Captive breeding of *Pelophylax* water frogs under controlled conditions indoors

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ABSTRACT - Water frogs, genus *Pelophylax*, are culturally and scientifically important taxa and as such are frequently collected and traded in large numbers. This, among other factors, has led to water frog species becoming both threatened in parts of the range of the genus and invasive aliens in others, and they are therefore of conservation interest. Captive breeding has been achieved in outdoor enclosures, but indoor breeding with its advantages of greater control over environment, escapes and pathogen movement, is not frequently achieved and is poorly documented. We present data concerning successful captive husbandry and reproduction of four species of *Pelophylax* (*P. perezi*, *P. shqipericus*, *P. lessonae* and *P. kurtmulleri*) at a private collection in the UK and at Norden's Ark, Sweden. Aquaterraria with flat land areas close to the surface of water were used to house frogs and warm, bright and UVB-radiation rich basking spots were provided, based where possible on field data. Frogs were annually environmentally cycled according to their native climate and reproduced in the spring following an increase in temperature and the end of winter dormancy. The critical temperature for breeding all four species was around 23 ˚C. Frogs were fed on gut-loaded invertebrates of appropriate size dusted with supplement powders and tadpoles were raised in aquaria and fed on mixtures of algae and animal protein. The similarity of husbandry requirements between species supports the use of closely related species as analogues in determining the husbandry requirements of target taxa. The methods presented here may be used to inform captive breeding programmes.

INTRODUCTION

Water frogs of the genus *Pelophylax* are distributed across most of Europe, the near and middle East, north Africa and south-east Asia. This speciose genus includes a number of klepton-forming taxa (e.g. Crochet et la., 1995; Dedukh et al., 2015; Sanchez-Montes et al., 2016), which, together with great abundance in the vicinity of human settlements in some species, has made it a frequent model organism for research in fields as disparate as ecotoxicology (e.g. Fasola et al., 2015; Marquez et al., 2011), evolutionary biology (e.g. Canastrelli & Nascetti, 2008; Pruvost et al., 2013; Dedukh et al., 2015) and development (e.g. Marracci et al., 2011). The genus is also culturally important in much of Europe, where it is the native taxon most frequently used in the preparation of frog legs dishes. Although some species are widespread and listed as Least Concern on the IUCN Red List, a number of forms are threatened with extinction and other local populations are also at risk. For example populations in Sweden and the UK represent the edge of the wide European range of *P. lessonae* and are in decline (Sjogren, 1991) or extinct (Sainsbury et al., 2016) and the focus of conservation efforts including captive elements (Buckley & Foster, 2005; Baker & Foster, 2015; Sainsbury et al., 2016). Other forms, are quite the opposite and present an important invasive alien threat in parts of Europe (Holsbeek et al., 2010; Harris et al., 2013); in some cases, threatened *Pelophylax* species may also act as alien invaders outside of their natural range (Domeneghetti et al., 2013).

Water frogs are frequently harvested from the wild in large numbers for the laboratory and culinary trades; a search (17/10/17) of the online wholesale trading website alibaba.com identified companies able to export 8 tonnes per month of *Pelophylax* cf. *ridibundus* from Turkey. The reproduction of *Pelophylax* in outdoors vivaria constituting enclosed ponds is well established, although little has been formally published on the subject. Reproduction of these frogs under controlled conditions indoors, however, is not frequently achieved and few if any published data exist. We present some data concerning the captive, indoors husbandry and reproduction of four species of *Pelophylax* in a private collection in the UK and at Norden's Ark, Sweden.

METHODS

Species and animal origins

Pelophylax perezi and *P. shqipericus* were obtained as eggs from and experimental population at the University of Aveiro, Portugal in 2016, and from a private breeder using outdoors enclosures in 2012, respectively, by CM. Larvae were reared under the conditions outlined, below.

Pelophylax lessonae and *P. kurtmulleri* were obtained by Norden's Ark, Sweden. *P. lessonae* was obtained under license (dnr 522-1586-08) as wild caught adults (5 pairs) from a wild population in Uppland, Sweden in 2008. *P. kurtmulleri* was collected during a field trip to the island of Milos in 2008. This island population is likely to represent a distinct taxon yet to be named (Vervust et al., 2013). The original group consisted of five adults, five subadults and 100 tadpoles.

