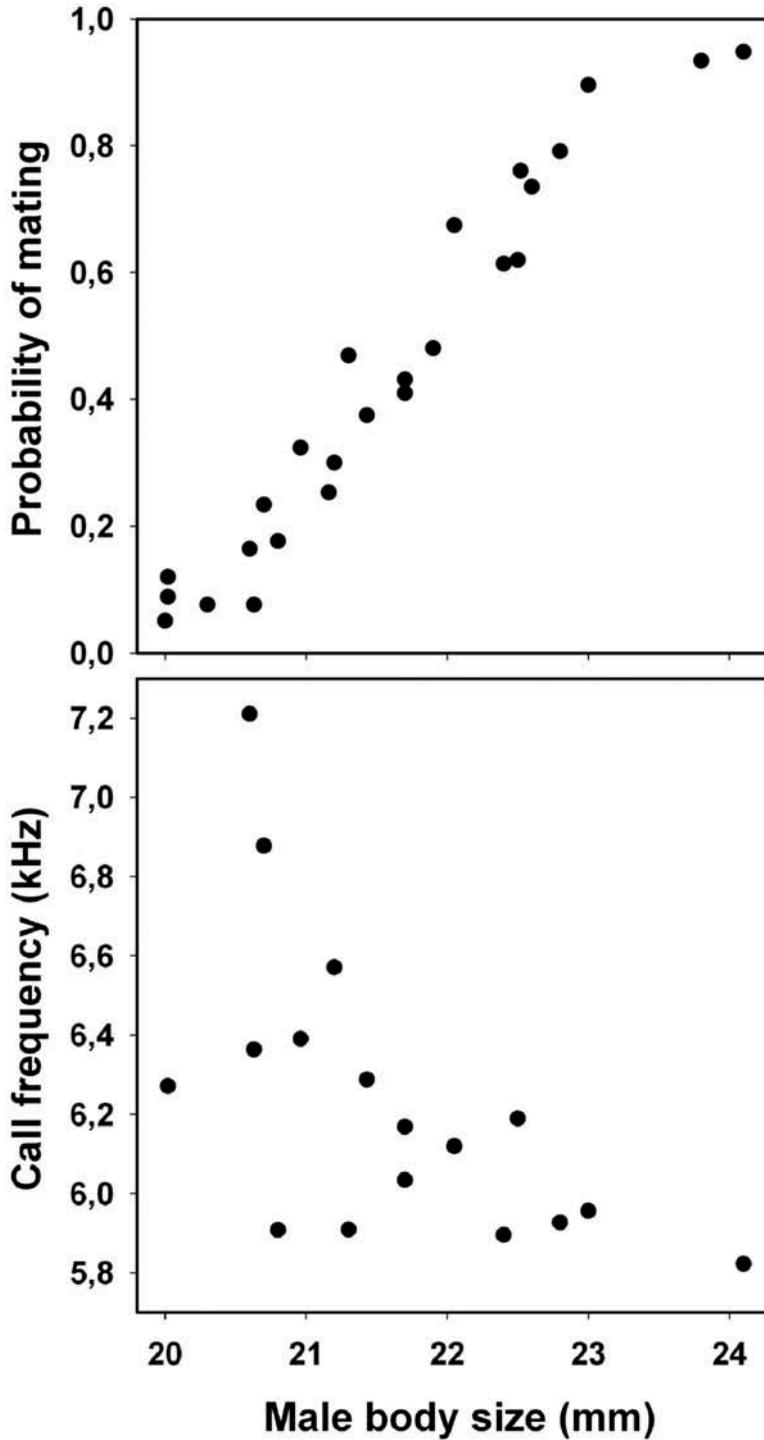


Figure 1. Relationship between male body size, mating success (above) and call peak frequency (below) in the glass frog *Centrolene savagei*.

