Austral ECOLOGY A Journal of ecology in the Southern Hemisphere

Austral Ecology (2021) 46, 683–686

Three is a crowd: Conspecific multi-male spawning in the Spotted Toad *Rhaebo guttatus* (Schneider, 1799)

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Abstract A case of multi-male spawning between two males and one female of *Rhaebo guttatus* is reported. An amplectant pair of *R. guttatus* was observed spawning in an ephemeral puddle located in a flooded area of a stream inside an open ombrophilous forest remnant on 31st January 2019, in the municipality of Cotriguaçu, north-western Mato Grosso state, Brazil. To our surprise, a conspecific male (secondary male) was submerged with its belly up, nostrils out of the water and clasping the female by the ventral region, apparently, trying to fertilise the eggs. For Neotropical anurans, multi-male spawning has been reported to occur in eight phyllomedusid and four leptodactylid species. Therefore, it is the first record of conspecific multi-male spawning for *R. guttatus*. These observations provide new insights into the breeding biology of *R. guttatus*. Further studies, however, are needed to better understand the reproductive features, focusing on genetic analyses of the offspring produced from multi-male spawning of *R. guttatus* to determine whether multiple paternity occurs in this toad species.

Key words: Amazon rainforest, bufonid toad, mating behaviour, multiple paternity, simultaneous polyandry.

The monophyletic genus *Rhaebo* Cope, 1862 (Anura: Bufonidae Gray, 1825) is composed of 13 species of medium- and large-sized toads (Frost 2020). Among these, the Spotted Toad *Rhaebo guttatus* is a large-sized (males: 121.3–146.9 mm, females: up to 174.3 mm), terrestrial and nocturnal species associated with primary forests in the Amazon basin, and it is found on the ground at the margins of rivers and streams or in the leaf litter on the forest floor (Azevedo-Ramos *et al.* 2010; Mueses-Cisneros *et al.* 2012). The species is widely distributed in the Amazon basin, occurring in Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Peru, Surinam and Venezuela (AmphibiaWeb 2019; Frost 2020). Although it is a common species across the Amazon, there is a lack of detailed information on the life history and ecology of this toad (Azevedo-Ramos *et al.* 2010). *Rhaebo guttatus* is an explosive breeder and breeds in ephemeral and permanent puddles and swamp areas inside the forest (Azevedo-Ramos *et al.* 2010). Males call at the edge of these water bodies, and females lay large clutches composed of a double row egg strand (Caldwell & Araújo 2005; Machado & Bernarde 2011; Marinho *et al.* 2019). Males also have low mate selectivity, exhibit active searches for females and can displace other males, leading to errors in the formation of couples, such as multiple and heterospecific amplexi between the sympatric toads *Rhinella marina* and *R. guttatus* (Machado & Bernarde 2011). However, the occurrence of simultaneous polyandry has not been reported for *R. guttatus*. Here, we report an observed case of conspecific multi-male spawning between two males and one female of *R. guttatus*.

The record was documented in a plot of the *Programa de Pesquisas em Biodiversidade* (*PPBio*) within an area of native forest inside the São Nicolau farm (9°48′51.66″S, 58°17′41.63″W), located at the western margin of the Juruena River – in the municipality of Cotriguaçu, north-western Mato Grosso state, Brazilian Central Amazonia (Noronha *et al.* 2015). The native vegetation is characterised by open and dense ombrophilous forest (Veloso *et al.* 1991). The region has been inventoried regularly for more than 10 years and contains at least 64 of the 240 anuran

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Accepted for publication January 2021.

doi:10.1111/aec.13014

species known in the Brazilian Amazon (Ávila-Pires *et al.* 2007; Rodrigues *et al.* 2011; Noronha *et al.* 2015). However, deforestation caused by agricultural expansion and the construction of roads and large hydroelectric power plants are among the main threats to biodiversity in the region (Rodrigues *et al.* 2011).

