

The territoriality, vocalizations and aggressive interactions of the red-spotted glassfrog, *Nymphargus grandisonae*, Cochran and Goin, 1970 (Anura: Centrolenidae)

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In many frog species, males defend a territory through direct male-to-male interactions and/or aggressive calling behaviour. We describe the site fidelity, vocalizations, aggressive interactions, and male combat behaviour of the glassfrog *Nymphargus grandisonae*. We show high specificity of males' calling and mating sites. We then describe the temporal and spectral differences for six types of vocalizations. We link these vocalizations to behavioural observations, describing their aggressive and reproductive contexts. Additionally, we show that combat is highly variable and includes three previously described and two unreported variations. We describe injuries resulting from combat and we report the first observation of a multiple night fight between the same two males. Our observations on site fidelity and aggression provide evidence for territoriality among males. Furthermore, our results suggest that combat behaviour in glassfrogs is more complex than previously hypothesized and that hypotheses on the evolution of combat behaviour need re-evaluation.

Keywords: advertisement call; antagonistic behaviour; combat behaviour; courtship call; distress call; encounter call; territorial call; release call; territoriality

Introduction

Resource defence mating systems in frogs have presumably evolved in response to limited reproductive resource availability (Wells 1977). Hence, males in some species have evolved adaptations that result in high site fidelity and active defence of a territory, ensuring exclusive access to the territory's resources (Wells 2007). These males

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defend resources (calling and oviposition sites) through aggressive calling and/or direct male-to-male conflict (Toledo and Haddad 2005; Wells 2007; Vilaça et al. 2011).

Frogs of the arboreal family Centrolenidae, commonly known as glassfrogs, generally reproduce and deposit their eggs on leaves above fast moving streams (Ruiz-Carranza and Lynch 1991a; Guayasamin et al. 2009). Males of many species defend their calling and mating territories against conspecifics, implying territoriality (e.g. McDiarmid and Adler 1974; Duellman and Savitzky 1976; Jacobson 1985; Bolívar et al. 1999; Savage 2002; Kubicki 2007). However, most of these studies lack evidence that this aggression is due to resource defence (but see: Greer and Wells 1980; Jacobson 1985). The variety of antagonistic behaviours reported in glassfrogs includes: corporal jerking motions, shaking of leaves, territorial and encounter calls, and combat behaviour (e.g. McDiarmid and Adler 1974; Jacobson 1985; Bolívar et al. 1999; Dautel et al. 2011).

Bolívar et al. (1999) suggested that combat behaviour is phylogenetically informative and resolves the relationships among glassfrog genera (*sensu* Ruiz-Carranza and Lynch 1991a). Bolívar et al. (1999) diagnosed two character states of combat behaviour: (1) a primitive state, where males fight in an “amplexus-like” position while wrestling on the surface of leaves, and (2) a derived state, where males grasp each other “venter-to-venter” while hanging upside down and holding the vegetation with their hind limbs. Following this, Guayasamin et al. (2009) proposed that the primitive state is present in the subfamily Hyalinobatrachinae, while the derived state is restricted to the subfamily Centroleninae. Recently, however, Rojas-Runjaic and Cabello (2011) reported both the primitive and derived states for *Centrolene daidaleum* (Ruiz-Carranza and Lynch 1991b), a member of Centroleninae, casting doubt on these previous hypotheses.

Herein, we assess the site fidelity and examine the vocal repertoire and aggressive interactions of the red-spotted glassfrog, *Nymphargus grandisonae* (Cochran and Goin 1970; Figure 1). Using distinctive red-spotted markings on individuals, we

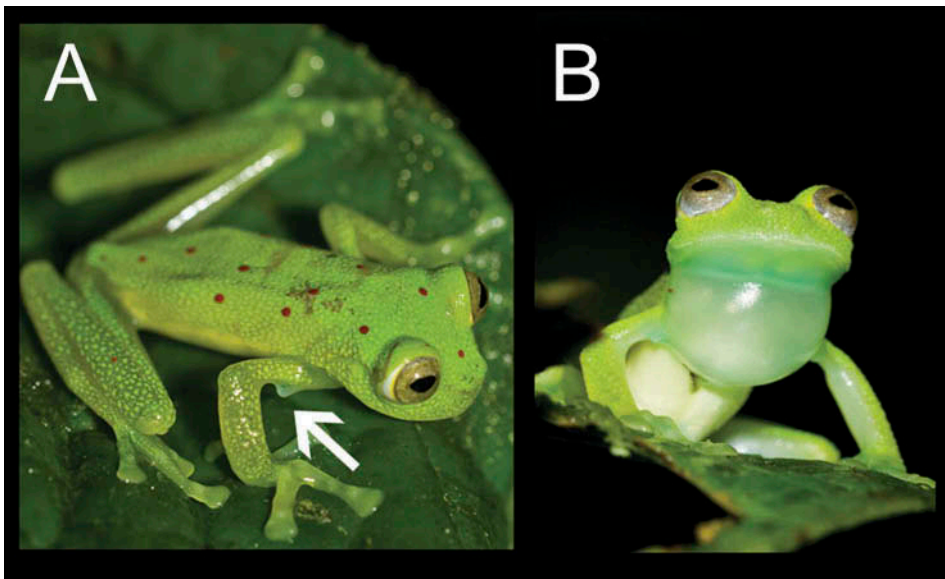


Figure 1. Male *Nymphargus grandisonae*. (A) The dorsal red-spotted pattern and humeral spines (white arrow) are shown. (B) A male is advertisement calling.

identified males and documented their specificity to reproductive sites. For the first time in Centrolenidae, we describe the full repertoire of male calls and their use during antagonistic interactions. We also describe the male combat behaviour in this species, and present information on the complexity, variation, and injuries associated with combat. Finally, based on our observations and those of Rojas-Runjaic and Cabello (2011), we recommend a revision of the phylogenetic hypotheses that use combat behaviour as a diagnostic character of subfamilies in Centrolenidae.

Material and methods

In Ecuador, we monitored a population of *Nymphargus grandisonae* along a 500-m transect on “Five Frog Creek” (00°00′33″ S, 78°44′15″ W; 2000–2150 m above sea level), in Reserva Las Gralarias, province of Pichincha, from 2 April to 3 July 2011. We conducted a mark–recapture study and identified individuals through their unique dorsal red-spotted pattern (Figure 1A). We recorded the presence of individual males and females, reproductive activity, and calling activity two to five nights a week. We measured the distance from the stream of captured individuals and the perch height of those located above the stream. Their capture locations were marked with yellow flagging tape to document prevalence at a given site. A site was considered an area that encompassed the movements of a single calling male, and no other calling males were present within this site.

In Ecuador, we recorded male vocalizations from 11 February to 14 June 2011. We observed male combat on 11 May and 1–2 June 2011 at “Five Frog Creek”. The site was located in primary forest. The two males from the first encounter were collected and deposited at the Museo de Zoología, Universidad Tecnológica Indoamérica, Quito, Ecuador (MZUTI 430–431). We also took time-stamped photographs and video recordings during combat to document the various forms and positions. Video is available as online supplementary material (SM) to augment text explanations.

In Colombia, we recorded calls on 3 May 2010 and observed male combat on 4 May and 3 October 2010 at “Aguas Claras Creek” (5°06′27″ N, 75°29′30″ W; 1833–2100 m above sea level), El Águila village, municipality of Manizales, department of Caldas. This site is located in fragmented secondary forest composed of small and broad-leaved herbaceous and shrub vegetation, consisting predominantly of *Heliconia latispatha*, *Calathea* sp., *Xanthosoma saggitifolium*, and *Oreopanax pallidum*. The two fighting males observed in May were collected and deposited at the Museo de Historia Natural of the Universidad de Caldas, Manizales, Colombia (MHN-UC 0250–0251).