Throughout the 20-min observation, the male was very active whereas the female remained motionless against the leaf surface. When the male finally approached the female, she jumped to another leaf and moved about 30 cm away. After 30 min, the female returned to the male, who had resumed calling. At this point, the male exhibited approximately the same behavioural sequence exhibited when the female approached the first time. As the male approached the female, she lowered her head (i.e. “crouched” in Hödl and Amézquita 2001). Then, the male mounted her head-to-vent, rotated on her dorsum and engaged in axillary amplexus. In the other two courtship interactions (Alto Bonito-Águila), we observed basically the same behavioural sequence as described above with some minor differences (Figure 2). When a female first approached a calling male, he increased the calling rate from 3 to 15 calls/min. Once on the same leaf, the male began to jump around the female while calling continuously. The male also quickly moved his arms in an up and down motion resembling limb shaking (Hödl and Amézquita 2001). During this time, the female remained motionless with her ventral surface pressed against the leaf surface. Later, the male positioned himself behind the female, calling continuously, and tapping the female dorsum with his head. Each courtship interaction, before amplexus, lasted between 45–60 min.

For calls recorded during the three pre-amplexus courtship events, a single male at Yotoco was recorded during 18 min and emitted two types of calls that differed in both duration and complexity (Figure 3). The complex and long calls were emitted 20 times and consisted of multiple units/pulses and semipulses with modulated amplitude and variable structure (Figure 3A). Our recordings captured two harmonics with frequency modulation. The less complex calls (Figure 3B) consisted usually of a single-note with amplitude and frequency modulation. Both types of calls were also recorded in two courtship interactions observed in Alto Bonito-Águila. A summary of the call traits recorded during each courtship interaction is presented in Table 2.

An interesting and unexpected behaviour was observed in four amplexant pairs at Yotoco (one pair), Filandia (one pair) and Piedramesa (two pair), when the observer approached the courting pair to record data and take photographs (Figure 4). The male interrupted amplexus, in one case jumping to an adjacent leaf, and then resumed courting with vigorous calling and body push-ups. The female crouched and the male mounted her from the head. The male rotated to grasp her in axillary amplexus and the pair moved across the leaf surface making several rotations before oviposition. In one out of four cases, the pair engaged in inguinal rather than axillary amplexus.

### ***Oviposition behaviour***

We observed 10 amplexant pairs (four in Yotoco, one in Filandia, two in Piedramesa and three in Alto Bonito-Águila); eight of them oviposited. The following observations are based on two pairs observed in Yotoco. Before oviposition, the amplexant pair frequently moved around the leaf surface for approximately 1 hour. At the moment of oviposition, the male raised the posterior part of his body and the female rapidly expelled her eggs (Figure 5A). Seconds later, the male ended amplexus and positioned himself immediately behind her (Figure 5B). Then, the female began to rhythmically raise and lower the posterior portion of her body as the male nudged her forward and began crawling on top of eggs. This synchronized

