



In male Emerald Glass Frogs, *Espadarana prosoblepon*, a bony spine projects from each humeral bone (visible here just to the inside of the arm). The function of these spines is not well understood, although they might be important during aggressive interactions. This individual was photographed on 24 June 2011 at Estación Biológica Las Cruces, San Vito de Coto Brus, Provincia de Puntarenas, Costa Rica.

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Body size, humeral spine size, and aggressive interactions in the Emerald Glass Frog, *Espadarana prosoblepon* (Anura: Centrolenidae) in Costa Rica

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ABSTRACT: Like many species of glass frogs, males of the Emerald Glass Frog (*Espadarana prosoblepon*) engage in intense bouts of fighting. During these fights, males have been observed using their humeral spines in a weapon-like context. Because fighting in glass frogs often has been observed opportunistically, however, we have little understanding of what causes glass frog aggressive behavior to escalate to fighting, or if the outcome of aggressive interactions is influenced by male body size or humeral spine size. Using a combination of field surveys and staged contests in *E. prosoblepon*, we investigated (1) variation in male body size and humeral spine size, (2) if the likelihood to engage in aggressive interactions depends on the abundance of males actively calling on a given night, and (3) if male body size or humeral spine size influences the outcome of aggressive interactions with other males. We found that male abundance varied nightly in our study area, and that male density influenced the likelihood that two males placed in an experimental arena would engage in aggressive interactions. We also found that male body size and humeral spine size varied among individuals; however, neither of these traits predicted the winner of aggressive interactions. These findings further our understanding of the factors influencing aggressive behavior and the outcome of aggressive interactions in *E. prosoblepon*.

Key Words: Aggressive behavior, body size, *Centrolene prosoblepon*, fighting, humeral hook, Las Cruces Biological Station

RESUMEN: Al igual que muchas especies de rana de vidrio, la Rana de Vidrio Esmeralda (*Espadarana prosoblepon*) participa en combates intensos con otros machos. Durante estas luchas, individuos de *E. prosoblepon* han sido observados utilizando las espinas humerales a manera de armas. Sin embargo, estos combates han sido observados de manera oportunista y, por lo tanto, tenemos poco conocimiento sobre las causas que llevan a que el comportamiento agresivo escale a un combate o si las interacciones agresivas son influenciadas por el tamaño del cuerpo de los machos o el tamaño de la espina humeral. Llevando a cabo una combinación de monitoreos en el campo y escenarios simulados, estudiamos en *E. prosoblepon* (1) la variación en el tamaño del cuerpo y la espina humeral en los machos, (2) si la probabilidad de verse envueltos en interacciones agresivas depende de la abundancia de machos vocalizando en la noche, y (3) si el tamaño de cuerpo del macho y la espina humeral influyen las interacciones agresivas entre los machos. Encontramos que la abundancia de machos varió en nuestra área de estudio cada noche y que la densidad de machos influyó la probabilidad de que dos machos colocados en un escenario experimental se enfrascaran en interacciones agresivas. También encontramos que el tamaño del cuerpo y de

la espina humeral varió entre individuos; sin embargo, ninguno de estos rasgos predijo al ganador de las interacciones agresivas. Estos hallazgos amplían nuestro conocimiento sobre los factores que influyen en el comportamiento agresivo y el desenlace de este tipo de interacción en *E. prosoblepon*.

Palabras Claves: *Centrolene prosoblepon*, comportamiento agresivo, espina humeral, Estación Biológica Las Cruces, gancho humeral, lucha, tamaño corporal

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INTRODUCTION

Male-male competition for access to mates occurs in the majority of anurans (Wells, 1977; Arak, 1983). During the breeding season, males may establish territories (Duellman and Savitsky, 1976; Wells, 1977, 1978; Given, 1988), and in most species effectively defend their territories using advertisement calls (Jacobson, 1985; Telford, 1985; Wilczynski and Brenowitz, 1988; Vockenhuber, 2008). High male density, however, can increase the intensity of male-male competition and lead to physical aggression. To date, physical combative interactions have been observed in at least 12 anuran families (reviewed by Wells, 2007).