To record vocalizations, we used an Olympus LS10 Linear PCM Recorder and Sennheiser M8 directional microphone. The vocalizations were recorded in PCM format at a sampling rate of 44 Hz. We recorded calls in the field 50–300 cm from calling males. We measured snout–vent length (SVL) of *in situ* live specimens with an analogue calliper. Recordings were processed with RAVEN PRO 1.4 (Bioacoustics Research Program 2011) on MAC OS X. Call parameter definitions follow Duellman and Trueb (1994), Cocroft and Ryan (1995), Dautel et al. (2011), and Hutter and Guayasamin (2012). We analysed oscillograms (waveforms), audio-spectrograms, and power spectra (fast Fourier transformations) for various temporal and spectral call components (Table 1). The fast Fourier transformation size was set to 512 and frequency grid resolution to 86.1 Hz. We measured temperature after each recording using an analogue thermometer and the temperature was between 13 and 15°C. The call data were not adjusted for temperature effects, as the variation was no more than 3°C (Brown et al. 2006). The digital recordings were deposited at MZUTI.

Table 1. Call variables measured for *Nymphargus grandisonae*. Variable definitions follow Cocroft and Ryan (1995), Dautel et al. (2011), and Hutter and Guayasamin (2012).

Parameter	Description
Note/call interval (s)	Time between a call/note.
Note/call duration (s)	Time from the start to the end of the call/note.
Call rate (calls/m)	(Total number of calls – 1)/time from the start of the first call to the start of the last call.
Number of pulses	Total number of pulses per note/call.
Pulse/note repetition rate (pulses/s)	(Total number of pulses/notes – 1)/time from start of first pulse/note to the start of the final pulse/note.
Pulse duration (s)	Time from start to end of one pulse for pulses at the start, middle and end of call/note.
Pulse/note/call rise time (s)	Time from start of a pulse/call/note to the point of maximum amplitude.
Pulse/note/call shape	(Rise time/duration); unitless variable that describes the overall shape of the amplitude envelope of the pulse. Right or left skewed pulses will have a rise time near the start or end of the call, respectively. This parameter allows comparison of pulses/notes/calls with differing lengths.
Pulse/note/call amplitude to peak amplitude ratio	Maximum amplitude of pulses/notes compared with the peak amplitude of the call. Measured between the start, middle, and end of call. Describes amplitude distribution and modulation throughout call.
Pulse/note/call amplitude change throughout call	Ratio of pulse/notes amplitude compared between: (1) the middle and beginning of call; (2) the end and beginning of call; and (3) the end and middle of call.
Lower frequency of the fundamental frequency (Hz)	Lower limit of the fundamental frequency.
Higher frequency of the fundamental frequency (Hz)	Upper limit of the fundamental frequency.
Dominant frequency (Hz)	Frequency of call that contains the greatest concentration of energy.
Frequency modulation (Hz)	Absolute value of the dominant frequency of the last 0.020 s of call subtracted from the dominant frequency of the first 0.020 s of call.
Harmonic frequencies (Hz)	Average frequency of the harmonic frequencies of the call.
Power	Measured for harmonic frequencies; proportion of maximum power in harmonic frequency compared with maximum power of call.

Notes were defined as the sound produced in a single exhalation of air. Specifically, notes were defined as individual units of sound containing single or multiple pulses, distinguished from other notes and calls by a return to the background noise, and a very short, constant interval therein. Notes were also categorized into two types,

pulsed and tonal (i.e. melodious). Pulsed notes had amplitude modulation and amplitude peaks. Tonal notes displayed no such modulation, and had relatively constant amplitude throughout the call (Dautel et al. 2011). We follow Duellman and Trueb (1994) for call-type definitions (i.e. advertisement, territorial, encounter, release, distress, and courtship calls). Call types are considered hypotheses and were categorized using their associated behavioural context (Duellman and Trueb 1994) and quantitative parameter differences. The call parameters used for this study are described in Table 1.

We analysed data using R version 2.15 (R Development Core Team 2012) and the MASS package (Venables and Ripley 2002). We tested for a relationship between body size (SVL) and the continuous call parameters. A principal component analysis (PCA) was used to differentiate call types and the major acoustic parameters that explain their differentiation. We performed two PCAs on all recorded call types; the first used multiple individuals and the second used only a single individual. An analysis using a single individual eliminates the variation among multiple individuals and more clearly reveals the differences between calls. The parameters used in the PCA analysis were dominant frequency (Hz), call duration (s), call shape, pulse rate (pulses/s), frequency modulation (Hz), amplitude change from the start to the end of call, and the amplitude change from the middle to end of call (PC loadings in SM Table S1, available online at <http://informahealthcare.com/doi/suppl/10.1080/00222933.2013.792961>). We used the broken-stick distribution (Jackson 1993) and found that PC1–2 explained more variation than expected by chance (PC1–2 combined variations: multiple individuals 64.8%; single individual 69.9%). Therefore, these axes were used in an analysis of variance (ANOVA) to test for a significant difference between call types.

We used a discriminant function analysis to test whether each call could be categorized correctly to our hypothesized type based on its call parameters. This analysis conducted a leave-one-out cross-validation of the calls. This procedure sequentially removed a single call from the dataset and then used the remaining calls and their categorization to train the classification model. Then each omitted call was tested to validate whether the model could classify the call correctly. A correct classification rate was then calculated. Initially, equal prior probabilities (i.e. the estimated probability of a male giving this call) were assigned to each call type. In a second analysis, the prior probability was assigned based on our behavioural data (number of calls recorded for each type divided by total number of calls).

Frequency data were adjusted for the effect of SVL for analyses that included multiple individuals by using the residuals from the linear regression. Statistical data shown throughout this work are referred to by the mean \pm one standard deviation of the mean, followed by the range.

Results

High site fidelity

In Ecuador, we identified 39 unique frogs (25 males; 14 females) from 244 captures. We documented 20 non-overlapping sites; males at these sites called in the same site three or more times. We recorded five additional males a single time each (near calling males); however, they did not call. We assessed site occupancy at 36.08 ± 26.8 (3–92) days, with 6.4 ± 4.5 (3–16) captures per individual ($n = 225$ captures). A proportion of 0.82 ± 0.17 (0.60–1.00) recaptures per individual were located above the stream.

The recaptures not located above the stream had a distance of 62 ± 42 (10–327) cm from the stream. The perch height of calling males above the stream was 296 ± 78 (17–567) cm. Additionally, on most survey nights, we did not recapture all known individuals or observe them calling. One time a male moved from its site to another, which resulted in combat behaviour (described below). Furthermore, females reproduced with males within the male calling sites. Females were documented in amplexus with the resident male ($n = 14$) and deposited clutches of eggs (32 total clutches; highest observed was four per site) above the stream within resident males' sites. Male egg attendance was not observed.