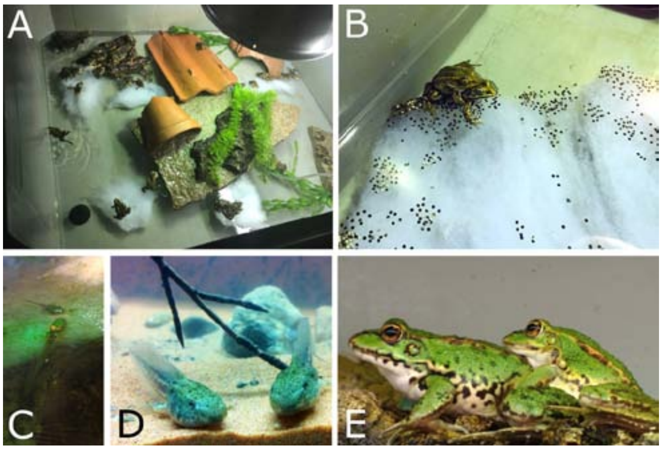

Figure 1. A. Enclosure used for housing *P. lessonae* B. Amplectant pair of *P. lessonae* amidst spawn on a mat of filter floss C. *P. perezi* tadpoles approaching metamorphosis basking under a UVB rich heat spot D. Advanced tadpoles of *P. lessonae* E. Amplectant pair of *P. perezi*

Environmental measurements

Surface temperatures were measured with non-contact infrared thermometers of various brands and models. Water quality was measured using Salifert re-agent kits. Ultraviolet Index (UVi) was measured using Solarmeter 6.5 handheld units (Solartech, USA). Water temperature was measured using probe thermometers of various brands and models.

Enclosures

The fundamental characteristics of enclosures used for housing all four species of *Pelophylax* covered here are summarised in Table 1; also see Figure 1A. All enclosures were essentially designed to provide a large amount of open water with low-lying islands allowing frogs to leave the water at both hot and cool ends of the enclosure. These islands were designed to be only just (several mm) above the water line to ensure ease of access. Live and/or plastic plants, as well as filter floss sheeting, provided structures in the water for shelter, calling, oviposition and resting, while plants above the water line provided overhead shelter. Larvae were reared in aquaria of varying size and varying design; water quality and temperature data are presented in Table 1. Water changes were performed as required (as often as daily) to remove waste.

Heating and Lighting

Frogs were provided with heating and lighting as summarised in Table 2, with overlapping Ultra-violet B radiation (UVB) and thermal gradients. Basking sites were concentrated over land areas and, for *P. shqipericus* and *P. perezi*, also shallow water above mats of algae, aquatic/plastic plants or filter floss sheeting. Ultra-violet index (UVi), collected following the methods used by Michaels and Preziosi (2013), and temperature readings, in June at the University of Aveiro in the ponds where the stock of *P. perezi* originated revealed basking frogs exposed to a maximum UVi of 6 and a basking surface temperature of 30-35 ˚C and are supported by field measurements of body temperatures (Meek, 1983). These data were replicated in captivity for *P. perezi*. *Pelophylax shqipericus* occurs at a similar latitude to *P. lessonae* in northern Italy and so the UVi data collected by Michaels and Preziosi (2013) (that is, a UVi up to 3.5) were used to guide maximal UVi exposure; a basking surface temperature of 30-35 ˚C was used for this species as well.

Pelophylax lessonae and *P. kurtmulleri* received similar basking site parameters, which are similar to those recorded for wild *P. lessonae* in France (Meek, 2011) and Italy (Michaels & Preziosi, 2013). Enclosures experienced a drop in both air and water temperature of several degrees, with air temperature falling more; exact changes depended on ambient conditions and time of year.

Tadpole aquaria did not receive direct lighting until animals began to metamorphose and exhibit juvenile, rather than tadpole, skin texture and colouration. Juveniles were maintained in small versions of adult enclosures, with UVB lighting being provided at the same levels and through the same methods as for adults once juvenile colouration was adopted.

Annual environmental variation and brumation

Annual variation in temperature for *P. perezi* and *P. shqipericus* was achieved through the use of aquarium heaters (various brands and models) connected to external thermostats (Inkbird; various models), allowing manipulation of temperature to 0.1 ˚C; room temperature was held somewhat lower than aquarium temperature to allow desired values to be achieved without a cooling mechanism. Local maximum/minimum temperature data and photoperiod data from worldweatheronline.com were used to determine the exact environmental values used. Photoperiod and ambient water temperature were adjusted monthly on this basis. Aquaria were also exposed to indirect natural sunlight and associated changing photoperiods. Basking lamps were turned on during the day all year, other than the months of December and January, where no basking sites were provided, to induce dormancy. Frogs were overwintered in the same aquaria in which they were housed the rest of the year.

