

# The role of waterbody structure and water condition in lemur leaf frog *Agalychnis lemur* breeding site selection

## Abstract

When assessing the extent of arboreal anuran habitat loss, pond environments may not be immediately considered. However, any dependence on water for reproduction creates an additional vulnerability. For the lemur leaf frog *Agalychnis lemur*, deforestation and climate change have reduced the availability of permanent pools for breeding in the Atlantic moist forest. As recent land regeneration in Costa Rica has made reintroduction a conservation priority, it is imperative to understand the abiotic determinants of *A. lemur* reproduction. To inform the selection and structure of optimal reintroduction environments, this study investigates the influence of waterbody structure and water condition on *A. lemur* breeding site preference. Thirty waterbodies were visited over 25 days (July - August 2023) in Limón Province, Costa Rica, constituting 125 observation hours. Breeding behaviours (calling, combat, and amplexus) were recorded during nightly spotlight surveys, egg masses were detected during daily systematic searches, and water chemistry analysis was conducted twice. A Generalised Linear Model was used to identify the drivers of breeding behaviour, and a Mann-Whitney U test was conducted to understand egg mass presence. Surface area had the greatest explanatory power over both response variables, with fewer detections as pool size increased. Egg presence was also influenced by pH, with oviposition occurring in near-neutral conditions. No correlation was found between reproduction and waterbody type, depth, nitrite, nitrate, phosphate, turbidity, conductivity, or dissolved oxygen concentrations. Conservationists should therefore focus on constructing waterbodies with surface areas <20m<sup>2</sup>, using tubs as a cheap and rapid means of establishing populations.

## Keywords

*Agalychnis lemur*, breeding preference, Costa Rica, lemur leaf frog, pH, surface area, water condition, water quality

## Introduction

With their complex life histories and niche habitat requirements, amphibians are the most rapidly declining taxonomic group (IUCN SSC Amphibian Specialist Group, 2022). Habitat loss, degradation, and fragmentation affect 93% of threatened species (Re:wild et al., 2023), compounding pressures such as disease and climate change (Pounds et al., 1999; Lips et al., 2006). Biphase anurans reliant on aquatic environments for reproduction face further challenges from hydrological change, and the diminution of naturally occurring waterbodies (Uribe et al., 2023). The Critically Endangered lemur leaf frog *Agalychnis lemur* is one such species, experiencing declines of 80-95% since 1998 (IUCN SSC Amphibian Specialist Group, 2020). Native to Costa Rica, Panama, and its border with Columbia, *A. lemur*'s declining abundance is primarily attributed to habitat loss and disease (Lips et al., 2006; IUCN SSC Amphibian Specialist Group, 2020). However, Costa Rica's commitment to restoring 1,000,000 hectares by 2030 under the Bonn Challenge saw the regeneration of 482,000 hectares between 2011-2020 (Nello et al., 2023). The silviculture and reforestation implemented to

date are thought to benefit amphibians more than any other taxonomic group, and with significant restoration occurring in *A. lemur*'s historic range, reintroduction is now considered a conservation priority (IUCN SSC Amphibian Specialist Group, 2020; Nello et al., 2023). As it is also hypothesised that *A. lemur* may have evolved to coexist with chytridiomycosis, reintroducing pathogen-resistant individuals from captive breeding programmes could also help to strengthen the in-situ population (Rosa, 2023). Consequently, to ensure the successful reestablishment of viable populations, reintroduction sites should be designed to best meet *A. lemur*'s habitat requirements and breeding preferences.

Frogs are thought to have the most diverse breeding behaviours of all terrestrial vertebrates in terms of where, when, and how they reproduce (McDiarmid, 2014). However, as most long-term ecological studies have focused on temperate species, relatively little is known about the life history and breeding ecology of many tropical anurans (Arguedas et al., 2022). From the limited literature available, *A. lemur* is known to be historically associated with undisturbed, sloping, premontane primary forest on Costa Rica's Atlantic versant, at elevations of 440m – 1,600m a.s.l (Duellman, 1970; Savage, 2002; Stuart et al., 2008). Whilst some amphibians are dependent on structural waterbody features for reproduction (Crump, 1991), *A. lemur* appears less sensitive to factors such as surface area, with breeding behaviours recorded at small plastic tubs (IUCN SSC Amphibian Specialist Group, 2020) and pools spanning 150cm - 35m width (Salazar-Zuñiga et al., 2019). Although *A. lemur*'s depth preference is unknown, increased breeding behaviours, egg mass deposition, and larval presence are associated with increased depth for other members of the Hylidae family such as Gunther's Costa Rican treefrog *Isthmohyla pseudopuma* (Crump, 1991), the stripeless treefrog *Hyla meridionalis* (Jakob et al., 2003), and the barking treefrog *Dryophytes gratiosus* (Button et al., 2022). *A. lemur* larvae have been found at waterbodies of 40cm depth in primary forest, and breeding has been observed at 7cm depth in captivity (Jungfer & Weygoldt, 1994); however, reproduction at this reduced depth may be reflective of proximal pool selection based on immediate need, rather than an indication of preference. As breeding behaviours can change under habitat modification scenarios (Liu et al., 2022), our historical understanding of *A. lemur* preference in primary forest may not be reflective of site selection in regenerating secondary forest, particularly with the loss of old-growth features such as phytotelmata which may present breeding opportunities for arboreal anurans (Burrow & Maerz, 2022). As post-agricultural reintroduction sites may now lack naturally occurring permanent pools, artificial and man-made waterbodies may be required to support the establishment and persistence of *A. lemur* populations (Button et al., 2022). Therefore, to help inform conservation strategies, it is important to understand whether *A. lemur* exhibits structural preferences when selecting breeding sites in secondary forest.

