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Out in the wind: wiping behaviour in the leaf-frog *Pithecopus ayeaye*, and first evidence of bladder-filling behaviour in the genus

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The colonisation of land by tetrapods marked a critical event in animal evolution, introducing desiccation risk as a pivotal selective pressure that influenced physiological, behavioural and morphological adaptations. Leaf-frogs (Phyllomedusinae) exhibit adaptations to mitigate water loss, but limited observations hinder our understanding of these behaviours' prevalence and consequently their evolutionary history. Here, we present novel observations of wiping and bladder-filling behaviours in the leaf-frog *Pithecopus ayeaye*. Wiping behaviour was recorded in both natural and captive settings, when frogs used their fore and hindlimbs to spread lipidic substances across the skin. Furthermore, we observed bladder-filling behaviour for the first time in this derived Phyllomedusinae genus. Our findings challenge existing paradigms regarding the evolution of these traits and emphasise the need for comprehensive studies across phyllomedusine genera to elucidate their phylogenetic distribution and adaptive significance.

Keywords: arboreal reproduction, Hylidae, monkey frogs, Neotropics, water loss mitigation

such as leaf-frogs (Hylidae: Phyllomedusinae; Faivovich et al., 2010). This subfamily of tree frogs, also called monkey frogs, presently comprises 67 species from eight genera (Duellman et al., 2016; Frost, 2024). A distinct characteristic of leaf-frogs is arboreal reproduction, with eggs deposited in open or folded leaves on bushes and trees pending above water bodies, where exotrophic tadpoles drop to complete their development (Haddad & Prado, 2005; Borges et al., 2018; Bastiani et al., 2024). The prolonged aerial exposure increases desiccation risks, to which phyllomedusines adapted. They may excrete uric acid instead of diluted urea (Shoemaker & McClanahan, 1975) and spread a lipid secretion in their skin using their limbs, which is known as wiping behaviour, and has been empirically linked with water loss mitigation (Blaylock et al., 1976; Lillywhite, 2006). Leaf-frogs have also adapted to prevent embryo desiccation by performing bladder-filling behaviour: the female, alone or while in amplexus, collects water from the water body into her bladder, which is released over the eggs right after oviposition (Pyburn, 1970). However intriguing and deeply associated with their life histories and challenges regarding water loss, these behaviours are described for a very limited number of species and genera (Faivovich et al., 2010).

Most phyllomedusines of the *Pithecopus hypochondrialis* group share an evolutionary history with close association with seasonal areas such as the Neotropical savannas (Magalhães et al., 2024), with a marked dry season. One example is *Pithecopus ayeaye* Lutz, 1966, a leaf-frog that occurs in the Cerrado of Minas Gerais state, south-eastern Brazil, and in transition areas with the Atlantic Forest (Nali et al., 2015; Magalhães et al., 2017). This species has lately attracted conservation attention, including molecular research that uncovered at least three evolutionary significant units across its distribution, and one of them is not effectively protected in Brazil (Magalhães et al., 2017). Moreover,

The colonisation of land by tetrapods is one of the most critical events in animal evolution, in which desiccation risk became a pivotal selective pressure that shaped physiological, behavioural and morphological adaptations (Bray, 1985; Amemiya et al., 2013; Finn et al., 2014). Exploring how tetrapods reduce water loss improves our understanding of their evolutionary history and has important implications for broader ecological and conservation efforts. A pertinent focal group in this field is amphibians, which thrive in both aquatic and terrestrial environments (Haddad & Prado, 2005), especially those with adaptations to reduce water loss,