Vocalizations

We recorded six different call types and 726 vocalizations from 22 males (summarized in Tables 2–4; expanded data summary in SM Table S2). We also recorded six call types from a single individual (summary: Table 5; expanded: SM Table S3). Dominant frequency was significantly negatively related to SVL ($r^2 = 0.782$; $p < 0.001$) (Figure 2A), lower fundamental frequency ($r^2 = 0.694$; $p < 0.001$), and upper fundamental frequency ($r^2 = 0.770$; $p < 0.001$) (Figure 2B). No other call parameters were related to SVL. The PCA revealed the degree of variation each call type exhibited (Figure 3). The call parameters that explained the most variation among call types were call duration(s), call shape, and pulse rate (pulses/s) (SM Table S1). For all individuals, PC1 differed significantly among call types (ANOVA: $F_{5,565} = 441.7$; $p < 0.001$), as did PC2 (ANOVA: $F_{5,565} = 100.2$; $p < 0.001$) (Figure 3A). For the single individual analysis, PC1 differed significantly among call types (ANOVA: $F_{5,138} = 190.2$; $p < 0.001$), and also PC2 (ANOVA: $F_{5,138} = 54.43$; $p < 0.001$) (Figure 3B).

Using equal prior probabilities, the discriminant function analysis assigned the advertisement, distress, encounter, and release calls to the correct type with high

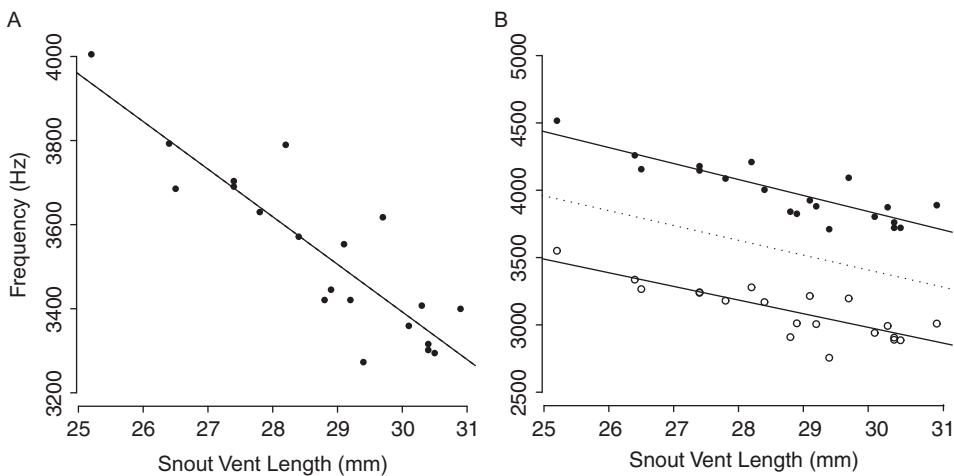


Figure 2. (A) The strong negative relationship between body size (SVL) and dominant frequency of the advertisement call in *Nymphargus grandisonae*. (B) The same body-size relationship is shown for lower fundamental frequency (open circles) and upper fundamental frequency (closed circle).

Table 2. Comparisons of call types recorded for *Nymphargus grandisonae*.

Parameter	Call type		
	Advertisement	Territorial	Encounter
<i>n</i> – calls (individuals)	417 (22)	30 (3)	19 (2)
Number of notes/call	1	1	1
Call duration (s)	0.115 ± 0.018 (0.056–0.158)	0.110 ± 0.015 (0.076–0.141)	0.115 ± 0.016 (0.091–0.148)
Call shape	0.521 ± 0.249 (0.034–1.0)	0.607 ± 0.274 (0.085–0.930)	0.744 ± 0.177 (0.439–0.943)
Pulse rate (/s)	143.2 ± 18.8 (107.9–320.8)	137.3 ± 17.1 (105.3–190.9)	131.4 ± 12.3 (115.7–169.8)
Dominant frequency (Hz)	3587 ± 189 (3100–4048)	3353 ± 101 (3100–3445)	3440 ± 142 (3186–3617)
Frequency modulation (Hz)	143.8 ± 107.9 (0–516.8)	212.5 ± 116.9 (0–430.7)	639.2 ± 135.6 (344.5–861.3)
Lower fundamental frequency (Hz)	3158 ± 184 (2694–3645)	2959 ± 118 (2700–3109)	2950 ± 117 (2700–3109)
Higher fundamental frequency (Hz)	4038 ± 210 (3441–4651)	3735 ± 136 (3527–4000)	3831 ± 165 (3527–4069)
Pulse-amplitude-peak ratio (first)	0.545 ± 0.203 (0.102–1.0)	0.509 ± 0.188 (0.261–0.963)	0.238 ± 0.156 (0.063–0.510)
Pulse-amplitude-peak ratio (middle)	0.812 ± 0.153 (0.403–1.0)	0.771 ± 0.149 (0.428–0.994)	0.628 ± 0.216 (0.273–0.939)
Pulse-amplitude-peak ratio (last)	0.616 ± 0.224 (0.165–1.3)	0.595 ± 0.289 (0.080–1.0)	0.932 ± 0.083 (0.746–1.0)
First harmonic (Hz)	7179 ± 409 (5857–8613)	6523.5 ± 296.0 (6029.3–7149.0)	6700 ± 234 (6201–7019)
Second harmonic (Hz)	10,697 ± 557 (8648–13,394)	10,304.4 ± 100.2 (10,077.5–10,508.2)	10,182 ± 382 (9431–10,723)
Third harmonic (Hz)	14,396 ± 627 (12,489–15,762)	13,532 ± 125 (13,350–13,695)	13,184 ± 649 (12,165–14,341)
Other frequency (Hz)	–	4657 ± 210 (4392–5168)	4750 ± 265 (4048–4995)

Note: Data are the mean ± standard deviation, and range (in parentheses).

accuracy (89.4–100%) (SM Table S4). However, the territorial and courtship calls were assigned correctly with less accuracy, 40.0% and 51.8%, respectively (mean of all call types = 89.3%). Prior probabilities based on the observed data led to improved classification: 56.7% for territorial call, 55.6% for the courtship call (mean of all call types = 91.6%).

We frequently heard the advertisement call along the survey area, where males presumably advertised their positions to conspecific males and females (Figure 1B). Therefore the advertisement call was the most frequently recorded call type ($n = 419$). To the ear, the call sounded similar to a whistle. The call was single noted, pulsed, and had some frequency modulation (Figure 4A; Table 2).

When a transient male approached a resident advertisement calling male, the resident male emitted the territorial call (interspersed with advertisement calls) in

Table 3. Comparisons of call types for *Nymphargus grandisonae*.

Parameter	Call type		
	Release	Distress	Courtship
<i>n</i> – calls (individuals)	48 (2)	30 (1)	27 (3)
Number of notes/call	18.2 ± 5.3 (9.0–34.0)	1	1
Call duration (s)	3.3 ± 0.877 (1.7–5.4)	0.020 ± 0.007 (0.010–0.037)	0.120 ± 0.023 (0.082–0.170)
Call shape	0.989 ± 0.011 (0.925–0.998)	0.513 ± 0.204 (0.158–1.0)	0.590 ± 0.211 (0.198–0.939)
Pulse rate (/s)	280.1 ± 75.3 (152.5–454.0)	145.3 ± 66.8 (55.6–333.3)	140.2 ± 12.1 (111.8–168.8)
Dominant frequency (Hz)	3267 ± 235 (2756–3531)	3080 ± 198 (2756–3445)	3541 ± 206 (3186–3876)
Frequency modulation (Hz)	228.4 ± 147.6 (0–602.9)	–	468.9 ± 198.9 (258.4–861.3)
Lower fundamental frequency (Hz)	2676 ± 307 (1882–3109)	2783 ± 182 (2536–3109)	3075 ± 218 (2615–3407)
Higher fundamental frequency (Hz)	3846 ± 227 (3255–4341)	3397 ± 217 (3074–3798)	4003 ± 197 (3720–4372)
Pulse-amplitude-peak ratio (first)	0.555 ± 0.336 (0.113–1.0)	0.786 ± 0.219 (0.271–1.1)	0.202 ± 0.091 (0.076–0.484)
Pulse-amplitude-peak ratio (middle)	–	0.892 ± 0.174 (0.477–1.0)	0.815 ± 0.148 (0.532–1.0)
Pulse-amplitude-peak ratio (last)	0.958 ± 0.145 (0.100–1.0)	0.785 ± 0.220 (0.281–1.0)	0.613 ± 0.231 (0.226–1.0)
First harmonic (Hz)	4959 ± 380 (4254–5512)	5735 ± 501 (4737–6546)	7056 ± 377 (6503–7752)
Second harmonic (Hz)	–	8494 ± 884 (6373–9216)	10,537 ± 719 (9431–11,714)
Third harmonic (Hz)	–	12,058 ± 498 (11,369–12,489)	14,211 ± 1929 (10,508–15,590)
Other frequency (Hz)	–	–	4929 ± 515 (3962–6115)