Beyond waterbody structure, water quality has also been shown to impact breeding site selection for certain anurans (Hecnar & M'Closkey, 1996; Brodman et al., 2003; Calderon et al., 2019). As oviposition site selection is thought to be a critical determinant of larval health and survival in certain species (Crump, 1991; Sparling, 2010), anurans may employ scent cues or engage sensory structures through dermal exposure to assess the chemical suitability of waterbodies for breeding (Brekke et al., 1991; Smith et al., 2007; Serrano-Rojas & Pašukonis, 2021). Whilst no study to date has examined the effect of water chemistry on *A. lemur*,

pollutants can cause a significant reduction in mass for some Hylidae (Egea-Serrano et al., 2012), with certain species actively avoiding fertiliser-contaminated pools (Takahashi, 2007). Although tolerance is variable by species (Odum & Zippel, 2008), elevated nutrient levels due to herbicides, pesticides, and fertilisers can lead to diminished hatching success, deformities, and lower larval survival rates (Marco et al., 1999; Fuentes et al., 2011). As such, reduced anuran breeding behaviours have been observed with increasing concentrations of nitrite, nitrate, and phosphate (Rouse et al., 1999; Brodman et al., 2003; Calderon et al., 2019). As residual agricultural chemicals can impact a site's water quality for a number of years post-contamination (McTammany, 2004), if elevated nutrient levels are found to influence *A. lemur* breeding site selection, it is essential to consider this when planning reintroductions.

In addition to the potential detrimental impact of inorganic chemicals, agricultural run-off can negatively affect the long-term health of waterways by increasing sediment deposition (Harding et al., 1999). As such, decreased adult and larval abundance have been observed with increased turbidity in some species (Hecnar & M'Closkey, 1996; Calderon et al., 2019), perhaps due to obscured predator visibility or impaired respiratory function (Stuart et al., 2008). In contrast, increased breeding behaviours have been observed with greater electrical conductivity for some anurans, as a certain ionic concentration is required for effective osmoregulation (Duellman & Trueb, 1994; McKibbin et al., 2008; Calderon, 2019). As insufficient regulation of plasma osmolality may increase the likelihood of contracting chytridiomycosis (Voyles et al., 2007), if *A. lemur* displays selectivity with water chemistry when choosing a breeding site, water condition may influence not only the suitability of sites for conservation action, but may also be key to *A. lemur* survival.

To enhance the effectiveness of future conservation efforts, it is imperative to provide practical, targeted strategies for delineating and structuring reintroduction sites (Moor et al., 2022). Although breeding site selection is also likely to be influenced by climatic factors such as temperature, humidity, and rainfall (Arguedas et al., 2022); abiotic factors such as light availability (Onorati & Vignoli, 2017), canopy cover (Sánchez-Ochoa et al., 2020), and perch suitability (Donnelly & Guyer, 1994); and biotic factors such as competition for breeding sites (Crump, 1991), and predator presence (Donnelly & Guyer, 1994), controlling these factors in reintroduction settings could be challenging. As habitat quality may be one of the more manageable factors in amphibian species recovery (Semlitsch, 2002), the aim of this study is to understand whether waterbody structure and water condition are determinants of *A. lemur* breeding site selection. As Dias et al. (2014) suggest that abiotic preferences may vary throughout the reproductive process, a secondary aim is to ascertain whether *A. lemur* oviposition and breeding site requirements differ. These aims serve to inform the selection and configuration of reintroduction sites, and to guide project timings based on the stage of habitat recovery.

## **Study site**

Research was conducted with an established population of *A. lemur* on a privately-owned reserve in Limón Province, Costa Rica. Situated on the Atlantic versant, the site follows the slope of the Turrialba Volcano

and spans elevations of 650m - 1,030m a.s.l. As a former plantation and cattle ranch taken out of commission in the early 2000's, the site now constitutes secondary forest with a series of modified, man-made, and artificial waterbodies. Earth- and concrete-based ponds have been constructed within the site's clay soil, and plastic and Ricalit (a cement and asbestos substrate) tubs have been positioned throughout the forest. Mean rainfall during the study period (11 July – 7 August 2023) was 11mm per day ( $\pm$  16mm), with a total of 331mm over 29 collection days. Although rainfall in this region dips in June and August (Kubicki, 2008), July would normally see significant rainfall reaching ~430mm (Instituto Meteorológico Nacional de Costa Rica, 2023). However, as this study was conducted at the end of a three-year La Niña event, warmer, drier conditions were prevalent across the Atlantic versant. Site temperatures therefore averaged 30° in the daytime, and nighttime temperatures averaged 24° with a mean humidity of 88%.

