Multimale amplexus, amplectant and advertisement calls, and tadpole development in *Ololygon perpusilla* (Lutz and Lutz, 1939)

Edelcio Muscat[1], Guilherme Augusto-Alves[2,3], Luís Felipe Toledo[2], Rafael M. Tanaka[1], and Daniel R. Stuginski[1,*]

Ololygon perpusilla (Lutz and Lutz, 1939) belongs to a group of bromeligenous hylid frogs endemic to the Atlantic forest (Alves-Silva and da Silva, 2009). The group is currently composed of 13 species, but its monophyly may still be further tested (Peixoto et al., 2016). All species in the *O. perpusilla* group present close association with terrestrial and epiphytic bromeliads, using phytotelmata tanks as egg-laying sites. The small number of eggs laid develop into exotrophic tadpoles with a non-selective omnivorous diet (Peixoto, 1995; Sabagh et al., 2012). One possible synapomorphy of the species group is the behaviour of laying eggs in multiple bromeliad tanks (Alves-Silva and da Silva, 2009). Such behaviour was interpreted as a strategy to decrease tadpole density in small water volumes and to provide better conditions for larval development (Alves-Silva and da-Silva, 2009). Another behaviour that seems to be linked to the improvement of larvae fitness is the non-random bromeliad tank selection, as noticed by Oliveira and Navas (2004), some abiotic and biotic factors seem to influence water tank choice.

Temperature is a ubiquitous abiotic factor that largely influences all amphibian life stages (Navas et al., 2013). However, due to the aquatic nature of many amphibian larvae and poor capacity of thermoregulation in such environments (due to the high thermal conductance of

the water), the effect of water temperature is especially strong for amphibian larvae (e.g., Hutchinson and Dupré, 1992; Edge et al., 2016). Extreme temperatures (high/ low or even huge thermal fluctuations) can affect tadpole hatching size, development time, size at metamorphosis, change in body proportions, increasing the number of malformations, or even decreasing survival of tadpoles (Kaplan and Phillips, 2006; Arrighi et al., 2013). These features are important for the species fitness and so it is expected that behavioural (e.g., microhabitat selection) or physiological (e.g., high thermal tolerance) strategies are employed to buffer such thermal challenges (Simon et al., 2015). In this context, bromeligenous frog species, such as those in the *O. perpusilla* group, are especially interesting. Since the species of the *O. perpusilla* group present larval development in small water bodies, they are probably more prone to face higher peaks and faster heating and/or cooling rates than species that use more stable thermal environments (like ponds and streams) as breeding sites.

Here, we describe the reproductive behaviour of *O. perpusilla* during a multimale amplexus, its advertisement call and report the bromeliad tank water temperature (at 07:30 am and 11:30 am) during the larval development of one clutch deposited inside it. All monitoring was made at the area of Projeto Dacnis, an NGO of Atlantic forest preservation, located near Ubatuba city, São Paulo, Brazil (-23.46291666, -45.1330333).

During a field survey on 23 November 2014 (10:00 am), we recorded an amplexus of two *O. perpusilla* males and one female inside the central water tank of the bromeliad *Neoregelia cruenta* (Fig. 1A). The bromeliad tank presented a diameter of 6 cm that was on top of a rock at 80 cm from the forest floor. Despite the presence of closed canopy and presence of other bromeliads few meters from this location, the frogs used, as their breeding site, a bromeliad in an area with open canopy

[1] Projeto Dacnis, São Francisco Xavier and Ubatuba, São Paulo, Brasil.

[2] Laboratório de História Natural de Anfíbios Brasileiros (LaHNAB), Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brasil.

[3] Programa de Pós-Graduação em Ecologia, Instituto de Biologia, Unicamp, Campinas, São Paulo, 13083-862, Brazil.