Note: Data are the mean ± standard deviation, and range (in parentheses).

response. This call was not observed in the absence of a transient male (i.e. during normal advertisement calling). Qualitatively, the territorial call sounded like an intense version of the advertisement call. When compared with the advertisement call quantitatively, the territorial call had higher frequency modulation, an additional frequency component, a shape skewed towards the end of the call, and it differed in the distribution and number of harmonic frequencies and their relative power (Figure 4B; Table 2). For a single individual, the territorial call had a shorter duration, lower dominant frequency, and greater amplitude change from the start to the end of the call compared with the advertisement call (Table 5).

The encounter call was emitted immediately before, during, or immediately after a combat incident; so it occurred less frequently than the territorial call (SM Video

Table 4. Comparisons of notes within the multiple note release call for *Nymphargus grandisonae*.

Par.	Start peep	Intermediate notes			End peep
		First note	Centre note	Last note	
<i>n</i>	33 (2)	49 (2)	49 (2)	49 (2)	49 (2)
PL	6.5 ± 1.9 (2.0–10.0)	17.7 ± 5.8 (7.0–32.0)	19.2 ± 5.8 (8.0–29.0)	20.4 ± 5.8 (8.0–32.0)	19.2 ± 10.3 (1.0–36.0)
PR	45.0 ± 22.2 (0.466–111.1)	270.9 ± 92.0 (82.7–463.8)	260.7 ± 94.8 (106.7–500.0)	285.1 ± 80.8 (111.1–524.6)	225.5 ± 123.5 (0.175–448.3)
ND	0.020 ± 0.016 (0.005–0.070)	0.069 ± 0.021 (0.033–0.133)	0.078 ± 0.021 (0.043–0.128)	0.073 ± 0.017 (0.042–0.129)	0.079 ± 0.043 (0.013–0.190)
NS	0.467 ± 0.205 (0.031–1.0)	0.291 ± 0.176 (0.019–0.789)	0.307 ± 0.164 (0.032–0.758)	0.297 ± 0.203 (0.032–0.958)	0.757 ± 0.217 (0.063–0.963)
DF	3047 ± 153 (2756–3359)	3206 ± 264 (2670–4220)	3280 ± 238 (2842–3703)	3334 ± 314 (2756–3876)	3292 ± 229 (2828–3703)
AP	0.555 ± 0.336 (0.113–1.0)	0.171 ± 0.084 (0.059–0.432)	0.191 ± 0.086 (0.046–0.399)	0.210 ± 0.109 (0.072–0.557)	0.982 ± 0.060 (0.677–1.0)
NI	–	0.132 ± 0.086 (0.013–0.321)	0.128 ± 0.087 (0.017–0.436)	0.083 ± 0.059 (0.007–0.342)	–

Notes: Data are the mean ± standard deviation, and range (in parentheses). Abbreviations: Par., call parameters; *n*, number of calls (number of individuals in parentheses); PL, Number of pulses; PR, Pulse rate (/s); ND, Note duration (s); NS, Note shape; DF, Dominant frequency (Hz); AP, Note amplitude to peak amplitude of call ratio; and NI, Note interval duration (s).

Table 5. Call comparisons for a single individual.

Call type	Parameter					
	<i>n</i>	Call duration (s)	Pulse rate (/s)	Dominant frequency (Hz)	Frequency modulation (Hz)	Amplitude change, start–end
AD	55	0.119 ± 0.011 (0.097–0.142)	150.3 ± 11.8 (130.8–201.9)	3792 ± 40 (3703–3876)	169.1 ± 66.2 (86.1–430.7)	1.4 ± 0.9 (0.4–3.5)
TR	10	0.094 ± 0.011 (0.076–0.115)	126.9 ± 12.1 (105.3–146.3)	3410 ± 44 (3359–3445)	232.6 ± 81.7 (86.2–344.5)	2.0 ± 0.9 (0.8–3.5)
EN	16	0.116 ± 0.017 (0.091–0.148)	129.5 ± 8.6 (115.7–148.9)	3483 ± 108 (3186–3617)	629.8 ± 136.1 (344.5–775.2)	6.2 ± 4.3 (1.6–15.8)
RL	25	3.5 ± 0.8 (2.1–5.4)	327.6 ± 58.1 (228.7–454.0)	3462 ± 43 (3359–3531)	300.2 ± 116.5 (0–602.9)	2.4 ± 1.6 (0.3–5.6)
DS	30	0.020 ± 0.007 (0.010–0.037)	145.3 ± 66.8 (55.6–333.3)	3080 ± 198 (2756–3445)	0	1.1 ± 0.5 (0.4–2.6)
CT	8	0.126 ± 0.011 (0.111–0.141)	144.3 ± 5.4 (134.9–153.2)	3779 ± 55 (3703–3876)	355.3 ± 55.2 (258.4–430.7)	4.0 ± 1.6 (1.6–6.6)

Notes: Data are the mean ± standard deviation, and range (in parentheses). Note the difference in dominant frequency and duration between the call types. The SVL of the individual is 26.4 mm. Abbreviations: *n*, number of calls; AD, advertisement call; TR, territorial call; EN, encounter call; RL, release call; DS, distress call; and CT, courtship call.

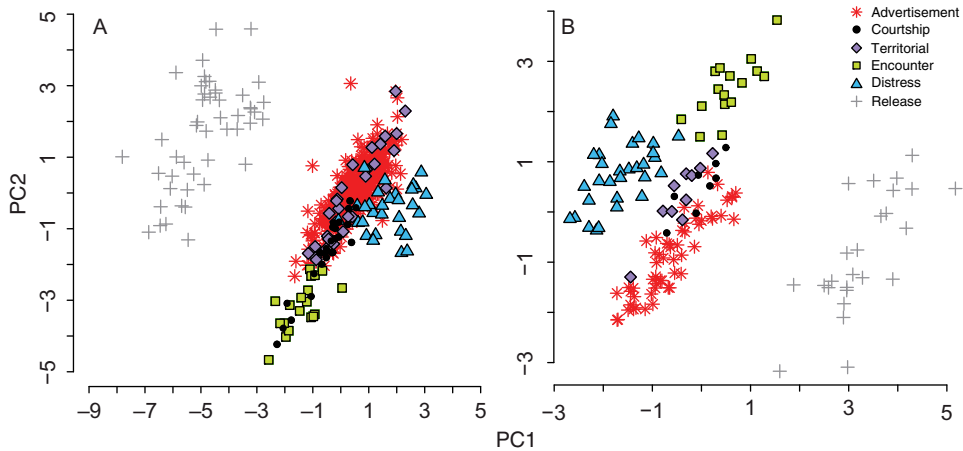


Figure 3. Principal component analyses (PCA) of call variables for the advertisement, territorial, encounter, distress, release and courtship calls of *Nymphargus grandisonae*. PC1 and PC2 are plotted against each other. (A) Plot for calls from all individuals; (B) plot for a single individual.