## **Methods**

### **Study variables**

Throughout the wet season, this nocturnal, arboreal frog descends from the canopy to breed, with males advertising their presence in short, single calls (Savage, 2002). As adult males are more likely to be observed than females because of this (Emmett et al., 2020), calling was taken as evidence of breeding behaviour as well as territorial displays, and pairs in amplexus (Abrunhosa & Wogel, 2004; Vilela et al., 2015). As it was not possible to mark individual frogs, as a proxy for breeding site preference, the frequency of individuals engaged in breeding behaviours was recorded as the core response variable indicating the number of times a waterbody was chosen for reproduction. As a secondary response variable, the presence of egg masses was also recorded. Structural predictor variables included surface area (m<sup>2</sup>), depth (m) and type (earth- and concrete-based pools, plastic and Ricalit tubs, lakes (i.e., waterbodies with a width >20m), and streams). Water condition metrics were represented by nitrite (mg/l NO<sub>2</sub><sup>-</sup>), nitrate (mg/l NO<sub>3</sub><sup>-</sup>), and phosphate (mg/l PO<sub>4</sub><sup>-</sup>) concentrations, turbidity (FTU), pH, dissolved oxygen (mg/l) and electrical conductivity (μS/cm).

### **Waterbody selection and structural data collection**

On arrival at the research site, all waterbodies were mapped on a GPS device. A total of thirty waterbodies were then selected for monitoring to allow for sufficient variability in the predictor variables, and to achieve a more robust sample (Jaeger, 2014). As only three Ricalit tubs were present, all were included within the study sample. All other waterbodies were categorised by type and allocated a number before a stratified random sample was chosen. Leveraging the knowledge that no researcher had ever detected *A. lemur* at the site's lakes or streams, three waterbodies were chosen for each using a random number generator. Seven earth-based pools were then selected, as well as seven concrete-based pools and seven plastic tubs, all within an elevational range of 744m – 839m a.s.l. The surface area (m<sup>2</sup>) and depth (m) of each waterbody were then measured, with maximum depth determined as close to the waterbody's centre as was possible to achieve.

### **Water condition data collection**

Aligning with similar studies, chemical readings were taken at intervals to generate mean values per parameter, per waterbody (Hecnar & M'Closkey, 1996; Brodman et al., 2003; Calderon et al., 2019). Samples were taken over two nights near the start of the study, and repeated again at the end. Water was collected in plastic bottles between 2000h – 2200h and analysed within five hours of collection. Where possible, samples were taken from the centre of the waterbody at a depth of 15cm (Hecnar & M'Closkey, 1996). Nitrite, nitrate, phosphate, and turbidity were analysed with a water quality photometer (Palintest Photometer 7100, Tyne and Wear, United Kingdom), and handheld meters were used to measure dissolved oxygen (Ohaus Starter Series ST-DO-11, New Jersey, USA), pH (Ohaus Starter Series ST320, New Jersey, USA), and conductivity (Hanna HI98192, Rhode Island, USA).

### **Observational data collection**

A pilot study was conducted prior to data collection, to determine the likelihood of increased behavioural detection with longer observational periods. Spotlight searches were conducted at six sites for 20 minutes each, establishing that after five minutes of observation, no further breeding behaviours were likely to be detected. All 30 sites could therefore be surveyed upon each observation night, achieving the maximum number of replicates over the 75 total observation hours, and delivering greater statistical confidence. Breeding behaviour surveys were then conducted over 25 nights during Costa Rica's wet season (11 July – 7 August 2023), with a standardised start time of 1900h (Emmett et al., 2020). The order of waterbody visitation rotated each night to reduce temporal effects, and to avoid systematic sampling bias. Spotlight surveys were conducted alongside call monitoring at each pool for five minutes, but only those individuals that were visually verified were recorded. To avoid double counting, searches were conducted systematically from left to right. Observations within a 5m radius of the waterbody were recorded (Vilela et al., 2015), and stream surveys were limited to a length of 10m. In addition, systematic egg mass searches were conducted each morning, inspecting substrates directly overhanging waterbodies and recording new egg mass presence. Time spent at each waterbody varied depending on the volume of vegetation, but all searches were completed within two hours constituting a total of 50 observation hours.

### **Data analysis methodology**

Analysis was conducted using the statistical software R (version 4.3.1, The R Foundation for Statistical Computing, 2023). Before breeding behaviour analysis commenced, all continuous predictor variables were scaled to allow for their direct comparison and to ensure those with larger values did not disproportionately influence the model. The Akaike Information Criterion corrected for small samples (AICc) was used for model selection (Burnham & Anderson, 2002), determining a Negative Binomial Generalised Linear Model (GLM) with a log link function to be the best fit. As potential multicollinearity was observed in a correlation matrix between conductivity, pH and phosphate, a variance inflation factor (VIF) analysis was performed using the 'car' package (Fox & Weisberg, 2019). To avoid conflating variables in GLM analysis, confidence intervals were also applied (Marcoulides & Raykov, 2019) with a tolerance level of five selected (James et al., 2021). The upper confidence interval for conductivity came in over that threshold at 5.7 (VIF: 3.7 (1.4, 5.7)) indicating

potential near multicollinearity with pH. As the  $r^2$  value was unaffected by its removal, conductivity was omitted from the global starting model without compromising model fit. A set of 20 biologically relevant candidate models were then generated (including conductivity, but never coupled with pH) and run against the global model using the 'mass' package (Venables & Ripley, 2002). The AICc and  $\Delta$ AICc were used to rank-order and select the most efficient final model (Cogălniceanu et al., 2012; Gonzalez Baffa-Trasci et al., 2020). Lastly, Spearman's rank correlation coefficient was performed on the sole significant predictor variable to determine its strength of effect. For the egg mass analysis, a non-parametric test of difference was performed comparing sites where breeding behaviours were observed, categorised by the detection or non-detection of egg masses (Stumpel & van der Voet, 1998). The contribution of each predictor variable was individually assessed using a Mann-Whitney U test, and a Cohen's d analysis was conducted to understand the magnitude of effect.

