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Toxicity impact of butachlor on the development of green toad *Bufotes viridis*

Zahra Mossadeghi, Zeinab Parvaresh, Nazihe Seddighi, Fatemeh Roushenas, Samira Rahimi, Elmira Hasani, Zahra Derakhshan & Mohsen Nokhbatolfoghahai

Biology Department, Faculty of Sciences, Shiraz University, Shiraz, Iran

Butachlor is the most commonly used herbicide on rice paddy fields in Asian countries. Paddy fields are habitats commonly used for reproduction by many species of amphibians. We examined the effects of butachlor on *Bufotes viridis* development. Amplectant pairs of *B. viridis* were kept in the laboratory in an aquarium overnight, and their spawn collected the next morning. Eggs were exposed to butachlor at different concentrations (0.1, 0.2, 0.8, 1, 2, 3.5, 7, 14µg/l), all lower than the concentration used in the area (rice paddy fields) by farmers. Eggs were allowed to develop to Gosner stage 24 and their developmental patterns compared with those reared in normal conditions (control). In order to examine whether jelly coats have a significant role in the protection of developing eggs from the toxin, another group of eggs were de-jellied and treated in the same procedure. The LC50 value of butachlor was calculated as 14µg/l and 7µg/l after 96h for jellied eggs and de-jellied eggs respectively. Butachlor lead to a range of external and internal body malformations. Butachlor concentrations of 2µg/l, 7µg/l and 14µg/l reduced embryonic growth and development. A high mortality rate and both internal and external abnormalities were observed at lower concentrations than used in fields, suggesting that butachlor can have significant negative effects on amphibians where this herbicide is used.

Key words: amphibian; Bufotes viridis; butachlor; embryo; embryonic development; de-jellied egg

INTRODUCTION

The decline of amphibian populations has become more dramatic in the last three decades (Pechmann & Wilbur, 1994; Beebee & Griffiths, 2005; Hoffman et al., 2010; IUCN, 2015). A recent report indicates that about 40% of amphibian species are threatened with extinction, more so than any other vertebrate class (Hoffman et al., 2010).

There are many factors that are influencing amphibian declines, including habitat loss, predation, competition, increasing doses of UV radiation as a result of atmospheric ozone degradation, infectious diseases, and pollution (Collins & Storfer, 2003; Beebee & Griffiths, 2005; Rohr et al., 2008). About 8% of threatened amphibian species are reported to be as a result of pollution (Alford, 2010). Human activities such as agricultural practices are believed to be among the causes of natural environmental contamination (Vos & Chardon, 1998; Lehtinen et al., 1999; Relyea, 2005; Johansson et al., 2006; Gurkan & Hayretdah, 2012). The high effectiveness of pesticides in controlling pest species both in aquatic and terrestrial environments has had a great role in promoting farm production and therefore improving human health (Jones, et al., 2010). Although the benefits of pesticides are well known and documented, there is less information about the possible side effects of pesticides on non-target organisms. There are many research projects around the most commonly used pesticides, but our knowledge is far from being complete (Fleeger et al., 2003; Forson & Storfer, 2006; Sparling & Feller, 2009; Egea-Serrano et al., 2012).

During agricultural activities, many chemical components are used that can have a negative impact on aquatic and terrestrial ecosystems (Van Dam et al., 1998; Garcia-Munoz et al., 2009; Emurotu & Anyanwu, 2016). Amphibians are under greater risk of chemical exposure than other vertebrates, as in many cases they use standing, temporary or shallow waters around agricultural fields for their reproductive activities and spawning (Duellman & Trueb, 1994; Tyler, 1994; McDiarmid & Altig, 1999; Rowe et al., 2003). They are highly sensitive and therefore vulnerable to chemical pollutants during their aquatic embryonic and larval stages (DeYoung et al., 1996; Murphy et al., 2000). As most amphibian life cycles encompass both aquatic and terrestrial environments, and amphibians have permeable and sensitive skin, they may be good bioindicators of changing environments (Wyman, 1990; Lips, 1998). Almost all amphibian species in temperate regions spawn at the time of year when pesticides are being used on agricultural land for controlling fungi, weeds, insects or other pests. These applications bring amphibians into high risk and vulnerability to the toxicity of the pesticides (Greulich & Pflugmacher, 2004; Pašková et al., 2011).

Previous studies have shown that some pesticides applied in agricultural activities have had an impact on the decline of amphibian populations (Davidson et al., 2001; Sparling et al., 2000; Davidson, 2004; Sparling & Fellers, 2007; Kang et al., 2008, Gill & Garg, 2014). Amphibians are known to be very sensitive to teratogenic effects by pesticide exposure including myoskeletal malformation, tail deformation and limb mal-differentiation (Fort et al., 2004a, b; Bacchetta et al., 2008). Nervous system and epidermal defects, oedemas, gut malformations (Degitz et al., 2003; Robles-Mendoza et al., 2009), high embryonic and larval mortality rate, extended hatching time, and growth retardation are among the other recognised teratogenic effects (Vismara et al., 2000, 2001a,b). Further studies show that a number of agrochemicals reduce the activity of the immune system of some organisms and cause amphibian decline by facilitating disease emergence (Rohr et al., 2008).

Although pesticide consumption in a number of countries in Europe, North America and Japan is decreasing, being restricted or even banned (EU Pesticides Database, 2016), global use of pesticides has increased 50 times since 1950 (PAN Germany, 2012). Butachlor (N-butoxy-methyl-2-chloro-2, 6'-diethylacetanilide) is the most commonly used herbicide on rice paddy fields in tropical and subtropical areas in South America, Africa, and Asia, specially throughout south east Asia. The information available in south and far east Asia shows that in the Philippines, Korea, Thailand, China, Taiwan, Japan and some other countries in the region, farmers use high concentrations of butachlor as an herbicide, either alone or in a mixture with other herbicides (Naylor, 1996). It is used to control a wide range of grasses and some broadleaf weeds in paddy fields (Senseman, 2007; Abrigail, 2015) and nonpaddy fields/dry-seeded rice fields (Jiang et al., 2014). Butachlor is thought to be an inhibitor for synthesising long chain fatty acids (Senseman, 2007). This pesticide is also genotoxic to toads where it causes DNA strand break induction in erythrocytes.

Paddy fields are one of the typical habitats that Bufonid species commonly use for spawning (Liu et al., 2011). Although there are studies that have investigated the toxic effects of butachlor on the embryos and tadpoles of different anuran species (Geng, et al., 2005b; Liu et al., 2011, Li, et al., 2016), the effects of butachlor on the embryos and tadpoles of the green toad Bufotes viridis (Laurenti, 1768) are unknown. Liu et al. (2011) assessed both the acute and chronic effects of butachlor at different concentrations on Gosner (1960) stage G26 tadpoles of a Ranid species Fejervarya limnocharis. At concentrations low enough to allow tadpoles to grow (≤ 0.2mg/l), tadpoles grew to metamorphosis at a similar size to controls but took significantly longer. At higher concentrations (≥ 0.4mg/l) survival was zero, with the time to death dependent on the concentration. Li et al. (2016) used Xenopus laevis embryos and exposed them to different butachlor concentrations ranging from 0.313 mg/l to 5 mg/l. They showed that butachlor caused developmental toxicity (e.g. eye malformation, precardial oedema, tail curvature) and thyroid endocrine disruption. Geng et al. (2005b) reported that the acute toxicity of butachlor to *Rana guentheri* tadpoles was high (LC50= 0.74 mg/l, 96 hrs). Yin et al. (2008) reported high levels of DNA damage in tadpole erythrocytes (*Bufotes bufotes gragarizans*) exposed to sublethal concentration of butachlor (LC50= 1.32 mg/l, 96 hrs).

The aim of this study was to determine the effects of butachlor on the development and growth of B. viridis embryos under laboratory conditions. We concentrated on B. viridis as this species is the only widely distributed species among amphibians in our area (southern Iran) where spawning happens solely in temporary pools including paddy fields. Bufotes viridis is a species complex with morphologically at least 14 related species widely distributed in many European, Asian and north African countries (Stock et al., 2006; Frost, 2016). The status of the green toad in southern Iran, Fars Province (study area) is unclear, and needing further research. One of the Bufotes surdus subspecies (Bufotes surdus annulatus) and probably Bufotes variabilis, which is retained here as *B. viridis*, are the two members of the *B. viridis* species group found in our study area (Fakharzadeh et al., 2014; Parvaresh, et al., 2016).

We hypothesised that butachlor has negative effects on the development and growth of embryos. In order to examine whether jelly coats have a significant role in development and in the response to butachlor, groups of eggs were de-jellied and treated with the same procedure as intact eggs. It is suggested that with removing the jelly coat, we are actually removing a protective barrier to chemical absorption: therefore, we hypothesised that butachlor would have more of an effect on de-jellied eggs compared to intact jellied eggs.

MATERIALS AND METHODS

Ethics Statement

The work carried out conformed to national legislation and guidance for animal welfare and conservation (BHS Ethics Policy and UK legislation). Our studies of the effects of butachlor on embryonic development did not proceed past the stage of onset of feeding (defined as Gosner stage 25) as required for unlicensed experiments (Animal – Scientific Procedures – Act, UK). We use the term 'embryo' rather than 'larva' for all these stages. Sample sizes were kept to a minimum and the experiments had no impact on the conservation status of wild populations of *B. viridis*.

Egg collection, incubation and hatching assessment

The green toad, *B. viridis*, is included in the conservation category of Least Concern, but populations of this species are decreasing (IUCN, 2015). This species is water-dependent during its reproductive season and stays in water for a long time. This toad generally uses slow-flowing and standing waters, seasonal ponds, and shallow pits filled with water for egg laying (Kinzelbach

& Kasparek, 1992). The eggs and tadpoles of this species can be observed in the seasonal ponds around agricultural fields and inside the fields.

Seven pairs of adult B. viridis were caught in the amplectant state in March-May 2013 in a seasonal pond at Bajgah field station in Fars Province, Iran. The toad specimens were brought to the laboratory in plastic containers and transferred to aquaria filled with aerated tap water to a shallow level. Each pair of toads was kept separately in an aquarium in the laboratory overnight. Their spawn was collected the next morning and transferred to polypropylene containers filled with de-chlorinated and aerated tap water. Mixed clutches of fertilised eggs (three clutches in total; mean clutch size = 6643 ± 1855) from different parents were used for all experimental treatments (parents were released back in the wild after spawning). The temperature, pH and dissolved oxygen levels of the water were measured daily with an Elmetron meter.

Experimental design

Butachlor (EC 60%, Karkhanejat Giahi Tehran, Iran, Butachlor) used in this study was considered as a toxic substance. A stock solution was prepared by dissolving the butachlor in dechlorinated tap water. A preliminary experiment was carried out with butachlor by using one of the clutches of the eggs. A broad range of butachlor concentrations was used to select the most suitable concentrations, and to avoid testing non-necessary concentrations for the actual experiment. Batches of 150 fertilised B. viridis eggs starting from Gosner stage 5 were transferred to plastic containers (25cm W. × 45cm L. × 3cm H.), each containing different concentrations of butachlor solutions (0.1, 0.2, 0.8, 1, 2, 3.5, 7, 14µg/l) as well as to the control container, with total volume of 2L water. The selected butachlor concentrations were chosen based on LC50 determination, and were lower than the concentration used in the study area in the paddy fields in Iran (4.8 mg/l). Table 1 presents a list of the treatments with details for each examination carried out in this study. Water in each container was aerated by an air pump. The developing embryos were kept in identical conditions of light (12h. light, 12h. darkness), temperature ($20^{\circ}C \pm 2.0$) and pH (7.71 ± 0.15). *Bufotes viridis* embryos were exposed to the different butachlor concentrations from G5 through to stages G23/24, and their developmental patterns were compared with the patterns exhibited by the control group. No pesticides were applied to the eggs in the control group and they were allowed to develop under identical environmental conditions as the eggs in the experimental groups. For the treatment groups, embryos were exposed to Butachlor just once at the beginning of the experiment, to each of three (*n*=150 per replicate).

The time and stage at which hatching occurred in each treatment was noted, and mean hatching time and stage were calculated both for the experimental treatments and the controls according to Gosner's (1960) developmental table. Hatching assessment was started after the first embryo hatched and hatching was observed regularly after that. Hatching time was calculated as the time when 50% of embryos were hatched in experimental and control groups.

In order to examine whether jelly coats have a significant role in development and in the response to butachlor, other groups of eggs (*n*=150 for each treatment) were de-jellied and treated to the same procedures as the intact eggs. The developmental patterns of the de-jellied eggs exposed to the butachlor were compared with the patterns found in normal conditions of de-jellied eggs (control). Eggs were de-jellied using cysteine solution (Viso & Khokha, 2012). To make the solution, 0.24g 20 M Tris buffer was dissolved in 80ml distilled water, and 2g cysteine was added, then the solution was conveyed to pH=8.1. The pH was adjusted to the desired value by using acid (HCl) and base (NaOH) solutions and by using pH probe.

Among the various butachlor concentrations tested in this study, the concentration at which 50% of the embryos in a container died by 96h was determined as the LC50. Those eggs that did not develop to further

Table 1. List of the treatments with details for each examination carried out in this study. Apart from the number of eggs used for experimental treatments, indicated in the table below, another 300 specimens in total were used for control treatments (jellied and de-jellied). G= Gosner stage

No	Type of treatment	Total number of specimens used	Butachlor concentrations (µg/I)	Developmental stages exposed to butachlor	Developmental stages examined	Examined characters
1	Embryo growth and developmental progress	2700	2, 7, 14	G5-G24	G10-G24	SVL, TAL, TL against time; Stages against time
2	Biometry of em- bryos	3600	0.8, 1, 3.5, 7	G5-G24	G24	SVL, TAL, TL
3	Mortality rate	1800 plus specimens reused from treatment number 1,2	0.1, 0.2, 0.8, 1, 2, 3.5, 7, 14	G5-G24	G5-G24	Dead and abnormal specimens
4	Morphological as- sessment	Specimens reused from treatment number 1	2, 7, 14	G5-G24	G10-G24	External organs in- cluding tail, trunk and head morphology
5	Histological assess- ment (sections)	Specimens reused from treatment number 1	2, 7, 14	G5-G24	G20	Internal organs includ- ing digestive tube; kidney and muscles

embryonic stages, that stopped their development and turned from black to white, or became motionless because of movement malformations were counted as dead samples.

Embryo growth and developmental progress analysis

To study the growth and developmental progress with time of embryos, we chose three of the concentration series ($2\mu g/I$, $7\mu g/I$, and $14\mu g/I$). For each concentration treatment examined ten developing samples at each stage (G10-24) were fixed in Bouin's solution (n=150). The collecting and fixation were carried out for jellied and de-jellied groups, as well as for the control jellied and dejellied groups. Observations of developmental progress were made for about 140h in total and the curves for developmental progress with time were then drawn. Total length (TL) of fixed samples both in jellied and dejellied groups at different times were also measured, and the curves for embryonic growth were drawn and analysed for the different treatments and the control.

Biometry analyses of embryos

Four embryos at stage G24 reared in each of four additional experimental tanks with concentrations 0.8, 1, 3.5, and 7µg/l of butachlor, as well as the control group, were randomly selected three times per treatment. After fixing the embryos in Bouin's solution, biometric measurements of the embryos (Altig & McDiarmid, 1999), were carried out immediately after the samples were stored in 70% alcohol. Snout-vent length (SVL), tail length (TAL) and total length (TL) of the embryos were measured to the nearest 0.01 mm with a digital calliper. Histograms for each character in both jellied and dejellied groups for different concentrations were then drawn.

Mortality percentage of embryos

During the developmental pattern experiments a number of embryos in different groups (0.1, 0.2, 0.8, 1, 2, 3.5, 7, $14\mu g/l$) became abnormal and did not continue to develop: embryos turned white in colour and showed abnormalities in body shape. To determine the embryonic mortality percentage, after each stage of development in each treatment, embryonic mortality was estimated. The final mortality percentage was calculated by adding together all mortality from all stages in each treatment. The mortality percentage in each treatment was estimated up to stage G24.

Morphological and histological assessment of embryos for abnormalities

During the developmental pattern experiments, fixed embryos at different stages (G10-24) were examined separately for a selection of treatment groups ($2\mu g/l$, $7\mu g/l$, and $14\mu g/l$) under a binocular microscope and any morphological abnormalities were recorded. Photographs of the embryos at different stages were taken under a binocular microscope with a digital camera. In addition, five surviving embryos were randomly selected at stage G20 from the control and each treatment group. Specimens were preserved in 70% alcohol after having been fixed in Bouin's solution for 10h. Afterwards, these five embryos were processed through a series of alcohol concentrations (i.e. dehydration), xylene, and paraffin. Serial cross-sections (5-7µm) of whole embryos were obtained from the samples and stained with haematoxylin and eosin (H&E). Microscopic slides were examined under a light microscope and over a range of magnifications, with images being recorded using a camera attached to the microscope (h550L, Nikon, Japan). Any abnormality observed in the internal organs of the treatment samples including digestive tube, kidney and muscles were recorded and compared to the control.

Statistical analyses

All statistical analysis was performed using SPSS, version 16.0 (SPSS Inc., Chicago, IL, USA). Differences between the control and treatments were compared by analyses of variance (ANOVA) and regression. Comparisons between the control and experimental groups were performed using Tukey tests. Statistical significance was accepted as α <0.05 (the acceptance level was set at P<0.05).

RESULTS

Hatching time and hatching stage

Hatching of 50% of the embryos occurred by stage G17-18 and 72h (three days) in the control group at lab temperature (20°C ± 2.0). Hatching of 50% of the embryos occurred by stage G19 and 96h (four days) in a selection of experimental groups (2µg/I, 7µg/I, and 14µg/I butachlor), all with intact jelly coats and at the same temperature. Hatching time and stage were not assessed at the lower butachlor concentrations (\leq 1µg/I) used in this study.