Within the glass frogs (Family Centrolenidae), aggressive interactions have been documented in at least 11 species (McDiarmid and Adler, 1974; Duellman and Savitsky, 1976; Greer and Wells, 1980; Jacobson, 1985; Restrepo-Toro, 1996; Bolívar et al., 1999; Savage, 2002; Guayasamin and Barrio-Amorós, 2005; Kubicki, 2007; Delia et al., 2010; Dautel, et al. 2011; Rojas-Runjaic and Cabello, 2011). Adult males of several species have a bony process called a humeral spine projecting from each humeral bone (Guayasamin et al., 2009). Observations of fighting males suggest that these structures could be used as weapons, since males grapple chest-to-chest or venter-to-venter in many of these species such that the spines could press into an opponent (Duellman and Savitsky, 1976; Jacobson, 1985; Guayasamin and Barrio-Amorós, 2005; Kubicki, 2007; Dautel et al., 2011; Krohn and Voyles, 2014). Injuries, presumably resulting from contact with the humeral spines, have been documented in at least two species (Bolívar et al., 1999; Hutter et al., 2013).

A humeral spine is present in *Espadarana prosoblepon*, and males have been observed engaging in intense bouts of fighting with other males that enter their territory (Jacobson, 1985; Kubicki, 2007). Fighting in *E. prosoblepon* may escalate to the point that males are hanging upside down from vegetation and wrestling venter-to-venter (Jacobson, 1985; Kubicki, 2007; Guayasamin et al., 2009). Kubicki (2007) hypothesized that the humeral spine may be used as a weapon in this species, and recent evidence demonstrating that the humeral spines may become interlocked while males wrestle (Krohn and Voyles, 2014) supports this hypothesis. Variation in humeral spine size and its influence on the outcome of aggressive interactions, however, has not been explored. Moreover, our current understanding of aggressive behavior largely is based on opportunistic observations (e.g., Jacobson, 1985; Kubicki, 2007; Krohn and Voyles, 2014), so the underlying causes of physical aggression in this species remain speculative.

In this study, we used a combination of field surveys and staged contests in *E. prosoblepon* to investigate (1) variation in male body size and humeral spine size, (2) if the likelihood to engage in aggressive interactions depends on the abundance of males actively calling on a given night, and (3) if male body size or humeral spine size influences the outcome of aggressive interactions with other males. We hypothesized that (H1) humeral spine size would be positively correlated with male body size, (H2) the intensity of aggressive interactions between males in staged contests would be correlated with the density of males calling on a given night, and (H3) the larger males with larger humeral spines would be the victor in staged contests.

MATERIALS AND METHODS

Study Site

Our research took place at Estación Biológica Las Cruces, San Vito de Coto Brus, Provincia de Puntarenas, Costa Rica, a research station run by the Organization for Tropical Studies. The study area entailed both sides of the road leading to the forest trails between the pump house and the apartments (8°47'04.07"N, 82°57'36.55"W; DMS; elev. 1,028 m). The vegetation consisted primarily of heliconias, gingers, and palms. A narrow ditch system crisscrossed through a large portion of the study area. *Espadarana prosoblepon* called from the vegetation surrounding these ditches and laid clutches of eggs along the moss-covered banks (H. Hedman and M. Hughey, pers. observ.).

Morphological Variation

From 1 to 4 July 2011 we measured morphological characteristics in 27 males of *E. prosoblepon* to assess population-level variation in male snout–vent length (SVL) and humeral spine size. We found males calling from the upper surfaces of leaves or occasionally while perched on fences. Males also called from the canopy, but all individuals used in this study came from less than 2 m above the ground. Upon collection, we housed individuals in 1 L plastic bags with a moist substrate. We collected males from a different section of the study area each night to avoid re-sampling the same individuals.