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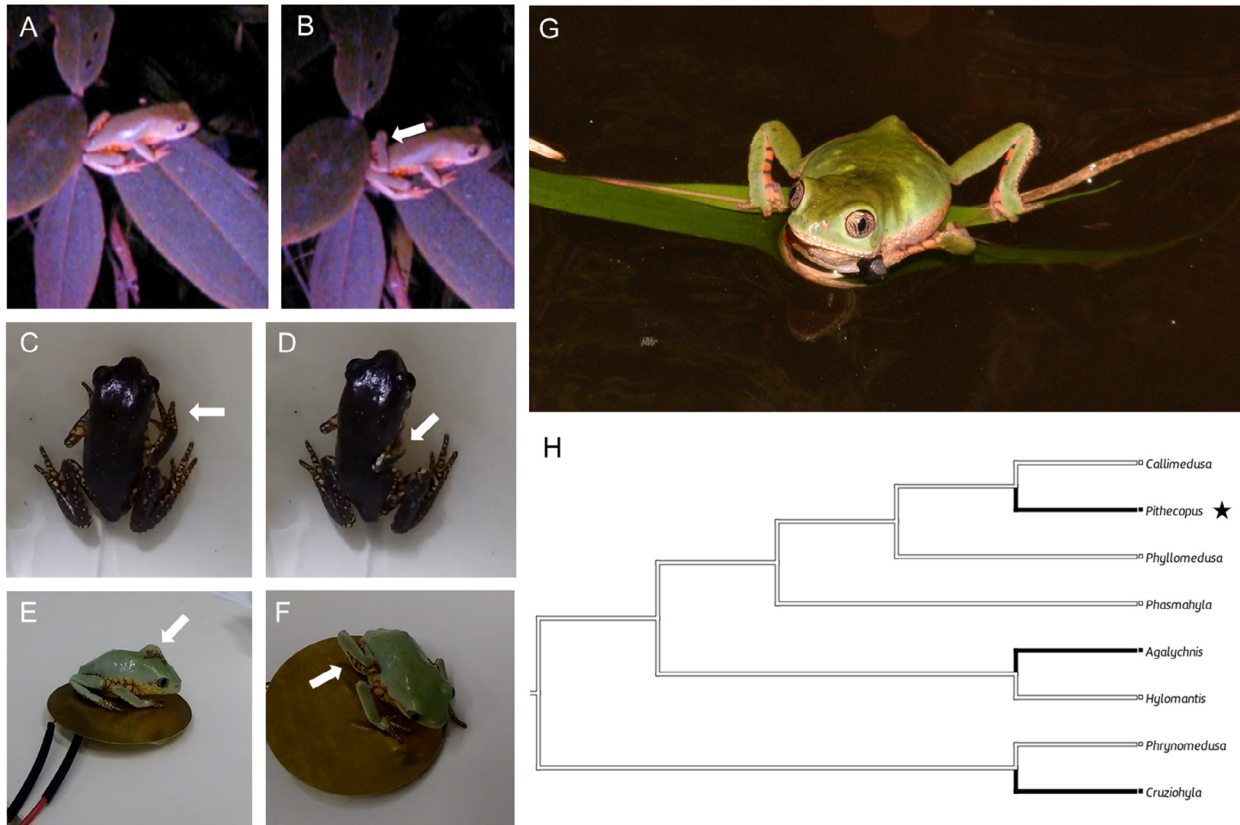


Figure 1. A–F. Wiping behaviour exhibited by the leaf-frog *Pithecopus ayeaye* in south-eastern Brazil, and G–H. bladder-filling behaviour in Phyllomedusinae leaf-frogs. **A, B.** In its natural habitat, a male demonstrates wiping behaviour by using its legs while emitting calls. **C, D.** The behaviour is replicated in captivity, with the frog using its arms. **E.** Noteworthy is the use of the arm to display wiping behaviour. **F.** The frog employs its leg to the side of the body in this behaviour. **G.** A gravid female of *Pithecopus ayeaye* exhibiting bladder-filling behaviour in the field (voucher specimen = CFBH 36031). **H.** Known cases of bladder-filling behaviour (black = presence; white = absence) across genera of the subfamily Phyllomedusinae, as reported by Faivovich et al. (2010), supplemented with a literature review and our new record (star). The phylogenetic framework is based on Duellman et al. (2016). Although parsimony analysis suggests independent evolution in three genera, our findings indicate potential bias due to limited observations of this uncommon behaviour.

present and future niche projections have uncovered that less than 5% of the suitable potential area of the species is protected, and that intraspecific diversity is not effectively preserved (Magalhães et al., 2017). Behavioural research on the species has significantly increased in the past decade (Nali et al., 2015; Oliveira, 2017; Borges et al., 2018), but many details on its natural history remain completely unknown. A thorough natural history study failed to report on either wiping or bladder-filling behaviours (Oliveira, 2017), the latter of which is known only for the phyllomedusine genera *Agalychnis* and *Cruziohyla* (Faivovich et al., 2010), underscoring the rarity of these observations overall. Herein, we describe wiping behaviour for the first time for the species, bladder-filling behaviour for the first time within the genus, and discuss the evolutionary implications of our findings.