Embryonic development: response to different concentrations of butachlor

The results for the development of the embryos from stage G5 (the stage at the start of the experiment) in three different butachlor concentrations $(2\mu g/l, 7\mu g/l,$ and $14\mu g/l$) showed that as the concentration of the toxin increased, most of the embryos stopped their development at early developmental stages. The rate of development in de-jellied groups was slower than in jellied groups when they were exposed to the same toxin concentration (Fig. 1). Figure 1 also shows that the embryos exposed to the $2\mu g/l$, $7\mu g/l$, and $14\mu g/l$ concentrations of toxin mostly stopped developing at stage G19/20 (only de-jellied group), G19 and G18 respectively in both jellied and de-jellied groups. There was no stop in development in the 2µg/l treatment jellied group. Figure 1 shows data only for embryos that survived for the total treatment period. Jellied and dejellied eggs in the control group continued their growth and development to stage 25 without any problem.

The results for the growth in length of embryos from stage G10 in three different butachlor concentrations $(2\mu g/I, 7\mu g/I, and 14\mu g/I)$ show that with the increasing concentration of the toxin, an increased number of embryos stopped their growth at a shorter length and

150

e

3 iellied

2

1

0

150

150

5

3 iellied

2

1

0

150

150

6

5

4

2

1

0

150

150

5

1

0

150

de-iellied

iellied

de-jellied

jellied

de-jellied

the embryos were always shorter in length in the dejellied group than the jellied group (not shown).

Figure 2 shows the mean sizes of the embryos at different treatments and in both jellied and de-jellied groups. The data are shown only for embryos that survived for the total treatment period. The growth rate and development of the embryos in the control group were normal, indicating that a density of 150 eggs presented no overcrowding issues. Removing the jelly coat from embryos did not affect the normal pattern of embryonic development in laboratory conditions.



Figure 1. The level of developmental progress with time in control (A) and in different concentrations of butachlor 2µg/l, 7µg/l, and 14µg/l in (B), (C) and (D) respectively and between jellied and de-jellied groups (10 samples were assessed at each sample point, but no variation in stage was observed amongst them).

Figure 2. The relationship between total embryo length and age in control (A) and in different concentrations of butachlor 2µg/l, 7µg/l, and 14µg/l in (B), (C) and (D) respectively as well as between jellied and de-jellied groups (n=4 at each sample point).

Biometry of embryos that successfully reached stage G24 Biometric results of embryos at stage G24 after exposure to different concentrations of butachlor (0.8, 1, $3.5\mu g/l$) and of very few survived embryos at stage G24 exposed to $7\mu g/l$ (according to the sensitivity of embryos in initial development to this concentration), are shown in figures 3A and 3B in jellied and de-jellied groups respectively.

The Tukey test showed that there were some significant differences (p < 0.05) in total length (TL), snout-vent length (SVL) and tail length (TAL) between the control and the four different concentrations of butachlor in both jellied and de-jellied groups in the embryos at stage G24. Details are as follows and the results are simplified in Table 2.

According to the data analysis, as the toxin concentration increased, TL, SVL and TAL were significantly lower in the treatment groups compared to the control group (p <0.05) in both jellied and de-jellied groups. There were no significant differences in TL, SVL and TAL between the jellied and de-jellied groups amongst the experimental groups or compared to the control groups. Results indicated some significant differences (p < 0.05) in TL, SVL, TAL of the embryos in the experimental group and between the experimental and the control group in the de-jellied group (Table 2). For every $l\mu g/1$ butachlor concentration increased, 0.1mm of the overall length was reduced. The results showed that TL and TAL of the embryos amongst the experimental groups and between the control and the experimental groups were significantly different (p < 0.05) in the jellied group (Table 2). For every lµg/1 butachlor increased, 0.6mm SVL and 0.9mm TAL were reduced.

LC50 value

LC50 values for a 96h exposure period in jellied and dejellied groups of *B. viridis* embryos were found at butachlor concentrations of $14\mu g/l$ and $7\mu g/l$ respectively.

Table 2. Tukey test results in jellied and de-jellied groups showing only significant differences amongst experiments and between control and experimental groups for total length (TL), tail length (TAL) and snout-vent length (SVL) in the embryos at stage G24.

	Character	Gro	oups	Sig
De-jellied	TL	3.5µg/l	0.8µg/l	0.001
		3.5µg/l	1µg/l	0.006
	TAL	Control	3.5µg/l	0.0001
		Control	7μg/l	0.014
		3.5µg/l	0.8µg/l	0.0001
		3.5µg/l	1µg/l	0.007
	SVL	Control	0.8µg/l	0.05
Jellied	TL	Control	0.8µg/l	0.001
		Control	3.5µg/l	0.0001
		Control	7µg/l	0.0001
		1µg/l	7µg/l	0.013
	TAL	Control	0.8µg/l	0.013
		Control	3.5µg/l	0.0001
		Control	7μg/l	0.0001



Figure 3. Sizes of embryos (snout-vent length, tail length, total length) at stage G24 after eggs were exposed to different concentrations of butachlor ($0.8\mu g/l$, $1\mu g/l$, $3.5\mu g/l$, $7\mu g/l$) and compared to control in (A) jellied and (B) de-jellied groups (n= 10 for each treatment).



Figure 4. Mortality rate comparison in between controls and eggs exposed to different concentrations of butachlor and between jellied and de-jellied groups. Mortality rate was assessed in each developmental stage from stage G5 to stage G24.

Mortality rate of embryos

In general, embryos at early stages exhibited higher mortality rates than later stages. The mortality percentages of embryos at low concentrations of butachlor (0.1, 0.2, and 0.8μ g/l) in the jellied group were slightly greater than in the de-jellied group; but at higher concentrations of butachlor (1, 2, 3.5, 7 and 14μ g/l), the mortality percentage of embryos was higher in the de-jellied group than in the jellied group (Fig. 4).

Teratology of butachlor (external morphology and histological section examination)

Figures 5 and 6 show the results for the effects of butachlor toxicity at different concentrations and in both



Figure 5. External morphology of embryos selected at stage G24 in the comparison between control (A) and the eggs exposed to different concentrations of butachlor ($2\mu g/l$, $7\mu g/l$, and $14\mu g/l$) showing various abnormalities (B) abdominal oedema, (C) Tail malformation, (D) tail and trunk curvature upward, (E) tail curvature sideward, (F) mouth and tail malformations (n= 5 for each treatment).

jellied and de-jellied groups in B. viridis from external (at stage G24) and internal (at stage G20) views respectively. Different types of malformations including abdominal oedema (55%), axial malformations (trunk and tail curves, 20% and 25% respectively) and deformities in mouth parts were observed at stage G24 exposed to $2\mu g/l$, $7\mu g/l$, and $14\mu g/l$ concentrations of butachlor (Fig. 5). The results from tissue examination for the impact of 7µg/l butachlor on the internal organs at stage 20 showed that the intestine and digestive component's quantity including digestive content in the experimental group was about 50% smaller than in the control group, and a significant part of the trunk space appeared empty of tissue because of trunk inflammation and reduction in the digestive component. Reduction of the number of renal tubules in one side of the trunk was also observed (Fig. 6A, 6B). Considerable reduction of muscle (50%) as well as curvature in dorsal and ventral fins of the tail (25%) was also observed in the experimental group in comparison to the control group (Fig. 6C, 6D).

DISCUSSION

Among the different kinds of pollution, pesticides are partially responsible for amphibian declines (Lehman & Williams, 2010). Although the data for global consumption of pesticides in general are available (PAN Germany, 2012), the quantitative data for each individual pesticide, including butachlor, are incomplete.

Butachlor is applied to rice paddy fields at the time of rice transplantation, as a means of controlling weeds. This

is also the time when the fields are flooded, which can act as a stimulus for breeding in local amphibian populations (Liu et al., 2011). Previous work on the effects of butachlor on amphibians has demonstrated different types of toxic effects on several species (Geng et al., 2005 a,b; Liu et al., 2011). Liu et al. (2011) examined the acute and chronic effects of butachlor at different concentrations on stage G26 tadpoles of the Ranid *Fejervarya limnocharis*. Liu et al. (2011) did not, however, examine effects on prehatching stages. In our experiments, we exposed the spawn of the *Bufonid Bufotes* viridis to butachlor from the start of development, and also assessed the potential role of the jelly coat in limiting the effects of butachlor.

A preliminary experiment was carried out with butachlor at higher concentrations than were ecologically relevant, similar to those used by Liu et al. (2011), but all specimens died soon after treatment. Our results with lower concentrations showed that the butachlor treated embryos took longer to hatch and that embryos hatched at stage G19 (about two stages later than the normal stage), whereas embryos in the control group hatched at stage G17. The delay in hatching may be due to the late development of the hatching gland cells, which are responsible for secreting enzymes and lysing the vitelline membrane and jelly coat (Nokhbatolfoghahai & Downie, 2007). Andrews & George (1994) reported that increasing concentrations of diazinon (an insecticide) cause a decrease in the hatching rate of the eggs of Polypedates maculatus.

In our experiments, the toxicity of butachlor had less impact on the jellied groups than de-jellied groups, and



Figure 6. Transverse sections of embryos at stage G20 (A) trunk region, jellied control- group, (B) trunk region, embryo treated with $7\mu g/l$ butachlor (jellied), (C) tail region, jellied control group, (D) tail region, embryo treated with $7\mu g/l$ butachlor. DF= dorsal fin, M= muscle, RT= renal tubules, S= spinal cord, ST= stomach, VF= ventral fin (deformation of body and tails of embryos showing by asterisks), H&E staining.

LC50 occurred at a higher concentration (14µg/l) in jellied groups compared to de-jellied groups (7µg/l). Pauli et al. (1999) exposed embryos and newly hatched embryos of four Ranid species (R. pipiens, R. clamitans, R. catesbeiana and R. sylvatica) to an insecticide (Mimict 240 LV) and concluded that egg jelly increases resistance and protects embryos from the insecticide in high concentrations. We showed that the jelly coat can protect the embryos from the toxic effects of low levels of butachlor, but has only a limited effect when higher concentrations of butachlor are applied. Although there is no report of the presence of eggs without a jelly coat in nature, the thickness and compositions of jelly coats are reported to differ among amphibian species (Duellman & Trueb, 1994), and this may give species different levels of protection against pollution (Marquis, 2006).

Liu et al. (2011) examined the impact of butachlor on an Asian frog Fejervarya limnocharis and reported LC50 as 0.87mg/l after 96h exposure to the toxin, which was much lower than the 4.8mg/l recommended application rate. Liu et al. (2011) used a range of butachlor concentrations (0.025, 0.05, 0.1, 0.2, 0.4, 0.8mg/l). The concentrations they used are much higher than in our experiment. Li et al. (2016) examined the impact of butachlor on Xenopus laevis and reported LC50 as 0.96 mg/l after 96h exposure to the toxin. One possible explanation for the different values of LC50 is that there is high variation in toxicity resistance between different anuran species (Geng et al., 2005a). In this comparison, the larvae of the species (F. limnocharis) that Liu et al. (2011) used and the embryos of the species (X. laevis) that Li et al. (2016) used have a higher resistance to butachlor than B. viridis.

Another suggestion for the difference may be because of differences in the butachlor manufacturer we used.

There was a negative relationship between butachlor concentration and the size of the embryos (Fig. 3). We also found that where significant differences in TL occurred, there were also significant differences in TAL but not SVL. Jung & Jagoe (1995) found that maximal swimming speed is positively correlated with a tadpole's total length. They concluded that swimming performance is reduced by small body size. Parichy & Kaplan (1995) examined morphology-performance in Bombing orientalis and concluded that larval amphibian vulnerability has a negative correlation to the length of the tail. Our data showed that the length of the tail contributes significantly to the total size of the embryos, which means that a longer tail is likely to have a significant effect on increasing swimming speed. Butachlor, in the higher concentrations that we used, inhibited embryonic growth probably because of its extensive side effects on tail structure and function. According to our results on tail structure, butachlor did not only affect tail length, but also tail morphology and internal tail structure. As we showed in Figure 6, butachlor has impacted on tail muscle development and has reduced muscle formation significantly (by about 50%), which would probably have a high impact on embryonic speed and performance.

The abnormality of the tail, especially curvature, was amongst the highest rate of abnormalities found. The reason may be because the tail in amphibians, including *B. viridis*, has a simple structure and the vertebral column does not extend to the tail. Therefore, the tails are more susceptible in the face of environmental change than other embryonic organs. Such plasticity, in most cases, plays a positive role as an adaptation for new conditions. However, under the presence of toxins such as butachlor, a change in tail structure occurs in such a way that the normal tail deviates from its straight form, which is a negative consequence.

CONCLUSION

This study illustrates that environmental pollutants can have complex effects that are not only concentrationdependent, but vary between different species. In fact, since butachlor (a widely-used herbicide) has environmental toxicity for aquatic animals including amphibians, farmers and growers are advised to replace butachlor (used in the first week after transplanting to eliminate weeds in rice) with an alternative non-chemical/ biological control if applicable. Herbicide mixtures with fewer side effects on non-target organisms are also suggested. Removing toad spawns from paddy fields and transferring them to a secured temporary pool before butachlor spraying is highly recommended.

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FULL PAPER



Amphibian species assemblages in a tropical forest of Bangladesh

Animesh Ghose¹, Jiban Chandra Deb^{1, 2}, Kwaku Brako Dakwa³, Jay Prakash Ray⁴ & AHM Ali Reza⁵

¹Department of Forestry and Environmental Science, Shahjalal University of Science and Technology, Bangladesh. ²School of Geography Planning and Environmental Management, The University of Queensland, Australia. ³Department of Entomology and Wildlife, School of Biological Sciences, University of Cape Coast, Ghana. ⁴Faculty of Veterinary and Animal Science, Sylhet Agricultural University, Bangladesh. ⁵Department of Biological Sciences, Delta State University, Cleveland, MS 38733, U.S.A.

Tropical forests are considered one of the most important biogeographic zones for amphibian species diversity. As a tropical country in Asia, Bangladesh implements different types of forest management practices in its forests, which might affect prevailing forest quality in the existing forest types. The current state of information on the impact of habitat alteration on amphibian species assemblages in Bangladesh is inadequate. To evaluate this, we conducted a study in Khadimnagar National Park (KNP) in north-eastern Bangladesh. We used a combination of several common amphibian study techniques in 15 pre-marked transects covering three major habitat types in KNP: forest edge, forest interior and swamp area. Twelve anuran species belonging to eleven genera and six families were recorded during the study period. *Euphlyctis cyanophlyctis* was the most abundant species, representing 51.7% of the recorded individuals, followed by *Fejervarya* spp. (18.9%); the remaining 10 species altogether recorded less than 30% of the total abundance. *Duttaphrynus melanostictus, Kaloula pulchra* and *Raorchestes parvulus* in particular occurred with very low abundance. Species richness, Shannon-Wiener diversity index, and evenness index value indicated that the amphibian species assemblage in the forest interior is more diverse than the forest edge and swamp area.

Key words: Amphibian, species assemblages, abundance, diversity, habitat types, tropical forest

INTRODUCTION

mphibians are among the planet's most threatened taxa, yet the most abundant vertebrate group in the tropical forest ecosystems, where they have essential roles in trophic dynamics (Hairston, 1987; Welsh & Droege, 2001). For example, they are an essential invertebrate predator in the ecosystem, and also an important part of the food web as prey species (Stebbins & Cohen, 1995). Amphibians are also highly efficient in converting energy into biomass in the entire food web (Stebbins & Cohen, 1995). However, tropical amphibian populations have undergone drastic population declines and extinctions in the recent decades. Nearly one-third of the world's 6,187 species of amphibians are threatened with extinction (Stuart et al., 2004). Declines and extinctions of amphibians have been more severe in the tropics than in other biogeographic regions. Population declines in recent decades have been especially severe, with up to five species going extinct each year (Stuart et al., 2004).

Causes of amphibian species decline are increasingly linked to human activities, and both the number and magnitude of threats has increased dramatically in modern times. Current threats to biodiversity include habitat loss (Young et al., 2001; Stuart et al., 2004), emerging infectious diseases (Daszak et al., 2003), invasive species (Vredenburg, 2004), increased UV-B radiation and chemical contaminants (Hayes et al., 2002; Blaustein et al., 2003; Davidson et al., 2001), and global climate change (Pounds, 2001; Thomas et al., 2004). In most cases, multiple factors are working synergistically to cause amphibian mortality and extinction (Mittermeier et al., 1998; Kiesecker et al., 2001).

Unless rapid and effective actions are implemented to halt the current wave of extinctions, it is likely that we will lose a significant proportion of amphibian diversity by the end of the century. Resolving this environmental crisis requires a combination of ecological, economic and socio-political solutions, which should be initiated on a local scale. Amphibian species diversity and relative abundance at the local scale is vital to monitoring community composition and providing fundamental information for conservation initiatives (Reza & Perry, 2015). Here, this study aims to investigate the amphibian assemblage structure across different habitat types in a tropical forest of Bangladesh in south-eastern Asia. This

Correspondence: AHM Ali Reza (areza@deltastate.edu)

work is specifically significant as no such study currently exists for Khadimnagar National Park in north-eastern Bangladesh.

MATERIALS AND METHODS

Study Site - The study was conducted in Khadimnagar National Park (24°56′-24°58′N latitude, 91°55′- 91°59′E longitude), located in the north-east region of Bangladesh (Fig. 1). Khadimnagar National Park (KNP) is located within the Indo-Burma Biodiversity Hotspot, and harbours some of the richest biota on earth (Mittermeier et al., 1998; Nishat et al., 2002). The Park was established as a Reserved Forest in 1957 and declared as a National Park in 2006 following the Bangladesh Wildlife (Preservation) Amendment Act, 1974 (currently known as Wildlife Conservation and Security Act, 2012). Prior to becoming a protected area of about 7 sq. kilometres, KNP suffered huge habitat alteration through logging and plantation. There was an attempt to recover the forest through mixed and monoculture plantation at the peripheries and along the main brook prior to its adoption as a national park. However, KNP is considered one of the important protected areas in Bangladesh due to its diverse flora and fauna (Sobuj & Rahman, 2011).