## Results

A total of 477 individuals were observed engaging in breeding behaviours at 50% ( $n=15$ ) of sampled waterbodies, with surface areas  $<17\text{m}^2$  ( $x = 4.4\text{m}^2$ ). No behaviours were detected at lakes or streams (Supplementary Fig. 1). Almost all recorded behaviours were calling events, with amplexus observed twice (once at a plastic tub, and once at an earth-based pool), and combat observed only once (at a concrete pool). Behaviours were always detected within 2m ( $x = 0.3\text{m}$ ) of waterbodies, and up to 3m ( $x = 0.7\text{m}$ ) above water. Where behaviours were observed, the waterbody with the lowest attendance was a plastic tub with a frequency of eight detections. However, plastic tubs had the highest overall occupancy rate with breeding behaviours observed at six out of seven (86%) tubs (Supplementary Fig. 2). The site with the most attendance was a concrete pool, which was occupied all 25 survey nights with a frequency of 93 behaviours. Water chemistry levels were relatively low across the study site. Ricalit and plastic tubs had some of the highest mean readings of all waterbody types (Supplementary Table 1); however, a VIF analysis showed no correlation between waterbody type and water condition, and breeding behaviours were observed across the full spectrum of conditions (Supplementary Table 2).

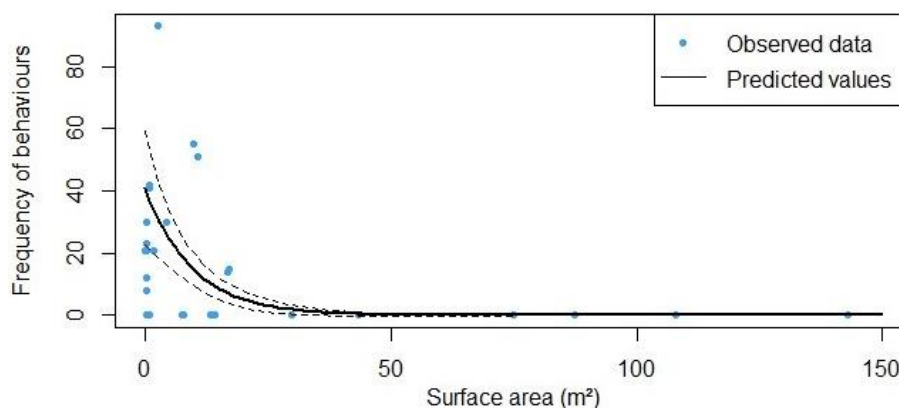


FIG. 1: Predicted influence of surface area on breeding behaviour frequency, extrapolated from the final model.

GLM analysis concluded that surface area was the only significant predictor of breeding behaviours ( $r^2 = 0.5$ ,  $F_{2,27} = 1872$ ,  $p = <0.01$ ). With increasing surface area, the frequency of observed behaviours decreased ( $LRT_{1,27} = 25.11$ ,  $p = <0.01$ ; Fig. 1). McFadden's pseudo- $r^2$  showed that the model explained 50% of variance in the response variable, and Spearman's rank correlation coefficient revealed a moderate-to-high magnitude of effect ( $r = -0.54$ ,  $p = <0.01$ ).

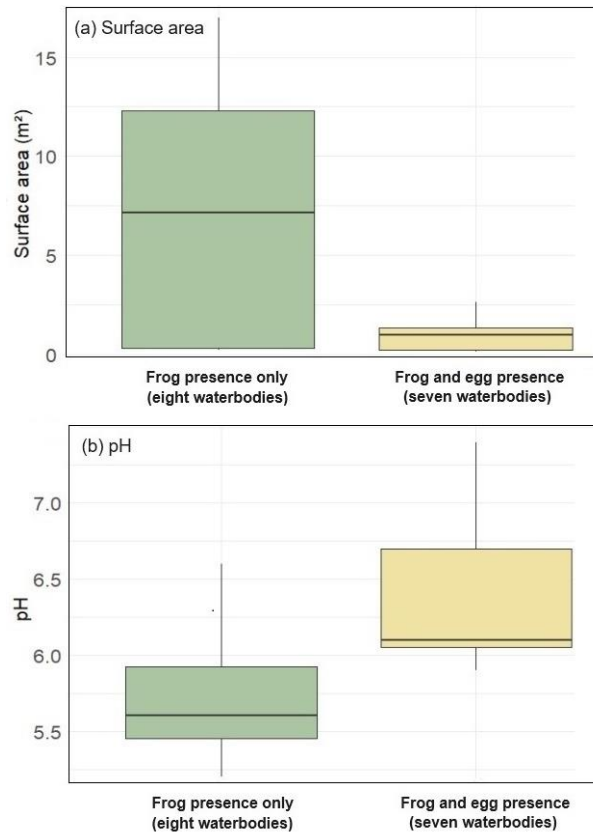


FIG. 2 Box plots demonstrating the influence of surface area (a) and pH (b) on egg mass deposition ( $n=14$  egg masses) at breeding sites.