The following morning we photographed each individual's right and left humeral spine and dorsal surface using a Nikon D40 camera fitted with a 90 mm macro lens, and included a ruler in each photograph to standardize measurements. We measured humeral spine height and area using ImageJ version 1.428 (Martin and Pfennig, 2010). We took measurements for the height of the spine from the center of the base of the spine to the center of the top of the spine (Fig. 1A), and for the area of the spine by outlining the perimeter of the spine (Fig. 1B). We measured both the left and right humeral spine of all individuals, and the SVL from the tip of the snout to the end of the body (Fig. 1C). For a given individual, the right and left humeral spine height and area were similar

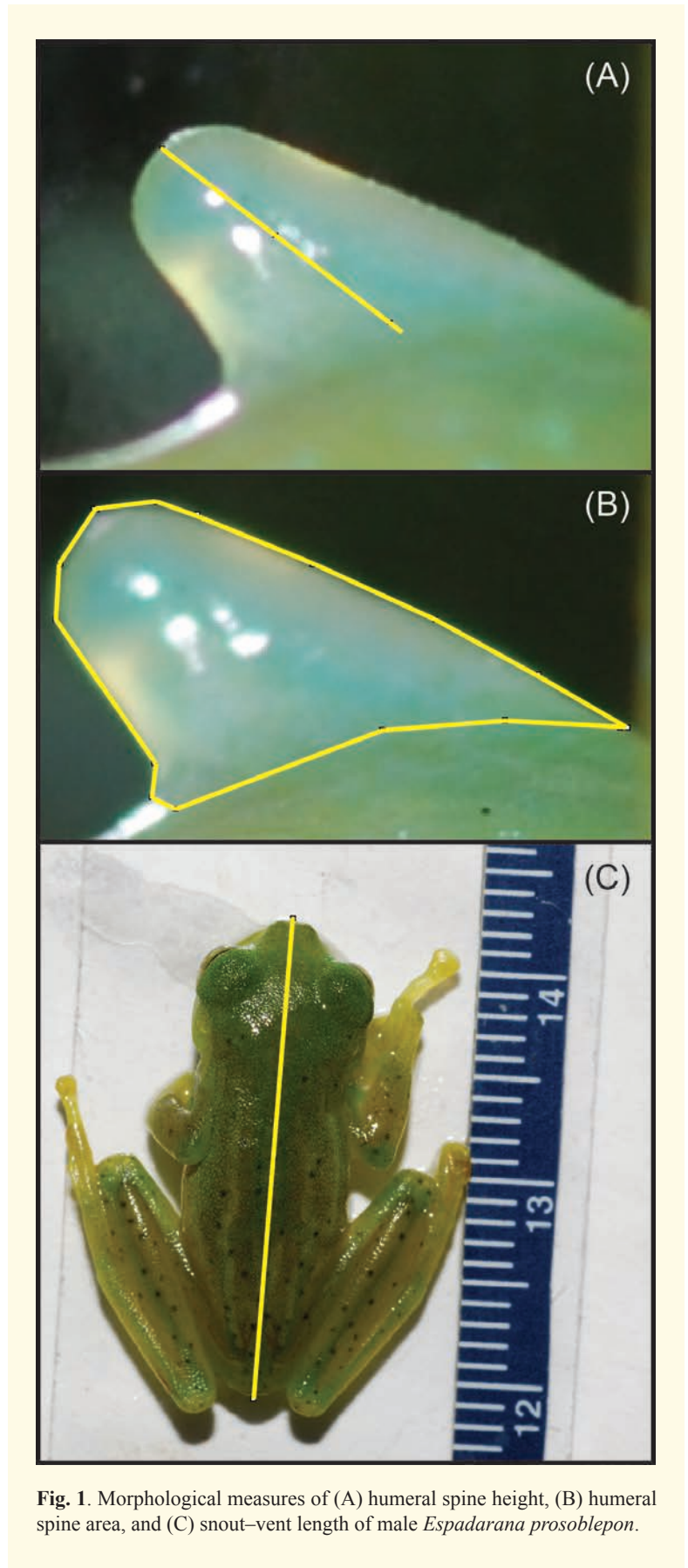


Fig. 1. Morphological measures of (A) humeral spine height, (B) humeral spine area, and (C) snout–vent length of male *Espadarana prosoblepon*.