Mean annual temperatures of KNP varies between 18.9°C and 30.7°C. The average annual precipitation is 3,400mm, most of which occurs between June and September (BBS/UNDP, 2005). The topography of the site is undulating with slopes and hillocks, ranging from 10 to 50 m asl (Sobuj & Rahman, 2011; Ghose & Bhuiyan, 2012). KNP is surrounded by seven tea gardens and

the anthropogenic disturbances resulting from the tea production activities might have some influence on the amphibian population of the national park.

Field Survey - We conducted fieldwork between April 2012 and March 2013, when a total of ~32 days were spent in the field covering all major seasons of Bangladesh: summer (hot and humid weather coincides with south-western wind from March to June), monsoon (mostly comfortable, rainy monsoon from July to October), and winter (cool, but very comfortable temperature with low precipitation from November to February). To maximise the data collection success, we used a combination of common amphibian population sampling techniques. We primarily used Visual Encounter Surveys (Crump & Scott, 1994; Doan, 2003) (total ~480 man-hours), supplemented by amphibian auditory or call surveys (Pierce & Gutzwiller, 2004), some trapping efforts (PVC pipes as artificial refuge and bucket traps), and opportunistic collection of specimens from local community members.

We categorised the study site into three major habitat types: i) Forest interior: the core area of the national park with heterogenic habitat types where plantation was initiated in late 1950s in a hilly landscape; ii) Forest edge: the dry peripheral land of national park that continued with tea plantation; iii) Swamp area: habitat composed of streams, seasonal perennial water bodies and adjacent fallen land. Streams in KNP have either north-south or east-west direction and the major source of household water for tea garden inhabitants.

During VES, we used a time-constrained active



Figure 1. Map of the study area, Khadimnagar National Park in Bangladesh.

search for amphibians in fifteen pre-marked transects throughout the three major habitat types: six transects in the forest interior, four transects in forest edge, and five transects in swamp area. The routes were selected based on the available habitat types proportionately with roughly 150 m length and were surveyed with a team of three members, which covered both day and night time searches.

We checked the PVC pipes as artificial refuge placed in different parts of the forest on a weekly basis and amphibians captured in the pipes were identified, recorded and released in the same location. For call survey, we positioned ourselves in a quiet spot in our study site at least half an hour after sunset and used a smartphone to record all the calls for 10 to 15 minutes at a time. We repeated the process up to three times depending on the amphibian activities of the night and used the recorded files later for species identification in the laboratory. When performing amphibian auditory or call surveys, in most cases, we were able to identify frog calls during their breeding season using some of the techniques discussed in Roy and Elepfandt (1993). We photographed each recorded species during our study and also the available captured ones during call surveys.

Data analysis - We calculated the average Shannon-Wiener diversity index (Magurran, 1988), evenness (the variation in the abundance of individuals per species within a community) and dominance (proportional importance of the most abundant species) (Magurran, 1988) for each habitat type by using PAST software (Hammer et al., 2001). We used the 'BiodiversityR' package of R software (Kindt & Coe, 2005) to extract rank abundance curves and species rarefaction curves. Kruskal-Wallis test (Spurrier, 2003), which is free of assumptions of normality, was used to determine if there were significant differences in abundance between habitat types using a significance level of p=0.05. Microsoft Excel[®] 2007 software was used to calculate and compare the percentage of occurrence of each species in the three major habitat types by dividing the number of plots where a particular species was present by the total number of plots.

RESULTS

We encountered a total of 497 individuals– all anurans – belonging to 6 families, 11 genera, and 12 species, (Table 1, Fig. 2). The families Dicroglossidae and Microhylidae (6 species) were the most abundant, followed by Megophryidae (2 species), Rhacophoridae (2 species) Bufonidae (1 species), and Ranidae (1 species). We noticed that the number of species recorded during our fieldwork is lower than the recorded species from other studies like Hasan and Feeroz (2014). No caecilian and salamander species were recorded from the study area during our fieldwork. The species rarefaction curves suggest a larger sample size would not have added many more species to our findings (Fig. 3).

Euphlyctis cyanophlyctis was the most abundant anuran species, representing 51.7% of the total abundance, followed by *Fejervarya* spp. (18.9%) (Fig. 4).



Figure 2. Some of the anuran species encountered in the Khadimnagar National Park: A) *E. cyanophlyctis*; B) *Fejervaya* spp.; C) *H. tigerinus*; D) *K. pulchra*; E) *H. leptoglossa*; F) *R. parvulus*; G) *M. ornata* and H) *L. smithi*.

Table 1. Amphibian species encountered in the differenthabitats of Khadimnagar National Park during the study[x = species recorded]

		F	labitat ty	ре
Family	Species	Forest edge	Forest interior	Swamp area
Bufonidae	Duttaphrynus melanostictus		х	х
Dicroglossidae	Euphlyctis cyanophlyctis	х	х	х
	Fejervarya spp.	х	х	х
	Hoplobatrachus tigerinus	х	х	
Megophyridae	Leptobrachium smithi	х	х	х
	Xenophrys parva	х	х	х
Microhylidae	Kaloula pulchra		х	
	Microhyla ornata	х	х	х
	M. rubra	х	х	
Ranidae	Hylarana leptoglossa	х	х	х
Rhacophoridae	Polypedates leucomystax	х	х	х
	Raorchestes parvulus		х	

There has been mounting evidence of this across Asia, and Islam et al. (2008) suggests that there could be four groups within a *Fejervarya* complex in Bangladesh.

However, we still used the traditional classification due to the difficulties of their identification in the field. The remaining species represented less than 30% of the total abundance, ranging from 2 to 29 individuals per species. Duttaphrynus melanostictus, Kaloula pulchra



Figure 3. Species rarefaction curve for the study area



Figure 4. Percentage occurrence of amphibian species in the study plots of all the habitat types combined.

and *Raorchestes parvulus* had relatively low abundances with five, three and two records respectively during our fieldwork (Fig. 4). We found *E. cyanophlyctis* to be the most common species in the study area, recorded in 12 out of 15 transects (highest frequency of sightings, Fig. 5). *Fejervarya* spp. and *Leptobrachium smithi* were found as the second and third most commonly found species (with 83 and 35 abundance score respectively) on the transects, while we found less than 20 individuals of the remaining nine species (Fig. 5).

E. cyanophlyctis was the dominant species across all habitat types with the highest abundance and highest percentage of occurrence in each habitat type, followed by *F.* spp.; *K. pulchra* and *R. parvulus* only the forest interior (Fig. 6). All 12 amphibian species were recorded from the forest interior, whereas only seven amphibian species were found in all three habitat types (Table 1, Fig. 6). Three amphibian species (*D. melanostictus, K. pulchra*,

and *R. parvulus*) were not recorded from forest edge, and four species (*Hopobatrachus tigerinus*, *K. pulchra*, *M. rubra*, and *R. parvulus*) were not found in the swamp area.

The evenness index showed that the highest value for the forest edge was 0.90, while for the forest interior and swamp area this was 0.82 and 0.85 respectively (Table 2). The forest edge was recorded as the lowest dominance score (0.16) as compared to those of forest interior (0.18) and swamp area (0.20; Table 2). However, the Kruskal Wallis test indicated that there was no significant differences among the three habitat types in the study area in terms of amphibian species abundance (Kruskal-Wallis $\chi^2 = 0.33$, p-value = 0.85) (Fig. 7).

Overall, the species richness, Shannon-Wiener diversity index, and evenness index value indicated that the amphibian assemblage in the forest interior was more diverse than the other two habitat types: forest edge and swamp area (Table 2).



Figure 5. Rank abundance curve of amphibian species in Khadimnagar National Park, Bangladesh.

	Forest edge	Forest interior	Swamp area
Richness	9	12	8
Individuals	40	47	41
Dominance	0.16	0.18	0.20
Shannon index	1.98	2.04	1.78
Evenness	0.90	0.82	0.86

Table 2.	Species	diversity	indices	of the	three	habitat	types
of the stu	udy area.						

DISCUSSION

Studies have addressed whether logging or habitat disturbance have any effect on the amphibian assemblages in different forest habitat types (Atauri & de Lucio, 2001; deMaynadier & Hunter, 1995; Dupuis & Steventon, 1999) but not many studies of this type exist for Bangladesh. As the richness of amphibians is more closely related to the abundance of certain land-use types (Atauri & de Lucio, 2001), the importance of habitat disturbances associated with logging and agriculture to the diversity, abundance, and distribution of amphibians in the KNP cannot be over-emphasised. The forest interior depicted the condition of the original forest prior to logging, while the forest edges portray the condition of adjacent agricultural land emerging from a secondary forest types. The original forest of the national park harbours all of the 12 species recorded during our study period, while the forest edge harbours 75% of them, and thus both habitat types support important richness of the amphibian species diversity. It has been suggested that many species can persist after intensive logging (Edwards et al., 2011; Gibson et al., 2011) and our current study results are consistent with this theory, and provided evidence that highly logged forests retain many of the species. It might be difficult to prove this theory in case of KNP amphibians as we do not have any reliable baseline data. The presence of *R. parvulus* and *K. pulchra* with low abundance only in the forest interior suggests that the populations might recover with better forest management practices.

Ribeiro et al. 2012 found a high number of species richness in the riparian zone (similar to the 'swamp area' of this study) compared to the non-riparian zone in an Amazonian forest. Our study results, on the other hand, indicated the opposite, with higher species richness in the forest interior compared with the forest edge and swamp area, possibly due to the year-round supply of water as well as damp habitat condition in the forest interior. The diversity of amphibians in the swamp area was low compared to the forest edge or forest interior. The swampy area of KNP has lost most of the original forest and has been taken over by teak plantations. However, swampy areas provide critical breeding habitat for amphibians, an important site to maintain healthy amphibian species diversity.

All 12 species of amphibians recorded during our study area are listed as 'Least Concern' by the IUCN Red List of Threatened Species (IUCN, 2017) in view of their wide distribution, tolerance of a broad range of habitats, and presumed large populations. These species are unlikely to be declining fast enough to qualify for listing in a more threatened category. While only E. cyanophlyctis is said to be fairly common in the study area, D. melanostictus, K. pulchra and R. parvulus are relatively rare in the park. E. cyanophlyctis is known to be very widespread in Bangladesh, and tolerates a high degree of habitat modification. There are no known threats to this species in other parts of Asia, e.g. Iran and Afghanistan (Khan et al., 2009). A complete explanation of the low abundance and low frequencies of occurrence of all other amphibian species at KNP remains uncertain.

H. tigerinus is a delicacy in Asia (Flores Nava, 2005), but the harvest data on this species is not available for KNP and it is difficult to estimate the exploitation pressure. van Dijk and Ohler (2009) described *R. parvulus* as a



Figure 6. Percentage occurrence of amphibian species in three different habitat types in the study plots.





Figure 7. Amphibian species abundance among different habitat types in Khadimnagar National Park, Bangladesh.

widespread species that primarily occurs in open habitats including forest edges, however, our study suggests that the species is only found in the forest interior in KNP. *R. parvulus* is a rare species in Bangladesh with very low abundance and has been added to the country's species list very recently (Ghose & Bhuiyan, 2012).

Protected areas harbour most of the amphibian species diversity in Bangladesh (Reza 2014) even though the species diversity is generally low in KNP, one of the 19 protected areas of the country. Some potential threats to the low amphibian species diversity include, but are not limited to, anthropogenic disturbances, pollution, infectious diseases, land use conversions, and climate change. In addition, conservation management of amphibians has not been on the priority list of the forest management practices in Bangladesh. Of course, conservation decisions are not only based on a single taxonomic group, so we need to incorporate amphibians and emphasise the importance of habitat heterogeneity in any future conservation initiatives for their long-term survival in Khadimnagar National Park.

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FULL PAPER



Effects of Chinese tallow leaf litter on water chemistry and surfacing behaviour of anuran larvae

Daniel Saenz¹ & Cory K. Adams

¹ Southern Research Station, USDA Forest Service, 506 Hayter Street, Nacogdoches, Texas 75965, USA.

The establishment of exotic invasive species, including plants, has been linked to the decline of some amphibian populations. Of particular concern with invasive plants, from an amphibian conservation perspective, is that they are disproportionately wetland or riparian species. Recent evidence suggests that Chinese tallow (Triadica sebifera), an exotic deciduous tree species, is expanding its range and becoming more abundant where it occurs in the United States. This is particularly relevant to amphibian conservation considering that Chinese tallow tends to invade wetlands, and recent studies have demonstrated that the leaf litter causes mortality of anuran eggs and larvae by reducing the dissolved oxygen and pH of water. The lethal effect of Chinese tallow leaf litter is short lived and concentrated soon after leaf fall, typically December through to February in the south-eastern United States. In this study, we were interested in determining the sub-lethal effects of Chinese tallow leaf litter on the surfacing frequency and air-gulping behaviour of overwintering anuran larvae. Lithobates catesbeianus and L. clamitans clamitans are two frog species that commonly overwinter as aquatic larvae and extensively overlap in range with invasive Chinese tallow, which may expose their tadpoles to the deleterious effects of the leaf litter. We conducted experiments where we exposed tadpoles to four different concentrations of tallow leaf litter and recorded water chemistry and tadpole surfacing frequency. We found that as Chinese tallow concentration increased, oxygen levels decreased. Both anuran species responded similarly to our treatments and dissolved oxygen levels, where tadpoles swam to the water's surface to air gulp at a significantly higher rate in the treatments with greater tallow concentration. Such changes in behaviour induced by Chinese tallow could have negative consequences on tadpole foraging efficiency and predator avoidance, ultimately reducing fitness. As biological invasions will continue to be an important part of global change, more attention should be given to sub-lethal impacts, as they pertain to fitness.

Key words: Anuran larvae; air gulping; Chinese tallow; leaf litter; invasive plants

INTRODUCTION

'he establishment of exotic invasive species has been linked to the decline of some amphibian populations (Doubledee et al., 2003; Ryan et al., 2009; Bucciarelli et al., 2014). Introduced vertebrates, such as fish and exotic amphibians, are known to compete with and induce behavioural changes in native amphibians, and reduce or eliminate amphibian populations through direct predation (Lawler et al., 1999; Crossland, 2000; Knapp & Matthews, 2000; Pyke & White, 2000; Gillespie, 2001; Smith, 2005). Even invasive invertebrates have been known to reduce the breeding success of native amphibians (Gamradt & Kats, 1996) and cause population declines by degrading foraging habitat (Maerz et al., 2009). Invasive plants can affect chemical and physical habitat features, influencing decomposition and nutrient dynamics that can alter the trophic structure of invaded ecosystems, which could ultimately affect native amphibian populations (Brooks et al., 2004; Maerz et al., 2005a; Brown et al., 2006; Maerz et al., 2010; Martin & Murray, 2011; Watling et al., 2011). Amphibian larvae exposed to Amur honeysuckle (Lonicera maackii) in

aquatic environments make more trips to the water surface (Watling et al., 2011; Hickman & Watling, 2014) and have shown reduced survival compared to larvae exposed to native plants, likely due to effects of phytochemicals acting on tadpole respiratory surfaces (Brown et al., 2006; Watling et al., 2011). Maerz et al. (2005b) speculated that gill tissue damage, caused by compounds in the extract of *L. maackii*, might compromise the ability of the anuran larvae to take up oxygen. Invasive plants not only influence amphibian biology directly, they can have strong indirect effects on amphibians through decreasing dissolved oxygen, as suggested in a study on the effects of Chinese tallow (*Triadica sebifera*) on amphibian egg hatching (Adams & Saenz, 2012).

Recent studies have shown that exposure to Chinese tallow leaf litter has negative effects on tadpole survival compared to leaf litter of native tree species (Leonard, 2008; Cotten et al., 2012). Of concern are the changes in water chemistry, particularly decreases in dissolved oxygen, caused by Chinese tallow leaf litter (Adams & Saenz, 2012; Saenz et al., 2013). Moderate concentrations of Chinese tallow leaf litter will decrease

Correspondence: Daniel Saenz (dsaenz@fs.fed.us)

pH and dissolved oxygen levels below the threshold for anuran egg survival (Adams & Saenz, 2012) and anuran larvae survival (Cotten et al., 2012; Saenz et al., 2013).

In addition to affecting survival, low dissolved oxygen has been linked to marked changes in behaviour in amphibian larvae (West & Burggren, 1982; Feder, 1983a; Wassersug & Feder, 1983; Crowder et al., 1998). Lithobates berlandieri (formerly Rana berlandieri) larvae exposed to hypoxia increased their aerial consumption of oxygen (Feder 1983a). Increased air-breathing was also observed in bullfrog (L. catesbeianus, formerly R. catesbeiana) larvae as dissolved oxygen levels decreased (Crowder et al., 1998) and West & Burggren (1982) found that L. catesbeianus took no air breaths at hyperoxia, had low levels of air breathing at normoxia, but took more breaths in more progressively hypoxic states. To date, we know of no studies that explicitly link invasive plants with hypoxia and altered behaviour of native aquatic amphibians.

Given that Chinese tallow leaf litter has been experimentally shown to decrease dissolved oxygen levels in water and cause mortality in anuran larvae (Adams & Saenz, 2012; Saenz et al., 2013) and lower oxygen levels are known to affect rates of air breathing in larval amphibians, we suggest the potential for induced behavioural responses by native amphibian species in the presence of sub-lethal concentrations of tallow leaf litter. We suggest that tallow will affect activity by lowering dissolved oxygen to levels where cutaneous and gill respiration by larval anurans would need augmentation with aerial oxygen uptake. In addition, aerial uptake of oxygen could affect the larva's relative position in the water column, as air-breathing affects buoyancy (Wassersug & Feder, 1983). The movement required for air-breathing is of particular concern because activity levels in anuran larvae have been associated with alerting predators to the prey's location, resulting in increased predation and reduction in foraging time (Feder, 1983b; Moore & Townsend, 1998; Watling et al., 2011; Hickman & Watling, 2014).