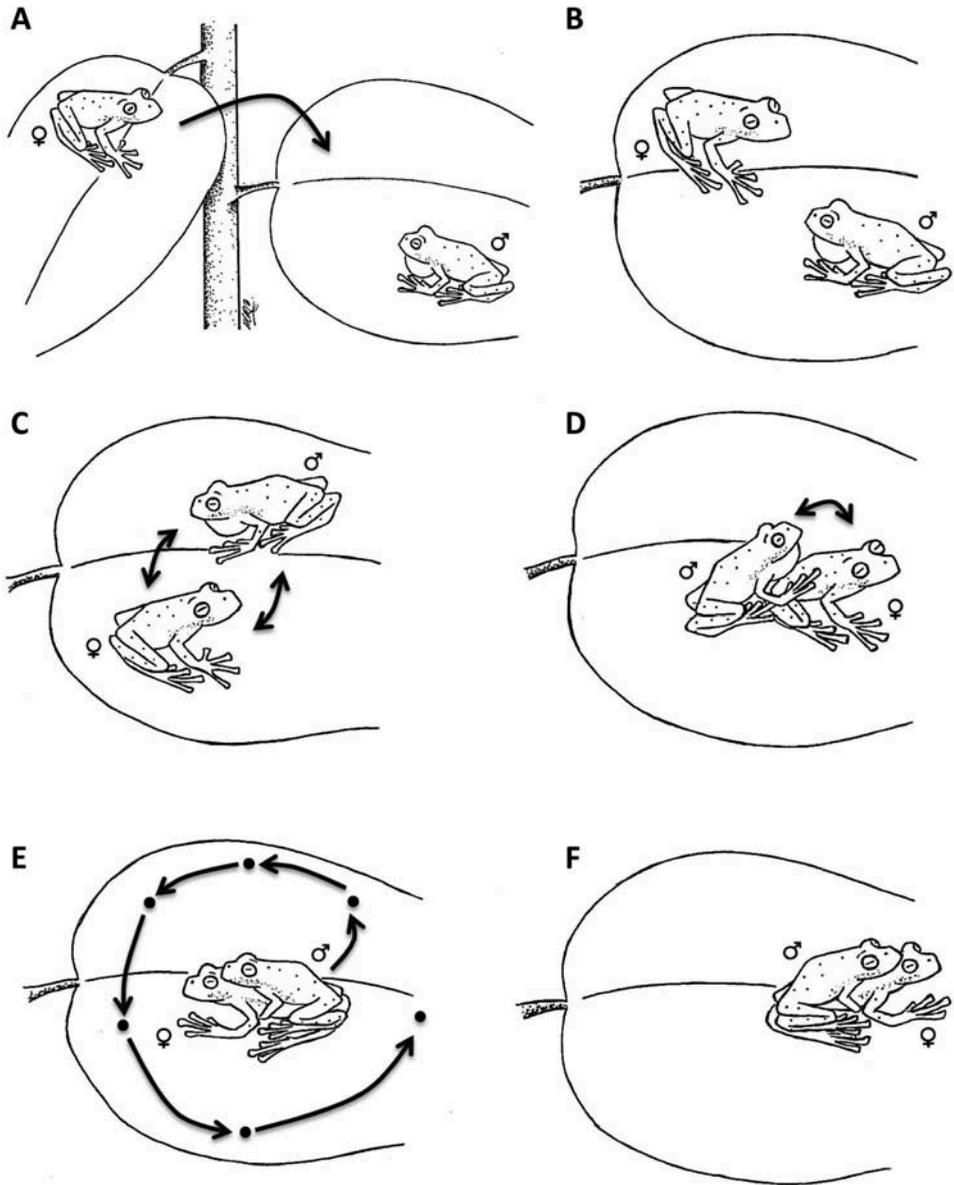


Figure 2. Behavioural sequence of courtship in the glass frog *Centrolene savagei* in a population at Alto Bonito-Águila ( $n = 2$ ). (A) Female approaching a calling male, (B) beginning of acoustic display by male, (C) jumping and movements by male, (D) tactile displays involving the male tapping his head on the female dorsum, (E) amplexus and movements on the leaf, (F) stationary phase of the amplexus. Illustration by M. Rivera-Correa.

behaviour continued until the male's body covered most of the clutch (Figure 5C, D). At that point, the female moved forward and jumped to another leaf, whereas the male remained in contact with the clutch (Figure 5E, F). On one occasion, the male emitted two calls while the female was leaving. Males remained motionless on