(height, paired t -test: $P = 0.8111$; area, Wilcoxon signed rank test: $P = 0.8589$). Thus, in all subsequent analyses we used only the right humeral spine height or area. To test for a relationship between body size and humeral spine size (H1), we used linear regression. We tested for a relationship between SVL and right humeral spine height and area separately.

Abundance of *Espadarana prosoblepon*

From 1 to 8 and 11 to 26 July 2011, one of us (HH) conducted timed surveys each night to document the relative abundance of visible calling males; on two occasions he was accompanied by a second individual, but to avoid any bias in data collection this individual did not assist in the surveys. Surveys of abundance began at approximately 1900 h each evening and ended at approximately 2000 h. During the surveys the observer(s) walked throughout the entire survey area (ca. 950 m²), searching and listening for calling males for 45 min, without retracing their steps. Thus, surveys were standardized to cover the same area in the same amount of time each night. The number of calling males, gravid females, amplexant pairs, and fighting males observed in the study area was recorded.

We used data from the Las Cruces weather station to try to better understand abiotic factors that might be influencing nightly male abundance. We tested for correlations between male abundance on a given night and measurements of (1) air temperature and (2) relative humidity recorded at the start of our survey period, as well as the (3) total afternoon rainfall (the total amount of rain that fell between 1200 and 1800 hrs each day).

Male-Male Interactions

Following the timed transect, we collected sets of two males haphazardly and placed them together in an experimental arena to document aggressive interactions. The experimental arena consisted of a small, potted ginger plant surrounded by black plastic to facilitate the location of escapees. The experimental plant was similar to the plants upon which males called naturally. To initiate interactions between males, we placed the two individuals simultaneously within five mm of each other on a single leaf. We observed males in complete darkness using the infrared Nightshot feature of the Sony Handycam video camera (DCR-DVD105). We ended the trials after 15 min if males remained on the plant but did not move or interact. If males interacted aggressively, we ended the trials when one individual fled the vegetation (max trial duration: 45 min). In total we staged 50 trials (number of trials per night: 2.17 ± 1.58 , \pm SD), and tested if the likelihood of males to engage in aggressive interactions was dependent on the density of calling males (H2) using logistic regression.

We recaptured both winners and losers and placed them in plastic bags. The following morning we photographed all individuals (as above) to measure body size and humeral spine size, and tested separately for differences in body size (SVL), humeral spine height, or humeral spine area of the winners and losers of aggressive interaction (H3). The SVL and humeral spine were distributed normally (Shapiro-Wilk test: $P > 0.05$), and we tested these using paired t -tests. The humeral spine area was not distributed normally, so we tested this using Wilcoxon signed rank tests. We excluded two trials from these analyses because one of the two males escaped, and we were unable to measure and compare the characteristics of the winner and loser of the interaction.

RESULTS

Morphological Variation

The SVL of calling males ranged from 20.4 to 23.2 mm (\pm SD: 22.0 ± 0.14 mm). The humeral spine height ranged from 0.21 to 1.74 mm (0.74 ± 0.07 mm). The humeral spine area ranged from 0.009 to 0.10 mm² (0.02 ± 0.003 mm²). The humeral spines of the larger males were larger in terms of height (linear regression: $F_{1,25} = 5.05$, $P = 0.03$, $r^2 = 0.14$; Fig. 2), but not area (linear regression: $F_{1,25} = 2.4$, $P = 0.13$, $r^2 = 0.05$).