Our primary objective was to determine if low concentrations of Chinese tallow leaf litter can cause behavioural changes in aquatic larval anurans. First, we explored the effects of different concentrations of Chinese tallow leaf litter on water chemistry. We then determined if increasing concentrations of tallow leaf litter increase air gulping behaviour of anuran larvae and affect their resting position in the water column. We then explored the relationship between dissolved oxygen and observed tadpole behaviour (see Moore & Townsend, 1998; McIntyre & McCollum, 2000), in the context of invasive plant/amphibian interactions.

MATERIALS AND METHODS

Study Species: Invasive Tree — The invasive Chinese tallow is a fast-growing deciduous tree native to China and Japan (Bruce et al., 1997). Currently, the invasive distribution of Chinese tallow includes much of the South Atlantic and Gulf Coastal Plain of the United States (Conner et al., 2002) and it is now the fifth most numerous tree species in the entire state of Louisiana

and the fifth most common species found in east Texas (Oswalt, 2010). The Chinese tallow has high reproductive potential and is known to replace native vegetation and produce monocultures, particularly in wetland habitats (Cameron & Spencer, 1989; Jubinsky & Anderson, 1996; Bruce et al., 1997).

All Chinese tallow leaves used in the study were collected from trees in the Stephen F. Austin Experimental Forest in Nacogdoches County, Texas, in the autumn of 2009. We collected newly senesced Chinese tallow leaves, those that had changed colour from green to red or orange, by stripping them directly from tallow trees or by raking freshly fallen leaves from the ground. Leaves were immediately air-dried in a dark climate-controlled room and stored in black plastic bags until used in experiments. Although leaves used in this study had been stored for over eight months, earlier work suggests that the leaves would not lose their "potency" in this amount of time and had similar effects on water chemistry as leaves that had been stored for shorter periods of time (Saenz et al., 2013).

Study Species: Native Anurans — American bullfrog (Lithobates catesbeianus) and bronze frog (L. clamitans clamitans) larvae were the focal animal subjects of this study. Both species breed in the summer months and regularly overwinter as larvae (Saenz et al., 2006). The ranges of both anuran species overlap significantly where Chinese tallow occurs (Conant & Collins, 1998; Conner et al., 2002), making them potentially vulnerable to negative effects of this invasive plant. All anuran larvae were collected in August 2010 by dipnet from ponds in the Davy Crockett National Forest in Houston County, and the Stephen F. Austin Experimental Forest in Nacogdoches County, Texas. Upon capture, we examined all tadpoles for signs of tail damage and kept only individuals that appeared undamaged. Tadpoles were housed in plastic bins filled with aged tap water at a density of approximately one tadpole per litre and provisioned with ground tropical fish flakes, ad libitum. Tadpoles were kept in the lab no more than one week before being used in behaviour trials.

Behaviour Trials — All behaviour trials were conducted in commercially available 5-gallon (18.93 L) glass aquaria in a 22 °C climate-controlled room. Ten litres of dechlorinated aged tap water, maintained at a constant temperature of 21.5 °C, was added to each aquarium resulting in approximately 12 cm of water depth. Mesh bags, resembling teabags, were added to each aquarium and provisioned with Chinese tallow leaves according to concentration levels for each treatment. The bags all sank to the bottom of each aquarium. Our study included four treatments: control (0.0 g tallow/L water, included an empty mesh bag), low concentration (0.15 g tallow/L water), medium concentration (0.25 g tallow/L water) and high concentration (1.0 g tallow/L water). For reference, a typical dry averaged-sized Chinese tallow leaf weighs approximately 0.25 g (Saenz, unpublished data). Bags with tallow leaves were allowed to soak for 48 hours prior to adding the anuran larvae. No aeration was added to the experimental aquaria at any time and the tadpoles were not fed during the trials. We measured dissolved oxygen (DO; mg/L), pH, and salinity of water

Table 1. Total length and Gosner stage of tadpoles exposed to four concentrations of Chinese tallow leaf litter (control = 0.0 g/L, low = 0.15 g/L, medium = 0.25 g/L, and high = 1.0 g/L). Within anuran experiments, different letters in a column represent significant differences across treatments

	Bullfrog			Bronze frog				
(n=128 tadpoles)			(n=120 tadpoles)					
Total length		Gos	ner	Total length Go		osner		
Treatment	SE		SI	E	SI	E	SE	E
Control	64.81 A	2.77	33.56 A	0.82	35.94 A	2.19	28.93 A	0.54
Low	65.28 A	2.90	33.16 A	0.85	34.49 A	2.06	28.57 A	0.48
Medium	65.52 A	2.79	33.41 A	0.81	35.13 A	2.20	28.93 A	0.55
High	64.53 A	2.74	33.25 A	0.77	35.18 A	2.09	28.63 A	0.52

in each trial immediately prior to behaviour trials using a Hach Hydrolab Quanta[®]. The Hach Hydrolab Quanta[®] uses the Practical Salinity Scale (PSS) to measure salinity. One tadpole was placed in each aquarium containing the Chinese tallow treatments, and was allowed to acclimate for 30 minutes prior to activity trials. Each tadpole was used only once in this study. All larvae were measured (total length) and developmental stage was determined after each trial (Gosner 1960). Developmental stage and total length did not differ among treatments for bullfrog (Gosner stage, F = 0.07, P = 0.9756, df = 3,117; total length, *F* = 0.04, *P* = 0.9899, df = 3,117) or for bronze frog larvae (Gosner stage, F = 0.17, P = 0.9175, df = 3,111; total length, F = 0.09, P = 0.9674, df = 3,111) (Table 1). Overall, bullfrog larvae averaged 65.04 mm (SE = 1.38) in total length with a mean developmental stage of 33.3 (SE = 0.40) while bronze frogs averaged 35.18 mm (SE = 1.06) in total length with a mean developmental stage of 28.8 (SE = 0.26).

Each aquarium setup was used for five trials before replacing with fresh water and tallow treatments. The five trials were considered a block. Trials consisted of a 10-minute observation period where the total number of air gulps taken by the tadpole and the relative resting position (when not actively swimming to the surface) in the water column were recorded. Air gulps were recorded only when a gas bubble was expelled from the mouth of the tadpole following surfacing behaviour of the animal. Trials were replicated 30 times per treatment for bronze frogs and 32 times per treatment for bullfrogs, resulting in 248 total trials.

In addition to laboratory trials, we sampled the water chemistry of 51 wetlands in the Davy Crockett National Forest in eastern Texas during 27-30 October 2009 as a reference to compare to our laboratory treatments. It is important to note that the samples were taken preceding typical annual leaf-fall and all sites sampled were devoid of the invasive Chinese tallow tree. We used a Hach Hydrolab Quanta[®] to measure dissolved oxygen, pH, and salinity from naturally occurring wetlands to use as a reference to compare to our laboratory treatments.

Analyses —For each anuran species, we compared water chemistry variables and tadpole size and developmental stage in the different treatments with a mixed effect, complete block ANOVA (block considered a random effect). The Tukey-Kramer adjustment was

used to control the error rate of pairwise comparisons of least-squares means. Air gulping rates across treatments were compared with a generalised linear mixed model (block considered a random effect) with a Poisson distribution. We modelled the relationship between gulp rate and dissolved oxygen with Poisson regression, including block as a random effect. A Cochran-Armitage test was used to determine if a trend was present in the relationship between buoyant behaviour and tallow leaf concentration. All results were considered statistically significant at the alpha < 0.05 level (version 9.1, SAS institute, 2003).

RESULTS

Dissolved oxygen and pH values differed significantly among treatments in the bullfrog (DO, F = 792.05, P< 0.0001, df = 3,117; pH, F = 1157.13, P < 0.0001, df = 3,117;) and bronze frog (DO, F = 955.08, P < 0.0001, df = 3,111; pH, F = 213.25, P < 0.0001, df = 3,111;) experiments with no block effect. Post hoc tests revealed that DO and pH levels were lower in treatments with greater Chinese tallow leaf litter concentrations. Salinity values had very little variation within treatments and were consistent across treatments (Table 2).

Bullfrog air gulping rates differed among treatments (F = 52.02, P < 0.0001, df = 3,117) with post hoc tests indicating that treatments with higher concentration of Chinese tallow leaf litter induced higher gulping rates with no block effect (Fig. 1), although tadpole response did not differ in the low concentration treatment in comparison to the control. Results for bronze frog air gulping trials (F = 50.44, P < 0.0001, df = 3,111) were very similar to the results from the bullfrog experiments (Fig. 1). When we considered only trials containing Chinese tallow leaf litter, Poisson regression indicated a significant relationship between air gulping and dissolved oxygen for bullfrogs and bronze frogs (Table 3; Fig. 2).

The typical resting position of the tadpoles of both species was at the bottom of the aquarium. A trend of floating behaviour, with increasing Chinese tallow concentration, was observed in bronze frogs with no block effect (Z = -5.73; P < 0.0001). Bronze frogs were never observed floating in the control and the low concentration treatments while 36% and 64% of the individuals were observed floating at the water's surface

Table 2. Water chemistry results from 51 wetlands from the Davy Crockett National Forest and from bullfrog and bronze frog experiments. Tadpoles were exposed to four concentrations of Chinese tallow leaf litter (control = 0.0 g/L, low = 0.15 g/L, medium = 0.25 g/L, and high = 1.0 g/L). Within anuran experiments, different letters in a column represent significant differences across treatments.

	Disso Oxyg	lved gen	рН		Salinity	
Source		SE		SE		SE
treatment						
Wetlands	6.63	0.16	6.06	0.07	0.03	0.01
Range	4.90-8	8.69	4.11-0	6.64	0.01-	0.05
Bullfrog exp	eriments	i				
Control	8.24 A	0.03	7.59 A	0.02	0.11	0.00
Low	3.23 B	0.25	6.72 B	0.04	0.11	0.00
Medium	0.64 C	0.09	6.51 C	0.03	0.11	0.00
High	0.12 D	0.01	5.46 D	0.06	0.12	0.00
Bronze frog	experime	ents				
Control	8.20 A	0.06	6.89 A	0.05	0.11	0.00
Low	3.15 B	0.23	6.49 B	0.04	0.11	0.00
Medium	0.64 C	0.07	6.28 C	0.03	0.11	0.00
High	0.17 D	0.01	5.49 D	0.05	0.12	0.00

in the medium and high concentration treatments, respectively. Floating behaviour was observed on only one occasion in the bullfrog experiments, in a high concentration trial; therefore, no analyses were performed.

DISCUSSION

Chinese tallow leaf litter concentration significantly influenced air gulping activity in the two species of anuran larvae in our study. Even at relatively low concentrations, submerged Chinese tallow leaf litter had significant effects on the behaviour of bullfrog and bronze frog larvae, suggesting that the results of interactions between the invasive plant and native anurans may be more complex than simple mortality versus survival outcomes demonstrated in other recent studies (Watling et al., 2012; Adams & Saenz, 2012; Cotten et al., 2012; Saenz et al., 2013).

We suggest that changes in dissolved oxygen levels, caused by the introduction of Chinese tallow leaf litter, was the driver that regulated gulping behaviour in our experiments. Results from many previous studies have demonstrated the link between dissolved oxygen and air gulping behaviour by amphibian larvae (West & Burggren, 1982; Feder, 1983a; Wassersug & Feder, 1983; Crowder et al., 1998). Even under normoxic aquatic conditions, some amphibian larvae species still regularly take air breaths (West & Burggren 1982). Our results were consistent with their findings, as we regularly





Figure 1. Bar graphs depicting the mean rate of air gulps taken by bullfrog and bronze frog tadpoles in each of the Chinese tallow treatments over a ten minute timeperiod. Different letters over the bars indicate statistical differences among treatments at the P < 0.05 level. Error bars represent Standard Error.



Figure 2. Predicted number of air gulps by bullfrog and bronze frog tadpoles over a ten minute time-period (as calculated with a Poisson regression model) in relation to dissolved oxygen. Points represent data for each trial from all four treatments.

Table 3. Results of regression analyses exploring the relationship between dissolved oxygen and gulp rate for tadpoles exposed to four concentrations of Chinese tallow leaf litter (control = 0.0 g/L, low = 0.15 g/L, medium = 0.25 g/L, and high = 1.0 g/L).

Bullfrog (<i>n</i> =128 tadpoles)			Bronze frog (<i>n</i> =120 tadpoles)					
Variable	ß	SE	t	Р	ß	SE	t	Р
Intercept	1.83	0.08	23.15	<0.0001	1.96	0.09	21.35	<0.0001
DO	-0.54	0.06	-8.85	<0.0001	-0.48	0.06	-8.60	0.0004

observed air-gulping behaviour in our control trials that averaged more than 8 mg DO per litre of water. The primary difference we found between treatments was in activity, not with position in the water column. Although the consistent trend was higher gulping frequency associated with higher concentration of leaf litter and lower oxygen (Figs. 1 & 2), statistically significant differences from the control were not apparent until compared with the medium concentration treatment of 0.25 g of leaf litter per litre of water. We suggest that a threshold for inducing a significant increase in the rate of gulping behaviour in amphibian larvae exists somewhere between 0.15 g and 0.25 g of Chinese tallow leaf litter per litre of water. Interestingly, our data showed a marked decrease in oxygen levels between the low and medium treatments, despite only a small difference in the amount of leaf litter (Table 2).

In addition to increased gulping rates, bronze frog tadpoles exposed to higher levels of Chinese tallow leaf litter had a significantly higher tendency to float at the surface of the water. This behaviour could be an indication of extreme stress induced by hypoxic conditions mediated by a positional change in the water column by the tadpoles in an attempt to seek more dissolved oxygen at the air/water interface. However, the position in the water column could simply be a result of changes in buoyancy caused by intake of air at the water's surface (Wassersug & Feder, 1983). The floating behaviour was never observed in the control or low concentration treatments, suggesting that the extreme conditions found in the other treatments were required to induce this particular behaviour. Lithobates pipiens (formerly Rana pipiens) tadpoles at earlier developmental stages were more apt to float near the surface than individuals at later stages (Wassersug & Siebert, 1975). Interestingly, the buoyant/floating behaviour in our study was primarily observed in bronze frogs and only once in 128 bullfrog trials. Animal size and developmental stage could be factors affecting our observations, as bullfrog larvae were much larger and more developed than bronze frog tadpoles (Table 1). Early stage tadpoles may lack the lung development and vascularisation to exchange oxygen via air gulping behaviour as efficiently as later stage tadpoles (Strawinski, 1956; Wassersug & Siebert, 1975), thus taking position near the water's surface may aid in obtaining oxygen subcutaneously if oxygen levels are higher there than deeper water.

Lung development also differs among species and could be an important factor affecting behaviour and survival of species that develop lungs later in the larval stage (Wassersug & Siebert, 1975). The differences in survival between the toad (Anaxyrus americanus) and the plains leopard frog (L. blairi) observed in the Watling et al. (2011) study could be related to ontogenetic differences in lung development between the taxonomic groups. Wassersug & Siebert (1975) dissected toads (Bufo woodhousii) and found that their lungs developed much later in the larval stage than in northern leopard frogs (*R. pipiens*). Both species in our study are in the Family Ranidae, as are the leopard frogs (Hillis & Davis, 1986), and are known to develop lungs early in the larval stage. Thus, ranids should be better suited to behaviourally compensate for low dissolved oxygen levels induced by Chinese tallow than some other native species of anuran such as toads. Fortunately, most of the anuran species that are likely to occur in the larval stage during the winter months, when Chinese tallow leaf litter is most likely to affect water chemistry (Saenz et al., 2013), are ranid species. The species most susceptible to low oxygen levels, the toads, are restricted to breeding in the summer months (Saenz et al., 2006) and will likely not be affected, as dissolved oxygen has been shown to rebound over time (Saenz et al., 2013). Time and energy costs are associated with air breathing, which suggests that changes in tadpole behaviour could have effects on fitness (Kramer, 1983). Based on extrapolations from our results, bullfrog larvae would average approximately 32 more forays to the water's surface per hour for aerial consumption of oxygen in the high concentration treatment (1.0 g tallow/L water) compared to the control, while bronze frog larvae in the high concentration treatments would surface, on average, 35 more times per hour than in the control aquaria. Obviously, natural wetlands would vary in depth, as would the energy expenditure to secure oxygen at the water's surface.

We argue that predation risk should be considered as an effect of Chinese tallow exposure, given the amount of time that larvae would be exposed to predators while engaging in surfacing behaviour, as it is well known that activity levels are positively correlated with predation in aquatic ecosystems (Woodward, 1983; Wolf & Kramer, 1987; Skelly, 1996; Bridges, 2002). Moore & Townsend (1998) found that lower dissolved oxygen increased surfacing by *L. clamitans* and increased predation by fishing spiders (Dolomedes triton) and Feder (1983b) showed that air breathing in R. berlandieri affected predation by the painted turtle (Chysemys picta), concluding that air breathing increased visual recognition by a visual predator. Despite the risk of predation, larval amphibians will continue engage in risk-prone surfacing behaviour when exposed to the leachate of some exotic plants (Hickman & Watling, 2014). The two anuran species in our study might be less susceptible to fish predators due to their unpalatable skin (Adams et al., 2011); however, they are still at risk of predation by invertebrate predators that are likely more abundant in ephemeral wetlands that could be at greater risk of oxygen depletion due to Chinese tallow leaf litter.

A closer look at water chemistry results revealed that dissolved oxygen levels in natural wetlands were approximately twice as high as in the lowest Chinese tallow treatment in our experiments; however, pH in our treatments was well within the normal range of naturally occurring wetlands in the study sites. This suggests that even modest invasion of Chinese tallow could significantly impact some water chemistry variables that could, in turn, affect the behaviour and survival of native amphibians. We do know, based on our results, that much variation occurs in the concentration of oxygen in natural wetlands, but we are not certain of the effects that Chinese tallow will have on water chemistry in nature.

