Received: 10 August 2021 | Revised: 4 February 2022 | Accepted: 22 February 2022

DOI: 10.1002/zoo.21688

RESEARCH ARTICLE

ZOOBIOLOGY WILEY

Developmental life stages of the Pickersgill's reed frog (*Hyperolius pickersgilli*) in an ex-situ environment at Johannesburg Zoo's captive breeding facility, South Africa

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Funding information

National Research Foundation (ZA), Grant/Award Number: 98404; University of KwaZulu-Natal (ZA); Ezemvelo KZN Wildlife (ZA); Johannesburg City Parks and Zoo (ZA)

Abstract

Globally, the threats of habitat loss and disease on amphibian survival have necessitated the creation of ex-situ insurance populations as a conservation tool. We initiated a captive breeding project to create an insurance population for the endangered Pickersgill's reed frog (*Hyperolius pickersgilli* Raw, 1982) at the Johannesburg Zoo from parents collected from KwaZulu-Natal Province, South Africa, in 2017. We found that this species has seven developmental life stages, each with unique management requirements. The quiescent tadpoles hatched 6–8 days after the eggs were laid and remained at this stage for 2 days. The next stage, the developing tadpoles, showed no form of cannibalism or carrion feeding. The external appearance of the first leg (the right hind) occurred 5–6 weeks after the tadpoles hatched, and the metamorph stage was reached after 7–8 weeks. The metamorph stage lasted 3–5 days, after which tail resorption was complete and the froglet stage reached. Froglets could not be sexed externally, although body color changed based on the amount of light present at the resting place. Sub-adults were 6 months and older with adult coloration and sex differentiation visible even with color change. Adults were older than 18 months and fully developed and sexually mature, displaying amplexus, oviposition, and external fertilization. A greater understanding of Pickersgill's reed frog's developmental stages and physiological and environmental needs can improve captive breeding and subsequent release of the frogs, facilitate captive breeding elsewhere, and improve the species' conservation status.

KEYWORDS

amphibian crisis, captive-breeding, diets, husbandry, metamorphism

1 | INTRODUCTION

Globally, amphibians face an extinction crisis originating from a range of causes, including climate change, habitat loss, and diseases such as chytrid fungus *Batrachochytrium dendrobatidis* infection (Gawor et al., 2012; Tapley et al., 2015). Consequently, captive breeding is being used as a conservation tool to prevent extinction (e.g., Banks et al., 2008; Becker et al., 2019; Bodinof et al., 2012; IUCN/SSC, 2014; Lee et al., 2006; McWilliams, 2008; Rorabaugh, 2005; Rorabaugh et al., 2020). Many amphibian species suit captive breeding and reintroduction programs because they reproduce relatively quickly, are small, and have relatively low maintenance

requirements, so allowing viable populations to be managed more cost-effectively than for many larger animal species (Dudgeon & Lau, 1999; Smith et al., 2020). The selection of amphibian species for captive breeding and reintroduction programs should assess if the species have a good chance of surviving after reintroduction. The threats to those species in the wild should be reversible or controllable to enable the long-term survival of the released frogs (Gawor et al., 2012; Griffiths & Pavajeau, 2008; Harding et al., 2016; Linhoff et al., 2021).

The Johannesburg City Parks and Zoo, Johannesburg, South Africa, created the Amphibian Research Project in 2006 to conduct research and assist with the conservation of selected South African endangered amphibian species. It was envisaged that the Amphibian Research Project would create and establish insurance breeding populations at the zoo for reintroduction or reinforcement of populations in the wild. The first endangered species bred in this project was the Pickersgill's reed frog (*Hyperolius pickersgilli* Raw, 1982) (Tarrant & Armstrong, 2017). When this species was first considered for the project, its IUCN Red List status was Critically Endangered, and it had the highest conservation priority ranking for South African amphibians (Measey, 2011).

The genus *Hyperolius* is among the most diverse sub-Saharan anuran genera containing 145 species (Frost, 2021). Only described as a species in 1982 by Raw (1982), *H. pickersgilli* is a relatively small (maximum snout-vent length of 29 mm) frog species endemic to the coastal region of KwaZulu-Natal, South Africa (Du Preez & Carruthers, 2017; Minter et al., 2004; Tarrant & Armstrong, 2013). Males emit soft chirps from dense reed beds, which may explain why this species was only relatively recently discovered (Raw, 1982). Its natural habitat has diminished rapidly because of extensive anthropogenic development, including urbanization, miniSeptember 2017 to 2019,ng, and wetland drainage (Minter et al., 2004; Tarrant & Armstrong, 2013). *H. pickersgilli* was listed as “Endangered” in 2016 because of its' small area of occupancy, the severe fragmentation of its preferred habitat, the extent, and quality of habitat, the number of locations and the continuing decline in the area of occupancy (IUCN/SSC, 2016; Tarrant & Armstrong, 2013).

A biodiversity management plan for *H. pickersgilli* was gazetted in 2017 (Tarrant & Armstrong, 2017) as per the provision of the National Environmental Management: Biodiversity Act (NEMBA) of 2004 (NEMBA, 2004). The biodiversity management plan proposed research on the habitat requirements, breeding biology, and general husbandry of *H. pickersgilli*, both in-situ and ex-situ, as a conservation approach. On this basis, a memorandum of understanding between the provincial conservation body Ezemvelo KZN Wildlife and the Johannesburg City Parks and Zoo was signed to set up an ex-situ breeding project for the species. Here we report on the developmental life cycle stages and behaviors of *H. pickersgilli* as observed ex-situ. We hope that a resultant captive breeding husbandry manual and protocol for the subsequent release of captive-bred offspring can be used as conservation tools for this threatened amphibian and facilitate similar conservation actions for other threatened South African frog species.

2 | METHODS

2.1 | Study area

Our study was conducted at the Amphibian Research Project facility at the Johannesburg Zoo (26.1678° S, 28.0379° E). The non-endangered (Minter et al., 2004) painted reed frog (*Hyperolius marmoratus* Rapp, 1842) was identified as a surrogate or control species to design the operating systems and protocols. These were subsequently modified and used for maintaining a captive population of *H. pickersgilli*.

2.2 | Study species collection

From 2017 to 2019, we collected parental stock (P1 generation) of *H. pickersgilli* from the eThekwini Metropolitan Area, KwaZulu-Natal, South Africa, including Mount Moreland (29.6383° S, 31.0978° E), Adam's Mission (30.0063° S, 30.8033° E) and Prospecton (29.9829° S, 30.9379° E) (Figure 1). During September 2017 to 2019, we collected breeding adults from the wild. We collected adults from Mount Moreland (5 males: 6 females) and Prospecton (7 males: 2 females) in 2017, from Mount Moreland (5:5) and Adam's Mission (5:5) in 2018, and Adam's Mission (5:4) and Mount Moreland (5:5) in 2019.