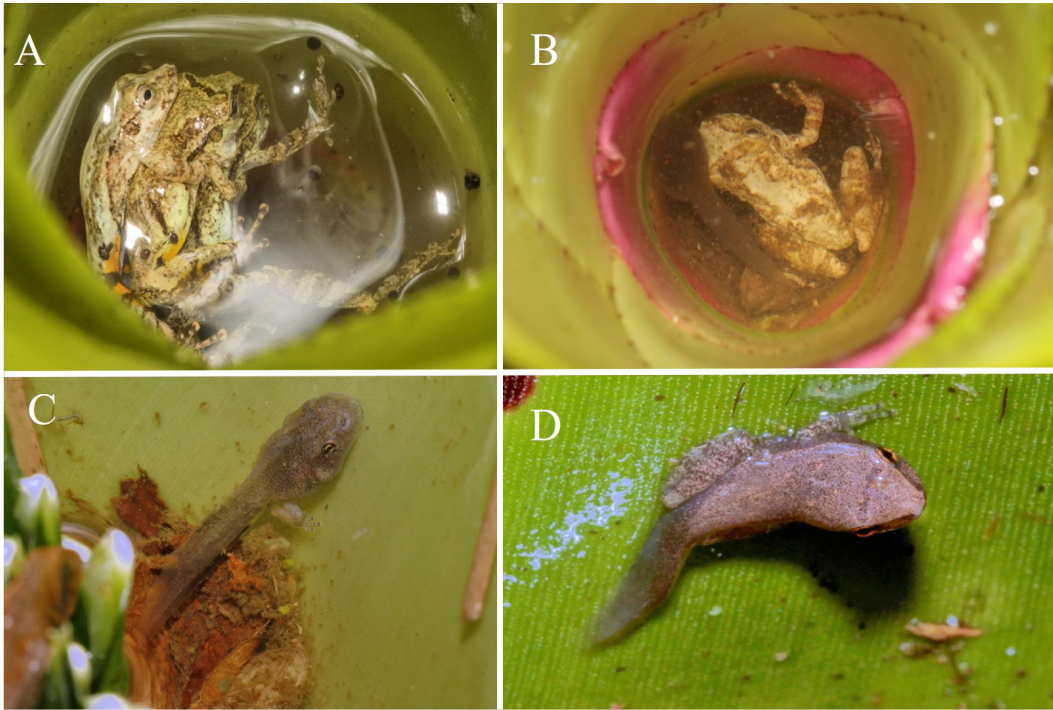* Corresponding author. E-mail: daniel.stuginski@gmail.com

Figure 1. Multimale amplexus among three individuals of *O. perpusilla* inside a bromeliad. Notice the presence of black and white eggs floating (A). An adult individual close to the first tadpole that hatched (B). A tadpole with developed hind limbs between Gosner stage 37-40 (C). One recently metamorphosed froglet, with the tail yet to be absorbed (D).

that received direct sunlight during four hours per day.

The male that was amplected with the female emitted a short vocalization that was recorded with a Nikon Coolpix p60 camera. In addition, we record and analysed the advertisement call of three different males during the morning (08:30 am) in 15 December 2015. Those advertisement calls were recorded with a smartphone (Motorola – Moto E), at approximately 30cm from the frogs (no air temperature information). All records were made at a sample rate of 44.1 kHz and a sample size of 16 Bits. Using Adobe Audition, we normalized the calls, removing DC offset (mean amplitude displacement from zero), centring on vertically and to the maximum amplitude of -1.0 dB. All sound analyses were performed in Raven Pro 1.4 (Bioacustic Research Program, Cornell Lab of Ornithology). Configurations adopted were: 65 % brightness, 50 % contrast, and Fast Fourier Transform length (FFT) of 128. We measured the following acoustic parameters: call duration; intercall interval; note duration; internote interval; note number per call; pulse number per note; pulse duration; call rate; peak of dominant frequency (peak frequency

function in Raven); minimum frequency (frequency 5 % function in Raven); maximum frequency (frequency 95 % function in Raven); and frequency bandwidth (BW 90% function in Raven). The audiovisual material was deposited at Fonoteca Neotropical Jacques Vielliard (FNJV), Museu de Zoologia "Prof. Adão José Cardoso" (ZUEC), Universidade Estadual de Campinas (UNICAMP), Campinas, Brazil (recordings of the advertisement call from three males: FNJV 40439–41; video of the multimale amplexus: ZUEC_VID 624). We followed the bioacoustical terminology and definitions presented by Köhler et al. (2017).