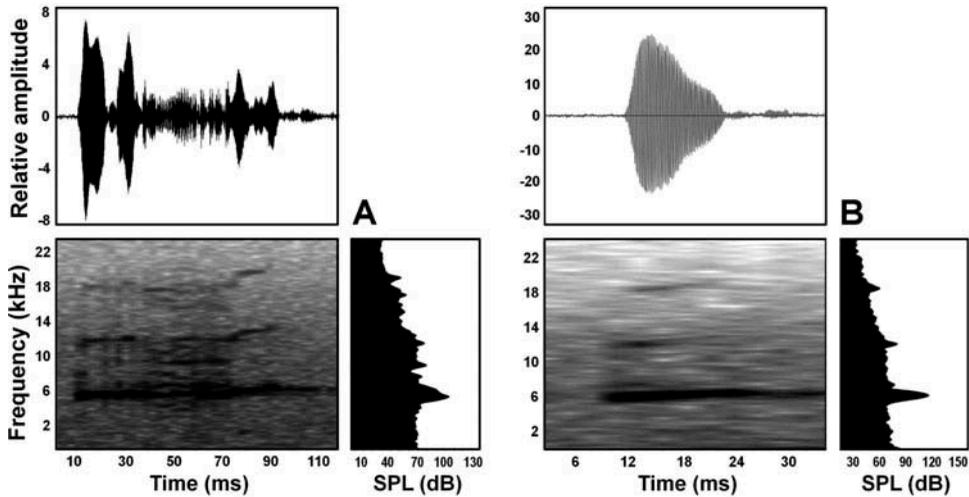


Figure 3. Oscillogram, spectrogram and power spectrum of calls emitted during courtship behaviour in *Centrolene savagei* at Bosque de Yotoco, Valle del Cauca, Colombia. Two kinds of calls were recorded: type I (A) and type II (B). Male body size = 22.6 mm; male body temperature = 18.3°C (see text and Table 2 for additional information).

top of the clutch for at least 24 h. Several days later, the males resumed calling, either above or very close to the clutch. In Alto Bonito-Águila, females laid one egg at a time, at intervals of 5–7 min, while the male was behind her (not in amplexus). Oviposition in pairs observed at this site lasted on average  $100 \pm 37$  min ( $n = 3$  females).

## Discussion

Our data reveal that larger males, with slightly lower pitch voices, have a higher probability of mating than smaller males. Also, the courtship involved the exchange of both acoustic signals and visual displays, the interruption of amplexus by the male, and a synchronized behaviour between the female and male after oviposition that imply the beginning of egg attendance.

## Mating success

Ours is the first study to reveal improved reproductive success in large male glass frogs compared with small males. A lack of such a relationship has been reported for other glass frogs (Greer and Wells 1980; Jacobson 1985; Salgado-Maldonado 2012). Given the negative relationship between peak call frequency and male body size, call frequency could be used by females of *C. savagei* as an honest indicator of male body size. Although this hypothesis received support from studies on various anuran species (reviewed in Sullivan et al. 1995; and Gerhardt and Huber 2002), it needs to be tested directly in our study species because the appreciable variance (error) in call frequency cannot be predicted from body size (Wells 2007). In addition, it will be interesting to evaluate the

Table 2. Summary of call traits for three pre-amplexus courtship events recorded at Yotoco and Alto Bonito-Águila.

Call trait	Yotoco		Alto Bonito-Águila		Alto Bonito-Águila	
	Call type I	Call type II	Call type I	Call type I	Call type I	Call type II
Peak frequency (Hz)	6049.22 ± 228.74 (5601.6–6375)	6153.25 ± 166.42 (5812.5–6562.5)	5825.69 ± 129.32 (5512.5–6029.3)	5830.22 ± 179.75 (5340.2–6201.6)	5901.69 ± 180.78 (5512.5–6201.6)	
Call duration (ms)	105.75 ± 16.79 (60–136)	24.077 ± 11.88 (11–49)	144.45–37.01 (44–170)	162.73–21.45 (125–236)	35.98–12.96 (13–65)	
Number of calls	20	26	11	45	54	
Male body size (mm)		22.6	22.65		22.45	
Temperature calling male (°C)		18.3	17.5		16.8	

Data are mean values ± standard deviation and range where pertinent. During acoustic courtship, males can exhibit two call types (see Figure 2 and text for details). All calls exhibit amplitude and frequency modulation.