Once caught, *H. pickersgilli* were housed in clear plastic 5 L containers with perforated screw-on lids and transported by road (~500 km) to Johannesburg Zoo. The relocation process was conducted at night to ensure lower temperatures and less carbon dioxide build-up in the holding jars. All *H. pickersgilli* specimens were fed before traveling. Feeding was not done during the transportation as the relocation took 8 h. Before traveling, each plastic container was cleaned with reverse osmosis (RO) purified water, and a clean paper towel that was misted with RO water was added to each. We conducted misting and cleaning once again near the halfway mark of the journey, about 3.5 h after departure. Ambient temperature in the vehicle was maintained at ~20°C.

2.3 | Captive conditions

On arrival at Johannesburg Zoo's facility, all the *H. pickersgilli* were removed from their containers and placed in vivaria (Exo-Terra, Montreal, Canada; 450 × 450 × 600 mm) in quarantine. We screened all the *H. pickersgilli* for chytrid fungus (*B. dendrobatidis*) via swabbing and polymerase chain reaction and for internal parasites via fecal floats (fecal floats were also done regularly after quarantine). After the chytrid fungus test results were received and were negative, the frogs were moved from quarantine in their vivaria and were housed in a biosecure building. The vivaria were made of glass with lockable, winged front doors, fixed front and side windows and a removable, stainless steel full-screen mesh at the top. Thick, clear plastic sheeting was placed over the light and mesh to prevent excessive

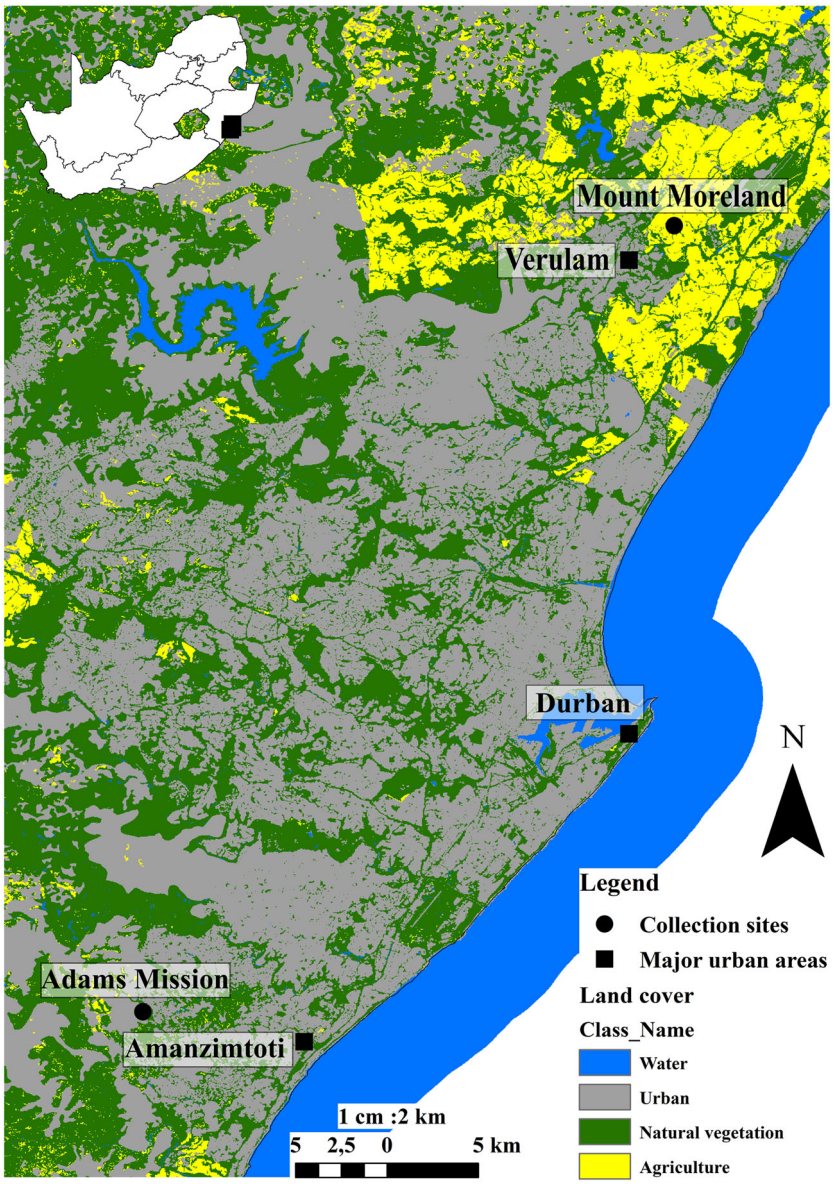

FIGURE 1 Collection sites of wild *Hyperolius pickersgilli* breeders in the eThekwini Metropolitan Area, KwaZulu-Natal, South Africa, for the present study [Color figure can be viewed at wileyonlinelibrary.com]

humidity and heat loss from each vivarium. The vivaria were angled forward at 30°, allowing for a pool of water (2.5–3 cm deep, maximum volume of 250 ml) at the enclosure's front and a dry area at the elevated back portion. Substrate (e.g., moss, leaves, etc.) were placed at the back of the enclosure. Sphagnum moss (*Sphagnaceae*) was preferred, but peat, fir bark, and coconut husk fiber were sometimes used. The substrate was changed monthly and prevented from being saturated with water, being separated from the water by stiff plastic mesh.

All environmental conditions in the facility where the *H. pickersgilli* were kept were maintained in the following ranges, simulating the natural habitat of the species as closely as possible: ambient temperature 16–28°C; humidity 60%–80%; water pH 6.5–7.0; water temperature 19–26°C; water NH_3, NH_4, NO_2, and NO_3 0 g/ml. Only RO water left stagnant for a minimum of 48 h was used in the facility. We filled two open-topped 200-l plastic drums with water from the RO filtration system installed in the building (Aqua-Home Unit). Each drum was fitted with 2 × 200 W submersible ViaAqua Aquarium Heaters (USA) set to 26°C. We measured certain water parameters (NH^3, NH^4, NO^2, NO^3, Cl, hardness, and pH) using sera testing kits (Sera) to prevent frog mortalities and used this water for cleaning, water changes, and misting the vivaria. Water quality was mostly monitored daily until the metamorph stage and then changed to twice weekly or at water changes.