A total of 14 egg masses were recorded at 23% ( $n=7$ ) of the 30 waterbodies, with surface areas  $<2.6\text{m}^2$  ( $x = 1\text{m}^2$ ). Seven masses (50%) were detected at plastic tubs, five (36%) at Ricalit tubs, and two (14%) at concrete-based pools. Oviposition ranged from heights of 0.02m – 0.5m above water ( $x = 0.2\text{m}$ ) with masses attached to the sides of tubs, shoe laces hung over waterbodies with low vegetation density, stems, branches, and abaxial and adaxial leaf surfaces. Categorising breeding sites by egg mass detection and non-detection, a significant difference with a large magnitude of effect was found between the two groups with surface area ( $z = 2.05$ ,  $p = 0.04$ ,  $d = 1.27$ ) and pH ( $z = -2.1$ ,  $p = 0.03$ ,  $d = 1.25$ ) (Fig. 2). The median surface area of pools not chosen for oviposition was  $7.1\text{m}^2$ , versus much a lower median of  $1\text{m}^2$  where eggs were detected. The median pH of pools not chosen for oviposition was pH 5.6, versus a slightly higher median of pH 6.1 where eggs were detected.

## Discussion

### Study summary

The objective of this study was to investigate whether waterbody structure and water condition influence *A. lemur* breeding site selection. Surface area emerged as the sole significant structural factor, showing negative correlation with increasing waterbody size. Notably, the sites selected for oviposition were significantly smaller than those chosen by calling males, highlighting the importance of variable pool size for *A. lemur* reproduction. Of the chemical conditions tested, pH was the sole significant parameter with a higher likelihood of egg mass deposition in near-neutral conditions. These differing breeding and oviposition requirements demonstrate the need to facilitate all stages of *A. lemur* reproduction on reintroduction sites.

### Waterbody structure and breeding site selection

Surface area has often been cited as one of the strongest determinants of anuran presence at waterbodies (Burne & Griffin, 2005; Werner et al., 2007; Semlitsch et al., 2015). Some species select larger pools for their increased breeding and larval feeding opportunities, or to reduce the risk of egg desiccation (Gonçalves et al., 2015; Gonzalez Baffa-Trasci et al., 2020). Others, such as the gray treefrog *D. versicolor*, Cope's gray treefrog *D. chrysoscelis*, and the boreal chorus frog *P. maculata*, prefer smaller waterbodies for the avoidance of predators (Shulze et al., 2013). As *A. lemur* breeding behaviours have previously been observed at both small plastic tubs (IUCN SSC Amphibian Specialist Group, 2020) and at pools spanning 150cm - 35m width (Salazar-Zuñiga et al., 2019), surface area sensitivity was not anticipated with this species. The non-detection of *A. lemur* breeding behaviours at waterbodies >17m<sup>2</sup> in the present study was therefore unexpected. However, it should be noted that the one large waterbody in Salazar-Zuñiga et al.'s study was situated in a mature, old-growth forest where vegetation levels and canopy cover were likely to be dense, and light availability was likely to be low. Consequently, *A. lemur*'s avoidance of large waterbodies in the present study may be explained by the higher exposure risk to both adverse weather and predation where vegetation may still be regenerating. In addition to surface area, depth has also been cited as a structural determinant of presence in other anurans (Sánchez-Ochoa et al., 2020; Knapp et al., 2003). Where *A. lemur* metamorphosis occurs over a prolonged period of up to 150 days (Skelton, 2012), a preference for deeper waterbodies was anticipated for the avoidance of pond drying. However, as this structural factor was insignificant in the present study, when preparing secondary forests for reintroduction, conservationists should focus on establishing waterbodies with surface areas <20m<sup>2</sup>.

Whilst the goal of this study is to offer practical guidance for waterbody construction on *A. lemur* reintroduction sites, the complex and uneven topography of this region may present challenges in establishing permanent pools (Kubicki, 2008). In such areas, the utilisation of plastic tubs is a pragmatic solution, having been successfully adopted by other anurans (Resetarits & Wilbur, 1991; Fegraus & Marsh, 2000) and by *A. lemur* in the present study. The confirmation that tubs effectively support reproduction demonstrates *A. lemur*'s adaptability to artificial waterbodies and equips conservationists with a quick, cost-effective, and efficient supplementary approach to reestablishing populations. In addition, given *A. lemur*'s vulnerability to fragmentation (Salazar-Zuñiga et al., 2019), tubs can be used to create habitat corridors, acting as stepping stones to enhance site connectivity. This not only facilitates dispersion but expands breeding opportunities,



promotes genetic diversity, and accelerates the process of recolonisation (Semlitsch, 2002; Burne & Griffin, 2005; Gonçalves et al., 2015).