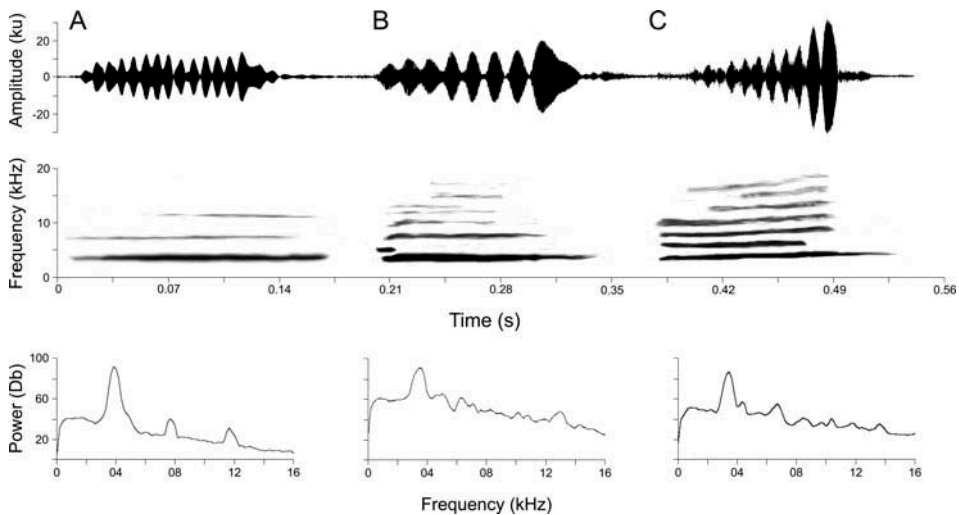


Figure 4. The oscillogram, spectrogram, and power spectra of calls in *Nymphargus grandisonae*. (A) Advertisement call; (B) territorial call; and (C) encounter call. Notice the increasing intensity of amplitude and power in the harmonics between the three call types.

S1). The encounter call sounded similar to, but was more intense than, the territorial call. The encounter call varied from the advertisement call in a similar manner to the territorial call, but the differences were more marked in the encounter call (Figure 4C; Table 2).

The release call occurred during combat behaviour, when the males grasped each other in various positions (see below, Figure 5A–I). The emitter's vocal sac inflated

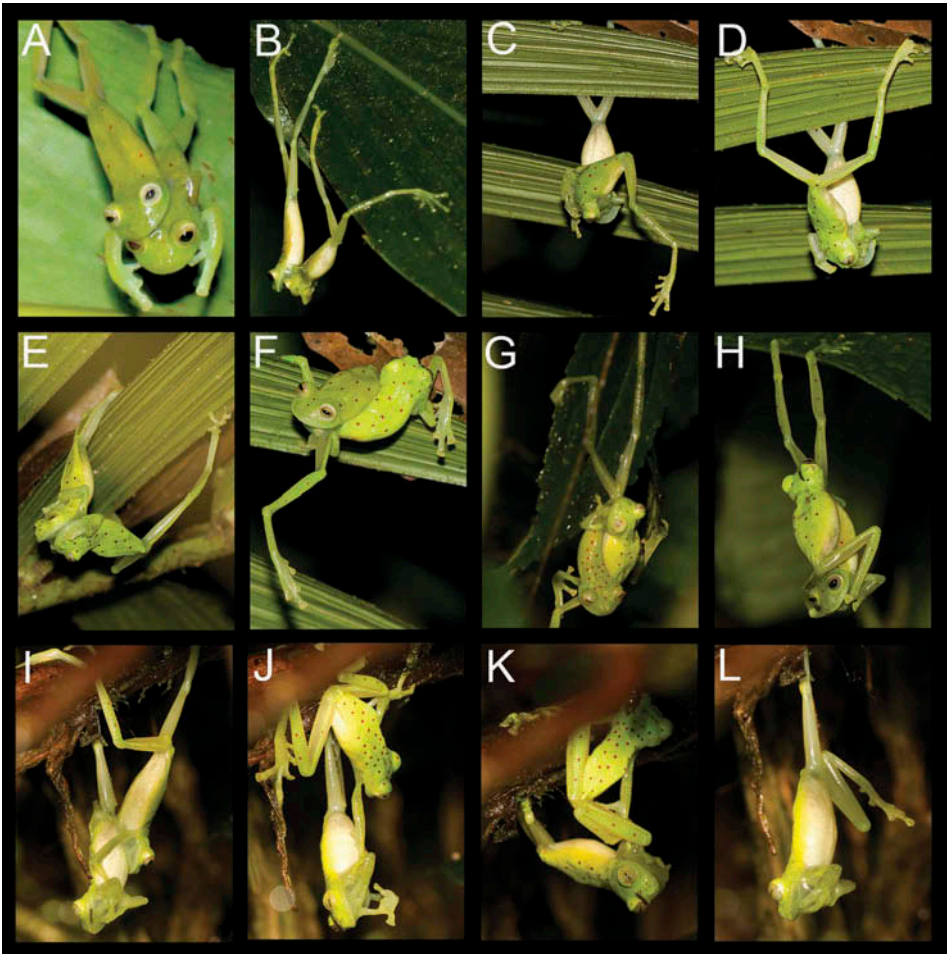


Figure 5. The variety of combat positions for *Nymphargus grandisonae*. Males fought through various combat forms: (A, B) dangling amplexus-like; (C–E) dangling venter-to-venter; (F, G) the head-to-vent wrestle; (H) reverse dangling amplexus-like; (I–K) behaviour at the end of a fight; and (L) the “throat-grasping” behaviour that occurred with the distress call.

when the call was given, and appeared to push the opposing male away from the emitter’s body. Bodily vibrations accompanied this call (SM Video S2). The release call was the only call with multiple notes; so it was a relatively longer call (Table 3). The call typically began with a loud peep (similar to its final note; Table 4), but sometimes this did not occur. A series of intermediate notes followed this initial peep and sounded like soft whimpers or squeaks. These intermediate notes were difficult to hear, and we needed to be 0.5–1 m away to hear and record the call. The release call always ended in an intense, heavily amplitude-modulated peep, which usually contained the peak amplitude (Figure 6; Table 4).

The distress call was presumably emitted as a response to pain or distress during or after long bouts of combat, when a male inflicted pain on another male (Videos

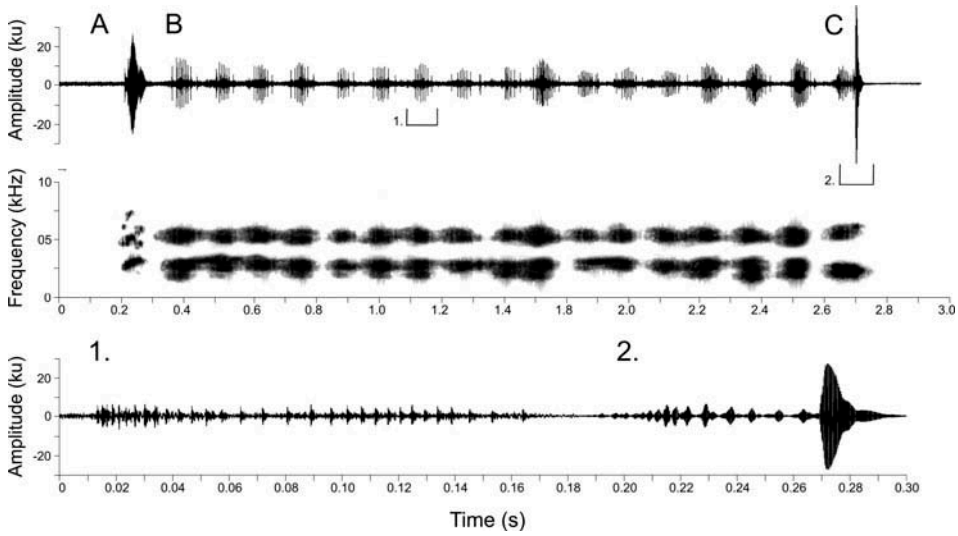


Figure 6. Release call of *Nymphargus grandisonae*. (A) First note peep; (B) intermediate notes; and (C) final peep. (1.) Enlarged view of an intermediate note and (2.) of the final peep.