H. pickersgilli typically lays its eggs on upright broad-leafed wetland plants and uses wetland or riparian vegetation to climb to higher perches in the in-situ environment (Raw, 1982; personal observation). Therefore, we used vertical vegetation structures in the vivaria. *Phragmites* spp. stems and *Strelitzia* spp. leaves (or similar, latex-free leaves) were placed vertically in the moss, providing refugia and serving as oviposition surfaces. We placed pebbles in the water, which served as resting and hiding spots. We washed all new additions to the vivaria with a scrubbing brush and RO water and/or a disinfectant (benzalkonium chloride and polyhexamethylene biguanide, diluted with

RO water at 1:250). We rinsed these well, left them to dry for 24 h, rinsed them once more and then placed them in the vivaria. We replaced leaves and sticks at least once a week.

We placed an ultraviolet (UV) light source (38 cm, 14 W tube light; output of 102 μW/cm^2 UVB, and 26 IU/min vitamin D at 10 cm, to 13 μW/cm^2 UVB, and 3 IU/min vitamin D at 40 cm; Exo-Terra Repti Glo 5.0) with no reflector on the removable woven mesh top of each vivarium. This emitted light similar to that found in shady environments and under which the frogs could and did bask during the daytime (personal observation). We manually measured the light output monthly using a digital UV meter (Solarmeter 6.2 UV meter). UV photoperiod was kept at 13 h on (06:00–19:00) and 11 h off. Natural light also filtered in through the windows of the facility. Ambient temperature was regulated to a range of 24–27°C in summer and 20–24°C in winter using room air-conditioning units and heating fans, and a household 7-blade oil heater. Owing to the size and construction of each vivarium, no thermal gradient was provided, although the colder water in the vivarium provided a mechanism for cooling. Ambient temperature and relative humidity inside each vivarium were measured continuously by a permanently fixed digital thermometer and hydrometer (Exo-terra, Hagan Group). Measurements were recorded once daily at the same time in the morning.

For adult frogs, we provided gut-loaded (fed) pinhead crickets (*Acheta* spp.) (primary food source) and fruit flies (*Ceratitis capitata and C. rosa*) (secondary food source as well as for enrichment). The crickets were fed carrots and maize ad libitum and the fruit flies were fed carrots and soft fleshy fruit ad libitum Carrots were left continuously in the vivaria as a food source for the introduced prey items. In addition, we dusted prey items with a calcium powder that includes vitamin D and D^{3+} (Exo-terra, Hagan Group). The ratio applied was one pinch of powder on ~50 insect specimens.

In the first phase of the project, a minimum of 10 *H. pickersgilli* individuals (5:5) were housed in each vivarium to mimic the natural process of mate finding and amplexus. The vivaria sides, leaves, and stones were cleaned and wiped down with a clean paper towel. We removed any leftover food from the enclosure before providing the next. We misted the vivaria with RO water before feeding to maintain a relative humidity range of 60%–80%. All husbandry activities conducted, standard environmental parameters (humidity and temperatures), and social, physical, or environmental changes, were recorded daily and evaluated weekly to improve the husbandry methods.

The second phase of the project focused on the species' well-being and natural environmental factors to maintain their breeding capacity without exceeding their tolerance of stress caused by any environmental changes outside their preferred conditions. The *H. pickersgilli* egg clutches were retained inside the vivaria for 24 h before being removed and placed into a hatching container.

We used one of two methods to remove the *H. pickersgilli* egg clutches, depending on whether the clutch was laid on a leaf (Figure S1) or the side of the vivarium. If the clutch was laid on the side of the vivarium (Figure S2), it was removed using a leaf portion that had been rinsed with boiling water and then RO water. We placed this leaf flat on the side of the vivarium and at the lowest point of the clutch. At the same time, we sprayed some RO water above the clutch, which then adhered to the clutch and caused it to move onto the leaf without dislodging the eggs from the jelly. Tadpoles were carefully removed from the vivarium and introduced to a secondary container dedicated to the temporary holding of the tadpoles from that particular vivarium (Figure S3). Each enclosure used to house froglets, quiescent tadpoles, and developing tadpoles had an aquatic habitat with up to 500 ml of RO water per 1000 ml holding tank or tub, without circulation for filtration to mimic the semi-stagnation of the water in the natural habitat (Raw, 1982). Water changes were performed every 24–48 h, with 100% of used water removed via siphoning.

We used the second method to remove the *H. pickersgilli* egg clutches when laid on a leaf placed in the enclosure. The leaf was removed slowly, tilted from the vertical to the horizontal position, placed inside the hatching jar, and softly sprayed with RO water. The hatching container was a transparent 5 L plastic container with a screw-on lid with a few holes of 4 mm in diameter for ventilation. Once a clutch was transferred to a hatching container, we sprayed the clutches daily with RO water. We used a 5-ml syringe to gather the free-swimming tadpoles from the hatching container to relocate them to a holding aquatic tank (20 cm × 15 cm × 10 cm) with RO water (total volume of 500 ml) with four pebbles and not more than 30 tadpoles per tank. We fed the developing tadpoles plant material (blanched Romain lettuce) and a fine mix of spirulina powder (Health Connection Wholefoods, South Africa) and fish flakes (DARO: sera, Germany) daily. No additional direct light was provided.

We continued to keep the adults together in each vivarium after breeding and only removed the eggs. The adult husbandry continued to be similar, but water changes were done every second day. During summer, misting was conducted a few times a day with RO water, and we provided pinhead crickets and fruit-flies daily. During winter, we decreased the misting and reduced the food offered by half. We monitored the body condition of adults to ensure feeding was sufficient. Before releasing the breeder adults and their offspring to the environment in their natural distribution range, we tested for internal parasites and chytrid fungus. All test results were negative.

2.4 | Data collection and analyses

Daily record-keeping focused on temperature and humidity fluctuations, social and physical preferences, and activities, feeding behavior, development, and breeding. A total of 18 *H. pickersgilli* egg clutches were laid between 2018 and early 2020. We made detailed observations of six clutches of eggs laid between 2018 and 2020 (Figure 2), from egg-laying to hatching, and through the various development stages until the froglets were classified as sub-adults. We took photographs of the egg clutches and counted the eggs from the photographs (Samsung S9 cellphone camera). We counted the number of hatched tadpoles and recorded mortalities. Data collected included egg-laying and fertilization dates, dates of hatching, and the dates the specimens were moved to age-appropriate enclosures.

Observations of physical changes were noted, and photographs were taken of changes considered unusual or different in some way. We used data sheets to improve and standardize data collection (several people conducted the daily husbandry). Data were captured in Microsoft Excel® and analyzed. The classification of life stages and development of *H. pickersgilli* was based on a set of broad, time-specific sequences that were readily observable by eye, each with an indicator to stipulate the end of a phase or the initiation of a new phase, and which may require different management approaches, rather than the stages in Gosner (1960).