During an anuran survey of a stream inside a flooded area of an open ombrophilous forest remnant on the 31st January 2019 at 20:57 p.m., an amplectant pair of *R. guttatus* was found spawning in an ephemeral puddle (Fig. 1a). To our surprise, a conspecific secondary male (which appeared smaller than the focal male) was submerged with its belly up, nostrils out of the water and clasping the female by the ventral region, apparently, trying to fertilise the eggs (Fig. 1b). The mating behaviour was observed for 5 min, and the toads did not seem disturbed by our presence. The anuran survey was continued, and after 23 min, we returned to the location. The toads were observed for another 5 min. During the two periods of observations, the toads remained in the same position. The male toads had not released the female by the end of the second observation period. Specimens were not collected.

Multi-male spawning occurs when one or more unpaired males join an amplexed pair to try to fertilise the egg clutch (Zamudio & Chan 2008; Roberts & Byrne 2011). This mating behaviour may be advantageous for females because it can increase fertilisation success by increasing sperm quality and number, as well as the genetic variability of the offspring (Prado *et al.* 2006). For secondary males, simultaneous polyandry may increase the chances of fertilising at least some of the eggs (Roberts *et al.* 1999). Despite the advantages, multi-male spawning has rarely been documented in anurans (Roberts & Byrne 2011). For Neotropical anurans, multi-male spawning has been reported to occur in eight phyllomedusid species: *Agalychnis callidryas*, *Agalychnis dacnicolor* (Pyburn 1970), *Agalychnis saltator* (Roberts 1994), *Phyllomedusa distincta* (Prado *et al.* 2006), *P. iheringii* (Oliveira *et al.* 2014), *Pithecopus azureus* [as *Phyllomedusa azurea*] (Dias *et al.* 2012), *Pithecopus megacephalus* [as *Phyllomedusa megacephala*] (Oliveira *et al.* 2012) and *Pithecopus rohdei* [as *Phyllomedusa rohdei*] (Wogel *et al.* 2005), and four leptodactylid species: *Engystomops pustulatus* (Ron *et al.* 2014), *Leptodactylus bufonius* (Faggioni *et al.* 2011), *L. macrosternum* [as *L. chaquensis*] and *L. podicipinus* (Prado & Haddad 2003).

Although multiple and heterospecific amplexi have been reported for *R. guttatus* (Machado & Bernarde 2011), spawning of heterospecific couples was not observed (P. S. Bernarde, pers. commun., 2020). Therefore, this is the first record of conspecific multi-male spawning for *R. guttatus*. Males in explosive

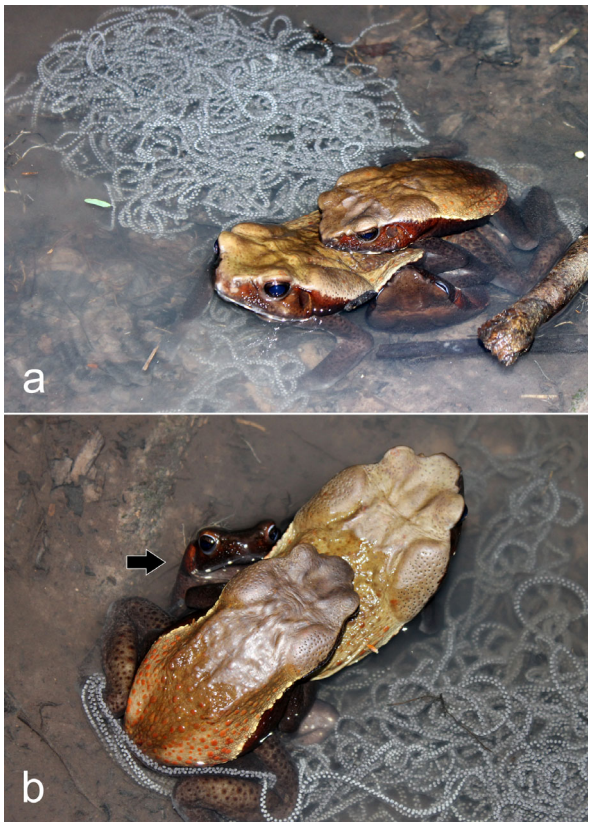

Fig. 1. Multi-male spawning between two males and one female of *Rhaebo guttatus*. (a) An amplectant pair of *R. guttatus* spawning in an ephemeral puddle; (b) Secondary male of *R. guttatus* submerged with its belly up, nostrils out of the water and clasping the female by the ventral region apparently trying to fertilise the eggs (black arrow). Photograph (a) by Lauren V. Ash, and photograph (b) by Fabrício H. Oda.