Biological invasions are an important part of global change, as suggested by Dukes & Mooney (1999), and it is a complex problem that is presenting challenges to native biota that we are only beginning to understand. Given that there are over 2,000 species of alien plants established in the United States alone (Vitousek et al., 1997) and invasive plant species are disproportionately wetland or riparian species (Zedler & Kercher, 2004), it is logical to conclude that Chinese tallow is only one of many invasive plants that pose a threat to native aquatic ecosystems. Our results revealed that extremely low levels of Chinese tallow could have profound impacts on species and potentially ecosystems. Small changes in the proportion of invasive trees and shrubs could have subtle sub-lethal effects that might go unnoticed but are still important ecologically. Given the recent laboratory studies results indicating lower amphibian survival when exposed to Chinese tallow and other invasive plant species, more investigation to assess the impact of invasive plants is warranted and more attention should be given to sub-lethal impacts, as they pertain to fitness.

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FULL PAPER



Social spacing of the montane lizard Tropidurus montanus

Conrado A. B. Galdino¹ Stefania P. R. Ventura¹, Clara Cabral Lisboa¹ & Robert J. Young²

¹ Programa de Pós-Graduação em Biologia da PUC-Minas, prédio 41. Av. Dom José Gaspar, 500 - Coração Eucarístico, 30535-610, Belo Horizonte-MG, Brazil.
² University of Salford Manchester Peel Building - Room G21 Salford, M5 4WT, U.K.

The way in which individuals use their surrounding space can be key to understanding species' sociobiology. We studied the social spacing of the lizard *Tropidurus montanus*. Males were found to have larger home ranges than females. Male body size was not associated with home range area, and the number of females associated with a male's home range was small when compared to other Iguanian lizards, thus forming small harems. The larger home range areas and overlaps found during the final period of the reproductive season might occur as a consequence of reduced social interactions at the end of the reproductive season. We provide evidence that the tropidurid *T. montanus* may be establishing short-term exclusive-use areas.

Key words: Home range, site fidelity, space use, Tropiduridade

INTRODUCTION

nimals generally use a restricted portion of the physical Aspace available to them to perform the activities necessary for survival and reproduction during their lives. This space used by individuals is termed the home range (Brown & Orians, 1970). Intraspecific interactions should predict how individuals are distributed in space, and therefore information on home ranges should provide insights into the ecology and conservation of a species (Kamler et al., 2003). In addition, the arrangement of individual home ranges in a given population are a key aspect in the understanding of a species' sociobiology (Hinze et al., 2013; Kaneko et al., 2014). For example, the occurrence of mutual avoidance is evidenced in cases where neighbouring individuals have few home range overlaps (Osterwalder et al., 2004; Kerr & Bull, 2006; Wegge & Mosand, 2015). The way animals use the space across time might also depend on whether species are territorial or not. Territoriality requires fidelity to the space used, thus territorial species are expected to use a relatively unchangeable location throughout time as found for gibbons (Hylobates), for example (Bartlett et al., 2015). Moreover, the arrangement of home ranges of males and females can also shed light on a species mating system. Thus, intersexual home range overlap can be indicative of whether males monopolise females resulting in the formation of harems, thereby revealing a polygynous mating system, or if they live in pairs, as expected for a monogamous system (Langergraber et al., 2013; Hibbitts et al., 2012). Hence, information about the home ranges of species is of importance to unveil aspects of social organisation in populations, especially in species that are difficult to observe.

sociability as it is assumed that species from this group of vertebrates present few social behaviours apart from dominance hierarchies and territorial behaviours (Stamps, 1977). However, studies on individual's spacing patterns have revealed complex social behaviours for squamates in relation to group formation and mating pair fidelity (Osterwader et al., 2004, Qi et al., 2012). Nonetheless, there is still a need for studies on squamata social behaviour (Chapple, 2003). In lizards, home range size is expected to differ by sex due to the different social behaviour of males and females. Thus, in territorial species it is common that males will have larger home ranges when compared to females (Baird et al., 2001; Ribeiro et al., 2009). These differences are frequently related to selective pressures for males to increase area use as a means to monopolise females, thereby increasing their reproductive success (Lappin & Husak, 2005). Therefore, in these cases, home range sizes can be related to male body size since larger males are able to dominate smaller ones, thus gaining access to a larger area with a greater number of females (Perry & Garland, 2002).

It has been shown that lizards change their social behaviour according to a population's breeding status. For example, the collared lizard Crotaphytus collaris exhibits low frequency of displays and patrolling at the onset of reproductive season, and territory sizes are also smaller during this period (Baird et al., 2001). Hence, the pattern of space use by individuals should change seasonally, according to reproduction, as observed by Liolaemus quilmes (Robles & Halloy, 2010). In this sense, one could expect for lizards that home range sizes will change in response to seasonal differences in behaviour. However, even considering temporal variation in home range size, individuals may use a relatively fixed space indicating that they are philopatric over time (e.g. Bull & Freake,

Squamates have been long neglected for studies of

1999). For neotropical lizards, home range sizes and their spatial arrangement are expected to increase during the reproductive period or during periods of decreased resource availability (e.g. Van Sluys, 1997).

The lizard *Tropidurus montanus* is an endemic species from rocky outcrop areas of the Espinhaço Mountain Range, south-eastern Brazil (Rodrigues, 1987). This species strictly uses rocky substrate during its daily activities (Filogonio et al., 2010) and as with most of the iguanian lizards, it can be characterised as a sit-and-wait forager species, feeding mainly on ants and termites (Kiefer, 1998). Reproduction of the species lasts from the middle of the dry season to the end of the rainy season (Van sluys et al., 2002).

Lizards from the genus *Tropidurus* have historically been termed as (or even presumed to be) territorial. However, there is still a lack of empirical evidence confirming territoriality in *Tropidurus* species: since to be considered territorial individuals have to be able to use exclusive areas and to maintain their territory area across time (e.g. Sheldahal and Martins 2000). In addition, taxonomic issues have confused even more the understanding of territoriality in this genus. In this study, we: i) evaluated if males and females had exclusive areas; ii) evaluated the persistence of individuals in the studied population across a two year period; iii) evaluated sex and seasonal differences in the home range sizes; and iv) evaluated the extent of intrasexual and intersexual home range overlap between wet and dry seasons.

METHODS

Study site

Fieldwork was conducted in rocky outcrop habitat at 1100 m above sea level at Serra do Cipó National Park (19°20'S and 43°40'W), in the southern portion of Espinhaço Mountain Range, Minas Gerais State, Brazil. At Serra do Cipó altitudes vary from 870 to 1400 m (above sea level), and above 1000 m the habitat is dominated by typical rocky outcrop vegetation forming open "Campos Rupestres" physiognomy (rocky meadows) (Giulietti et al., 1987) with the predominance of shrubs and herbs (Giulietti et al., 1987). Climate in the region is highly seasonal with monthly average temperature varying between 17°C and 23.5°C and monthly rainfall averaging between 11.9 mm and 281.1 mm. Rains occur between October and April and the dry season lasts from May to September.

Procedures

We sampled lizards from July to September in the years 2011 and 2013. This period corresponds to the middle dry period, when *T. montanus* starts its reproductive season. We also sampled individuals during the rainy season from December 2013 to January 2014 at the end of reproductive season for this species (Van Sluys et al., 2002). In the study site, we delimited a 2 ha grid partitioned into 10m x 10m quadrats by attaching flags on the vegetation and by painting coordinate points on rocks. The spatial data on *T. montanus* were obtained by intensively sampling the grid area (31 days from July to September 2011, 17 days through July to September 2013 and 25 days in December 2013 to January 2014).

Lizards found within the grid were captured by noose and permanently marked using coloured beds strung with surgical nylon monofilament at the base of each lizard's tail (Galdino et al., 2014). Captured lizards had their body size measured (snout-vent-length) to the nearest 1mm. The sex of individuals was assigned based on the presence of dark colouration of the ventral face of the thigh and anal flap (marks present only in adult males).

Spatial data of individuals *T. montanus* were obtained by censusing the grid during the main activity period of the species (1000-1500h) (following Filogonio et al., 2010). During each census, we recorded a lizard's position in relation to the grid limits (used as a cartesian axis reference). As a means to decrease temporal dependency in the spatial data, we took care to not perform the same sample route within the grid during the same sample week. In addition, for a given individual we only registered more than one point on the same day of observations if locations were separated by a minimum interval of two hours.

Spatial organisation of T. montanus was evaluated by estimating the area and location of their home ranges by the minimum convex polygon (MCP) analysis. For each individual, we removed 5 percent of the points farthest from the centroid of the cloud of locations before estimating home ranges (HR) (Bath et al., 2006; Calenge, 2006). This procedure excludes extreme location points, generally associated with an individual's occasional sallies. Home range areas and mapping were obtained by using adehabitatHR package (Calenge, 2006) in R (R Core Team Development, 2015). As it is known that HR areas increases with the number of locations (Stone & Baird, 2002), we plotted HR size against number of individual sightings (Ln transformed) to evaluate the minimum number of locations necessary to estimate unbiased areas (e.g. Sheldahl & Martins, 2000; Halloy & Robles, 2002). Home range overlaps were estimated by calculating the overlaps of the polygons using the R package rgeos (Bivand & Rundel, 2013). Spatial analyses were performed by using the "HunteR" script (https:// github.com/NeoLiBE/NeoLiBE/), an implementation of routines of the aforementioned packages from Passos et al. (2015).

We also used the Half Weight Index (HWI) (Whitehead, 2008) as a measurement of temporal association between pairs of individuals. The index is defined as:

HWI = x/(x+Yab+((Ya+Yb)/2))

where: x= number of sampling days when both individual "a" and individual "b" were observed, Ya= number of sampling days when just individual "a" was observed, Yb= number of sampling days when just individual "b" was observed, Yab= number of sampling days when both, individual "a" and individual "b" was observed but not in association. The HWI varies between 0 (no association) to 1 (maximum association). Therefore, we only considered spatial home ranges overlaps for individuals with HWI > 0.5 as a form to exclude spatial overlaps that did not reflect temporal associations among lizards. For HWI calculations we used the program SOCPROG (Whitehead, 2009).

We evaluated data normality using Shapiro-Wilk test. The effect of sex and reproductive period on HR areas was evaluated by a two-way ANOVA. The association among male HR sizes (log10 transformed values) and body size was evaluated by the Pearson's correlation test. We used the G-test to evaluate differences in the proportion of males with overlapped home ranges. In addition, we used Mann-Whitney U-tests to evaluate differences in the intrasexual home ranges overlaps, and to evaluate the number of intersexual home ranges overlaps and HWI values between reproductive seasons (one test per dependent variable). For all tests the significance level was set at 0.05, and the descriptive statistics throughout the text are mean ± one standard deviation.

RESULTS

We sampled 146 individual T. montanus, 53 males and 70 females, for whom we obtained a total of 877 spatial points. For each of the sampled seasons, home ranges estimated using at least seven points were less variable than those with fewer locations (Fig. 1), thus we only considered individuals that had seven or more sightings. During 2011 we marked 34 lizards, 17 of both sex with a mean of 7.16±4.70 spatial locations per individual. For this period 19 individuals had more than seven visual recaptures. In the beginning of the reproductive season of 2013 we marked 39 T. montanus with a mean of 6.85 ± 3.34 spatial points per lizard and were able to estimate the home ranges of 22 lizards for this period. We sampled 50 individuals during the end of reproductive season of 2013, with a mean of 5.5 ± 4.8 spatial points, resulting in the estimated home ranges for 17 lizards.

We found that home range of males and females

The proportion of males that had their home range overlapped by at least one other male was greater at the end of reproductive season (0.90) than for the start of the reproductive season (0.44); however, no difference was found for the proportion of males with overlapped home ranges between seasons (G test, p > 0.05). The mean number of intrasexual home range overlaps at the start of the reproductive period for males was 0.78 \pm 1.09 (Fig. 2) and at the end of reproduction was 3.56 ± 1.59 (Fig. 2), this difference being significant between seasons (Mann-Whitney U-test, p = 0.04). The mean number of female overlapping home ranges with males was 1.56±1.13 at the beginning of reproduction in 2013 and 1.44 ± 1.23 in the end of the reproductive period (Fig. 2) with no difference found between sampling periods (Mann-Whitney U-test, p>0.05). Concerning the time associations of pairs of individuals overlapping their home ranges, the mean HWI for male-male associations from the beginning of reproduction was 0.4 ± 0.18 (n = 4) while that of male-female pairs was 0.29 ± 0.25 . We found at the end of reproductive season four pairs (male-female), which despite having overlapped home ranges were not time-associated (i.e. HWI = 0). At the beginning of the reproductive season in 2013, HWI malemale time associations were 0.39±0.05, whereas that of male-female pairs was 0.49±0.15. Values of HWI for malefemale time association differed between periods with values at the start of the reproductive season in 2013 being significantly higher than those from at the end of reproduction in the same year (Mann-Whitney U-test: W = 49.5; p = 0.03).

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Figure 1. Relationship between home range area (In transformed) and number of sightings for individual *T. montanus* at Serra do Cipo, southeast Brazil.

were both smaller at the start of reproductive season of 2013 (526.40 \pm 564.43m²; 142.83 \pm 131.39m², respectively) compared to the end of reproductive period of 2013 (males = 988.56 \pm 861.02 m²; females = 307.43 \pm 333.28 m²) (Fig. 1) (p = 0.02). However, male home ranges were larger than those of females during both the beginning and the end of reproduction in 2013 (Fig. 2) (p = 0.006), but with no significant result for the interaction term (p = 0.40). Home range size was not associated with male body size in both sampled periods of 2013 (p > 0.05).

Regarding individual persistence in the sampled



Figure 2. Spatial distribution of the home ranges of *T. montanus*. A) Map of the home ranges at the beginning of reproductive season. B) Map of the home ranges at the end the reproductive period. Lines in bold: Males; thin lines: Females

population: no marked lizard from July - September 2011 was recaptured in July - September 2013 or in December 2013 - January 2014. However, 21% (n = 62) individuals from July - September 2013 were observed in December 2013 - January 2014. Unfortunately, due to our method to estimate *T. montanus* home ranges, we were only able to estimate the fidelity of space use for two individuals among those lizards that persisted in the area. One individual presented a home range overlap of 68% from July - September 2013 to December 2013 - January 2014.

DISCUSSION

For T. montanus, the size of home ranges differed between the sexes and also between the reproductive period. Males and females used smaller areas during the initial period of reproduction, increasing their use of space (i.e. larger home ranges) at the end of the reproductive period. In addition, the proportion of males that had their home range overlapped by other individuals did not different between periods, however, the number of neighbouring lizards overlapping their home ranges with a given male was greater at the end of reproduction in comparison with the beginning of the reproductive season. Body size of males was unrelated to home range size. In addition, the majority of individuals did not persist in the studied population, thus we observed a high turnover of individuals across the total sampling period from 2011 to 2013.

We showed that males of T. montanus had larger home ranges than females. A similar pattern was observed for other *Tropidurus* species from the torquatus group (e.g. T. itambere, Van Sluys et al., 1997; T. torquatus, Ribeiro et al., 2009; T. hispidus, Melo et al., in prep). In general, selective pressures for males to monopolise a larger number of females, thereby increasing their reproductive success by enlarging their home ranges, explains the sexual dissimilarities in the size of home ranges (Aragón et al., 2001; Haenel et al., 2003a). Therefore, a territorial and polygynous mating system with males forming harems of exclusive use would be expected in this case. Nevertheless, concerning the mating system of T. montanus, one might consider that the mean number of females associated to a male's home range was small if compared to a typical polygynous lizard species. For example, in Crotaphytus collaris the mean number of females overlapping their ranges with males was 5.4 (Lappin & Husak, 2005), while a mean of 3.7 was found for Sceloporus undulatus (Haenel et al., 2003a). Therefore, our data suggest that male T. montanus form small harems, thus reducing the pressure for them to enlarge their home ranges to increase reproductive success due to the consequent increase in the number of females in their home range. The sex ratio of the studied population is 1:1, with females being settled spatially apart from each other. Thus, we suggest that males T. montanus would have great energetic expenditures to maintain larger harems, thus favouring the maintenance of small harems by males.

Despite the increase of home range size of males at the end of the reproductive period, we observed no proportional change in the number of females that overlapped their home ranges with males. This result is unexpected if males increase their reproductive success by copulating with as large number of females as possible (e.g. Haenel et al., 2003b). In this sense, although December and January correspond to the end of reproductive season of the species, females might be apt to reproduce in these months as they can still be found with mature gonads (Van Sluys et al., 2002). On the other hand, December and January are the last month before the non-reproductive season of T. montanus starts (Van Sluys et al., 2002). Hence it would be expected that males might decrease their performance of behaviours related to reproduction during December/January with a consequent reduction in social activities. The aforementioned prediction is supported by the larger values of time associations (HWI) among males and females at the beginning of reproduction compared to the end of the reproductive period. Therefore, social interactions between individuals of both sexes have more chance to occur during the first months of the reproductive season. Hence, we suggest that the larger home range size of males during December/January might be related to a release in social activities of males (i.e. maintenance of exclusive areas of use for courtship and mating) imposed by the ending of the reproductive period. This release in social activities of males was supported by the increase in the number of home range overlap per male at the ending of reproduction, which in turn might reflect reduced intraindividual avoidance. Therefore, the increase in home range overlaps in December/January might be viewed as a consequence of reduced social interactions during a period in which individuals of both sexes are reducing their reproductive activity.

We expected that home range size would be related to the body size of males, however, no relationship was found. Such a relationship would be expected in cases where intrasexual competition for mates favoured larger males who are expected to use larger areas and thereby gain access to a greater number of females when compared to smaller males (Haenel et al., 2003a). Indeed, male body size can be related to their fitness due to attracting a greater number of females (e.g. Robles & Halloy, 2009) and thus impacting on the number of offspring an individual can sire (Salvador et al., 2008). However, our results point to males having small harems, which reduces the pressure for them to have large home ranges.