3 | RESULTS

The mortality rate observed during the first phase of the project (the keeping of adult breeders together) was 0%. We observed *H. pickersgilli* in amplexus (mating) and producing fertile eggs 3–5 days after introducing them to the biosecure facility after quarantine. The sequence of development observed in the second phase of the project followed the pattern indicated in Table 1. Based on the recorded life cycle of *H. pickersgilli*, we identified seven broad and distinctive developmental stages which required different management approaches.

3.1 | Developmental Stage 1: Egg stage

The egg clutches of *H. pickersgilli* ($n = 6$; Table 2) had a mean size (±SD) of 96.8 ± 4.02 eggs and a mean hatching rate of 66.3 ± 5.5 %. This first stage, the "amphibian spawn" or "egg stage," lasted a maximum of 8 days (Table 1). The breeding females used the leaves and the sides of the vivaria as oviposition sites (Figures S1 and S2). The clutches were laid during the night. The color of the eggs changed from creamy white when newly laid to a two-toned color

FIGURE 2 An example of one of the *Hyperolius pickersgilli* clutches laid in captivity during the present study [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Summary information on six clutches from P1-generation adult female Pickersgill's reed frogs (*Hyperolius pickersgilli*) laid between 2018 and 2020 during the months of January, February, and March

Parameter	Clutch size	Hatch rate %	Proportion males	Tadpole mortality %
Mean	96.8	66.3	0.902	17.2
Std error	1.64	2.3	0.011	1.8
Std deviation	4.02	5.5	0.027	4.4
Minimum	90	57	0.867	10
Maximum	100	72	0.947	22

TABLE 1 The observed sequence of broad developmental life stages of the Pickersgill's reed frog *Hyperolius pickersgilli* bred in captivity at the Johannesburg Zoo, South Africa

	Life stage	Development indicators and time frames
1.	Eggs	Fertile eggs display a two-tone color: whitish dorsally and dark brown to black ventrally. The fertile eggs hatched from 6 to 8 days after laying. (The number of eggs varied between clutches, and the percentage that hatched per day also varied).
2.	Quiescent tadpoles	Newly hatched tadpoles were black in color and remained dark for the first 24–48 h. They were relatively inactive.
3.	Developing tadpoles	After 48 h, the tadpoles changed to a sandy-beige color. After 6 weeks, the tip of the tail turned black for a few days, before returning to its normal sandy-beige color. This was the first indication that limb development had started. From 6 to 8 weeks, developing tadpoles were characterized by swimming upside down, on the left side and then on the right side in line with lung development and limb development. The legs develop in a particular sequence: right hind, then left hind, then left fore, and then right fore.
4.	Metamorphs	Lung development necessitated air-breathing from about 8 weeks. The metamorph could climb and move about freely and the tail was absorbed within 3–5 days.
5.	Froglets	The froglet stage occurred from about 9 weeks to 6 months. The sexes could not be distinguished.
6.	Sub-adults	From 6 to 18 months, the sub-adult frogs developed their mature coloration, and males started calling without reproducing.
7.	Adults	Sexual maturity was reached at 18 months. Lifespan in captivity for males was 4–6 years, while for females, it was 10–12 years.

(white and dark brown to black) within 48 h. Embryo development was observed in the transparent jelly until Day 6 when the first tadpoles hatched. Hatching continued until Day 8, after which no further hatching occurred, the remaining eggs being infertile. Mortality of five entire clutches that were housed in plastic 1 L containers was recorded after as little as 0.005 ppm chlorine came through in the water because of a component failure in the RO system.

3.2 | Developmental Stage 2: Quiescent tadpole stage

The second developmental life stage of *H. pickersgilli*, called the quiescent tadpole stage, was the shortest. The newly hatched tadpoles were only known as quiescent tadpoles for 48 h, whereafter they were called developing tadpoles. The quiescent tadpoles were dark to black in color, only 3 mm in total length and relatively inactive. These hatched and initially remained stuck within the jelly substance. A soft spray of RO water assisted the tadpoles in freeing themselves. They started swimming in the shallow water that accumulated from the daily spraying in the hatching container. The tadpoles did not eat during this stage, and no fecal matter was seen. This was the most vulnerable phase as they appeared not to resist water parameters outside their narrow tolerance range. Development was directly affected by the water temperature, and mortalities were recorded below 16°C and above 27°C; thus, these were the tolerance range limits with optimum development at about 25–26°C (personal observation, Figure S4).

3.3 | Developmental Stage 3: Developing tadpole stage

Developing tadpoles were classified as individuals that were at least 3 days old and, for 8 weeks, went through morphological changes (Table 1). These developing tadpoles changed color after 3 days from the dark black color of the quiescent tadpole to a brown-beige color with silver speckles along the dorsal and ventral fin margins (Graphical abstract; Du Preez & Carruthers, 2017). The developing tadpoles started to feed on aquatic plant material, and we provided a fine mix of spirulina powder and fish flakes throughout this developmental life stage. Tadpoles fed blanched romaine lettuce showed muscle weakness at the metamorph stage and could not jump. As a result, the lettuce was removed from the tadpoles' diet altogether.

After 2 weeks, the tadpoles floated motionless upside down on the surface of the water for long periods, indicating lung development, and their mouthparts broke the water surface to allow breathing (Figures S5 and S6). If we caused slight ripples in the water or touched the developing tadpoles, they turned around and swam down to the tank's bottom. These observations were only recorded to take place during the day with water temperature exceeding 23°C. Lower water temperatures were found to reduce activity, and the developing tadpoles stayed motionless at the bottom of the tank for hours.

The developing tadpoles grew in size without any behavioral change up to week 5 or 6 when the tip of the tail turned black, the first indication that the limbs were developing (Figure 3; Table 3). The developing tadpoles started swimming on their sides, leaning to their right side for 48 h when the right hind leg became visible and able to be moved freely (Table 3; Figure S7). The developing tadpoles were unbalanced and swam with the right side hanging downwards because of the limb's weight. The hind left limb developed 48 h after the right hind limb was fully developed. The developing tadpoles were then able to swim correctly again as the body was balanced with both hind limbs developed (Figure 3). At this point, the water volume was lowered to 400 ml so that the pebbles provided resting surfaces for the developing tadpoles to breathe air and exercise their hind limb muscles. The front left limb developed in the next 48 h (week 6 or 7; Table 3) and caused the developing tadpoles to swim leaning to the left because of the additional weight on that side. The front right limb developed after 48 h, and the developing tadpoles were able to swim horizontally again (Figure 3). Development was faster at 25°C than at 20°C.

The developing tadpoles can exhaust themselves with continuous swimming; the pebbles placed in each enclosure prevented the developing tadpoles from drowning. The developing tadpoles could move freely in and out of the water at this stage. Their diet continued to be the same, but their habitat preference changed as they became more terrestrial than aquatic. Tail absorption started slowly without any visible effect on the developing tadpoles.