Five calls from one amplected male were analysed. This vocalization is composed by a single non-pulsed note, with mean of dominant frequency of 1.80 kHz (Fig. 2A; Tab. 1) and can represent an amplectant or release call (*sensu* Toledo et al., 2015). During posterior fieldworks, we stimulated the emission of release call in other 8 males (holding males gently), without success. Thus, we suggest that the vocalization recorded is an amplectant call, with probable function to stimulate the female to release the eggs (e.g., Zornik and Kelly, 2011;
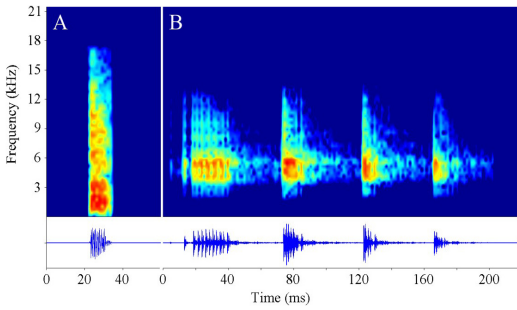
Figure 2. Spectrogram (above) and waveform (below) of the amplectant call (A) and advertisement call (B) of *Ololygon perpusilla,* recorded in the municipally of Ubatuba, state of São Paulo, southeastern Brazil.

Toledo et al., 2015). To describe the advertisement call, we analysed 45 calls from three males. The advertisement call is composed of 2 – 8 pulsed notes, with mean of dominant frequency of 5.40 kHz (Fig. 2B;

Tab. 1). The advertisement call of *O. perpusilla* was previously described by Pombal and Bastos (2003) from the municipally of Rio de Janeiro. As described for the Ubatuba population (present study), the Rio de Janeiro population presents advertisement call composed by pulsed notes. The call parameters analysed between those populations are roughly similar, but with some differences: the Ubatuba population presents longer calls, with longer notes, more pulses per note, and longer intercall interval (Tab. 1). Geographic variation on call parameters is observed for several species (e. g. Bernal et al., 2005; Pimenta et al., 2008; Ohmer et al., 2009; Tessarolo et al., 2016; Zornosa-Torres and Toledo, 2019), including for *Ololygon* genus (Pombal Jr. et al., 1995; Pontes et al., 2013). This variation between populations can be related with environmental factors, such as temperature, plant coverage or microhabitat available; community structure; or even geographic and genetic isolation (Pröhl et al., 2007; Ohmer et al., 2009; Forti et al., 2016; Köhler et al., 2017).

Table 1. Temporal and spectral parameters of *Ololygon perpusilla* advertisement and putative amplectant calls. Values presented as mean ± standard deviation (range; sample size).

	Ubatuba (SP) - Present study		Rio de Janeiro (RJ) - Pombal & Bastos, 2003
	Advertisement call	Amplexus call	Advertisement call
Call duration (ms)	208.96 ± 73.39 (87 - 429; 45)	11.6 ± 0.89 (11 - 13; 5)	140 ± 27 (92 - 174; 10)
Intercall interval (ms)	1681.10 ± 648.67 (473 - 4578; 42)	252 ± 33 (226 - 299; 4)	937 (776 - 1067; 10)
Note duration (ms)	28.67 ± 18.97 (9 - 132; 184)	-	14 ± 2 (7 - 18; 43)
Internote interval (ms)	29.03 ± 9.43 (3 - 74; 138)	-	26 ± 7 (9 - 59; 33)
Note number per call	4.09 ± 1.2 (2 - 8; 45)	-	4.3 ± 0.8 (3 - 6; 10)
Pulse number per note	9.76 ± 6.26 (1 - 42; 1796)	-	3.5 ± 0.9 (3 - 5; 43)
Pulse duration (ms)	3.41 ± 2.04 (0.48 - 18.4; 185)	-	3 ± 1 (2 - 5; 43)
Call rate (calls/minute)	30.9 ± 12.08 (15.17 - 43.5; 3)	-	-
Peak of dominant frequency (kHz)	5.40 ± 0.22 (4.82 - 5.86; 45)	1.80 ± 0.09 (1.70 – 1.89; 5)	4.82 ± 0.09 (4.55 – 4.86; 10)
Minimum frequency (kHz)	4.06 ± 0.14 (3.79 – 4.13; 45)	0.89 ± 0.07 (0.86 – 1.03; 5)	-
Maximum frequency (kHz)	6.74 ± 0.47 (6.20 - 7.58; 45)	8.03 ± 0.58 (7.06 – 8.44; 5)	-
Frequency bandwidth (kHz)	2.69 ± 0.47 (2.07 – 3.44; 45)	7.13 ± 0.65 (6.03 – 7.58; 5)	-