Since Werner (1977), Tropidurus lizards had been considered as territorial (e.g. Wiederhecker et al., 2003; Kohlsdorf et al., 2006; Ribeiro et al. 2009). Not withstanding, for a species to be considered as territorial, individuals should possess a fixed area, which in turn is actively defended against intruders by behavioural acts, thus securing exclusive use of that space (Brown & Orians, 1970). However, T. montanus showed low persistence in the population with a huge turnover of individuals within a two-year period and a considerable loss of individuals within six months. A pattern that differs for other iguanian, for example, the lizard Uta stansburiana presents a marked inter-annual fidelity in its use of space (Scoular et al., 2011), and Phtynocephalus vlangalii shows high site fidelity between seasons (Qi et al., 2012). Alternatively, the high turnover of individuals we observed might be

accounted for by mortality. Nonetheless, we intensively sampled the study area through both periods and did not observe any predatory events on T. montanus. In addition, no dead individuals were found during the sampling periods. These considerations are critical for samples in 2013 in which 79% of the individuals that were observed from July to September were not found in the grid in December 2013. Hence, we suggest that mortality is probably playing a minor role in the low lizard persistence found. Thus, it seems that males of T. montanus used a fixed area during a short time period. The used areas by T. montanus seemed to have some degree of exclusive use by individual, at least at the beginning of reproduction since during this time there were few home range overlaps. Hence, our results provide evidence that dispersion might be playing a role in the social spacing of *T. montanus* with residents establishing short-term exclusive-use areas.

Finally, home range overlaps are important in the studies of social behaviour. Our results point to the importance of the measurement of time associations to ensure that spatial overlap is corroborated by the occurrence of individuals at the same time in space. For example, we had two cases of females that were spatially overlapped with males that were not time associated. Thus, to provide a time (or even better, behavioural) association metric would be of importance to produce unbiased home range overlap estimates.

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FULL PAPER



Tadpole species richness within lentic and loticmicrohabitats: an interactive influence of environmental andspatial factors

Lilian Sayuri Ouchi de Melo^{1,4,5,} Thiago Gonçalves-Souza², Michel Varajão Garey³ & Denise de Cerqueira⁴

¹ Pós-Graduação em Biologia Animal, Universidade Estadual Paulista (UNESP), R. Cristóvão Colombo, 2265, Jd. Nazareth, CEP 15054-000, São José do Rio Preto, São Paulo, Brazil.

² Departamento de Biologia, Área de Ecologia, Universidade Federal Rural de Pernambuco (UFRPE), Rua Dom Manoel de Medeiros s/n, Dois Irmãos, CEP 52171-900, Recife, Pernambuco, Brazil.
 ³ Instituto Latino-Americano de Ciências da Vida e da Natureza, Universidade Federal da Integração Latino-Americana (UNILA), Avenida Tarquínio Joslin dos Santos, 1000. Jd.

Universitário, CEP 85870-901, Foz do Iguaçu, Paraná, Brazil

4 Departamento de Zoologia e Botânica, Universidade Estadual Paulista (UNESP), R. Cristóvão Colombo, 2265, Jd. Nazareth, CEP 15054-000, São José do Rio Preto, São Paulo, Brazil. 5 Corresponding author. Lilian Sayuri Ouchi de Melo. Programa de Pós-Graduação em Biologia Animal, Departamento de Zoologia e Botânica, Instituto de Biociências, Letras, Ciências Exatas, Universidade Estadual Paulista (UNESP), R. Cristóvão Colombo, 2265, Jd. Nazareth, CEP 15054-000, São José do Rio Preto, SP, Brazil.

Anurans inhabiting lentic and lotic water bodies show distinct responses to environmental and spatial variables due to dispersal by adults and microhabitat selection by tadpoles, which creates a hierarchical structure in these metacommunities. Aiming to understand the influence of tadpole microhabitat selection and adult dispersal on species richness distribution, we tested the influence of microhabitat environmental variables and habitat spatial variables on tadpole richness in lentic and lotic water bodies located in the Atlantic Rainforest. We sampled tadpoles in 99 lentic microhabitats and 288 lotic microhabitats for seven months. We performed a Hierarchical Partitioning Analysis to test the influence of environmental and spatial variables. The percentage of aquatic vegetation within microhabitats and the main spatial gradient (dbMEM1) affected species richness in lentic water bodies. Sand percentage, aquatic vegetation, and depth in the microhabitat and small-scale spatial gradient (dbMEM4) affected species richness in lotic water bodies. Spatial processes indicate an influence of adult dispersal limitation in search of reproductive habitats. The influence of microhabitat variables was mostly related to the amount of aquatic vegetation, indicating the influence of environmental processes on the larval phase of anuran life cycle. In conclusion, both environmental and spatial processes are driving the species richness in microhabitats inside lentic and lotic water bodies in the Atlantic Rainforest.

Key words: Amphibians; biodiversity hotspot; microhabitat; species number; Tropical Rainforest.

INTRODUCTION

nderstanding how environmental and spatial processes operate in community assembly of species with complex life cycles (CLC) is a challenge for ecologists. CLC species are those whose individuals undergo two distinct development phases (larval and adult) in an abrupt morphological, physiological, and behavioral change, usually associated with the use of different habitats such as aquatic and terrestrial (Wilbur, 1980; Duellman & Trueb 1994; Moran, 1994). Amphibians are the only tetrapods with CLC strategy (Wells, 2007); therefore, their different development phases are expected to respond differently to environmental and spatial processes. Adults colonise and choose breeding sites according to abiotic (Eterovick & Barata, 2006; Prado et al., 2009; Both et al., 2011; Vasconcelos et al., 2011) and biotic variables (Both et al., 2009, 2011). However,

the patchy distribution of aquatic habitats (Resetarits et al., 2005) together with reduced dispersal ability of some individual anuran species (Smith & Green, 2005) could impede new habitat colonisation by adults. Breeding site selection seems to determine the occurrence of tadpoles in each habitat (e.g., Provete et al., 2014). However, inside habitats (i.e., at the water body scale) tadpoles may select their occurrence sites according to environmental microhabitat characteristics (e.g., Kopp et al., 2006). Habitat and microhabitat selection strategies of anurans are linked to the relative influence of adults and tadpoles on anuran species distribution, and consequently, the relative influence of these different developmental phases on the species richness pattern of anurans.

Previous studies have shown that microhabitat selection by tadpoles in ponds and streams is influenced by food availability (Warketin, 1992; Govindarajulu & Anholt, 2006), predation risk (Wilbur & Fauth, 1990;

Correspondence: Lilian Sayuri Ouchi de Melo (sayuriouchi@gmail.com)

Hero et al., 2001; Teplitsky et al., 2003), and physical and chemical variables such as dissolved oxygen, pH, turbidity, and conductivity (Evans et al., 1996; de Oliveira & Eterovick, 2009). Specifically in ponds, depth and amount of aquatic vegetation increase microhabitat availability, favouring species occurrence and survival from predation, which thus increase tadpole species richness (Fairchild et al., 2003; Kopp & Eterovick, 2006; Prado et al., 2009). In tropical streams, water flow, number of potential predators, stream volume and depth, and type of substrate are microhabitat characteristics selected by tadpoles and other co-occurring organisms, such as fishes and macroinvertebrates (Power, 1984; Beisel et al., 1998; Brown, 1991; Zweimüller, 1995; Eterovick & Barata, 2006; Strau β et al., 2013). In addition, strategies for fixation in the substrate are of great importance in streams, preventing tadpoles from being carried away by the water current. Hence, substrate heterogeneity of streams could harbour species with different morphological adaptations to inhabit different substrate types (Hoff et al., 1999). For example, rheophilic tadpoles of Vitreorana uranoscopa bury themselves in the sand (Hoff et al., 1999), and tadpoles of genus Thoropa have a modified abdomen as an adherent ventral disk, for fixation on rocks covered only by a film of water (Rocha et al., 2002).

Several studies have already compared anuran communities at the habitat (e.g., Vasconcelos et al.,

2011; Provete et al., 2014) and microhabitat scales (e.g., Eterovick & Barata, 2006; Kopp & Eterovick, 2006). However, these studies focused only on one type of water body (lentic or lotic). Moreover, there is a lack of studies encompassing the microhabitat scale in pristine areas of Atlantic Rainforest. Therefore, we intend to detect the key factors driving microhabitat species richness within tadpole communities in ponds and streams of the Atlantic Rainforest. For this purpose, we investigated the influence of microhabitat environmental variables inside water bodies, which only influence tadpole distribution across the different types of microhabitats, and spatial location of ponds and streams, which influence adult tadpole occurrence in the habitat because of breeding site selection by adults. A higher influence of environmental variables on species richness variation might indicate that microhabitat selection by tadpoles plays a structuring role. On the other hand, a higher influence of the spatial location of water bodies might indicate that colonisation by adult dispersal is the most important process in determining species richness distribution of tadpole communities. As ponds and streams represent two distinct aquatic systems, we expect that the drivers of species richness in lentic and lotic microhabitat will be different. Thus, we expect that higher species richness may be associated to microhabitats in ponds with higher amount of aquatic vegetation, since they represent an important refuge from predators (Kopp et al., 2006).



Figure 1. Spatial distribution of the water bodies sampled in the Atlantic Rainforest, state of São Paulo, Brazil.



Figure 2. Illustration of environmental structure and species occurrence in lentic (a and b) and lotic (c and d) water bodies (WBs). (a) top view of lentic WB's and (b) the illustration of vegetation and substrate structure creating heterogeneity across the pond, (c) top view of lotic WB's and (d) the illustration of the stream and how the water current exerts a pressure and even tadpoles adapted to currents tend to stay in the backwaters, either attached to rocks or buried in sand at the bottom.

In streams, we expected to find a strong relationship between species richness and microhabitats with different types of substrate because of the high degree of tadpole specialisation required to deal with water flow (Hoff et al., 1999).

MATERIALS AND METHODS

Study area and sampling

We conducted this study at Parque Estadual da Serra do Mar Núcleo Picinguaba (PESM hereafter; 23°21'36''S, 44°50'52''W, datum WGS84), in the state of São Paulo, municipality of Ubatuba, south-eastern Brazil (Fig. 1). The total area (47,500 ha) of PESM is covered by Atlantic Rainforest (distributed from the sea level to 1,340 meters of altitude), and is legally protected by the Brazilian law. The climate in this region is tropical humid with no dry season (Peel et al., 2007).

We sampled tadpoles in 288 microhabitats, 99 located in six lentic water bodies (WBs), three in open areas, and three inside the forest, and 189 microhabitats in seven lotic WBs (all inside the forest). We only sampled streams inside the forest because there are no streams in the open areas of this site. We carried out sampling once a month during the breeding period of most anuran species, from September 2011 to March 2012, between 8. a.m and 6 p.m with a randomised WB sampling sequence on each visit. We selected unconnected WBs with different environmental variables (different types of substrates, depths, and amount of vegetation in the WB), located in lowlands between 1m and 150m above sea level to reduce the influence of elevational effects. Microhabitats were sites inside WBs with different environmental variables (Figs 2a, 2b, 2c, and 2d). We sampled tadpoles in microhabitats distributed throughout the area of lentic WBs, whereas in lotic WBs, we sampled tadpoles along 100 meters. We sampled at least four microhabitats in each WB, except for the uncommon dry months (January to March 2012), when waters were very shallow. In this case, we sampled one to three microhabitats; this number varied according to the water volume available.

Microhabitat

We quantified three environmental variables which generally affect tadpole distribution in each microhabitat (Kopp & Eterovick, 2006; Strauß et al., 2013) and these variables will be considered here as determinants of environmental processes. (i) percentage of vegetation in the microhabitat, (ii) percentage of substrate types (clay, rock, sand, gravel, organic matter, mud, or leaf litter), and (iii) maximum depth (in centimeters). Microhabitats selected were spaced at a minimum distance of two meters apart from each other, in order to include sites with different characteristics and represent the variation in environmental variables throughout the water body.

We sampled tadpoles using the Alford & Crump (1982) method in lentic WBs; according to this method, each sampled microhabitat is delimited by a metal cylinder (32 cm diameter and 70 cm length) open at both ends. We submerged the metal cylinder in the WB with a quick movement and buried its edge in the substrate, delimiting the selected sampling microhabitat. After this procedure, we checked for the existence of gaps between the cylinder and the substrate through which tadpoles could escape. If we found any space, we cancelled the sampling and selected a new microhabitat. We collected tadpoles contained in the cylinder with an 18cm x 10cm (length and width) rectangular-shaped net with wire mesh of 3mm², moving it through the cylinder area 20 times. For each collected tadpole, we started counting the number of dipnet sweeps. Sampling ended after 20 dipnet sweeps without capturing any tadpoles.

Metal cylinder sampling was impracticable in lotic WBs due to their rocky substrate, which does not allow for burying the cylinder in the substrate in order to prevent tadpoles from escaping. Thereby, we visually defined the microhabitat. We collected tadpoles that could be detected within three minutes in each microhabitat using the same wire net mesh described above. Due to water transparency, it was possible to visualise tadpoles on the stream substrate. When we could not capture tadpoles, we disregarded that sample and selected a new microhabitat.

In each type of WB, we performed microhabitat characterisation according to the following sequence. (i) determining percentage vegetation in the microhabitat; (ii) determining the types of substrates; and (iii) determining maximum depth with a measuring tape. To quantify the first two descriptors, we divided the space occupied by the microhabitat into four quadrants. We visually categorised the sum of vegetation and substrate types in each quadrant as follows. 1-20%, 21-40%, 41-60%, 61-80%, and 81-100%.

We anesthetised tadpoles with benzocaine, and fixed them with a formalin (15%) and ethanol (70%) solution (1.1) in the field. We deposited all tadpoles in the DZSJRP Amphibia-Tadpoles Scientific Collection at the "São Paulo State University, Campus of São José do Rio Preto, Brazil".

Data analysis

We performed a Variance Inflation Factor (VIF) analysis to check and exclude collinear variables from the environmental data. In the VIF analysis, each variable is regressed with all the other variables in the system and a coefficient of determination is noted (Legendre & Legendre, 2012). Variables with high VIF coefficients must be investigated and might be dropped from the group of explanatory variables (Legendre & Legendre, 2012). Among the different values proposed to identify non-collinearity and to drop variables, we chose VIF > 3 for a more conservative choice of environmental variables (Zuur et al., 2010). All environmental variables showed collinearity (Online Appendix Table 1); thus, we performed further analyses including all the environmental variables in the system. Due to the different measure units and scales, we transformed the values of the environmental variables into unit variance so that they had a mean of zero and a standard deviation of one (Legendre & Legendre, 2012).

We used distance-based Moran's Eigenvector Maps (dbMEMs; Dray et al., 2012; Legendre & Legendre, 2012) to create spatial variables (eigenvectors) based on the Euclidean distance matrix of WB geographical coordinates. The dbMEM (formerly called Principal Coordinates of Neighbor Matrices, PCNMs) is an ordination method that produces orthogonal eigenvectors used to represent the spatial relationships among habitat samples; in our case, the WBs (Dray et al., 2012). This analysis first computes a pairwise Euclidean distance matrix between samples (waterbodies), and then, builds a truncated distance matrix based on a choosen threshold, and, finally, performs a principal coordinate analysis (PCoA) of the truncated distance matrix creating the orthogonal eigenvectors that are used as spatial predictors in multivariate analysis (Borcard & Legendre, 2002; Dray et al., 2006).

To evaluate how the spatial configuration and the distance among water bodies influence species richness,

we considered WB spatial eigenvectors as predictor variables. To select spatial explanatory variables we used forward selection (Blanchet et al., 2008). For lentic water bodies, we selected dbMEM 1 and dbMEM 4, and for lotic water bodies, we selected dbMEM 2 and dbMEM 4. We used a Hierarchical Partitioning Analysis (Chevan & Sutherland, 1991) to assess which set of variables (microhabitat environmental variables or water body spatial variables) influences species richness in lentic and lotic WBs. The output of Hierarchical Partitioning analysis is a list of predictor variables and their independent (I) and joint (J) influences on response variable Y (Mac Nally, 2002). In our analysis, we randomised the predictor database 1000 times to calculate the distribution of "I" for each predictor variable and "J" for all joint effects of each variable (Mac Nally, 2002). Results of Hierarchical Partitioning analyses are expressed as a Z-score with statistical significance based on 95% confidence limit and Z-score value higher than 1.65 (Mac Nally, 2002). To minimise the high effect of zeros and extreme values

Table 1. Percentage of each anuran family in the total tad-pole abundance in lentic and lotic water bodies.

Waterbody type	Family	Abundance Percentage
Lentic	Leptodactylidae	81
	Bufonidae	12.5
	Hylidae	6.5
	Microhylidae	0.02
Lotic	Hylidae	58
	Hylodidae	33
	Cycloramphidae	9
_	Centrolenidae	0.1

Table 2. Hierarchical Partitioning, Z-score values, I. independent contribution of predictors, J. interactions between each predictor and the others, and I (%). explanation percentage of each variable and significance value of environmental and spatial variables of species richness in lentic and lotic water bodies.

	Variables	I	1	I (%)	Significance Z-score
Lentic	Leaf litter	0.009	0.017	3.30	-0.08
	Sand cover	0.002	-0.001	0.68	-0.62
	Clay cover	0.003	0.002	1.19	-0.50
	Mud	0.006	0.002	2.11	-0.25
	Organic matter	0.014	-0.004	5.44	0.34
	Aquatic vegetation	0.080	0.064	30.08	5.17*
	Depth	0.012	0.013	4.51	0.16
	dbMEM1	0.112	0.035	42.12	6.77*
	dbMEM4	0.028	0.006	10.57	1.28
Lotic	Rock cover	0.004	0.000	2.19	-0.20
	Sand cover	0.028	0.020	15.35	3.17*
	Leaf litter	0.002	-0.001	0.95	-0.47
	Mud	0.001	0.001	0.72	-0.50
	Aquatic vegetation	0.025	-0.010	13.75	2.60*
	Depth	0.064	0.037	35.28	8.43*
	dbMEM2	0.015	0.008	8.14	1.19
	dbMEM4	0.043	0.002	23.60	5.18*

*Asterisks indicate significant values
in the original species composition matrix due to the high abundance of some tadpole species, we used the Hellinger transformation (Legendre & Gallagher, 2001). We also performed a Pearson correlation with significant environmental variables and species richness to find out whether influence among the dependent and independent variables was positive or negative, when Hierarchical Partitioning did not show the type of relationship. We did not correlate the spatial eigenvectors because they already represent spatial structure in species richness.