Our observations arose from individuals of a single population that reproduce in a temporary stream in the municipality of Sacramento, Minas Gerais state, Brazil (GPS co-ordinates 20° 13'09.9" S; 47° 06'21" W; 880 m altitude). This temporary stream is located within the Cerrado, a Brazilian savannah with a marked dry season

(Borges et al., 2018). Wiping behaviour was recorded in at least three different occasions and conditions. We recorded environmental temperatures with a digital thermometer to the nearest 0.1 °C in the field, and the temperature was kept approximately constant with air conditioning in the lab. On 27 December 2010 at approximately 23:00 h we observed and recorded a video of a male (uncollected) performing wiping behaviour on a leaf in the field (air temperature = 21.7 °C and water temperature = 24.2 °C; Fig. 1A & B). The male executed stereotypical movements solely with its hind limbs in the posterior region, consistent with wiping behaviour and not visual signalling. During this behaviour, the male also vocalised, including likely aggressive vocalisations aimed at male competitors (long notes; Nali et al., 2015). In July 2019 (air temperature ~25 °C) we observed wiping behaviour in an individual ca. 1.5 years old (collected as a tadpole), which metamorphosed within a terrarium at the Department of Animal Morphology and Physiology, UNESP, Jaboticabal, Brazil (GPS co-ordinates 21° 14' S, 48° 17' W; Fig. 1C & D). The animal started wiping behaviour laterally towards its back and belly using the right hind limb, followed by head and belly wiping using both

forelimbs, and finished with belly wiping using the left hind limb. We made a similar observation on 11 July 2019 at 17:00 h (air temperature ~ 25 °C) after we removed one individual from the terrarium and placed it outside. The individual also used its fore- and hindlimbs to spread lipidic substance on the skin, in the head region and across the body (Fig. 1E & F). The videos of wiping behaviour are deposited in FIGSHARE under the link <https://dx.doi.org/10.6084/m9.figshare.25126997>. Maintenance of individuals in captivity occurred to conduct experiments in physiology that were unrelated with the scope of this work, led by KCB. These experiments were authorised by the Ethics Committee on Animal Use of the UNESP, Jaboticabal, Brazil, under protocol # 010260/19. The frogs were housed in a 70-litre plastic enclosure lined with moistened paper towels that were sprayed once or twice daily, which provided hydration. The enclosure featured artificial plants and bromeliads for shelter, as well as ropes for climbing.

We observed bladder-filling behaviour in the evening of 6 November 2013. Reproductive activity of *P. ayeaye* was intense in the stream, with more than ten calling males and the presence of gravid females. A gravid female descended from the vegetation to the stream and placed her ventral region on the water while holding onto a partially submerged grass; she then moved her hands as if she were to push water towards her body, with her legs open (Fig. 1G). Afterwards, she walked up the grass staying approximately 5 cm above the water and then kept moving towards the ground. The female was then collected and paired with a male within a plastic bag. They began and remained in amplexus in the bag for a few hours, but oviposition did not occur. The female was measured with a digital caliper and weighted with a dynamometer, euthanised and fixed following Heyer et al. (1994), and dissected to confirm the presence of mature oocytes. It was then deposited in the Célio F.B. Haddad Amphibian Collection, Rio Claro, São Paulo, Brazil (snout-vent length = 42.7 mm, body mass = 4.9 g, voucher = CFBH 36031).

Wiping behaviour can be associated with secretion of lipids in specialised integument glands and is not exclusive to phyllomedusines (Christian & Parry, 1977; Lillywhite et al., 1997). It is considered however more elaborated in this group (Barbeau & Lillywhite, 2005; Lillywhite, 2006), despite being only known for the genera *Pithecopus* and *Phyllomedusa* (e.g. *Pithecopus hypochondrialis*, *Phyllomedusa sauvagii*, *Phyllomedusa iheringi*, *Phyllomedusa boliviana*), all of which are normally referred to as "waterproof frogs" (Blaylock et al., 1976; Gomez et al., 2006; Faivovich et al., 2010; Rota et al., 2017). In Phyllomedusinae, lipid secretion, the presence of a complex wiping behavior with front and hind limbs, and perch selection have been linked with a very low evaporative water loss (Blaylock et al., 1976). Therefore, its complexity in this subfamily is comprehensible, given that not only mating behaviour, but also oviposition itself, occurs outside the water (Barbeau & Lillywhite, 2005; Haddad & Prado, 2005; Oliveira, 2017; Borges et al., 2018; Bastiani et al., 2024). The studied population