A variation in development was recorded in 2019 and 2020 when clutches were laid at the end of March. The eggs hatched within the stipulated time, and the quiescent tadpoles developed into developing tadpoles. But a lower water temperature of between 19°C and 20°C was recorded from May to August of each year, resulting in delayed development. No physical changes in any of the specimens were recorded, and all survived till the end of August each

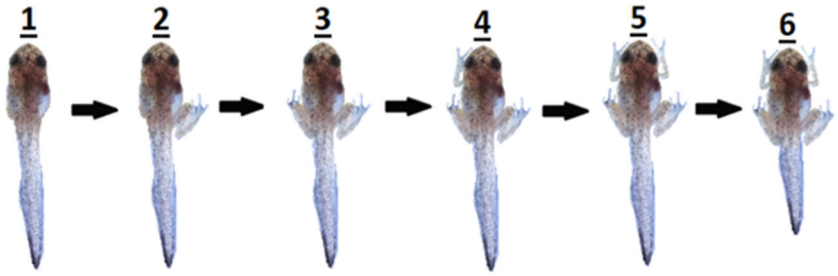

FIGURE 3 Limb developmental stages from Weeks 6 to 8 in Pickergill's reed frog *Hyperolius pickersgilli* developing tadpoles in captivity [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Summary information on the development rates of tadpoles from six clutches laid by P1-generation female Pickersgill's reed frogs (*Hyperolius pickersgilli*)

Year clutch laid	Site of the capture of breeder	Median period (weeks) until right hind leg appearance	Median period (weeks) until left front leg appearance	Median period (weeks) until metamorph
2018	Mount Moreland	5	6	7
	Adam's Mission	6	7	8
2019	Mount Moreland	6	7	8
	Adam's Mission	5	6	7
2020	Mount Moreland	6	7	8
	Adam's Mission	6	7	8

Note: Median period from hatching, calculated as the period at which 50 % of the tadpoles per clutch reached the given development when the water temperature was between 20°C and 25.4°C.

year. However, when water temperature increased above 20°C, development resumed.

3.4 | Developmental Stage 4: Metamorph stage

The metamorph stage (Figure 4) is the transition between aquatic and arboreal organisms. The developing tadpoles became metamorphs at 7 or 8 weeks of age (Table 3) and started climbing using their four limbs. The tail was still present but served only for stability and nutrition. The *H. pickersgilli* metamorphs (Figure S8) were housed in a vivarium with a wet paper towel and *Strelitzia* spp. leaves until the tail was completely absorbed. They had no dietary requirement as their tails were being absorbed, gaining enough nutrition to sustain them over the 3–5 days of the metamorph period. The metamorphs were kept moist by a soft RO water spray twice daily. They could walk and jump up to 30 cm from one side to the opposite side of the enclosure. The body color changed from light brown to cream-beige, yellowish, or even dark brown throughout the day. Sex determination from external characteristics was not possible because of the absence of adult body coloration and markings (Figure 5).

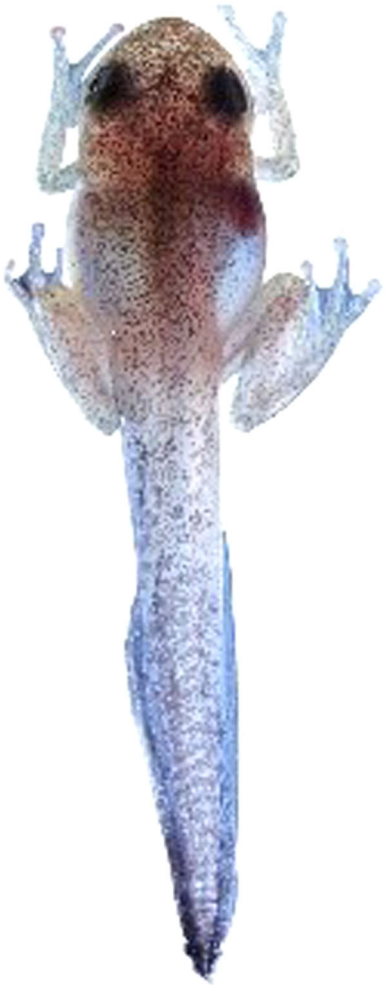

FIGURE 4 An example of the metamorph development stage of *Hyperolius pickersgilli* [Color figure can be viewed at wileyonlinelibrary.com]

3.5 | Developmental Stage 5: Froglet stage

Once the tail was absorbed completely, the metamorph's physical appearance was similar to the sub-adult *H. pickersgilli*; this was called the froglet stage (Figures 5 and S9). The froglets preferred to be solitary at the lower, denser foliage level but were not antagonistic to others. Opportunistic feeding was common. They would sit and wait for suitable prey to come into the near vicinity before jumping towards and grabbing it with a single bite. Once the froglets' body lengths exceeded 5 mm and were deemed to be in good health, they were relocated to a vivarium as described earlier, with options for them to choose from the terrestrial and aquatic microhabitats. The terrestrial portion also had foliage to allow them to climb and exhibit natural behavior. The social structure observed indicated that no feeding competition was present. Body color changed throughout the day based on the amount of light present inside each enclosure and the location of the resting place. The sexes could still not be distinguished.

3.6 | Developmental Stage 6: Sub-adult stage

Sub-adult male *H. pickersgilli* were 6 months and older with sex differentiation visible. The male's lateral line stretched from the nostrils to the groin, as Raw (1982) described. Females were lime green to dark green dorsally and were without vocal sacs; however, their full adult coloration took longer to appear. Sub-adults preferred to spend more time higher up, away from the water surface, and occupied similar heights on plants as the breeding adults. At this stage, they were not sexually mature and were similar in behavior to the adults but without engaging in mating. Sub-adults hunted actively by moving around the vivarium until they noticed prey, after which they would jump towards and catch and consume the prey. The freely moving prey encouraged the sub-adults to behave naturally in sourcing food.

3.7 | Developmental Stage 7: Adult stage

Adult *H. pickersgilli* were older than 18 months, fully developed and sexually mature (Figure S10). Although adults were active day and night, males only called during the period from dusk to dawn. Both sexes used the entire vivarium, including the different substrates, and during the daily husbandry routine, swam for short periods or hid. They hunted actively and opportunistically. Males were found to use the higher portions of the vivaria later in the day before calling. When a female was attracted, the male attached himself to the female's back by grasping the female with his front legs in the axillary amplexus position and fertilized the eggs as they were released from the female's body (Figure 6). Females were observed to react to and move toward the males. Once a suitable breeding mate was located, they would engage in amplexus for one day. The female would lay one or two clutches of eggs that the male would fertilize. The breeding behavior was stimulated by misting in the enclosure at ambient temperatures of between 23 and 30°C, providing a suitable spawning site is available.