Abundance of *Espadarana prosoblepon*

The density of calling males ranged from 3 to 18 individuals per night during the study period ($n = 22$ nights; Fig. 3). We observed unpaired gravid females on zero nights, but observed pairs in amplexus on eight nights. Female density always was low ($n \leq 3$ individuals), and there was no relationship between the number of males out calling and the number of females observed on a given night (Pearson's product-moment correlation: $P = 0.76$). We observed a

single natural fight, on the third day of our survey (marked with an *, Fig. 3).

During the study period, the air temperature ranged from 17 to 20°C, the relative humidity from 75 to 96%, and the amount of afternoon rainfall from 0 to 23 mm. The abundance of males on a given night was marginally negatively correlated with air temperature (Pearson's product-moment correlation: $r = -0.38$, $P = 0.07$). We found no associations between nightly male abundance and relative humidity or total afternoon rainfall ($P = 0.61$ and 0.37 , respectively).

Male-Male Interactions

Over the course of the study period, we conducted 50 staged trials. Males engaged in aggressive interactions in 10 trials, on six out of 23 nights. Aggressive behaviors included calling, tackling and mounting, wrestling venter-to-venter, and chasing (Table 1, Supplementary Video 1). The density of calling males in the area influenced the likelihood that two individuals would engage in aggressive interactions in staged trials (logistic regression, $\chi^2 = 7.2$, $P = 0.007$).

The SVL of winners ranged from 20.9 to 23.0 mm (22.0 ± 0.3 mm), and of losers from 20.6 to 22.8 mm (21.7 ± 0.3 mm). Neither body size (paired t -test, $P = 0.4$) nor humeral spine size (height: paired t -test, $P = 0.7$, area: Wilcoxon signed rank test, $P = 0.7$) influenced the outcome of aggressive interactions.

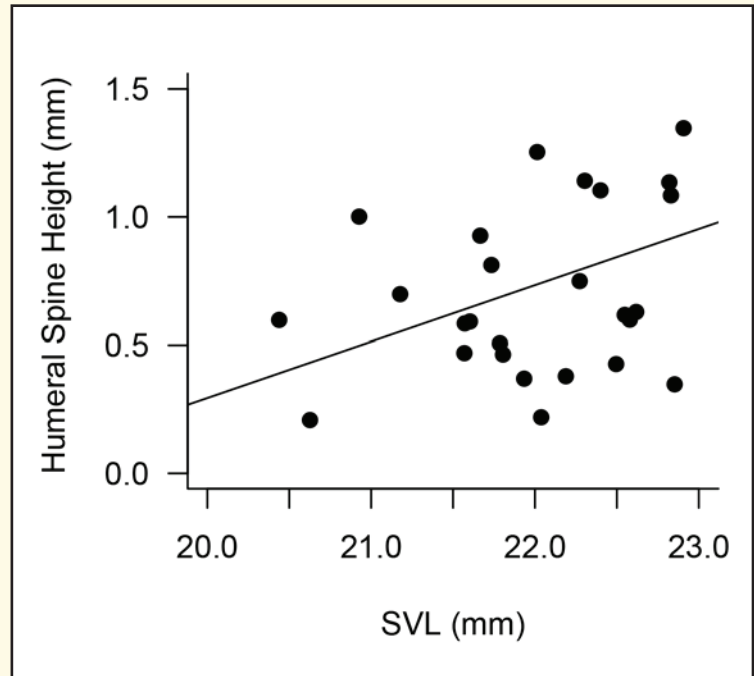


Fig. 2. Relationship between snout-vent length and humeral spine height in *Espadarana prosoblepon*. Line is best fit line from linear regression ($F_{1,25} = 5.05$, $P = 0.03$, $r^2 = 0.14$).