mating aggregations display physical competition for the possession of females (see example in Machado & Bernarde 2011), where unpaired males struggle to displace males already in amplexus (Wells 2007). The intense competition among males may result in group spawning, during which large males often acquire mates by displacing smaller males (Howard & Young 1998). However, in multi-male spawning, secondary males may adopt alternative mating tactics, such as different amplectant positions (Byrne & Roberts 2004; Roberts & Byrne 2011) to attempt to displace the focal male (Prado & Haddad 2003; Wogel *et al.* 2005). In the common toad *Bufo bufo*, ventral and dorsolateral positions by secondary males have been reported in group spawning events (Sztatecsny *et al.* 2006). In these groups, secondary males can also release sperm simultaneously for egg fertilisation, leading to sperm competition and multiple paternity within a single egg clutch (e.g. D'Orgeix & Turner 1995; Laurila & Seppä 1998; Roberts *et al.*

doi:10.1111/aec.13014

1999). Using genetic analyses, Roberts *et al.* (1999) demonstrated that during group spawning, secondary males of *Crinia georgiana*, amplexed dorsally and ventrally with a single female, had an equal share of paternity with the focal male. Thus, we believe that the ventral position adopted by the secondary male of *R. guttatus* seems to avoid intense direct competition with the focal male, increasing the probability of fertilising the eggs. Considering that explosive breeding and reproductive aggregations are common features in bufonid species (e.g. Machado & Bernarde 2011; Sodré *et al.* 2018), multi-male spawning and multiple paternity may be underestimated in this family. Further studies, therefore, are needed to better understand these reproductive features, focusing on genetic analyses of the offspring from multi-male spawning of *R. guttatus* to determine whether multiple paternity occurs in this toad species.

ACKNOWLEDGEMENTS

The authors would like to thank Roberto Stofel for providing field assistance, the *ONF Brasil* who granted us access to *São Nicolau farm* and for providing logistic support, the *Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq* for providing financial support (process n° 428478/2016-6), and P. S. Bernarde for their valuable help with the information on heterospecific couples and of multiple amplexi between *Rhaebo guttatus* and *Rhinella marina*. Fabrício H. Oda received a postdoctoral fellowship from *Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico/Coordenação de Aperfeiçoamento Pessoal de Nível Superior – CAPES* (process n° 88887.162751/2018-00). Samuel F. dos Anjos and Jonatha E.P. Lima received fellowships from *CAPES* (process n° 88887.199355/2018-00, process n° 1519468). Domingos J. Rodrigues is on a *CNPq* grant of research productivity fellowship (process n° 307650/2019-7). Finally, we would like to thank the *Instituto Chico Mendes de Conservação da Biodiversidade/Sistema de Autorização e Informação em Biodiversidade*, which provided the collecting permit (process n° 30034).

AUTHOR CONTRIBUTIONS

Fabrício Hiroiuki Oda: Conceptualization (lead); Investigation (equal); Writing-original draft (equal); Writing-review & editing (equal). **Samuel Ferreira dos Anjos:** Investigation (equal); Writing-original draft (equal). **Jonatha Edson de Paula Lima:** Investigation (equal); Writing-original draft (equal). **Lauren V. Ash:** Investigation (equal); Writing-original draft (equal); Writing-review & editing (equal). **Karla Magalhães Campião:** Investigation (equal); Writing-original draft (equal); Writing-review & editing (equal). **Domingos de Jesus Rodrigues:** Writing-original draft (equal); Writing-review & editing (equal); Project administration (lead).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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doi:10.1111/aec.13014