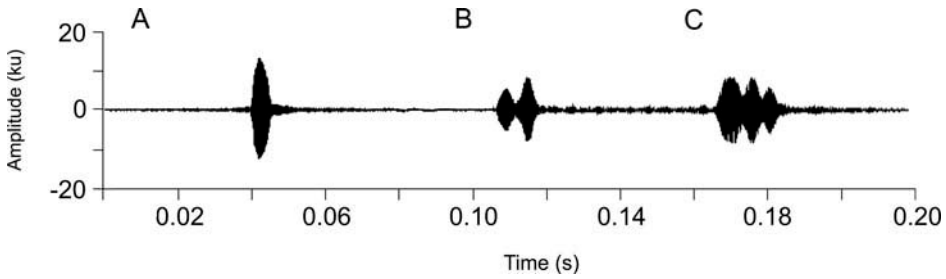


Figure 7. Distress calls of *Nymphargus grandisonae*. The pulse variation in the distress call is shown in A–C.

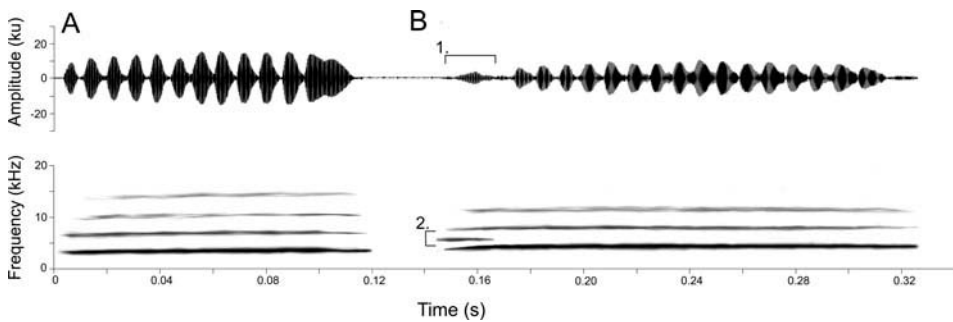


Figure 8. Oscillogram and audio-spectrogram of (A) the advertisement and (B) the courtship calls of *Nymphargus grandisonae*. (1.) Modified pulses and (2.) the additional frequency component of the courtship call are indicated.

S2–S3). The call also occurred when a male dangled alone and grasped its vocal sac, after losing a fight (Figure 5I–L; SM Video S3). The call sounded like a short squeak and could be heard several metres away. The distress call had the shortest duration and was pulsatile (Figure 7; Table 3). The single individual analysis also revealed it to have a lower dominant frequency than the advertisement call (Table 5).

Males gave the courtship call when a female was present nearby, on the same leaf. In this situation, the male increased the rate of advertisement calling and frequently emitted the courtship call *c.*30 s before amplexus; no calls occurred during amplexus. The courtship call was the most similar to the advertisement call (Figure 8A), except the first few pulses were of lower amplitude and frequency, it had stronger frequency modulation, and there was an additional frequency component between the fundamental frequency and the first harmonic (Figure 8B; Table 3).

Combat behaviour

We documented six combat events that were all observed mid-conflict except for Event 6, which we witnessed in full (Table 6). We observed high variability between combat events. We recorded five “forms” of combat: (1) dangling amplexus-like (Duellman and Savitzky 1976; Figure 5A, B; SM Video S1); (2) dangling venter-to-venter (Bolívar et al. 1999; Figure 5C–E; SM Video S2); (3) amplexus-like (McDiarmid and Alder 1974); (4) a new form, the head-to-vent wrestle: each male’s head was positioned towards the other’s vent, and each male grasped its opponent around the waist with its forelimb (Figure 5F, G; SM Video S4); and (5) a second new form, reverse dangling amplexus-like: two males dangled from the substrate, facing venter to dorsum, with one male’s head positioned near the other’s venter (Figure 5H; SM Video S5). The form of combat with the longest duration was the head-to-vent wrestle position. Additionally, the second longest duration was the dangling venter-to-venter position. The form with the shortest duration was amplexus-like, which occurred briefly before the males tumbled to the margin of the leaf (Table 6). The release call was observed in the long duration combat modes (Videos S1, S2, S4–S6).

Before combat, we observed escalated aggressive encounters (see fig. 1 in Martins et al. 1998). When a transient male (Male B) approached a calling male (Male A), both frogs moved closer to each other, and the calling male increased its calling rate. A fight began when the two males came into contact; Male B jumped onto the dorsum of Male A, which resulted in an amplexus-like position. Male A jerked his body and jumped around the leaf to throw off Male B. Shortly after, the frogs rolled towards the margin of the leaf and Male A fell and remained attached to the leaf with his hind limbs while Male B dangled from Male A with his forelimbs. Male A climbed up the dorsum of Male B, which resulted in a dangling reverse amplexus-like position (Figure 5H). Male A struggled to climb over Male B and they both fell into the vegetation below. The males then fought in the head-to-vent wrestle position on the surface of a leaf (Figure 5G; SM Video S4). Combat ended when the two males separated (Figure 5I–L); the presumed “winner” began advertisement calling (SM Video S1). Combat was observed to end in one of two ways: (1) the “loser” fell into the vegetation or stream below (SM Video S7); or (2) the “loser” silently moved away from the “winner” after separation. The “loser” sometimes attempted to initiate another fight.

Table 6. Observations from each combat event for *Nymphargus grandisonae*.

Observation	Event 1	Event 2	Event 3	Event 4	Event 5	Event 6	Summary
Location	Colombia	Colombia	Ecuador	Ecuador	Ecuador	Ecuador	—
Date (mm/dd/yy)	04/05/10	03/10/10	11/05/11	01/06/11	01/06/11	2/06/11	—
Time	2227 h	2148 h	0044 h	0341 h	2129 h	2237 h	—
Combat duration (min)	25	3	248	15	92	302	114.2 ± 129.5
Other observations (min)	0	0	32	22	36	78	28.0 ± 28.9
Temperature (°C)	—	—	13.5	13.5	13.5	13.5	13.5
Weather	Rain	Rain	Clear	Clear	Fog	Fog	—
Substrate	<i>Heliconia</i> sp.	<i>Calathea</i> sp.	Palm	Aroid	Branch	Small leaf	—
Substrate height (m)	1.9	1.3	4	3	1	0.5	1.95 ± 1.32
Combat mode				Durations (min)			
Dangling amplexus-like	25	—	—	15	—	—	20.0 ± 7.07
Dangling venter-to-venter	—	3	190	—	70	—	87.7 ± 94.7
Amplexus-like	—	—	—	—	—	1	1
Head-to-vent wrestle	—	—	370	—	—	299	334.5 ± 50.2
Reverse dangling amplexus-like	—	—	—	—	—	1	1

We observed numerous bouts of consecutive combat between the same two individuals, which lasted a considerable amount of time (Table 6, Events 4–6). Between Males A and B, Event 4 was observed mid-conflict for 15 min and observations ceased 22 min after the fight ended. The next night, Event 5 was observed mid-conflict, 17 h 34 min after Event 4; Event 5 occurred along with Event 6 for a total duration of 6 h 34 min. Male A was the “winner” (described above) after Event 6. Assuming combat occurred in our absence, the total potential duration of these encounters could have lasted 24 h 58 min (additional combat that may have occurred before Event 4 would add to this time). The following night, Male B was advertisement calling at the combat site. Male A was absent, suggesting more combat incidents may have occurred after Event 6. Therefore, the potential duration of the conflict may have been 42 h 19 min. Male A was not observed to return to this location on subsequent nights. It is also unknown whether combat occurred during the day; however, it is clear that combat can occur between the same males on consecutive nights.