During the multimale axillar amplexus, all three frogs were partially submerged, and we recorded 6 eggs at the bottom of the bromeliad tank (Fig. 1A). The first frog to leave the breeding group was the external amplected male, and after a few minutes each individual took a different direction, finishing the interaction. Seven days later (4 December 2014) we recorded the first larvae inside the bromeliad water tank. At that time, we registered one adult specimen inside the same water tank (Fig. 1B), however, it was not possible to determine if this adult was part of the previous breeding group or a new specimen using the same site. The adult specimen was not vocalizing, and it was not observed inside the bromeliad tank any other day. We did not search for other tadpoles inside the bromeliad tank, in order to not physically alter the bromeliad. Thirty-nine days after the amplexus observation (1 January 2015), we recorded the first tadpole with visible hindlimbs (Gosner stage 37-40 – Fig. 1C; Gosner 1960). At day 64 (25 January 2015) we recorded the first metamorph of the clutch (Fig. 1D) and other three metamorphs were detected in the following days. We could not be sure if all six eggs completed the development into post-metamorphic individuals.

The breeding behaviour of the species of the *O. perpusilla* group was studied by Alves-Silva and da Silva (2009) and most parts of our data corroborates their observations. Similarly, we recorded reproductive activity in a bromeliad on a rock and the axillary amplexus inside the water tank of the bromeliad. However, Alves-Silva and da Silva (2009) did not detect the presence of a multimale amplexus. It is also important to note that the authors recorded multiple clutches deposited in different bromeliads tanks with few eggs in each deposition (maximum three eggs). In our observation, we did not observe multiple depositions and the clutch was deposited at once (six eggs). Multimale amplexus seem to be uncommon in bromeligenous species since these frogs are considered typical resource defensive species (Wells, 2007). *Ololygon perpusilla* is a prolonged-breeder (calling activity extending throughout the rainy season), with a resource defensive behaviour, so it is expected that females chose their mates based on the quality oviposition sites. Males defend their resources (bromeliad tanks) from other males, keeping them distant and decreasing the chance of a multimale amplexus occurs during breeding activity. In the opposite way, in species with explosive breeding mating system, especially those with scramble competition behaviour, multimale amplexus, sperm competition, and multiple paternity are more common because of the high density

of males in proximity and poorly possibility for females to choose their mates (Wells, 2007).

During the monitoring period, we measured water temperature twice a day every 2 to 4 days (Thermofocus model 01500/A3). Temperature measurements were made during early morning (at 07:30 am) and late morning (at 11:30 am). The average temperature at 07:30 was 22.6 ± 0.66 °C with a minimum of 21 °C and a maximum of 23 °C. During measurements at 11:30 temperature average was 34.5 ± 3.4 °C with a minimum of 28.3 °C and a maximum temperature of 37.3 °C. The thermal variation between these periods was in average 11.8 ± 3.0 °C and a maximum of 15.4 °C (Fig. 3).

As tadpole development and survivorship is related to the thermal environment, extreme temperatures and high-temperature fluctuations can be a stressor to frog communities (Duarte et al., 2012, Arrighi et al., 2013). In this context, the use of small water volumes inside bromeliad tanks by *O. perpusilla* group, as with other bromeliginous frog species, makes them especially prone to face such thermal challenges. Taking this into account, together with the fact that bromeliad choice is not-random and seems to be based on biotic and abiotic factors that could improve tadpole fitness (Oliveira and Navas, 2002), we predicted that bromeliads in open areas would be avoided, especially when other more suitable sites are available. Nevertheless, our record was made in a bromeliad exposed to sunlight.