### **Water condition and breeding site selection**

Beyond waterbody structure, breeding site selection is also influenced by a waterbody's hydroperiod and its water condition (Borges Júnior & Rocha, 2013). Whilst water condition has proven to be a significant predictor of calling in various frog species (Hecnar & M'Closkey, 1996; Brodman et al., 2003; Calderon et al., 2019), its impact is variable and as such, no such influence was observed in this study. This lack of significance may indicate *A. lemur*'s inability to sensorially determine water suitability for breeding, rather than implying its lack of importance. Alternatively, it could be hypothesised that *A. lemur* demonstrates resilience to extreme nutrient elevations, as some Hylidae species exhibit high tolerance to elevated nitrite and nitrate levels. For instance, *H. meridionalis* has shown only moderate effects to a nitrite concentration of 20 mg/l (Shinn et al., 2008), well-surpassing the recommended threshold of 1 mg/l for amphibians (Whitaker, 2001). Similarly, the Pacific treefrog *Pseudacris regilla* has exhibited high tolerance to a nitrate concentration of 25 mg/l in laboratory tests (Marco et al., 1999). However, the lack of significance in the present study may also be attributed to the lower comparative concentrations on this site, which generally fell within naturally occurring ranges (Supplementary Table 3). With the site being free from agricultural use for approximately 20 years, any residual chemicals appear to have been recycled. Consequently, conclusions about *A. lemur* breeding preference in environments with elevated nutrient levels cannot be drawn.

Similarly, limnological parameters such as turbidity, conductivity, and dissolved oxygen yielded comparatively low readings across the site. It was anticipated that *A. lemur* may exhibit a preference for lower turbidity, given its negative correlation with larval and adult abundance and its deleterious impact on larval development in other studies (Hecnar & M'Closkey, 1996; Wood & Richardson, 2009; Calderon et al., 2019). However, contrary to expectation, this preference was not observed. In addition, where conductivity and dissolved oxygen levels were considered to be predictors of breeding site selection, neither emerged as significant. Although dissolved oxygen should ideally remain >4 mg/l for amphibians (Odum & Zippel, 2008), certain species can tolerate lower concentrations provided levels stay above a critical reading of 2 mg/l (Wassersug & Seibert, 1975). For instance, the map treefrog *Boana geographica* and the Demerara Falls treefrog *B. cinerascens* have thrived in environments with highly depleted dissolved oxygen levels ( $x = 1.75$  mg/l – 2.48 mg/l) (Gascon, 1991). With the uniformly low readings observed on this site; likely attributable to an inhibited oxygen absorption potential resulting from a prolonged La Niña event; it is uncertain whether dissolved oxygen is truly an insignificant factor, or whether preference would be exhibited with greater variability. As water chemistry is intricately linked to wider environmental and climactic conditions, given the spatial and temporal restrictions of this study, longer-term research employing constant passive water sampling is recommended across all known *A. lemur* populations to further assess the relationship between water chemistry and breeding site selection. In conjunction, structured laboratory testing is recommended to elucidate *A. lemur*'s tolerance levels, and identify any deleterious developmental impacts arising from severely depleted or elevated conditions.

## Waterbody structure, water condition, and oviposition site selection

With gender-specific breeding preferences observed in various species (Resetarits & Wilbur, 1991; Silva & Giaretta, 2008), the secondary aim of this study was to discern whether *A. lemur* exhibits divergent site requirements for breeding and oviposition. Results indicate that variations exist in surface area and pH preference across the reproductive process. Whilst males favoured waterbodies with slightly larger surface areas of up to 17m<sup>2</sup> for calling; possibly for improved sound transmission or to meet space requirements for territorial purposes; the notable reduction in waterbody size for oviposition may again be reflective of the heightened risk of predation in regenerating secondary forests resulting from lower densities of vegetation and canopy cover. Bolstering this theory, for *A. lemur* congeners *A. moreletii* and *A. callidryas*, a positive correlation has been observed with canopy cover and clutch abundance (Sánchez-Ochoa et al., 2020). The observed preference for *A. lemur* oviposition at sites with near-neutral pH aligns with expectations based on the behaviours of other anurans (Cummins, 1989; Tavares-Junior et al., 2020), and the diminished reproductive success induced in some Hylidae species by a lower pH (Warner et al., 1991). These differences demonstrate the importance of accommodating all stages of reproduction when designing reintroduction sites. Management plans should therefore incorporate the construction of waterbodies with surface areas <2m<sup>2</sup>, tubs should be included to provide extra opportunities for oviposition, and where sites with exceptionally low pH may be otherwise suitable for reintroduction, proactive strategies can be implemented to reverse acidification such as the treatment of targeted waterbodies with calcium (Sparling, 2010).

## Conclusion

When preparing sites for *A. lemur* reintroduction, optimising waterbodies for reproduction is crucial for conservation success. This study reveals important insights into *A. lemur*'s preference for smaller pools in secondary forest, with a higher likelihood of egg mass deposition at near-neutral pH. As such, it is recommended that waterbodies of graduated size are constructed, encompassing surface areas of up to 20m<sup>2</sup>. Tubs should also be integrated across the site to enhance ecological connectivity, to fortify oviposition site choice, and to facilitate recolonisation. For sites with pronounced acidity, targeted water treatment can create more conducive pH conditions for oviposition. Further field research is recommended, employing passive water sampling to understand the influence of water condition on reproductive behaviours. This should be supported with laboratory research to understand water chemistry tolerances. These recommendations offer conservationists clear, practical, and actionable strategies for *A. lemur* reintroduction, supporting the restoration of viable in-situ populations.