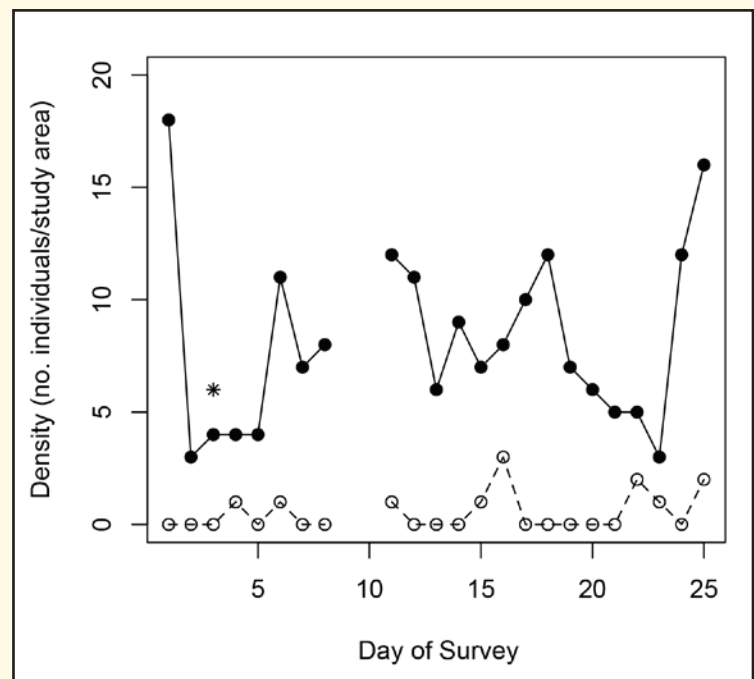


Fig. 3. The number of calling males (black circles, solid lines), pairs in amplexus (white circles, dashed lines), and natural fighting events (star) observed each night during a field survey of *Espadarana prosoblepon* reproductive activity. The survey took place from July 1 to 8 and 11 to 26, 2011, at Estación Biológica Las Cruces, San Vito de Coto Brus, Provincia de Puntarenas, Costa Rica.

Table 1. Frequency and type of aggressive behaviors observed during staged contests between pairs of male *Espadarana prosoblepon*, excluding trials where no aggressive behavior occurred. Male density refers to the number of calling males observed in the study area during nightly surveys of male abundance. Visual examples of all behaviors are provided in Supplementary Video 1, available at: <https://youtu.be/MD91u-yPLB8>.

Date	Trial	Male Density	Aggressive Behaviors				Trial Duration (min)
			Calling	Tackling and Mounting	Chasing	Venter to Venter Wrestling	
1 July 2011	1	18	3	1	1	1	0:45
6 July 2011	6	11	2	0	1	0	0:15
11 July 2011	10	12	3	0	0	0	0:33
16 July 2011	21	8	13	0	0	0	0:45
20 July 2011	33	6	0	0	0	1	0:04
25 July 2011	41	12	0	0	1	0	0:20
26 July 2011	47	16	6	0	0	0	0:19
26 July 2011	48	16	2	0	0	1	0:05
26 July 2011	49	16	2	0	1	1	0:11
26 July 2011	50	16	0	0	1	0	0:12

DISCUSSION

Physical contact between competing male anurans rarely is observed in nature, as vocalizations generally are adequate to attract mates, establish territories, and resolve disputes (Greer and Wells, 1980). In the tropics, however, where many species undergo an extended breeding season, males sometimes are present in high densities at breeding sites for prolonged periods of time, which can lead to greater levels of territorial and aggressive behavior (Duellman and Savitzky, 1976; Halloy and Espinoza, 2000; Tsuji and Matsui, 2002).

Also in the tropics, species in several families of anurans have evolved structures that likely function as defensive weaponry, including the Leptodactylidae, Hylidae, Ranidae, Myobatrachidae (reviewed by Shine, 1979; Emlen, 2008). Evidence is accumulating that weapon-like structures also exist in the Centrolenidae, although whether these structures are used to inflict damage or in a more ritualized context remains unclear for many species. For example, male *Espadarana prosoblepon* have been observed with their humeral spines interlocked during venter-to-venter wrestling (Krohn and Voyles, 2014) with no resulting injury, while other centrolenids have demonstrated the use of humeral spines to injure opposing males, including *Centrolene buckleyi* (Bolívar et al., 1999) and *Nymphargus grandisonae* (Hutter et al., 2013). Clearly, additional studies focused on understanding aggressive behavior and the role these structures play during aggressive interactions are needed. In this study, we examined inter-individual variation in the size of the humeral spine in *E. prosoblepon* and its relationship to male body size. We tested if male density in the environment influenced aggressive behavior, and if male body size or humeral spine size influenced the outcome of aggressive interactions.