Injuries

Nymphargus grandisonae and some populations of *Nymphargus griffithsi* are the only species in the genus *Nymphargus* with humeral spines (Figure 1A; see fig. 13 in Guayasamin et al. 2009). Duellman and Savitzky (1976) hypothesized that humeral spines are used during combat, which may lead to injury. We observed several injuries, possibly resulting from combat (Male B; Events 4–6). During combat, males squeezed their opponent, which appeared to drive the humeral spines into the opposing male. We photographed an injured male 3 days before and a day after combat (Figure 9A–C). However, there was some uncertainty as to the cause of injury, as the individual was not examined immediately before combat. The specific location of injuries may have resulted from humeral spine positioning during different combat forms (Figure 5).

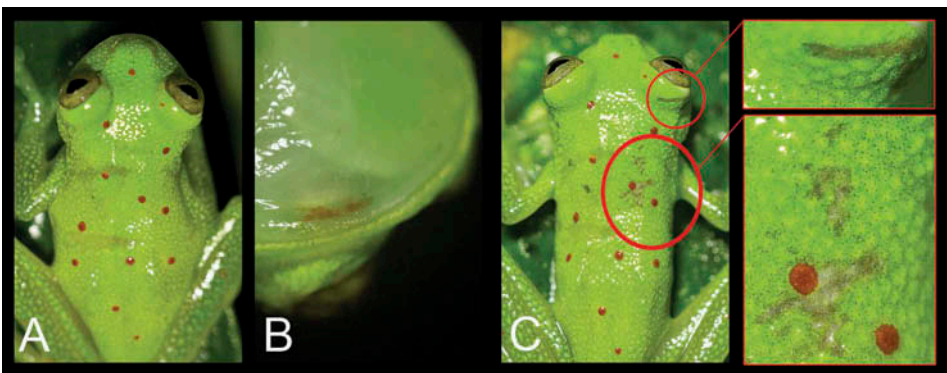


Figure 9. Evidence of injury from combat in males of *Nymphargus grandisonae*, possibly from humeral spines. (A) Photograph taken 3 days before combat incident and (B, C) photographs taken 31 hours after combat.

Discussion

High site fidelity

We showed high site fidelity of male *N. grandisonae* across a 3-month period. Male frogs advertisement called and reproduced within their sites, excluded other males from reproductive resources, and were aggressive towards transient males through calling and combat behaviour. This result is concordant with two other mark–recapture studies of site fidelity and aggression in glassfrogs (Greer and Wells 1980; Jacobson 1985). With these results, and our observations of aggressive responses and combat behaviour towards transient males, we hypothesize that *N. grandisonae* is a territorial species. Furthermore, we hypothesize that all glassfrog species where combat behaviour has been reported are also territorial species.

Vocalizations

We describe the advertisement, courtship, territorial, encounter, distress, and release calls of *N. grandisonae* for the first time. For these calls, we also infer their intraspecific communicative functions and their use during antagonistic interactions from behavioural observations. Despite our categorizations, we emphasize that these call definitions remain hypotheses until they can be validated through behavioural experiments (e.g. Ryan and Rand 1990; Ibáñez 1993).

The advertisement call has been described for 21% of Centrolenidae (SM Table S5), whereas in *Nymphargus* it has been described in five species (Table 7). Many of the temporal and spectral attributes of the advertisement call of *N. grandisonae* are similar to those described in other species of *Nymphargus* (Catenazzi et al. 2009; Hutter and Guayasamin 2012). All the calls described in *Nymphargus* are single noted. The

Table 7. Described advertisement calls for the genus *Nymphargus*.

Parameter	Species			
	<i>N. griffithsi</i>	<i>N. lasgralarias</i>	<i>N. truebae</i>	<i>N. pluvialis</i>
<i>n</i> – calls	48	119	8	6
Call duration (s)	0.122 ± 0.009 (0.103–0.148)	0.026 ± 0.006 (0.016–0.044)	0.181 (0.177–0.192)	0.170 (0.132–0.217)
Notes/call	1	1	1	1
Number of pulses/note	2.4 ± 0.5 (Tonal, 1–3)	1.5 ± 0.6 (1–3)	12.4 (11–13)	5.4 (4–8)
Dominant frequency (Hz)	4107 ± 105 (3789–4306)	3691 ± 131 (3445–3962)	3738; 4143 (3488–4177; 4005–4392)	3842; 4044 (3617–4048; 3875–4144)
Frequency modulation (Hz)	199.3 ± 116.2 (0–344.6)	30.4 ± 65.9 (0–172.3)	405 (215–603)	212 (43–431)
Reference	Hutter and Guayasamin (2012)		Catenazzi et al. (2009)	

Notes: Data are the mean ± standard deviation, and range (in parentheses; when available). The dominant frequency for *N. truebae* and *N. pluvialis* is shown as the dominant frequency at the start and the end of the call (entire call was unavailable).

call of *N. grandisonae* shares a similar duration with *N. griffithsi* and a similar number of pulses with *N. truebae*. Dominant frequency and overall call structure appear to be conserved in *Nymphargus* (Table 7). In a cladistic analysis of calls in the genera *Smilisca* and *Pseudacris* (Hylidae), Cocroft and Ryan (1995) found similar patterns of conservatism (dominant frequency and call structure) and divergence in temporal components (length, pulse rate, and amplitude modulation). The evolution of temporal call divergence remains poorly understood but the study of female preference can reveal whether sexual selection mechanisms are involved in call divergence (e.g. Ryan and Rand 1990; Ron 2008).

Courtship calls are particularly complex in frogs (Wells 2007). For example, males of some species lengthen their advertisement calls in response to females (Wells 2007), others increase their calling rates (Ibáñez 1993), and some may modify their advertisement call (Owen et al. 2006). In certain cases, courtship calls are distinct from the advertisement call (Wells 2007). In Centrolenidae, the courtship call has only been described for *Hyalinobatrachium fleischmanni* (Greer and Wells 1980). The males of *H. fleischmanni* use two types of calls (that differ from the advertisement call) during courtship, mews and chirps, depending on male proximity to the female (Greer and Wells 1980). Conversely, our results suggest that the courtship call of *N. grandisonae* is a modified advertisement call; however, further observations are needed, especially in courtship call variation from female proximity. Additionally, we recommend female choice experiments using the recorded “courtship” call to test whether its function is related to female mate choice (e.g. Ryan and Rand 1990; Ibáñez 1993).