Two other variations of external fertilization were observed in captivity. First, the female laid eggs in the absence of the male but returned afterwards with the male who mounted her back and passed sperm onto the ova, thus fertilizing them. Second, the female laid eggs again without a male's presence (Figure S11). The male later passed sperm over the eggs in the absence of the female. It is unknown whether these variations occur in situ.

The proximity of too many males or too many females prevented optimal breeding as competition between males, or a lack of mature males were negative stressors. The maximum life span of adults in captivity was found to be 6 years in males to more than 10 years in females (unpublished Zoo collection data since January 2012). The F1 generation to the F2 generation was bred in 2019. We released most of the F1 generation back into the environment within the native distribution range. The breeding adults collected were released back to the location where they were collected after 2 years as their breeding behavior stopped in captivity.

FIGURE 5 An example of the froglet development stage of *Hyperolius pickersgilli* [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

H. marmoratus was introduced in the ex-situ environment as a pioneer species to implement the baseline husbandry methods for the genus before *H. pickergilli* was introduced to the Amphibian Breeding Project at Johannesburg Zoo. Notwithstanding differences between

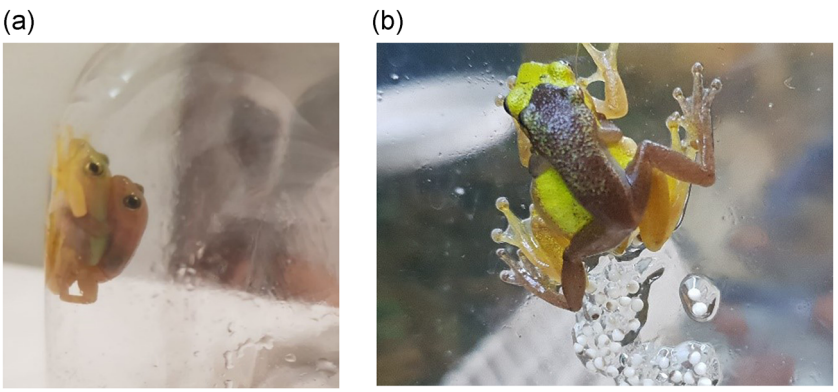

FIGURE 6 Examples of *Hyperolius pickersgilli* adults in amplexus in captivity at the Johannesburg Zoo, South Africa [Color figure can be viewed at wileyonlinelibrary.com]

the species, for example, *H. marmoratus* tadpoles have a relatively high tolerance to ammonia levels (Schmuck et al., 1994), the basic husbandry methods developed sufficed for *H. pickersgilli*. The latter has a single genetic population and relatively good heterozygosity (Kotze et al., 2019). We attempted to maintain genetic heterozygosity ex-situ by obtaining founder individuals from several sites in the natural distribution range of the species. We observed seven developmental life stages in *H. pickersgilli* with distinctive management requirements and characteristics in the ex-situ environment. The developmental sequence and time intervals were elucidated, with clear feeding and environmental preferences.

Whether clutch sizes in captivity are similar to those in the wild for *Hyperolius* species is unknown. Clutch size varies in *Hyperolius* species. The clutch size of wild-caught *H. marmoratus* is double to six times the size of clutches of *H. pickersgilli* in captivity (Table 2; Telford & Dyson, 1990). In contrast, the clutch size of the tropical *H. rubrovermiculatus* (="*mitchelli*") in captivity (50–100 eggs) is more similar (Channing & Crapon de Caprona, 1987). However, developmental periods may differ between captivity and the wild. *H. marmoratus* tadpoles hatch in less than a week and develop into small froglets within 2 months, but in captivity, metamorphosis takes 64–100 days (Channing, 2001).

The periods of development of various stages in the life history of the few *Hyperolius* species that are relatively well known are summarized in Table 4. Notwithstanding the differences in the distributions of and environmental conditions each species faces, they have similar periods for the earlier life history stages. The tadpoles of some *Hyperolius* species with distribution ranges that overlap that of *H. pickersgilli* (*H. semidiscus*, *H. tuberilinguis*, *H. poweri*, *H. marmoratus*, and *H. pusillus*) wriggle free from the egg masses within 5 days (Wager, 1986). However, plasticity in the timing of hatching of various species of frogs, including some *Hyperolius* species, as a response to environmental conditions, predators or pathogens is known (Warkentin, 2011). The diets of the tadpoles and adults are similar, although cannibalism in captivity has been noted for the tadpoles of *H. marmoratus* (Du Plessis et al., 2019; personal observation), but not for *H. pickergilli*.

Tadpoles fed blanched romaine lettuce did not need to use their limbs to propel themselves to the surface to feed as they had to do for the crushed fish flake and spirulina powder because the lettuce was kept at the bottom of the tanks by pebbles. This resulted in muscle weakness at the metamorph stage owing to inactivity during the developing tadpole stage while feeding. Therefore, blanched romaine lettuce was discontinued as part of the diet of developing tadpoles.

The eight tropical montane species of *Hyperolius* from Rwanda in the study of Sinsch and Dehling (2017) had lower recorded longevity than the maximum longevity of the subtropical *H. pickersgilli* in captivity. This may be explained by the findings of Stark and Meiri (2017) that longevity in amphibians decreases with an increase in mean annual temperature and is greater in captive than in wild frogs. The life expectancy of species such as *H. nitidulus* is relatively low owing to the extreme environmental conditions it faces, such as unpredictable rainfall (Lampert & Linsenmair, 2002).

TABLE 4 Summary information on time taken to complete various life stages in *Hyperolius* species

Hyperolius spp.	Env.	Eggs (d)	Tadpoles (w)	Diet	Metamorphs (d)	Froglets (m)	Sub-adults (m)	Adults (m)	Diet	Lifespan (y)	Reference
H. marmoratus	E	5-7	6–9	A/H	?	3	?	?	I	4 (M), 10 (F)	Channing (2001); Passmore and Carruthers (1995)
H. nitidulus	I	2–5	6–8	A	?	?	2–4	4–12	I	1 (M, F)	Lampert and Linsenmair (2002)
H. pickersgilli	E	A6–8	6–8	A/H	3–5	4	12	18	I	6 (M), 12 (F)	Present study
H. viridiflavus	I	2–5	8	A/H	?	?	4	>4–12	I	2 (M)	Sinsch and Dehling (2017)

Abbeviations: ?, unknown; A, algae; d, days; DD, Data deficient; E, ex-situ; Env, Environment; F, Female; H, herbivorous; I, insectivorous; I, in-situ; m, months; M, Male; T, absorption of tail; w, weeks.