For *P. lessonae* and *P. kurtmulleri*, from February until end of September the photoperiod was kept at 14 hours. To induce dormancy two months prior to hibernation (October and November) the photoperiod was shortened to 10 hours and basking lamps were turned off. From December until the end of January, *P. lessonae* was moved to hibernation with males and females in separate containers (Samla 78x56x43 cm, IKEA) with ventilation holes drilled in the lid and with a deep layer of moist sphagnum moss as substrate. The temperature was gradually lowered from 15 ˚C to 5 ˚C over a five day period and was then kept at this temperature for two months. During the same period the males and the females of *P. kurtmulleri* were maintained in the same boxes as used the rest of the year, but separated by sex. The ambient and water temperature was not reduced for *P. kurtmulleri*, but no basking light was provided, photoperiod was reduced to 10:14 and feeding frequency was reduced.

Table 1. Enclosure characteristics for *Pelophylax* species

Species	Adult enclosure type	Adult water depth (cm)	Adult enclosure footprint dimensions (cm)	Adult enclosure furnishings	Filtration	Water chemistry	Maximum adult stocking density (frogs per L)	Maximum larval stocking density (tadpoles per L)
Pelophylax perezi	Glass aqua-terraria (front opening)	15	150x50	Floating cork bark; blocks of filter foam breaking water line; live plants (*Scindapsus*, *Elodea*, *Hydrocotyle* mats of algae); filter floss	Air-stream sponge filters and internal box filters	Tap water; Alkalinity 180-220mg/L, pH 7.5-8, treated with dechlorinator	0.25	1
P. shqipericus	Glass aquarium (top opening)	15	60x40	Weathered concrete slab resting on bricks to break surface; live plants as above.	None	Tap water; alkalinity<20mg/L; pH 6.5-6.8; treated with dechlorinator	0.3	1
P. lessonae	Plastic box	10-15	100x100	Large flat rocks just above water line; plastic plants; filter floss		Tap water; alkalinity <20mg/L, pH 6,5-6,8, non chlorinated	0.25	1
P. kurtmulleri							0.5	1

Table 2. Heating and lighting used for *Pelophylax* species

<table>
<tr><th>Species</th><th>Lighting array</th><th>UVi Gradient (summer maximum)</th><th>Basking site temperature (˚C)</th><th>Age post-metamorphosis at sexual maturity (months)</th><th>Winter temperature (˚C)</th><th>Critical spawning water temperature (˚C)</th><th>Larval rearing temperature (˚C)</th></tr>
<tr><td>P. perezi</td><td>T5 fluorescent tubes (D3+ T5, Arcadia; Lightwave T5, Growth Technology) 80W Mercury Vapour lamps (Arcadia).</td><td>0-6</td><td rowspan="4">30-35</td><td>8</td><td>10</td><td rowspan="2">20-26</td><td>23</td></tr>
<tr><td>P. shqipericus</td><td>T8 fluorescent lamp (ZooMed 10% UVB) 80w incandescent lamps, arranged such that great and Uvb gradients were correlated.</td><td>2-3</td><td>12</td><td rowspan="2">3-5</td><td>23 (but overwintering down to at least 10 possible)</td></tr>
<tr><td>P. lessonae</td><td rowspan="2">T5 lamps (Arcadia D3 6%; Arcadia Plant Pro)
Halogen spots (Osram Halopar Alu 75W)</td><td rowspan="2">3-5</td><td>9</td><td>20</td><td rowspan="2">20-23</td></tr>
<tr><td>P. kurtmulleri</td><td>12</td><td>10-15</td><td>23-24</td></tr>
</table>

Diet

Pelophylax perezi and *P. shqipericus* were fed a variety of commercially available insects (mainly *Gryllus* and *Acheta* crickets, *Schistocerca* locusts, *Lumbricus* earthworms), dusted (apart from the earthworms) with calcium and multivitamin/mineral powder (Nutrobal, Vetark), which were placed on the islands for frogs to capture. Food was offered daily during the active months and once a week during cooler periods where frogs were largely dormant. After tadpoles of these species had absorbed yolk sacks, larvae were fed on a mixture of *Spirulina* and *Chlorella* algae, fish flakes (various brands) and spinach until metamorphosis. Initial diets were more plant/algae heavy and slowly the protein content became dominant.