The advertisement, territorial, and encounter calls may represent a spectrum of parameter differences that change in response to the degree of aggression the emitter was attempting to convey. The context of the encounter call might be peak aggression (i.e. before combat), whereas the territorial call might represent a wider range of aggression levels leading up to peak aggression. This might explain why the discriminant function analysis incorrectly categorized the territorial call c.50% of the time. Despite this, we consider the encounter and territorial calls different types, as their relative behavioural contexts differ in a biologically relevant manner (Duellman and Trueb 1994). Greer and Wells (1980) quantitatively described the only other glassfrog encounter call for *H. fleischmanni*. This call had a longer duration and a lower dominant frequency than the advertisement call (SM Table S6), which we also show. Male frogs may artificially signal that they have a larger body size by lowering the dominant frequency of their call, which other males may interpret as aggression (Wells 2007; Figure 2). This pattern has been observed in other frogs: such as *Leptodactylus albilabris* (Lopez et al. 1988), *Rana virgatipes* (Given 1999), and *Acris crepitans* (Wagner 1992).

We describe the release call for *N. grandisonae*, which fighting males emitted during combat. This may be the first release call description for glassfrogs; however, Bolívar et al. (1999) described a call recorded during combat that may have been the encounter or release call. Nevertheless, Bolívar et al. (1999) hypothesized that the vocal sac inflation from a call given during combat loosens its opponent’s grip and assists the emitter in winning a physical fight. We observed males calling during combat for several hours, and these calls did not appear effective in loosening the grip of their opponent. In other species of frogs (e.g. *Bombina variegata*; Schneider et al. 1986), it has been suggested that this call communicates to the clasping male that he has grasped an inappropriate

mate (Wells 2007). Furthermore, the release call of *N. grandisonae* shows several similarities with the release call of *Bufo biporcatus* and *Duttaphrynus melanostictus* (*Bufo melanostictus* auctorum) in regards to duration (c.2.5 s) and multiple notes (Márquez and Eekhout 2006). This apparent conservatism across distantly related frogs might suggest that the release call faces little selective pressure to diverge, as it may not play a significant role in species recognition and/or sexual selection.

Lastly, we describe the distress call, which a male emitted as a response to pain from another male during a fight (Figure 5I–L; SM Video S2). Bogert (1960) hypothesized that the distress call is a response to a predator causing pain and distress. The call also may be used to startle the predator and aid escape (Bogert 1960). Despite this definition being specific to predatory harm, if the distress call is a response to the stimuli of pain and distress, we consider that it would be reasonable to extend the definition to conspecifics causing the same harm.

Combat behaviour

We describe the combat behaviour of *N. grandisonae* for the first time. Several unreported combat forms for glassfrogs are documented in addition to the longest measured duration of combat in Centrolenidae (previous longest length: 2 h 30 min, *Cochranella granulosa* [Kubicki 2007]), and any frog. All other recorded glassfrog durations lasted less than an hour (e.g. McDiarmid and Adler 1974; Duellman and Savitzky 1976; Jacobson 1985; Bolívar et al. 1999; Kubicki 2007; Dautel et al. 2011; Rojas-Runjaic and Cabello 2011; see SM Table S7 for a summary). Further, we provide the first observations of multiple-night fights in a frog.

We suggest that aggressive interactions in *N. grandisonae* are escalated. As observed in other frogs (e.g. Kluge 1981; Martins et al. 1998), escalated aggressive encounters offer males the option of abandoning a fight if they perceive continuing as costly, avoiding the energetic and physical risks associated with combat (see Fig. 1 in Martins et al. 1998). We also documented extensive fighting occurring across multiple nights; but the explanation for these extensive fights is unclear. In territorial experiments in butterflies, Davies (1978) suggested that repeated fights occurred when both residents thought they were the territory holder. This may be the case for *N. grandisonae*. We observed that during the string of combat incidents, both males advertisement called within the same territory, which is only done by the territory holder (Wells 2007). As observations on the full length of fights in frogs are limited, it is not clear what factors lead to long durations and repeated fights in the combat behaviour of *N. grandisonae* (and possibly other frogs). However, an experimental approach might elucidate these factors (e.g. removal or introduction experiments; Davies 1978).

We provide evidence of injury resulting from combat (Figure 9); only one other glassfrog study has also provided such evidence (Bolívar et al. 1999). In *Centrolene buckleyi*, Bolívar et al. (1999) reported injuries following combat, proposing that humeral spines caused the injuries; however, they did not examine the frog before combat, leaving the origin of the injuries in doubt. Additionally, male *Centrolene geckoideum* often have scarification on their head and body, suggesting that the large humeral spines of conspecifics caused these injuries during combat (Bolívar et al. 1999). Other species of frogs possess ornamentation hypothesized for use in combat; for example the prepollex spines of gladiator frogs (*Hypsiboas*; Wells 2007), and

of the Otton frog *Babina subaspera* (Iwai *in press*), the fangs of Indonesian fanged frogs (*Limnonectes*; Wells 2007), and the keratinized nuptial spines in moustache toads (*Leptobrachium*; Hudson et al. 2011). However, direct observations of injury that resulted from these ornamentations are limited (but see observations in *Babina* and in *Leptobrachium*; Hudson et al. 2011; Iwai 2013).

It is clear that fights can occur in various forms as additional combat observations are reported (Delia et al. 2010; Rojas-Runjaic and Cabello 2011; this work). We offer multiple new descriptions of variation in combat (e.g. various forms of dangling and positioning on leaves), which appeared to arise randomly and as a result of opportunity (also see Delia et al. 2010; Dautel et al. 2011). Therefore, behavioural contexts and hypotheses regarding combat forms are difficult to infer. Insight can also be acquired from other frogs, where wide variation in combat has been observed. Similar to glassfrogs, dangling venter-to-venter, wrestling, and amplexus-like combat have been described in *Phyllomedusa* (Wogel et al. 2004; Vilaça et al. 2011). In addition, pushing, lunging, and grasping have been observed in poison-dart frogs (Hermans et al. 2002). Using incomplete observations as a diagnostic character of subfamilies in Centrolenidae may be invalid as combat in glassfrogs exhibits more complexity and variation than previously hypothesized (Bolívar et al. 1999; Guayasamin et al. 2009; Delia et al. 2010; Rojas-Runjaic and Cabello 2011; this work). Therefore, we recommend hypotheses using limited behavioural data be cautioned against, and the hypotheses that use combat behaviour as a diagnostic character state of subfamilies in Centrolenidae be revised.

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Supplemental data

Supplemental data for this article can be accessed <http://dx.doi.org/10.1080/00222933.2013.792961>.

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Appendix

Videos of combat events

(SM Video S6 recorded by Julián A. Rojas-Morales, all others recorded by Carl R. Hutter)

SM Video S1. One male throws the other male off the leaf and encounter calls.

SM Video S2. One male squeezes another, who is unable to squeeze in return.

SM Video S3. A male dangles and grasps his throat while distress calling.

SM Video S4. The head-to-vent wrestle combat position and audible release calls.

SM Video S5. A fight in the venter-to-venter position.

SM Video S6. The dangling amplexus-like position.

SM Video S7. Two males separate from each other and combat ends.

Additional supporting information

Table S1. Principal component analysis loadings for the different call types.

Table S2. Complete data summary for all call types and individuals.

Table S3. Complete data summary for all call types for a single individual.

Table S4. Expanded discriminant function analysis results.

Table S5. Advertisement calls described in Centrolenidae.

Table S6. Comparisons of calls emitted during combat by other glassfrogs.

Table S7. Summary of combat behaviour observations for glassfrogs.