We found that the *H. pickersgilli* individuals changed color in different developmental life stages. Amphibian specimens change color in response to age, lighting, and radiation levels (Perry et al., 2008). According to Raw (1982), juveniles and males of *H. pickersgilli* have Phase J coloration while females change from Phase J to Phase F coloration after a certain size is reached (20–22 mm snout-vent length). Juvenile *H. viridiflavus* have a different coloration than adults, referred to as Phase J, which is light brown to green in color. Sexually mature males frequently maintain juvenile coloration throughout adulthood. Phase F, the adult phase, maybe a highly variable color pattern with distinct morphs (Grafe & Linsenmair, 1989; Schiøtz, 1999). The present study demonstrated that the coloration of adult *H. pickersgilli* females is relatively uniform.

The preferred in-situ habitat occupied by *H. pickersgilli* consists of dense wetland vegetation, limiting the potential to study the complete life cycle of the species there (Raw, 1982). Captivity facilitated observations and recording of morphological changes with age and changes in the social behavior and dietary and habitat preferences of the developing and breeding *H. pickersgilli*, resulting in some understanding of the life cycle in situ. The study enabled the establishment of suitable husbandry methods to ensure the survival of each developmental stage and the development of a husbandry manual for *H. pickersgilli*.

Numerous ex-situ amphibian conservation projects have been initiated globally in response to the decline of populations of amphibian species (e.g., Banks et al., 2008; Bodinof et al., 2012; Dudgeon & Lau, 1999; Griffiths & Pavajeau, 2008; Harding et al., 2016; Lee et al., 2006; Nahonyo et al., 2017; Rorabaugh, 2005; Rorabaugh et al., 2020; Sharifi & Vaissi, 2014; and references in Linhoff et al., 2021). Most amphibian captive breeding conservation projects aim to breed specimens in managed ex-situ environments as insurance populations. These provide offspring that can be reintroduced into their natural environment to ensure and promote sustainability and survival of the species to maintain a natural ecological balance. With the resources available from zoological institutions and universities, the research and husbandry can be closely monitored, recorded and analyzed. The continued use of ex-situ captive breeding for different species and the systematic record-keeping of researchers can promote the method for many other amphibian species in the future as demonstrated in the present study and other studies (e.g., Wildenhues et al., 2012).

ACKNOWLEDGEMENTS

We are grateful to the Johannesburg City Parks and Zoo (ZA), Ezemvelo KZN Wildlife (ZA) and the University of KwaZulu-Natal (ZA) for the facilitation of this project and the supply of all required resources. CTD and CP are grateful to the National Research Foundation (ZA, Grant 98404) for funding. We thank two anonymous reviewers for their comments that improved the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICS STATEMENT

The authors obtained ethical clearance to conduct the study from the Johannesburg City Parks and Zoo Animal Ethics Committee.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Banks, C. B., Lau, M. W. N., & Dudgeon, D. (2008). Captive management and breeding of Romer's tree frog *Chirixalus romeri*. *International Zoo Yearbook*, *42*, 99–108.

Becker, C. A., Du Plessis, I., Malepa, P. L., & Netsianda, T. (2019). Observation on the impact of nutrition and feed presentation on leg development in the Pickersgill's reed frog. *Amphibian Ark Newsletter*, *47*, 13–14.

Bodinof, C. M., Briggler, J. T., Junge, R. E., Beringer, J., Wanner, M. D., Schuette, C. D., Ettling, J., Gitzen, R. A., & Millspaugh, J. J. (2012). Post release movements of captive-reared Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*). *Herpetologica*, *68*(2), 160–173.

Channing, A. (2001). *Amphibians of Central and Southern Africa*. Comstock Publishing Associates–Cornell University Press.

Channing, A., & Crapon de Caprona, M.-D. (1987). The tadpole of *Hyperolius mitchelli* (Anura: Hyperoliidae). *South African Journal of Zoology*, *22*(3), 235–237.

Dudgeon, D., & Lau, M. W. N. (1999). Romer's frog reintroduction into a degraded tropical landscape, Hong Kong, P.R. China. *Re-introduction News*, *17*, 10–11.

Du Plessis, I., Marais, E. C., & Armstrong, A. (2019). Field observations at Mount Moreland, KwaZulu-Natal South Africa. Unpublished report.

Du Preez, L., & Carruthers, V. (2017). *Frogs of Southern Africa – A complete guide*. Penguin Random House.

Frost, D. R. (2021). Amphibian species of the world: An online reference, version 6.1. Retrieved January 25, 2022 from https://amphibiansoftheworld.amnh.org/index.php. American Museum of Natural History, New York, USA. https://doi.org/10.5531/db.vz.0001

Gawor, A., Rauhaus, A., Karbe, D., van der Straeten, K., Lotters, S., & Ziegler, T. (2012). Is there a chance for conservation breeding? Ex situ management, reproduction, and early life stages of the Harlequin toad *Atelopus flavescens* Duméril and Bibron, 1841 (Amphibia: Anura: Bufonidae). *Amphibian and Reptile Conservation*, *5*, 29–44.

Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, *16*(3), 183–190. http://www.jstor.org/stable/3890061

Grafe, T. U., & Linsenmair, K. E. (1989). Protogynous sex change in the reed frog *Hyperolius viridiflavus*. *Copeia*, *1989*(4), 1024–1029.

Griffiths, R., & Pavajeau, L. (2008). Captive breeding, reintroduction, and the conservation of amphibians. *Conservation Biology*, *22*(4), 852–861.

Harding, G., Griffiths, R. A., & Pavajeau, L. (2016). Developments in amphibian captive breeding and reintroduction programs. *Conservation Biology*, *30*(2), 340–349.

IUCN/SSC. (2014). Guidelines on the use of ex situ management for species conservation. Version 2.0. Gland, Switzerland: IUCN Species Survival Commission. Retrieved August 29, 2014, from www.iucn.org/about/work/programmes/species/publications/iucn_guidelines_and__policy__statements/

IUCN/SSC Amphibian Specialist Group and South African Frog Re-assessment Group (SA-FRoG). (2016). Hyperolius pickersgilli. The IUCN Red List of Threatened SpeciesHyperolius pickersgilli.IUCN. Retrieved October 6, 2016, from. http://www.iucnredlist.org/details/10644/0

Kotze, A., Ralph, T. M. C., Barrow, L. N., Tarrant, J., Du Preez, L., Madisha, M. T., & Dalton, D. L. (2019). Lack of phylogeographic structure in the endangered Pickersgill's reed frog; *Hyperolius pickersgilli* (Raw,1982). *African Journal of Herpetology*, *68*, 1–17.

Lampert, K. P., & Linsenmair, E. K. (2002). Alternative life cycle strategies in the West African reed frog *Hyperolius nitidulus*: The answer to an unpredictable environment? *Oecologia*, *130*(3), 364–372.