The humeral spines of larger males of *E. prosoblepon* tended to be longer, but we did not identify a relationship between the outcome of aggressive interactions and either male body size or humeral spine morphology (height or area). Larger size contributes to fighting success in other combative species of anurans (e.g., Given, 1988). For example, in Tusked Frogs (*Adelotus brevis*), larger males successfully excluded smaller males from calling sites (Katsikaros and Shine, 1997) and, in Fanged Frogs (*Rana kuhlii*) larger males typically won aggressive encounters between two males (Tsuji and Matsui, 2002). For *E. prosoblepon*, other factors we did not measure, such as individual motivational state (Wells, 1988, 2007; Valencia-Aguilar et al., 2012), might be more important determinants of the outcome of aggressive interactions.

Nightly male abundance fluctuated throughout the study period, indicating that different numbers of individuals might have been descending from the canopy or entering the study area on different nights. Although *E.*

prosoblepon is territorial, individuals do not show the same degree of site fidelity as in some other species of glass frogs (Jacobson, 1985; but see Robertson et al., 2008), so we did not necessarily expect to encounter the same individuals in the same locations night after night. Additionally, nights of intense aggression (i.e., nights of high male density) might require subsequent recovery nights for some individuals (Savage, 2002), which could explain the drop in male abundance that often occurred after nights of high activity (i.e., when > 10 individuals were observed; Fig. 3). Our study was designed to be non-invasive, and thus we did not individually mark males, which would have enabled us to track which individuals were active from night to night.

Local weather conditions, specifically air temperature, might have affected male activity. We found a marginal negative association between nightly male abundance and air temperature at the time of our survey. This finding was somewhat unexpected, because the activity of amphibians, as ectotherms, should increase with increasing temperature. A thorough assessment of how weather influences *E. prosoblepon* activity was beyond the scope of this study; however, the wealth of information present in the weather station data certainly merits further exploration.

The abundance of male *E. prosoblepon* on a given night influenced the likelihood that we would observe aggressive behavior in our staged trials. Density was low on many nights of our study, and the majority of our staged trials ended with no displays of aggressive behavior. On these nights, males might not have been motivated enough to interact under our experimental conditions. On other nights, however, males began calling at one another almost immediately. Increased aggression with increased density has been observed in other prolonged breeding anurans. For example, in a laboratory setting male *Hyperolius marmoratus* demonstrated greater levels of aggressive behavior in the presence of higher density choruses (Dyson and Passmore, 1992).

In general, glass frogs are an excellent system for studying the natural history, ecology, and evolution of aggressive behavior and weapon use in anurans. As a whole, this group displays a wide variety of antagonistic behaviors and great diversity in humeral spine morphology (Guayasamin et al., 2009). Moreover, these traits have been important for understanding phylogenetic relationships within the Centrolenidae (Bolívar et al., 1999; Guayasamin et al., 2009; Rojas-Runjaic and Cabello, 2011; Hutter et al., 2013). To our knowledge, this study is the first to investigate aggressive behavior in glass frogs under more controlled settings. By demonstrating the feasibility of this approach, this study opens up possibilities for exciting new avenues of research. Future directions include examining the physiological basis of aggressive behavior, conducting more detailed studies of ritualized aggressive behavior and weapon function, and assembling comparative data for different species.

Supplementary Video 1. A montage of aggressive behaviors observed during staged contests between pairs of male *Espadarana prosoblepon* is available at: <https://youtu.be/MD91u-yPLB8>.

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