The selection of a bromeliad in an open area resulted in a huge thermal variability and, as far as we know, the widest thermal variability and thermal peak during larvae development recorded for a species of the *O. perpusilla* group. Moreover, this is even larger than the temperature recorded in 676 bromeliads used by six other
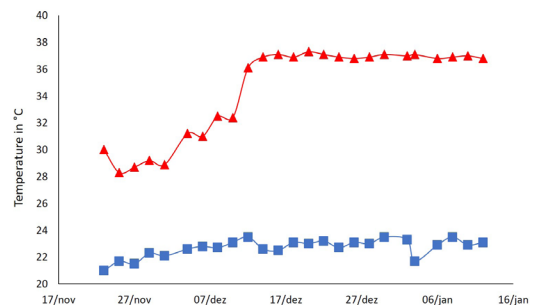

Figure 3. Water temperature inside the bromeliad water tank during development of *O. perpusilla* tadpoles. Squares and blue line represent temperatures measured at 07:30 am and triangles and the red line represents temperature measured at 11:30 am.

species (Schineideri and Teixeira, 2001). However, it is important to note that even in an environment presenting high mean water temperature, huge water temperature variation and relative fast heating rates (reaching 3.8 °C/hour), the tadpoles' development and survivability seemed to occur without any apparent problem. None of the frogs recorded presented any signal of malformation and no tadpole or froglet were found dead during the study. Hence, we suggest that *O. perpusilla* tadpoles could be thermal tolerant, possibly presenting high upper thermal limits and/or high warming tolerance. In fact, Duarte and colleagues (2012) showed, for a sample of 47 amphibian species, a positive relationship between upper thermal critical limits and maximum temperature of different ponds, suggesting a co-evolutionary process on these traits.

Finally, the deep association between *O. perpusilla* breeding activity and bromeliad water tanks could represent a "trade off" between advantages (protection against pond predators and decrease in competition), and disadvantages (unpredictability of water availability, space limits and temperature control). However, the ecophysiology mechanism behind such lifestyle remains poorly understood. Future studies addressing tadpoles' upper thermal limits and warming tolerance of close related bromeligenous and non-bromeligenous species could be helpful to elucidate how these mechanisms are associated to the reproductive biology of *O. perpusilla* and help to predicts the future of the complex in a global warming context.

References

Alves-Silva, R., Da Silva, H.R. (2009): Life in bromeliads: reproductive behavior and the monophyly of the *Ololygon perpusillus* species group (Anura: Hylidae). Journal of Natural History **43**: 205–217.

Arrighi, J.M., Lencer, E.R., Jukar, A., Park, D., Phillips, P.C., Kaplan, R. (2013): Daily temperature fluctuations unpredictability influence development rate and morphology at a critical early larval stage in frog. BMC Ecology **13**: 18.

Bernal X.E., Guarnizo C., Lüddecke H. (2005): Geographic variation in advertisement call and genetic structure of *Colostethus palmatus* (Anura, Dendrobatidae) from the Colombian Andes. Herpetologica **61**: 395–408.

Duarte, H., Tejedo, M., Katzemberger, M., Marangoni, F., Baldo, D., Beltrán, J.F., Martí, D.A., Ricther-Boix, A., Gonzalez-Voyer, A. (2012) Can amphibians take the heat? Vulnerability to climate warming in subtropical and temperate larval amphibian communities. Global change biology **18**: 412–421.

Edge, C.B., Houlahan, J.E., Jackson, D.A., Fortín, M. (2016): The response of amphibian larvae to environmental change is both consistent and variable. Oikos **125**: 1700–1711.

Forti, L.R., Costa, W.P., Martins, L.B., Nunes-de-Almeida, C.H.L., Toledo, L.F. (2016): Advertisement call and genetic structure conservatism: good news for an endangered Neotropical frog. PeerJ **4**: e2014.

Gosner, K.L. (1960): A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica **16**(2):183–190.

Hutchison, V. H., Dupré R. K., (1992): Thermoregulation. In Environmental Physiology of the amphibians, p. 206–249 Ferder, M.E, Burggren, W.M, Eds., The University of Chicago Press, Chicago.

Kaplan, R.H., Phillips, P.C. (2006): Ecological and developmental context of natural selection: maternal effect and thermally induced plasticity in the frog *Bombina orientalis*. Evolution **60:** 142–156.

Köhler, J., Jansen, M., Rodríguez, A., Kok, P.J.R., Toledo, L.F., Emmrich, M, Glaw, F., Haddad, C. F. B., Rödel, M. O. & Vences, M. 2017. The use of bioacoustics in anuran taxonomy: theory, terminology, methods and recommendations for best practice. Zootaxa **4251**: 1–124.