Lee, S., Zippel, K. C., Ramos, L., & Searle, J. (2006). Captive-breeding programme for the Kihansi Spray Toad *Nectophrynoides asperginis* at the Wildlife Conservation Society, Bronx, New York. *International Zoo Yearbook*, *40*, 241–253.

Linhoff, L. J., Soorae, P. S., Harding, G., Donnelly, M. A., Germano, J. M., Hunter, D. A., McFadden, M., Mendelson, J. R., III, Pessier, A. P., Sredl, M. J., & Eckstut, M. E. (2021).*The IUCN guidelines for amphibian reintroductions and other conservation translocations* (1st ed.). IUCN. https://portals.iucn.org/library/node/49485

McWilliams, D. A. (2008). Nutrition recommendations for some captive amphibian species (Anura and Caudata). *The Canadian Association of Zoos and Aquariums Nutrition Advisory and Research Group (GARNNARG)*. http://www.amphibianark.org/wp-content/uploads/2018/07/Amphibian-nutrition-report-CAZA-2008.pdf

Measey, G. J. (Ed.) (2011). Ensuring a future for South Africa's frogs: A strategy for conservation research. *SANBI Biodiversity Series 19*. Pretoria: South African National Biodiversity Institute.

Minter, L. R., Burger, M., Harrison, J. A., Braack, H. H., Bishop, P. J. & Kloepfer, D., (Eds). (2004). *Atlas and Red Data book of the frogs of South Africa, Lesotho and Swaziland*. Avian Demography Unit, University of Cape Town.

Nahonyo, C. L., Goboro, E. M., Ngalason, W., Mutagwaba, S., Ugomba, R. S., Nassoro, M., & Nkombe, E. S. (2017). Conservation efforts of Kihansi spray toad *Nectophrynoides asperginis*: Its discovery, captive breeding, extinction in the wild and re-introduction. *Tanzanian Journal of Science*, *43*, 23–35.

NEMBA. (2004). National Environmental Management: Biodiversity Act 10 of 2004. *Republic of South Africa Government Gazette No. 26436, Notice No. 700*.

Passmore, N. I., & Carruthers, V. C. (1995). *South African frogs, a complete guide*, (Revised ed). Southern Book Publishers Pty. Ltd/ Witwatersrand University Press.

Perry, G., Buchanan, B. W., Fisher, R. N., Salmon, M., & Wise, S. E. (2008). Effects of artificial night lighting on amphibians and reptiles. In J. C. Mitchell, R. E. Jung Brown, & B. Bartholomew (Eds.), *Urban Herpetology Herpetological Conservation 3, Society for the Study of Amphibians and Reptiles* (pp. 239–256). Salt Lake City.

Raw, L. R. G. (1982). A new species of reed frog (Amphibia: Hyperoliidae) from the coastal lowlands of Natal, South Africa. *Durban Museum Novitates*, *13*(9), 117–126.

Rorabaugh, J. C. (2005). Re-establishment of the Tarahumara frog into Arizona. USA. *Re-introduction News*, *24*, 43–44.

Rorabaugh, J. C., Owens, A. K., King, A., Hale, S. F., Poulin, S., Sredl, M. J., & Lemos-Espinal, J. A. (2020). Reintroduction of the Tarahumara frog (*Rana tarahumarae*) in Arizona: Lessons learned. *Herpetological Conservation and Biology*, *15*(2), 372–389.

Schiøtz, A. (1999). *Treefrogs of Africa*. Edition Chimaira.

Schmuck, R., Geise, W., & Linsenmair, K. E. (1994). Life cycle strategies and physiological adjustments of reedfrog tadpoles (Amphibia, Anura, Hyperoliidae) in relation to environmental conditions. *Copeia*, *1994*, 996–1007.

Sharifi, M., & Vaissi, S. (2014). Captive breeding and trial reintroduction of the endangered yellow-spotted mountain newt *Neurergus microspilotus* in western Iran. *Endangered Species Research*, *23*, 159–166.

Sinsch, U., & Dehling, J. M. (2017). Tropical anurans mature early and die young: Evidence from eight Afromontane *Hyperolius* species and a meta-analysis. *PLoS One*, *12*(2), e0171666.

Smith, R. K., Meredith, H., & Sutherland, W. J. (2020). Amphibian Conservation. In W. J. Sutherland, L. V. Dicks, S. O. Petrovan, & R. K. Smith (Eds.), *What Works in Conservation* (pp. 9–64). Open Book Publishers.

Stark, G., & Meiri, S. (2017). Cold and dark captivity: Drivers of amphibian longevity. *Global Ecology and Biology*, *2018*, 1–14. https://doi.org/10.1111/geb.12804

Tapley, B., Bradfield, K. S., Michaels, C. J., & Bungard, M. (2015). Amphibians and conservation breeding programmes: Do all threatened amphibians belong on the ark? *Biodiversity Conservation*, *24*, 2625–2646.

Tarrant, J., & Armstrong, A. J. (2013). Using predictive modelling to guide the conservation of a critically endangered coastal wetland amphibian. *Journal of Nature Conservation*, *21*(5), 396–381.

Tarrant, J., & Armstrong, A. J. (2017). Biodiversity management plan for Pickersgill's reed frog (*Hyperolius pickersgilli*). *Government Gazette*, *624*(No. 40883), 184–222. http://www.gpwonline.co.za/Gazettes/Gazettes/40883_2-6_NationalGovernment.pdf

Telford, S. R., & Dyson, M. L. (1990). The effect of rainfall on interclutch interval in painted reed frogs (*Hyperolius marmoratus*). *Copeia*, *1990*, 644–648.

Wager, V. A. (1986). *Frogs of South Africa: Their fascinating life stories*. Delta Books.

Warkentin, K. M. (2011). Plasticity of hatching in amphibians: Evolution, trade-offs, cues and mechanisms. *Integrative and Comparative Biology*, *51*(1), 111–127.

Wildenhues, M., Rauhaus, A., Bach, R., Karbe, D., van der Straeten, K., Hertwig, S. T., & Ziegler, T. (2012). Husbandry, captive breeding, larval development and stages of the Malayan horned frog *Megophrys nasuta* (Schlegel, 1858) (Amphibia: Anura: Megophryidae). *Amphibian & Reptile Conservation*, *5*, 15–28.

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Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: du Plessis, I., Armstrong, A., Malepa, P. L., Kanengoni, A. T., Price, C., & Downs, C. T. (2022). Developmental life stages of the Pickersgill's reed frog (*Hyperolius pickersgilli*) in an ex-situ environment at Johannesburg Zoo's captive breeding facility, South Africa. *Zoo Biology*, 1–11. https://doi.org/10.1002/zoo.21688