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## Supplementary material

TABLE 1 Waterbody structure and conditions expressed as mean values, standard deviation, and range (min - max) per waterbody type

	All sites (n=30)	Earth pool (n=7)	Concrete pool (n=7)
Surface area (m <sup>2</sup> )	83.3 ± 191.8 (0.1 - 750)	27.6 ± 24.6 (7.6 - 75)	8.8 ± 6.1 (1.7 - 17)
Depth (m)	0.1 ± 0.3 (0.1 - 1.3)	0.7 ± 0.3 (0.3 - 1.3)	0.4 ± 0.1 (0.2 - 0.5)
Nitrite (mg/l NO <sub>2</sub> <sup>-</sup> )	0.013 ± 0.011 (0 - 0.046)	0.014 ± 0.011 (0.002 - 0.036)	0.012 ± 0.007 (0 - 0.021)
Nitrate (mg/l NO <sub>3</sub> <sup>-</sup> )	1.65 ± 2.66 (0.09 - 13.2)	1.5 ± 2.36 (0.09 - 6.75)	0.63 ± 0.29 (0.19 - 0.92)
Phosphate (mg/l PO <sub>4</sub> <sup>-</sup> )	0.48 ± 0.51 (0.07 - 2.25)	0.38 ± 0.5 (0.08 - 1.38)	0.27 ± 0.2 (0.09 - 0.56)
Turbidity (FTU)	29 ± 8 (16 - 48)	25 ± 7 (16 - 34)	26 ± 5 (18 - 31)
pH	6.2 ± 0.7 (5.2 - 8.4)	5.6 ± 0.2 (5.3 - 5.8)	6.7 ± 0.8 (6 - 8.4)
Dissolved oxygen (mg/l)	2.34 ± 0.37 (0.94 - 2.97)	2.36 ± 0.09 (2.23 - 2.52)	2.34 ± 0.4 (1.6 - 2.81)
Conductivity (µS/cm)	55 ± 60 (18 - 269)	27 ± 8 (18 - 36)	55 ± 26 (22 - 90)

	Ricalit (n=3)	Plastic (n=7)	Lake (n=3)	Stream (n=3)
Surface area (m <sup>2</sup> )	0.95 ± 0	0.2 ± 0.1 (0.1 - 0.3)	633.3 ± 104.1 (550 - 750)	122.8 ± 28.1 (87.5 - 143)
Depth (m)	0.65 ± 0.4 (0.4 - 1.1)	0.4 ± 0.2 (0.1 - 0.7)	1 ± 0.3 (0.6 - 1.2)	0.5 ± 0.3 (0.2 - 0.7)
Nitrite (mg/l NO <sub>2</sub> <sup>-</sup> )	0.022 ± 0.009 (0.012 - 0.03)	0.013 ± 0.016 (0 - 0.046)	0.01 ± 0.008 (0.002 - 0.018)	0.010 ± 0.003 (0.007 - 0.013)
Nitrate (mg/l NO <sub>3</sub> <sup>-</sup> )	2.49 ± 2.81 (0.82 - 5.74)	3.32 ± 4.48 (0.5 - 13.2)	0.56 ± 0.28 (0.4 - 0.89)	0.75 ± 0.09 (0.67 - 0.85)
Phosphate (mg/l PO <sub>4</sub> <sup>-</sup> )	1.07 ± 1.07 (0.17 - 2.25)	0.78 ± 0.44 (0.16 - 1.24)	0.18 ± 0.18 (0.07 - 0.89)	0.2 ± 0.11 (0.07 - 0.28)
Turbidity (FTU)	22 ± 9 (16 - 32)	37 ± 8 (28 - 48)	35 ± 4 (32 - 39)	29 ± 6 (26 - 36)
pH	7.2 ± 0.2 (7.1 - 7.4)	5.9 ± 0.5 (5.2 - 6.7)	6.3 ± 0.2 (6.2 - 6.5)	6.1 ± 0.3 (5.8 - 6.3)
Dissolved oxygen (mg/l)	2.42 ± 0.1 (2.34 - 2.49)	2.23 ± 0.21 (1.98 - 2.53)	2.74 ± 0.2 (2.61 - 2.97)	2 ± 0.92 (0.94 - 2.55)
Conductivity (µS/cm)	215 ± 58 (154 - 269)	38 ± 17 (23 - 66)	27 ± 10 (18 - 38)	30 ± 9 (22 - 39)

TABLE 2 The range of structural and water condition measures across all waterbodies, waterbodies where breeding behaviours were detected, and waterbodies where egg mass presence was detected.

	Total range across all waterbodies	Range of breeding behaviour detection	Range of egg mass presence
Surface area (m <sup>2</sup> )	0.11 – 750	0.11 – 17.01	0.11 – 2.63
Depth (m)	0.05 – 1.34	0.05 – 1.1	0.17 – 1.1
Nitrite (mg/l NO <sub>2</sub> <sup>-</sup> )	0 - 0.046	0 – 0.025	0.002 – 0.025
Nitrate (mg/l NO <sub>3</sub> <sup>-</sup> )	0.09 – 13.2	0.09 – 13.2	0.48 – 5.74
Phosphate (mg/l PO <sub>4</sub> <sup>-</sup> )	0.07 – 2.25	0.08 – 2.25	0.14 – 2.25
Turbidity (FTU)	16 – 48	16 – 48	18 - 48
pH	5.2 – 8.4	5.2 – 7.4	5.9 – 7.4
Dissolved oxygen (mg/l)	0.94 – 2.97	1.98 – 2.67	1.98 – 2.53
Conductivity (µS/cm)	18 – 269	18 – 269	22 - 269

TABLE 3 Mean water condition values from the present study compared with those of others analysing hydrid abundance, richness, and breeding behaviours, presented alongside the critical recommended limits for amphibians.