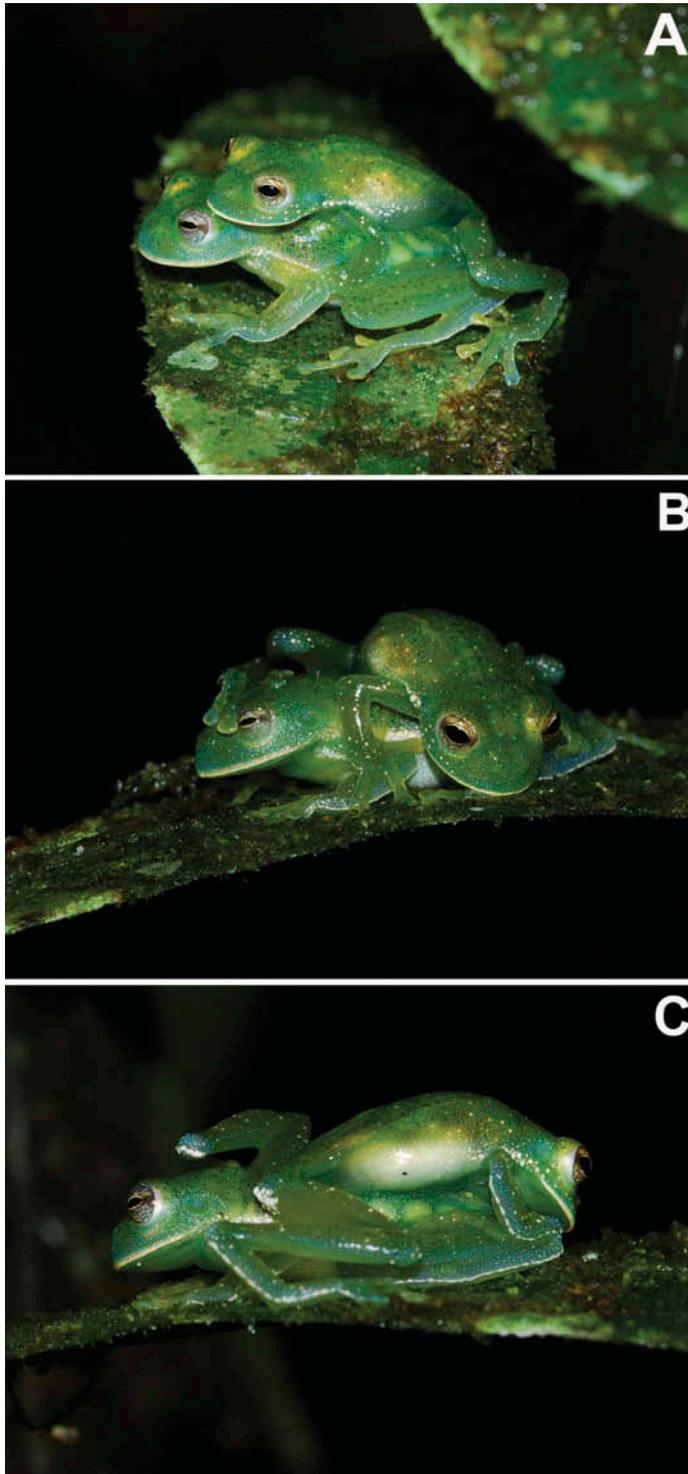


Figure 4. Male behaviour during amplexus in the glass frog *Centrolene savagei*; Yotoco, Valle del Cauca, Colombia. (A) Amplexant pair (body size male = 22.58 mm, female = 26.24 mm); (B) and (C): male rotation on the female dorsum. Photos by F. Vargas-Salinas.