We adopted α = 0.05 and 1000 permutations in the tests of all analyses. We conducted all the analyses using R (version 3.0.2) software (R Development Core Team, 2013), with the following packages. ade4 (Dray & Dufour, 2007), hier.part (Walsh & Mac Nally, 2012), gtolls (Warnes et al., 2013), vegan (Oksanen et al., 2012), car (Fox & Weisberg, 2011), packfor (Dray et al., 2011), PCNM (Legendre et al., 2013), and spdep (Bivand, 2014).

RESULTS

We recorded 5,394 tadpoles of 21 anuran species (Online Appendix Table 2); 4,401 individuals of 15 species in ponds and 993 individuals of seven species in streams. Hylidae was the richest family in lentic WBs, represented by twelve species, and the most abundant family was Leptodactylidae, although it was represented by only one species (Table 1, Online Appendix Table 2). No families stood out in lotic water bodies regarding species richness; however, Hylidae was the most abundant family, followed by Hylodidae (Table 1, Online Appendix Table 2).

In lentic WBs, species richness was influenced by the percentage of aquatic vegetation inside the microhabitats and by dbMEM 1 (Table 2, Online Appendix Figure 1). Large amount of vegetation increased tadpole species richness (Pearson r = 0.380). In lotic WBs, species richness was influenced by sand percentage in the substrate, aquatic vegetation and depth inside the microhabitats, and by dbMEM 4 (Table 2, Online Appendix Figure 1). Amount of sand (Pearson r = 0.22), aquatic vegetation (Pearson r = 0.12), and depth (Pearson r = 0.32) positively affected species richness in stream microhabitats.

DISCUSSION

Environmental and spatial processes affected species richness distribution of anuran larvae in both ponds and streams. Especially in ponds, spatial process had a higher influence on species richness than environmental process, emphasising that the oviposition site selected by adults is the main determinant of tadpole occurrence. Provete et al. (2014) had already found evidence of the influence of behavioural decisions of adults on tadpole distribution in a higher area of the Atlantic Forest. On the other hand, Almeida et al. (2015) found the opposite pattern in Central Amazonia, i.e., a lower influence of spatial variation on tadpole species richness than the number of ponds per plot, which was the major determinant. Almeida et al. (2015) assumed that the lower influence of spatial variables was caused by the low dispersal limitation of anurans in the Amazon forest. Therefore, different processes probably drive species richness in the Atlantic Rainforest and in the Amazon rainforest. In streams, their natural spatial proximity seems to explain the higher influence of small-scale spatial gradient (dbMEM4) on the distribution of tadpole richness in lotic water bodies. Thus, the higher influence of spatial variables in ponds and of environmental variables in streams seems to be related to the scale at which each type of water body is inserted. Ponds encompassed a larger spatial scale than streams and this more likely increases the influence of longer distances (dbMEM1) on species richness.

Our results indicated that a high amount of vegetation inside microhabitats sheltered higher species richness in tadpole communities of both ponds and streams. Aquatic vegetation increases habitat complexity and suitability, which can increase food availability for tadpoles (Peltzer & Lajmanovich, 2004). It also provides a higher amount of shelter against predators (Baber & Babbitt, 2004; Kopp et al., 2006), which increases tadpole survival thus favouring species richness (Diaz-Paniagua, 1987; Baber & Babbitt, 2004). The positive effect of aquatic vegetation on tadpole species richness has been reported in lentic habitats (Kopp & Eterovick, 2006; Hamer & Parris, 2011, Peltzer & Lajmanovich, 2004). However, to our knowledge, this is the first study that shows that aquatic vegetation also positively affects tadpole species richness in lotic habitats (but see de Oliveira & Eterovick, 2009; for the influence of aquatic vegetation on variation in species composition in lotic habitats). The strong and positive influence of the amount of aquatic vegetation on microhabitats suggests that habitat complexity promotes the coexistence of species at the microhabitat scale.

Despite the favourable influence of aquatic vegetation on species richness, lotic water bodies generally have low amounts of aquatic vegetation. In these environments, the main sources of food for tadpoles are sediment and microalgae deposited in the substrate (Peterson & Boulton, 1999; Wells, 2007). Especially diatoms are deposited in sand substrates with a mild water current, because strong currents prevent the establishment of these algae (Grimm & Fisher, 1984; Atkinson et al., 2008). Accordingly, stream-dwelling tadpoles in Madagascar that feed on microalgae deposited in the sand were associated to less intense water currents (Strauß et al., 2013). Our results and those by Strau β et al. (2013) reinforce the hypothesis that sandbanks located in backwaters have higher tadpole richness. Thus, we suggest that higher tadpole richness in microhabitats of lotic water bodies could be influenced by a combination of deeper water column and lower water current, as a larger amount of sand can accumulate and diatom establishment might occur, increasing food availability. Accordingly, we only found a positive relationship between tadpole richness and microhabitat depth in streams.

In conclusion, we have demonstrated that species richness of tadpole communities in the Atlantic Rainforest of south-eastern Brazil is influenced by spatial and environmental processes. Spatial processes indicate an influence of adult dispersal limitation in search of reproductive habitats. The influence of microhabitat variables was related mainly to the amount of aquatic vegetation, indicating influence of environmental processes (e.g. predation pressure, competition) in the larval phase of anurans life. Furthermore, our results bring new information about anuran species ecology in Atlantic Rainforest, one of the most threatened biomes of the world. In this way, this knowledge could ensure to help new theoretical insights and drive conservation efforts to conserve not just the forested area, but also keep the breeding habitats of anurans in good quality preserving its intrinsic environmental features.

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FULL PAPER



Evolutionary patterns in life-history traits of lizards of the genus *Xenosaurus*

J. Jaime Zúñiga-Vega¹, Jesualdo A. Fuentes-G.², J. Gastón Zamora-Abrego³, Uri O. García-Vázquez⁴, Adrián Nieto-Montes de Oca⁵ & Emília P. Martins^{2,6}

¹Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, Universidad Nacional Autónoma de México. Ciudad Universitaria, Distrito Federal, 04510, México. ²Department of Biology, Indiana University. Bloomington, Indiana, 47405, United States.

³Departamento de Ciencias Forestales, Facultad de Ciencias Agrarias, Universidad Nacional de Colombia. Medellín, Antioquia, 050034, Colombia.

⁴Facultad de Estudios Superiores Zaragoza, Universidad Nacional Autónoma de México. Batalla 5 de Mayo s/n, Ejercito de Oriente, Iztapalapa, Distrito Federal, 09230, México

⁵Laboratorio de Herpetología, Departamento de Biología Evolutiva, Facultad de Ciencias, Universidad Nacional Autónoma de México. Ciudad Universitaria, Distrito Federal, 04510, México.

Life histories are directly related to fitness and, hence, are the focus of strong selective pressures. However, different life-history traits may evolve at different paces and may respond differentially to particular selective pressures. We examined patterns of evolutionary change in the following life-history traits of xenosaurid lizards: size at maturity, average size of adult females, litter size, neonate size, and relative litter mass. We used a phylogenetic hypothesis of the genus Xenosaurus and different phylogenetic comparative methods to search for evolutionary relationships between traits as well as to estimate ancestral states, rates of evolution, and the amount of phylogenetic signal on each trait. In addition, we searched for differences in these life-history traits among the different environments where these lizards inhabit (cloud forest, tropical forest, oak-pine forest, and xeric scrub). We found an evolutionary relationship between size at maturity and average adult size, with larger species maturing at larger sizes. We also found an evolutionary trade-off between litter size and neonate size. Ancestral state reconstructions revealed differences among traits in the relative timing of diversification. Litter size and neonate size began diversification early in the history of the genus. In contrast, size at maturity and relative litter mass remained phenotypically invariant for a long time period before diverging into distinct phenotypic values. Litter size exhibited significant phylogenetic signal because the diversification history of this trait has tracked the phylogeny closely. The observed variation among species in neonate size also showed some trace of the phylogenetic relationships. The remaining three traits diverged throughout time without a clear phylogenetic pattern. In addition, litter size and relative litter mass exhibited the highest evolutionary rates whereas average adult size and neonate size exhibited the lowest rates. Litter size was the only trait that differed significantly among environments, with largest litters in cloud forests. We discuss potential hypotheses to explain the observed differences among life-history traits in the tempo and mode of evolution.

Key words: ancestral state reconstructions, evolutionary rates, life histories, phylogenetic signal, trade-offs, xenosaurid lizards.

INTRODUCTION

ife-history traits are strongly linked to individual fitness (Roff, 2002). The particular combination of age and size at maturity, body growth rates, adult size, number and size of offspring, and lifespan directly determines the total reproductive output of all living organisms. Hence, natural selection is usually strong on these phenotypic traits and changes in the local conditions may promote adaptive responses that quickly lead to new phenotypic optima (Lande, 1982; Crozier et al., 2008; Zhu et al., 2014). Life-history traits may also vary among different environments as a result of phenotypic plasticity (e.g., Karjalainen et al., 2016; Osores et al., 2017). However, within particular lineages, the evolution of life-history traits might also be driven by random processes such as genetic drift, which promote gradual changes that accumulate throughout time. In this case, variation

among species and populations in life-history traits may be better explained by their phylogenetic relationships, with closely related species or populations being more similar to each other compared to distant species or populations (i.e. phylogenetic signal; Blomberg & Garland, 2002; Revell et al., 2008). However, similarity between closely related species might also occur when they inhabit similar environments. In this case, resemblance in their life histories might be explained by parallel adaptations to the same ecological conditions rather than by shared ancestry (Losos, 2008). Several studies focused on the evolution of life-history traits have attempted to understand how much of the phenotypic variation is accounted for by the ancestor-descendant relationships within the focal lineage and how much can be associated with selection operating in local environments (e.g., Staggemeier et al., 2010; Brandt & Navas, 2011; Vukov et al., 2014; Salguero-Gómez et al., 2016).

Correspondence: J. Jaime Zúñiga-Vega (jzuniga@ciencias.unam.mx)

However, we can expect differences among life-history traits in how much of the variation among taxa can be explained by local adaptation versus phylogenetic history, as well as in the timing and amount of evolutionary change (Gittleman et al., 1996; Ackerly, 2009; Adams, 2013). Such differences in how these traits evolve can arise from several causes, such as constraints caused by particular trade-offs with other traits (Poos et al., 2011), differences among traits in both the amount of genetic variability and degree of phenotypic plasticity (Houle, 1992; Osores et al., 2017), specific selective pressures arising from the local conditions that affect one particular trait but not others (Kingsolver et al., 2001), and differences in their relative impact on the fitness of individuals (Stearns, 1992; Burns et al., 2010). For example, age and size at maturity usually have the strongest impacts on fitness such that, under a set of particular conditions, minimal differences in these traits from the optima are rapidly selected against (Stearns & Koella, 1986; Oli & Dobson, 1999; Crozier et al., 2008). Both theoretical and empirical studies have demonstrated that individual fitness is strongly correlated with age and size at maturity, such that slight changes in these life-history traits may have profound impacts on the lifetime reproductive output (Heino & Kaitala, 1997; Oli et al., 2002; Verdú, 2002; Kinnison et al., 2011). In this way, changes in the selective environment should quickly promote changes in these two traits (assuming sufficient genetic variability and no constraints). Therefore, we expect lower phylogenetic signal in age and size at maturity compared to other lifehistory traits such as litter size, size at birth, adult body size, or longevity. However, if closely related species experience similar selective pressures affecting age and size at maturity, we would observe high phylogenetic signal in the ecological conditions and, in consequence, high phylogenetic signal in age and size at maturity (Losos, 2008; Revell et al., 2008).

Trade-offs might also account for differences among life-history traits in the tempo and mode of evolution. For example, numerous studies have documented a physiological trade-off between number and size of offspring (e.g., Kolm et al., 2006; Walker et al., 2008; Warne & Charnov, 2008). Producing a large number of offspring usually comes at the cost of decreasing their individual size because energy and resources are limited. Similarly, producing large individual offspring usually causes a reduction in their number. In viviparous organisms, this trade-off between litter size and size at birth can also result from space restrictions within the female reproductive tract (Bleu et al., 2013; Ford & Seigel, 2015). In many oviparous species, the total number of offspring may also be constrained by the size of each individual egg, which in turn is apparently constrained to an optimal volume (larger eggs cannot pass undamaged through the pelvic girdle and smaller eggs may not contain enough nutrients to support embryo development; Congdon & Gibbons, 1987; Rollinson & Brooks, 2008). Even when there is strong directional selection for changing the number or size of offspring, the tight link between these two traits may restrict their response to selection, leading to a negative evolutionary correlation between number and size of offspring.

Evidence for different evolutionary patterns and tradeoffs between life-history traits can be found in diverse taxa such as fishes (Rochet et al., 2000), amphibians (Gomez-Mestre et al., 2012), birds (Ricklefs, 2000), and mammals (De Magalhães et al., 2007). However, the tempo and mode of life-history evolution have not been examined in several lineages of reptiles, including several snakes and lizards which are under-represented in the literature on this topic (Bauwens & Díaz-Uriarte, 1997; Clobert et al., 1998; Shine, 2005; Scharf et al., 2015; Mesquita et al., 2016). In this study, we used different phylogenetic comparative methods to estimate and compare evolutionary patterns in life-history traits of lizards in the Neotropical genus Xenosaurus. Xenosaurid, or knob-scaled, lizards are small, live-bearing, and crevice-dwelling animals, closely related to the anguid alligator and legless lizards. Xenosaurid lizards are only found in restricted habitats of Mexico and Guatemala (Lemos-Espinal et al., 2012). We focused on: (1) searching for evolutionary relationships (e.g., trade-offs) between traits, (2) reconstructing life-history traits for the common ancestor of the genus, and estimating (3) the amount of phenotypic change throughout time (i.e. evolutionary rates) as well as (4) the amount of phylogenetic signal in each trait and in the different environments that these lizards inhabit.

In addition, given that similar environmental conditions may have promoted convergent evolutionary changes in the life histories of these lizards, we examined if different types of environment (i.e. cloud forest, tropical forest, oak-pine forest, and xeric scrub) have had different evolutionary impacts on the life-history traits. We addressed this additional question also using a phylogenetically-informed approach.

MATERIALS AND METHODS

Study species, life-history data, and phylogeny

Lizards of the genus Xenosaurus are members of the monotypic family Xenosauridae and are viviparous, crevice-dwelling lizards found only in restricted areas of eastern and southern Mexico and western and central Guatemala (Lemos-Espinal et al., 2012). Currently, eight species have been formally described. One of them, X. grandis, has five subspecies, some of which have been treated as species in other studies (Nieto-Montes de Oca et al., 2013). In addition, other populations of uncertain taxonomic status may also represent undescribed species (Zamora-Abrego, 2009; Nieto-Montes de Oca et al., 2017). We conducted an extensive literature search from which we gathered life-history data for nine recognised taxa (six species and three subspecies of X. grandis) and five populations whose taxonomic status is still uncertain (Table 1). We analysed the following five life-history traits: female minimum size at sexual maturity (mm snout-vent length, SVL), average adult female size (mm SVL), litter size (number of newborns), average size of the neonates (mm SVL), and relative litter mass (RLM, which is the proportion of the female mass that is devoted to reproduction) (Table 1). In those cases

where data was available from two different localities, we calculated a weighted average using the sample size of each locality as the weighting factor. Only in one case (*X. tzacualtipantecus*) data was not available for all five life-history traits (Table 1; Fig. 1). We log-transformed these variables before analyses to place them on a common scale and to homogenise variances (this is particularly important for estimating evolutionary rates; Adams, 2013).

We based our comparative analyses on the phylogenetic hypothesis for the genus Xenosaurus recently inferred from RADseq data with maximum likelihood methods by Nieto-Montes de Oca et al. (2017). Branch lengths were estimated as the number of expected substitutions per site. We trimmed the phylogeny to the 14 taxa for which life-history data were available (Table 1), and created an ultrametric tree (Fig. 1) by using a semi-parametric smoothing method based on penalised likelihood (Sanderson, 2002) implemented in the R package 'ape' (Paradis et al., 2004; R Development Core Team, 2008). We used a smoothing parameter equal to 0.1, which is a conservative value (Revell & Reynolds, 2012). For our study we did not differentiate between recognised species (or subspecies) and populations of uncertain taxonomic status. Instead, we treated them all as evolutionary independent units because all the known populations of these lizards are geographically isolated and because xenosaurid lizards are highly phylopatric and exhibit remarkably restricted movement (Zamora-Abrego, 2009; Lemos-Espinal et al., 2012). Hence, gene flow among populations is likely negligible (Nieto-Montes de Oca et al., 2017). We used information on the topology and branch lengths from the ultrametric tree as estimates of expected evolutionary divergence (Fig. 1).

Evolutionary relationships

We followed two approaches to search for evolutionary relationships between pairs of life-history traits. First, we

estimated correlation coefficients using phylogenetically independent contrasts (Felsenstein, 1985) calculated with the R package 'ape' (Paradis et al., 2004). Felsenstein's (1985) method assumes that traits evolve along a phylogeny with random fluctuations occurring at a constant rate, such as when phenotypes diverge predominantly under random genetic drift. This mode of evolution can be described by a Brownian motion model in which the expected phenotypic difference between sister species grows in direct proportion to the time of divergence from a common ancestor. Using Felsenstein's independent contrasts (FIC), we calculated a phylogenetically-corrected correlation coefficient (r) to describe the magnitude of the evolutionary relationship between each pair of traits (forced through the origin as per Martins & Garland, 1991). We also calculated a non-phylogenetic correlation coefficient for each pair of life-history traits using the observed data. This nonphylogenetic correlation assumes a 'star' phylogeny, with phenotypes evolving without any trace of the phylogenetic relationships between taxa.