	Present study	Calderon et al.	Hecnar et al.	Brodman et al.	Critical limits
Nitrite (mg/l)	0 – 0.05	N/A	N/A	N/A	Remain below 1 mg/l (Whitaker, 2001)
Nitrate (mg/l)	0.09 – 13.2	0.62 – 2.33	0.1 – 77.4	0.41 ± 0.48	Remain below 50 mg/l (Whitaker, 2001)
Phosphate (mg/l)	0.07 – 2.25	0.01 – 0.73	0.1 – 4	0.56 ± 0.98	Remain below 10 mg/l (Odum & Zippel, 2008)
Turbidity (FTU)	16 – 48	N/A (Reading NTU / JTU)	N/A	55.5 ± 29.6	
pH	5.2 – 8.4	7.4 – 8.7	7.2 – 10.2	6.8 ± 0.3	pH 6.5 – 8.5 (NB: if species specific requirements are unknown, aim for pH 7) (Whitaker, 2001)
Dissolved oxygen (mg/l)	0.94 – 2.97	7.2 – 9.4	0.5 – 20	9.5 ± 1.0	Remain above 4 mg/l (ASTM 1988, cited in Sparling 2010)
Conductivity (µS/cm)	18 – 269	155 – 781	124 – 3100	N/A	Remain between 50 – 500 (USEPA 2008, cited in Sparling 2010)

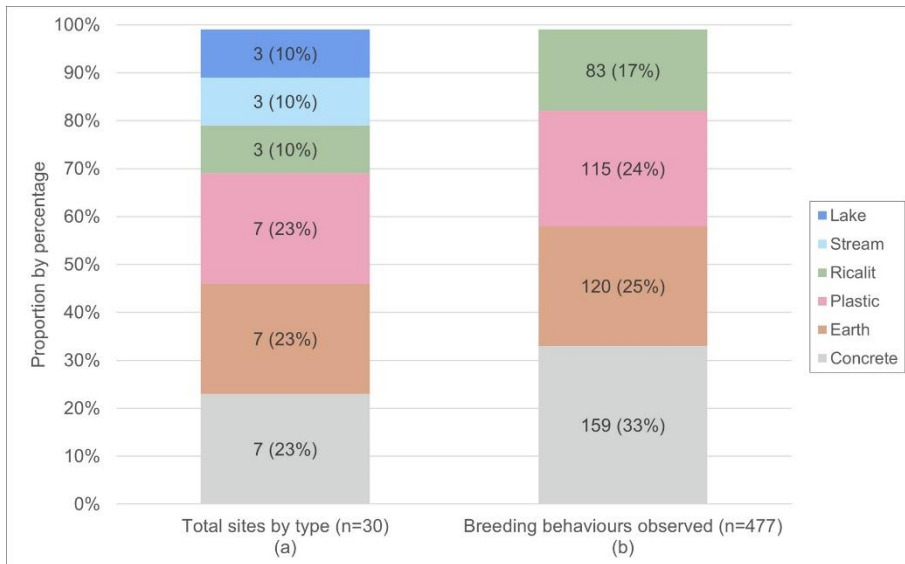


FIG. 1: Proportion of waterbodies by type (a) (n=30 waterbodies), versus proportion of breeding behaviours by type (b) (n=477 behaviours)

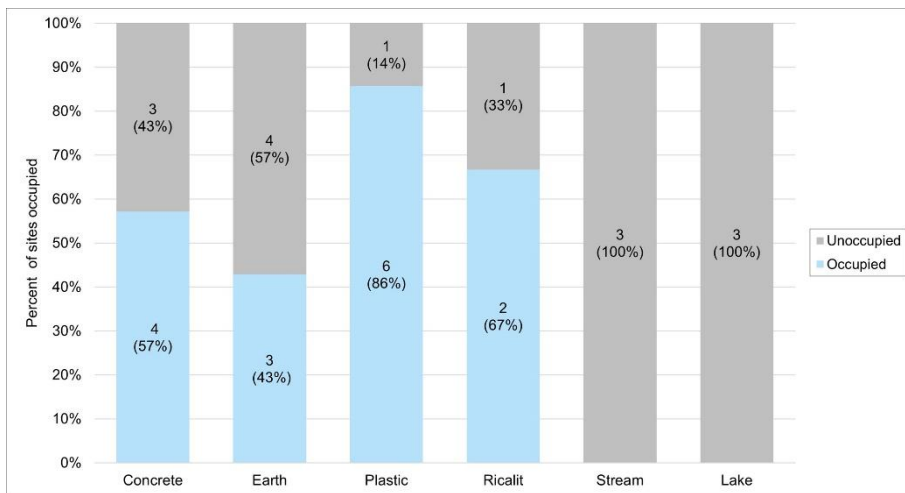


FIG. 2: Proportion of waterbodies occupied by frogs engaged in breeding behaviours, categorised by type (n=30 waterbodies)