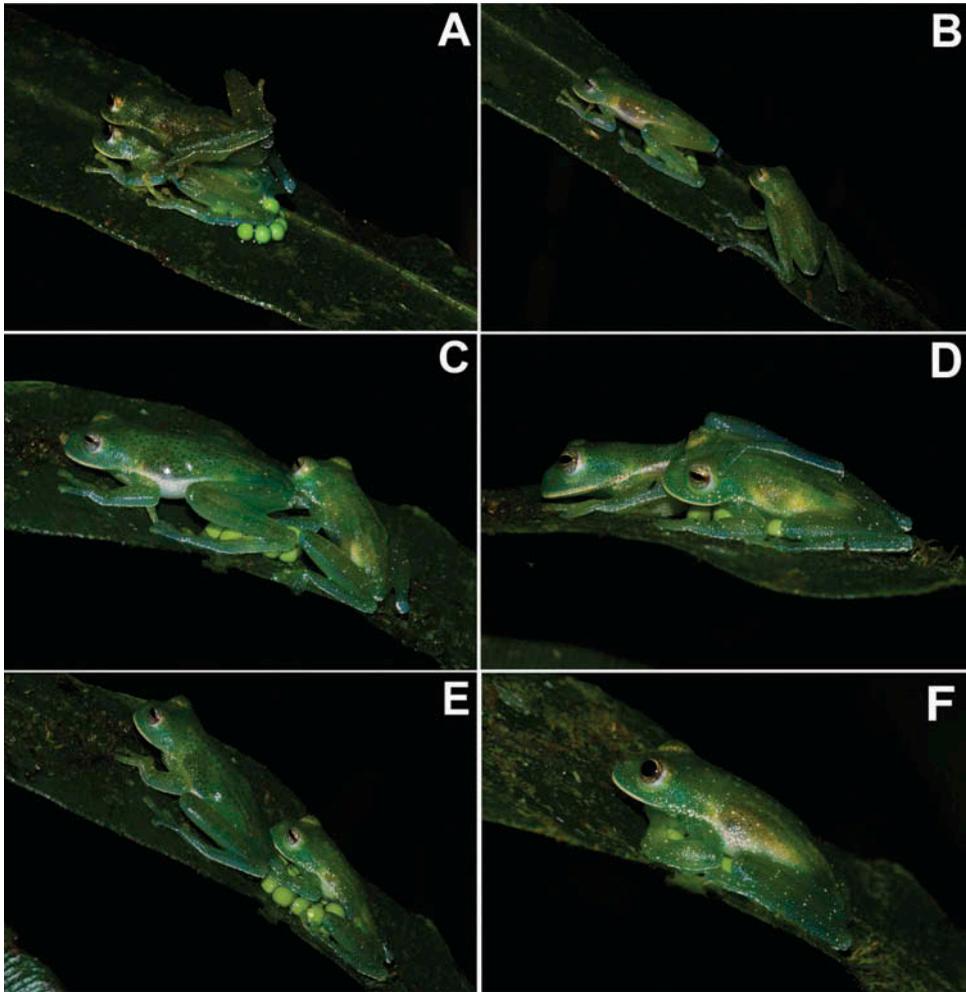


Figure 5. Oviposition and parental behaviour in the glass frog *Centrolene savagei*. (A) Oviposition and possible fertilization of eggs; (B, C) female raising posterior part of her body and male starting to position himself between her belly and the egg clutch; (D) male accommodation above eggs, (E, F) female leaving the oviposition site and male engaging in parental behaviour. Photographs by F. Vargas-Salinas.

potential benefits of mate choice for female offspring. Nevertheless, indirect evidence from our and other studies further supports a case for female choice in *C. savagei*. First, the mating season appears to be prolonged and males appear to remain on the same perches; both characteristics facilitate mate sampling by females (Sullivan et al. 1995; Wells 2007). Second, there is a prolonged courtship with exchange of numerous signals and females appear to take the critical decisions that lead to amplexus. Third, the direct benefit for choosy females may be male attendance of the clutch and thereby the increased survivorship of egg and embryos (Sullivan et al. 1995; Vockenhuber et al. 2009). In any case, the reproductive bias among male *C. savagei* is similar to that in other glass

frogs (e.g. *Espadarana prosoblepon* Jacobson, 1985; *Centrolene lynchi* Dautel et al., 2011) and less biased than species where a single male simultaneously guards 0–11 clutches (e.g. *Hyalinobatrachium fleischmanni* Greer and Wells, 1980; *Hyalinobatrachium valerioi* Vockenhuber et al., 2008; *Hyalinobatrachium orientale* Lehtinen and Georgiadis, 2012). Additional data and analyses are necessary to understand the ecological conditions underlying those differences.

In contrast with *H. fleischmanni* (Greer and Wells 1980; Jacobson 1985), the height of calling perch did not predict male mating success in *C. savagei*. Males of *H. fleischmanni* usually call from ventral surfaces of horizontal broad leaves, which attenuate acoustic signals and/or reflect the sound energy towards the ground (Wells and Schwartz 1982); hence, males calling from low perches may experience lower propagation of their calls and therefore lower probability of attracting a mate. Males of *C. savagei* call from the upper surface of small and medium-sized leaves. Because the higher number of recaptures in our study was relatively low (three), we cannot discard an effect of the number of nights calling on male mating success. Future studies should monitor calling males over time to create an integrated measure of call effort, including call duration, call rate and number of nights calling.