Navas, C.A., Carvajalino-Fernández, J.M., Saboyá-Acosta, L.P., Rueda-Solano, L.A., Carvajalino-Fernández, M.A. (2013): The body temperature of active amphibians along a tropical elevation gradient: patterns of means and variance and inference from environmental data. Functional ecology **27**: 1145–1154.

Ohmer, M.E., Robertson, J.M., Zamudio, K.R. (2009): Discordance in body size, colour pattern, and advertisement call across genetically distinct populations in a Neotropical anuran (*Dendropsophus ebraccatus),* Biological Journal of the Linnean Society **97**: 298–313.

Oliveira, F.B., Navas, C.A. (2004): Plant selection and seasonal patterns of vocal activity in two populations of bromeligen tree frogs *Scinax perpusillus* (Anura, Hylidae). Journal of Herpetology **38**(3): 331–339.

Peixoto, O.L. (1995): Associação de anuros a bromeliáceas na Mata Atlântica. Revista da Universidade Rural Série Ciência da Vida **17**: 75–83.

Peixoto, M.A., Guimarães, C.S., Lacerda, J.V.A., Leal, F.R., Rocha, P.C., Feio, R.N. (2016): Vocal repertory of *Scinax v-signatus* (Lutz 1968) (Anura, Hylidae) and comments on bioacustical synapomorphies for *Scinax perpusillus* species group. Acta Herpetologica **11**(1): 53–57.

Pimenta, B.V.S., Wachlevski, M., Cruz, C.A.G. (2008): Morphological and acoustical variation, geographic distribution, and conservation status of the spinythumb frog *Crossodactylus bokermanni* (Anura, Hylodidae). Journal of Herpetology **42**: 481–492.

Pombal Jr., J.P., Bastos, R.P. (2003): Vocalizações de *Scinax perpusillus* (A.Lutz & B.Lutz) e *S. arduous* Peixoto (Anura, Hylidae), com comentários taxonômicos. Revista Brasileira de Zoologia, **20**(4): 607–610.

Pröhl, H., Hagemann, S., Karsch, J., Höbel, G. (2007): Geographic variation in male sexual signals in strawberry poison frogs (*Dendrobates pumilio*). Ethology **113**: 825–837.

Sabagh, L.T, Ferreira, G.L, Branco, C.W.C, Rocha, C.F.D, Dias, N.Y.N. (2012): Larval diet in bromeliad pools: a case study of tadpoles of two species in the genus: *Scinax* (Hylidae). Copeia **4**: 683–689.

Schineider, J.A., Teixeira, R.L. (2001): Relacionamento entre anfíbios anuros e bromélias da restinga de Regencia, Linhares, Espírito Santo, Brasil. Iheringia, (Zoología) **91**:41–48.

Simon, M.N., Ribeiro, P.L., Navas, C.A. (2015): Upper thermal tolerance plasticity in tropical amphibian species from contrasting habitats: Implication for warming impact prediction. Journal of thermal biology **48**: 36–44.

Tessarolo, G., Maciel, N.M., Morais, A.R.; Bastos, R.P. (2016): Geographic variation in advertisement calls among populations of *Dendropsophus cruzi* (Anura: Hylidae). Herpetological Journal **26**: 219–22.

Toledo, L.F., Martins, I.A., Bruschi, D.P., Passos, M.A., Alexandre, C., Haddad, C.F.B. (2015): The anuran calling repertoire in the light of social context. Acta Ethologica, **18**(2): 87–99.

Wells, K.D. (2007): Mating system and sexual selection in anurans. In The ecology and behaviour of amphibians, p. 338–402, The University of Chicago Press, Chicago.

Zornik, E., Kelley, D. B. (2011): A neuroendocrine basis for the hierarchical control of frog courtship vocalizations. Frontiers in Neuroendocrinology, **32**: 353–366.

Zornosa-Torres, C., Toledo, L.F. (2019): Courtship behavior and advertisement call geographic variation of *Bokermannohyla luctuosa* (Pombal Jr. and Haddad, 1993) (Hylidae, Cophomantini). South American Journal of Herpetology **14**: 71–79.