Pelophylax kurtmulleri and *P. lessonae* were fed commercially available crickets (*Gryllus bimaculatus*). These were gut-loaded (Superload insect gutload formula, Repashy) 48 hours prior to feeding the frogs. Before being offered to the frogs they were dusted with calcium (Repti Calcium without D3, Zoomed) and multivitamin/mineral powder (Nutrobal, Vetark). Food was offered three times a week and crickets were placed on the islands for the frogs to catch. After tadpoles of these species had absorbed yolk sacks, larvae were fed on a mixture of turtle food (ReptoMin, Tetra), algae food for fish (Algae wafers, Hikari) and fish fry food (Micron, Sera). Also available until metamorphosis were pieces of cuttlefish bone.

Collection and treatment of spawn

Spawn of all species was moved to separate aquaria heated to 20-23 ˚C, with aquarium heaters if ambient conditions were not warm enough. Spawn was moved without substrate if possible; otherwise small pieces of plant or filter floss sheeting were moved with the spawn and removed, if necessary, once larvae were free swimming. Air-stream sponge filters provided filtration for *P. perezi* and *P. shqipericus*; the other two species were not kept under filtered conditions.

RESULTS

Pelophylax shqipericus* & *P. perezi

Calling began in both *P. perezi* and *P. shqipericus* after the end of winter cooling, during which frogs became largely dormant, stopped feeding and rested on the bottom of the tanks underwater. When water temperature began to increase and the basking lamps were turned back on, frogs became active and fed again. Amplexus (Figure 1E) occurred once water temperatures reached 18-20 ˚C during the day and spawning occurred both during day and night when water temperatures remained between 23-26 ˚C. Spawning could be readily induced by increasing water temperature to 23-26 ˚C and heavily feeding animals; dropping temperature again to below 21 ˚C interrupted breeding behaviour, thereby allowing spawn to be produced on demand throughout the early summer. In *P. perezi,* air temperatures above 28 ˚C during the late summer caused a reduction in and eventual cessation of calling. Falling temperatures in early September led to a brief resumption of chorusing, but spawn was not produced.

Spawn was deposited in clumps of c. 20-100 eggs on aquatic vegetation and filter floss matting. Spawn hatched within one week and tadpoles began feeding 4-5 days after hatching, having absorbed yolk sacs and become free swimming. Tadpoles were raised successfully in both hard (alkalinity c. 180ppm, pH 7.5-8) and soft (alkalinity <20ppm, pH 6.5) water with comparable success. For *P. perezi*, harder water was used in preference to replicate field data from the source population (a concrete lined pond with alkalinity >200ppm and pH 8). Metamorphosis occurred as quickly as 6-8 weeks in *P. perezi*; tadpoles of *P. shqipericus* sometimes metamorphosed just as quickly; some other individuals overwintered in water as cold as 10 ˚C before metamorphosing the following spring. Tadpoles, especially those nearing metamorphosis and having begun to adopt adult colouration, frequently basked in shallow water (Figure 1D) with a water temperature of 26-28 ˚C and a UVi of up to 3. Mortality in tadpoles and juveniles was negligible and largely restricted to animals that were not viable. Animals reached sexual maturity within 6 months, just prior to winter cooling, and reproduced successfully the following breeding season. *P. perezi* juveniles began producing simplified, shortened vocalisations in July at a snout-to-vent length of around 30mm.

Pelophylax lessonae* & *P. kurtmulleri

Calling began in *P. lessonae* shortly after being moved to water with a temperature of 18-19 ˚C without any extra heating provided. Amplexus occurred once the basking lamps were turned on and the water temperatures reached 21-22 ˚C. Once filter floss was placed in the water the spawning began. *P. kurtmulleri* needed the basking lamps turned on again and for water temperatures to reach 21 ˚C and ambient temperature of 22-23 ˚C to start calling. Amplexus and spawning occurred in *P. kurtmulleri* once water temperatures reached 22-23 ˚C and with ambient temperature around 23-25 ˚C.

Pelophylax lessonae spawn was deposited in clumps of c. 20-100 eggs on aquatic vegetation and filter floss matting (Figure 1B), while *P. kurtmulleri* also used stones and roots to deposit their eggs on. Spawn hatched within one week and metamorphosis was achieved within 2 to 3 months. Tadpoles (Figure 1D) were raised without issues.