### **Courtship behaviour**

The courtship behaviour in *C. savagei* involves the exchange of numerous acoustic signals and visual displays. During courtship, males utter calls that are longer and structurally more variable than advertisement calls (Figure 3A). Courtship calls appear to be modifications of advertisement call in other anurans (Rosen and Lemon 1974; Greer and Wells 1980; Wells 1980; Owen and Tucker 2006) including glass frogs (Hutter et al. 2013). As in *H. fleischmanni* (Greer and Wells 1980; Jacobson 1985) and other anurans (Owen and Tucker 2006), courtship calls in *C. savagei* were interspersed with advertisement calls during courtship. In addition, limb shaking and body push-ups during courtship suggest that *C. savagei* also use visual signals during courtship. Courtship behaviour including more than one sensory modality have been recorded in several anuran families (Limerick 1980; Pombal et al. 1994; Zina and Haddad 2007; Grafe et al. 2012) including glass frogs (calls and tactile cues; see Greer and Wells 1980; Jacobson 1985). The possible widespread presence of visual displays in glass frogs needs to be further studied, but is feasible given the occurrence of this behaviour in other nocturnal frogs and its potential importance in short-distance communication (Hartmann et al. 2005; Zina and Haddad 2007; Lipinski et al. 2012), especially those breeding in noisy habitats like streams (Hödl and Amézquita 2001).

To our knowledge, ours is the first record of an anuran male temporarily interrupting amplexus to vocalize. Males of other species (*H. fleischmanni* and *E. prosoblepon*) re-initiate amplexus when experimentally disrupted by a researcher (Jacobson 1985). In *C. savagei*, disruption may have been caused by artificial lighting (Cadle and McDiarmid 1990; Buchanan 2006). Under this scenario, disturbed males would be led to call again to insure re-coupling. Moreover, at Alto Bonito-Águila, where we used red light for behavioural observations, amplexus interruption was not recorded.

### ***Oviposition and parental behaviour***

It is intriguing that males in one of the studied populations (Alto Bonito-Águila) positioned themselves behind the female before oviposition, whereas in another population (Yotoco), they remained in amplexus during oviposition (Figure 5A). Although we cannot yet explain the apparent geographic variation in male behaviour, males of *C. savagei*, like *H. fleischmanni* males, appear to hydrate the eggs following oviposition, causing the eggs to expand rapidly in size (Jacobson 1985; Delia et al. 2010; Rojas-Morales and Escobar-Lasso, personal observation).

The presence of males near or above developing clutches has been documented in some glass frogs and it has been suggested that this is a mechanism of egg attendance (see Wells 2007). Male care of eggs has been observed in *Centrolene geckoideum* and *Centrolene petrophilum* (Lynch et al. 1983; Ruiz-Carranza et al. 1991b), *Centrolene antioquiense* and *Centrolene peristictum* (Gutiérrez-Cárdenas, personal observation), *Centrolene lynchi* (Dautel et al. 2011), and several species of the genus *Hyalinobatrachium* (e.g. *H. fleischmanni*, *H. chirripoi*, *H. colymbiphyllum*, *H. talamancae*, *H. valerioi*, *H. vireovittatum*; see Kubicki 2007; *H. orientale*, Lehtinen and Georgiadis, 2012; Murphy et al., 2012; and *H. ibama*, Rada et al., 2007). Furthermore, a similar behaviour has been reported for *Celsiella vozmediano* (Guayasamin et al. 2009). The egg brooding described in *C. savagei* (Vargas-Salinas et al. 2007; Rojas-Morales et al. 2011; this work) has also been observed in its sister species *Centrolene daidaleum* (Rada et al. 2007; Guayasamin et al. 2008, 2009; Cardozo-Urdaneta and Señaris 2012). Besides the molecular evidence that suggests a close relationship between *C. daidaleum* and *C. savagei* (Guayasamin et al. 2008), the ventral protection of eggs by adult males is considered here as a putative behavioural synapomorphy that supports the close relationship between these two species. However, egg attendance is an evolutionary novelty that has arisen several times in the family Centrolenidae and in other anuran taxa (e.g. Terrarana; see Wells 2007). Our observations should be combined with comparable studies in other taxa of glass frogs, to get a more comprehensive understanding of the ecological conditions that favoured the evolution of egg attendance.

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