of *P. ayeaye* reproduces in a temporary stream within the Brazilian Cerrado, i.e. it dries out during the dry season (Borges et al., 2018). New evidence suggests that the typical droughts in the Brazilian Cerrado, especially hydrological droughts, are likely to intensify with climate change (Rodrigues et al., 2020). For instance, sampling efforts in January 2021 found no adults or tadpoles in the same stream (GL, KCB and CPAP, personal observation), highlighting the threats faced by individuals of *P. ayeaye* in this seasonal environment. Although morphological evidence suggests that wiping behaviour may be present in other phyllomedusine genera, such as *Agalychnis* (Rota et al., 2017), it remains undocumented. Therefore, the observation and report of this type of behaviour contributes to understanding the links between morphology, physiology and behaviour in this group.

Some frogs may control water absorption through their ventral region in seat patches (Lemanager et al., 2022), a mechanism that could be confounded with bladder-filling behaviour. Seat patches have been primarily associated with imminent desiccation risks in terrestrial frogs or regulating water intake in aquatic frogs (Lemanager et al., 2022), and occur in both males and females. We found a gravid female submerging the ventral region in the water during a breeding event (Fig. 1G), similar to what is described for bladder-filling behaviour (Pyburn, 1970), which was not observed for males, despite many that were active at the moment. This female later entered in amplexus. While we did not observe water release in the eggs due to the collection of the pair, the circumstances of this report strongly indicate bladder-filling behaviour. Bladder-filling behaviour was only known in *Agalychnis* and *Cruzeirohyla*, (Fig. 1H), which led Faivovich et al. (2010) to hypothesise that it had evolved independently in these two instances. Our novel observation of bladder-filling behavior in one of the most derived genera in the subfamily raises an important discussion regarding the evolution of this trait (Faivovich et al., 2010; Fig. 1H). The first possibility at this time, supported by parsimony, is that bladder-filling behaviour has evolved independently three times within Phyllomedusinae (Fig. 1H). However, because (1) bladder-filling is an adaptation against desiccation for embryos that develop outside the water, (2) species from all phyllomedusine genera show this type of oviposition, and (3) we now confirmed its presence in this derived lineage, we argue that bladder-filling behaviour may have originated just once at the common ancestor of Phyllomedusinae. Consequently, it could be present throughout the group's phylogeny, and our interpretation of three independent origins might simply be an artifact due to the limited observations of this rare behavior. This calls for a deeper investigation on the natural history of all genera within Phyllomedusinae.

The documented wiping and bladder-filling behaviours uncover some adaptive strategies of *P. ayeaye*, a species that remained in the IUCN Red List status of Critically Endangered until 2018 (ICMBio, 2018; Marcondes et al., 2020). This species is currently classified as Least Concern, but it has a severely fragmented and declining population (IUCN SSC, 2023). This suggests a need for

further studies, including assessments of genetic diversity, to better evaluate its conservation status (Magalhães et al., 2017). Our observations challenge existing paradigms regarding the evolution of such traits within the broader phylogenetic context of the charismatic Phyllomedusinae (Faivovich et al., 2010). These findings underscore the importance of detailed studies on reproductive behaviour across phyllomedusine genera (e.g. Röhr et al., 2020) and call for a more comprehensive exploration of amphibian adaptations, providing essential knowledge for conservation initiatives and advancing our comprehension of the evolutionary dynamics of these organisms in arboreal environments.

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Author contributions

Lead writing: RCN; supporting writing: MMB, GSL, LZ, KCB, CPAP; overall supervision: CPAP, KCB; observations and recordings in the field: RCN; MMB; observations and recordings in the lab: GSL, LZ, KCB; conceptualisation: RCN, MMB, GSL, LZ, KCB, CPAP.

Data accessibility

The videos of wiping behaviour are deposited in FIGSHARE under the link <https://dx.doi.org/10.6084/m9.figshare.25126997>. The collected female is deposited in the Célio F. B. Haddad Amphibian Collection, Unesp, Rio Claro, Brazil (voucher = CFBH 36031). Any data can be provided upon reasonable request to the corresponding author.

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