DISCUSSION

Pelophylax sp. rely on well warmed, sunny areas of relatively still water with rafts of floating vegetation and rarely stray far from water. They are heliophiles and actively bask, exposing themselves to the heat and UVB irradiation of direct sunlight (Michaels & Preziosi, 2013). Historically, indoors enclosures for amphibians were typically lacking in UVB provision and thermal gradients. With increasing understanding of amphibian lighting requirements and the availability of technology to meet them, indoors husbandry for water frogs is now much more easily achievable. Our captive enclosures were designed to recreate the UVB rich, brightly lit and warm environments inhabited by water frogs in nature and these conditions proved successful in maintaining and breeding this genus indoors. Using these methods, we were able to achieve success in maintaining, breeding and rearing *Pelophylax* frogs indoors in captivity. Frogs did not exhibit any noticeable health problems, and did not display any symptoms of metabolic bone disease, a condition which is common in ranid frogs and may result from inadequate calcium or UVB provision (Wright & Whitaker, 2001; Michaels et al., 2015).

Although some field data were available from the microhabitat of *Pelophylax*, which could be used to underpin the husbandry approach taken for housing these taxa, they did not cover the whole year and so some anthropocentric climate data had be integrated into husbandry. Although these sorts of climate data may not even remotely reflect the microclimates used by some amphibians (Michaels et al., 2014), the habitats of *Pelophylax* are typically exposed and so anthropocentric climate databases may broadly reflect the conditions used by frogs. Nevertheless the extremes of anthropocentric climate data were avoided as these may not be the reflected in pool frog microhabitats (Michaels & Preziosi, 2013).

Overwintering temperatures resulting in successful brumation and subsequent reproduction were based on such modified anthropocentric data, and varied according to species reflecting differing geographic origins. However, similar optimal spawning and tadpole rearing of around 23 ˚C were found in all species, reflecting the similarity in habitat between species. Animals could also be housed under relatively high densities with success, and mortality of tadpoles, juveniles and adults was extremely low. These characteristics are highly favourable to production of large numbers of animals indoors and these methods may prove useful for the generation of captive bred stock for conservation and research. Juveniles did prove to be highly cannibalistic of frogs substantially smaller than themselves, but provided that froglets were size sorted and well fed, cannibalism was avoided.

Indoors reproduction allows animals to be held under controlled environmental conditions, and for more effective barriers to pathogens, predators and escapes to be put in place. Moreover, many laboratory experiments requiring tadpoles produced in captivity resort to artificial crossing requiring the deaths of both parental animals (e.g. Pruvost et al., 2013). By using natural reproduction, parental animals may be kept alive to reduce animal use, to provide the potential for replicate clutches from the same parents, and to improve the quality and viability of progeny, which may be reduced in artificially inseminated spawn (Browne & Zippel, 2007).

The high degree of overlap in the husbandry of these species lends support to the concept of analogue species (Michaels et al., 2014), whereby closely related species may be used to predict the husbandry of closely related taxa. This is likely due to the fact that all European, North African and Near Eastern *Pelophylax* species inhabit similar habitats and speciation is a result of geographic isolation rather than invasion of new niches, which can lead to very different captive requirements (Michaels et al., 2016). Bearing in mind the need to adjust husbandry to reflect subtle differences in local climates, including the overwintering temperature tolerated by different species, relatively common taxa with low extinction risk (here, *P. lessonae*; *P. perezi*; *P. ridibundus*; Bosch et al., (2009); Kuzmin et al. (2009a; b)) may be used as a predictor and training tool to prepare husbandry resources for threatened species (here, *P. shqipericus*; Uzzell & Ivailovic (2009)). It is also important to note that *P. perezi* and *P. lessonae* have very large distributions, incorporating a large range of altitudes and latitudes, and so captive husbandry parameters, especially optimal overwintering temperature, may vary between populations. For example, *P. perezi* in this study originated from coastal Portugal, where winter temperatures rarely fall much below 10 ˚C and snow or frost are absent; the same species extends into mountainous northern Spain where populations will tolerate snow and ice throughout the winter. The data presented here should therefore be extrapolated with some caution to other populations and species of *Pelophylax*, with field or at least local weather/climatic data, especially surrounding overwintering temperatures, integrated into husbandry strategies.

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