Second, we used phylogenetic generalised least squares (PGLS; Martins & Hansen, 1997) to search for potential evolutionary relationships between life-history traits. PGLS explicitly incorporates the phylogenetic information as part of the error term of the regression model. We implemented this procedure using different microevolutionary assumptions. First, we assumed that traits coevolve in a way that is well-described by a Brownian motion model (PGLS-BM), such that changes accumulate steadily through time, leaving substantial phylogenetic signal. Hence, PGLS-BM provides the exact same result as would fitting a least-squares regression using Felsenstein (1985) contrasts as input variables (Rohlf, 2001). Second, we assumed that phenotypic evolution is better described by an Ornstein-Uhlenbeck model of evolution, as would, for example, phenotypes experiencing random genetic drift with some stabilising

		Size at maturity (mm SVL)	Adult size (mm SVL)	Litter size (neonates)	Neonate size (mm SVL)	Relative litter mass
	– X. mendozai	92.0	106.9	2.1	51.5	0.27
	– X. newmanorum	107.0	117.6	2.6	50.0	0.25
	 X. platyceps 	100.4	111.3	2.3	50.3	0.24
	– X. tzacualtipantecus	-	101.9	5.0	-	-
	– X. sp. San Lucas Camotlan	110.0	113.0	5.0	41.4	0.35
	– X. grandis rackhami	99.0	110.9	4.5	44.5	0.23
	– X. sp. Sierra de Juarez	107.0	110.2	5.7	40.6	0.2
	– X. sp. Puente de Fierro	108.0	114.9	4.6	45.7	0.28
	– X. grandis grandis	100.0	113.0	5.1	49.0	0.35
4 1	– X. sp. Zoquitlan	109.0	112.3	4.3	47.9	0.24
	- X. rectocollaris	79.9	97.0	2.6	47.2	0.27
	– X. sp. Concepcion Papalo	100.0	108.6	2.4	47.9	0.25
Ц <u>г</u>	– X. phalaroanthereon	112.7	122.7	2.2	51.1	0.16
-	– X. arandis agrenon	97.7	106.2	2.8	47.0	0.24

Figure 1. Phylogenetic hypothesis for 14 distinct taxa of the lizard genus *Xenosaurus*. Populations of uncertain taxonomic status are identified by the name of the type locality. We used this phylogeny to implement all phylogenetic comparative methods. Branch lengths represent relative time. We show for each taxon the five life-history traits that we analysed. In those cases where data was available from two different localities (see Table 1), the values that we show correspond to weighted averages across localities.

Table 1. Minimum female size at maturity, average adult female size, litter size, neonate size, and relative litter mass for 14 distinct taxa of lizards of the genus *Xenosaurus*. These data represent six species, three subspecies, and five populations of uncertain taxonomic status (identified by the name of the type locality within parentheses). Relative litter mass is the proportion of female mass devoted to reproduction. The type of ecosystem and altitude (m above sea level) are also shown. The codes are used in figures to identify each taxon. For some taxa and some traits data was available from two different localities. Standard errors can be found in the original sources, and are not shown here because they were not included in the phylogenetic comparative analyses. SVL = snout-vent length.

Taxon	Code	Locality and Mexican state	Size at maturity (mm SVL)	Adult size (mm SVL)	Litter size (neonates)	Neonate size (mm SVL)	Relative litter mass	Altitude (m)	Type of environ- ment	References
X. sp. (Zoquitlán)	Xzoq	Zoquitlán, Puebla	109	112.3	4.3	47.9	0.24	2000	Cloud forest	Zamora-Abrego et al. 2007
X. grandis grandis	Xgra	Cuautlapan, Veracruz	100	113.0	5.1	49.0	0.35	1100	Tropical forest	Ballinger et al. 2000
X. sp. (Puente de Fierro)	Xpfi	Puente de Fierro, Oaxaca	108	114.9	4.6	45.7	0.28	1108	Tropical forest	Zamora-Abrego et al. 2007
X. sp. (Sierra de Juárez)	Xsju	Sierra de Juárez, Oaxaca	107	110.2	5.7	40.6	0.20	1750	Cloud forest	Zamora-Abrego et al. 2007
X. sp. (Concepción Pápalo)	Хсра	Concepción Pápalo, Oaxaca	100	108.6	2.4	47.9	0.25	2097	Oak-pine forest	Zamora-Abrego et al. 2007
X. phalaroan- thereon	Xpha	San Juan Acaltepec, Oaxaca	109	119.2	2.3	51.1	0.16	2130	Oak-pine forest	Zamora-Abrego et al. 2007
		Santa María Ecatepec, Oaxaca	117	125.0	2.0	_	_	2185	Oak-pine forest	Lemos-Espinal & Smith 2005; Sheetz et al. 2010
X. grandis agrenon	Xagr	San Gabriel Mixtepec, Oax- aca	98	107.0	2.7	47.0	0.24	724	Tropical forest	Zamora-Abrego et al. 2007
		San Juan	97	105.0	3.2	46.3	_	1000-	Tropical	Lemos-Espinal
		Lachao, Oaxaca						1470	forest	et al. 2003
X. rectocollaris	Xrec	Zapotitlán de	93	102.9	2.6	47.2	0.27	2200	Xeric scrub	Zamora-Abrego
		las Salinas, Puebla								et al. 2007
		Tehuacán Val- ley, Puebla	69	92.1	2.6	_	_	2100- 2400	Xeric scrub	Lemos-Espinal et al. 2012; Woolrich-Piña et al. 2012, 2014
X. sp. (San Lucas Camotlán)	Xslc	San Lucas Camotlán, Oaxaca	110	113.0	5.0	41.4	0.35	1800	Cloud forest	Zamora-Abrego et al. 2007
X. grandis rackhami	Xrac	Ocozocuautla, Chiapas	99	110.9	4.5	44.5	0.23	838	Tropical forest	Zamora-Abrego et al. 2007
X. newmanorum	Xnew	Xilitla, San Luis Potosí	107	117.6	2.6	50.0	0.25	780	Tropical forest	Ballinger et al. 2000
X. platyceps	Xpla	El Madroño, Tamaulipas	101	110.7	2.5	50.9	0.26	1210	Oak forest	Ballinger et al. 2000; Rojas- González et al. 2008a, 2008b
		Gómez Farías, Tamaulipas	100	111.7	2.1	49.6	0.21	420	Tropical forest	Rojas-González et al. 2008a, 2008b
X. mendozai	Xmen	Tilaco, Queré- taro	92	106.9	2.1	51.6	0.27	1184	Oak forest	Lemos-Espinal et al. 2004; Zamora-Abrego et al. 2007
		El Pinalito, Hidalgo	—	108.0	4.0	48.5	-	1600	Oak forest	Reaño-Hernán- dez et al. 2016
X. tzacualtipantecus	Xtza	La Mojonera, Hidalgo	_	101.9	5.0	_	_	1900	Cloud forest	Woolrich-Piña & Smith 2012; García-Rico et al. 2015

selection towards a fixed optimum (PGLS-OU). This second regression model includes an additional parameter (α) that could be interpreted as a measure of the strength of selection acting on the phenotypes. When α is small (close to zero), the model depicts a situation similar to Brownian motion evolution, with weak selection and phenotypic change accumulating as a function of time. In this case, closely-related species would be more similar to each other than they would be to more distantly-related species. When α is large (close to 20 for ultrametric trees scaled to a total length of 1), the PGLS-OU model describes a situation in which selection is strong and phenotypes adapt quickly to the local environment, leaving behind no trace of the phylogenetic relationships. Third, we conducted an ordinary least squares regression (OLS) that does not account for the phylogenetic relationships and, hence, assumes a 'star' phylogeny.

We fit these three regression models (PGLS-BM, PGLS-OU, and OLS) to each pair of life-history traits and compared their fit to the data using the Akaike Information Criterion adjusted for small sample sizes (AICc). The lowest value of the AICc indicates the bestfitting model and a difference between two models in AICc values (Δ AICc) larger than two indicates a clear difference in their fit to the data (Burnham & Anderson, 2002). These regression models are explicitly directional. Thus, we conducted each analysis twice, using one variable as explanatory (X) and the other as response (Y) in the first analysis, and then repeating with reversed variables because we had no a priori hypotheses about potential causality. In all cases, reversing the order of the explanatory and response variables yielded qualitatively similar results, so we report only one set of results below.

Ancestral state reconstructions

We reconstructed ancestral states of the five life-history traits using maximum likelihood (Schluter et al., 1997), as implemented by the R package 'phytools' (Revell, 2012). We focused on estimating the ancestral value at the root of the phylogeny. However, we also estimated ancestral states for all the internal nodes. Again here, we assumed and tested two different evolutionary processes: Brownian motion (BM) and Ornstein-Uhlenbeck (OU). As explained in Martins (1999) and Rohlf (2001), ancestral states estimated using maximum likelihood and assuming a BM model are identical to those resulting from leastsquares parsimony (Maddison, 1991), PGLS (Martins, 1999) and Felsenstein's (1985) independent contrasts on the root node. We used AICc to compare the fit of BM and OU versions, and report ancestral estimates derived from the best-fitting model.

Rates of evolution

To estimate the rates of evolutionary change for the studied life-history traits, we used maximum likelihood procedures (O'Meara et al., 2006) implemented in the R package 'mvMORPH' (Clavel et al., 2015). In particular, we used multivariate approaches for estimating evolutionary rates simultaneously for a set of continuous characters, applying both BM and OU models of

evolution, as described above (Bartoszek et al., 2012; Adams, 2013). Again, we used AICc to compare the fit of these two evolutionary models to our data. We report here evolutionary rates derived from the best-fitting model. To compare these rates among life-history traits, we used a Monte Carlo simulation procedure, generating 100 data sets based on the original conditions (i.e. the estimated rates, 13 or 14 taxa depending on the particular life-history trait, and our topology and branch lengths), and calculating evolutionary rates for each simulated data set. We then used a Kruskal-Wallis test and posthoc pairwise comparisons (as per Siegel and Castellan, 1988) to determine which life-history traits exhibited the fastest and slowest rates of evolution.

Phylogenetic signal

We estimated the amount of phylogenetic signal (i.e. the amount of resemblance among species due to their shared evolutionary history) for each of the five lifehistory traits using two different methods implemented in the R package 'phytools' (Revell, 2012). First, we calculated λ (Pagel, 1999), which is a scaling parameter that weights the influence of the phylogeny in explaining the observed phenotypic data. Second, we calculated K (Blomberg et al., 2003), which measures the strength of phylogenetic signal as the ratio between the mean squared error of the observed data and the mean squared error based on the variance-covariance matrix derived from the given phylogeny. For both λ and K, values close to zero indicate independence from the phylogeny, values equal to one indicate that the phylogeny explains substantial variation among species, and values larger than one indicate a stronger similarity between related species than that expected under BM. To assess whether λ was significantly different than zero (i.e. significant phylogenetic signal), we used a likelihood ratio test that compares the model that accounts for the observed λ against a model in which λ is set equal to zero. For K, we used a randomisation test that permutes several times the observed phenotypic values across the tips of the tree, calculates new values of K, and compares the observed K against the distribution of K values obtained under random trait variation (Münkemüller et al., 2012).

In addition, we estimated the amount of phylogenetic signal in the type of environment where these lizards inhabit (Table 1). We used the R package 'geiger' (Harmon et al., 2008) to implement continuoustime Markov models of trait evolution, from which a maximum likelihood estimate of λ can be obtained for discrete characters. Before estimating λ , we compared the fit of three different Markov models. First, an 'equalrates' model, where all transition rates between types of environment share the same value. Second, a 'symmetric' model, in which forward and reverse transition rates between two particular environments share the same value. Third, an 'all-rates-different' model, where each particular transition has a different value. We compared the fit of these models using AICc and then used the bestsupported model to calculate λ . To assess whether λ was significantly different than zero, we used a likelihood ratio test to compare a model that accounts for the estimated λ against a model that does not incorporate the phylogenetic information (i.e. λ = 0).

Differences among environments

To compare the life-histories of xenosaurid lizards among different environments (i.e. cloud forest, tropical forest, oak-pine forest, and xeric scrub), we used the adaptationinertia model as implemented in the R package 'slouch' (Hansen, 1997; Hansen et al., 2008). This comparative method fits an explicit evolutionary model that accounts for phylogenetic relatedness and time spent in different selective contexts using an Ornstein-Uhlenbeck model of evolution. This model envisions several competing selective forces, some of which may have stronger impacts on phenotypic evolution than others. We implemented this method by treating each type of environment as a different selective scenario that may promote convergent adaptive evolution in the life histories of xenosaurid lizards. We used the adaptation-inertia method to fit two different models for each life-history trait. First, we fit a model that estimates a different optimal value for each type of environment and, second, we fit a model that estimates a single optimum for all taxa across all environments. We used AICc to compare the fit of these two models.

This comparative method also estimates the strength of phylogenetic inertia by means of the phylogenetic half-life ($t_{1/2}$), which is the time taken for a trait to evolve halfway towards its adaptive optimum (Hansen et al., 2008). If $t_{1/2}$ is small (close to zero), adaptation to the optimum is fast and the trait does not exhibit phylogenetic inertia. As $t_{1/2}$ increases and approaches infinity, traits retain the influence of their ancestral values and the evolutionary process corresponds to a Brownian-motion model.

To apply the adaptation-inertia method, we assumed previous knowledge about the evolutionary history of the putative selective agent across the phylogeny (Hansen, 1997; Hansen et al., 2008). Thus, we inferred the relative amount of time that each species spent evolving in each type of environment, using an ancestral reconstruction based on maximum likelihood implemented in the program MESQUITE 3.04 (Maddison & Maddison, 2015).

RESULTS

Evolutionary correlations

Using the raw data (non-phylogenetic correlation), average adult size and minimum size at sexual maturity were positively correlated (r = 0.90; Fig. 2a). When using FIC, the positive correlation coefficient between these two traits was smaller (r = 0.78; Fig. 2a), although still significant as compared to the critical value of $r_{0.05} = 0.55$ for n = 13. In contrast, we found a negative relationship between litter size and neonate size as indicated by both non-phylogenetic and phylogenetic (FIC) correlations (r = -0.77 and -0.58, respectively; Fig. 2b). All other pairwise correlations using both the raw data and FIC were not significant (-0.55 < r < 0.55 in all cases).

Evolutionary regressions

We found a significant, positive relationship between adult size and size at maturity using both OLS and PGLS-BM models (Table 2; Fig. 2a). The OLS model provided a better fit to the data than did the PGLS-BM model (Δ AICc = 8.3), indicating little or no phylogenetic signal in the residuals of this bivariate relationship. We were unable to obtain estimates of this relationship using the PGLS-OU model because the optimisation algorithm failed to converge.

We also found a significant, negative relationship between litter size and neonate size using all three regression models (OLS, PGLS-BM, and PGLS-OU; Table 2; Fig. 2b). Again in this case, the OLS model provided the best fit, although both PGLS-OU and PGLS-BM models resulted in similar fit compared to the top model (Δ AlCc = 1.9 and 2.0, respectively). We found no other significant relationships between life-history traits, regardless of the evolutionary model being used (Table 2).

Ancestral state reconstructions

We estimated the following states for the common ancestor of xenosaurid lizards at the root of the phylogeny (± SE): 100.2 ± 7.06 mm SVL for minimum size at maturity, 109.5 ± 4.36 mm SVL for average adult size, 3.2 ± 0.47 neonates for litter size, 47.8 ± 2.30 mm SVL for neonate size, and 0.25 ± 0.044 for relative litter mass (Fig. 3). Two clades (including X. grandis grandis, X. g. rackhami, and four of the undescribed taxa), as well as X. tzacualtipantecus, evolved litter sizes that are substantially larger than that of the root ancestor (Fig. 3c). Some of these taxa with larger litters (X. g. rackhami, X. sp. San Lucas Camotlán, and X. sp. Sierra de Juárez) also evolved much smaller neonates than were present in the root ancestor (Fig. 3d). In contrast, two clades retained relatively small litters (Fig. 3c), and one of these clades (including X. newmanorum, X. platyceps, X. mendozai) along with X. phalaroanthereon evolved large offspring (Fig. 3d). These patterns also suggest a possible evolutionary trade-off between number and size of offspring.

Our ancestral reconstructions also find that the relative timing of diversification varied among life-history traits. Litter size (Fig. 3c) and neonate size (Fig. 3d) began to diverge early in the history of the genus, whereas size at maturity (Fig. 3a) and relative litter mass (Fig. 3e) apparently remained relatively constant for a long time period and began divergence quite a bit later. The history of litter size appears to have tracked the phylogeny closely (Fig. 3c). In the case of neonate size, the clade including *X. mendozai*, *X. platyceps*, and *X. newmanorum*, as well as their common ancestor, evolved relatively large neonate sizes early in the history of the genus (Fig. 3d). All other traits diverged throughout time without a clear phylogenetic pattern.

For all five traits, the log likelihood of the BM and OU models was quite similar. Given that OU models have one additional parameter (α), AICc penalised these models and indicated that BM provided a better fit when estimating ancestral states. Moreover, in size at maturity, litter size, and neonate size the estimated α was quite



Figure 2. Relationships between (log-transformed) size at maturity and adult size (a) and between litter size and neonate size (b) for distinct taxa of the lizard genus *Xenosaurus*. The continuous line corresponds to an ordinary (non-phylogenetic) least squares regression. The dashed line corresponds to a phylogenetic generalised least squares regression (PGLS) assuming a Brownian motion model of evolution. In (b) the additional dotted line corresponds to a PGLS assuming an Ornstein-Uhlenbeck model of evolution. In both (a) and (b) *r* is the non-phylogenetic correlation coefficient and FIC *r* is the correlation coefficient between phylogenetically independent contrasts.

small (< 0.001), indicating that a BM model sufficiently explained variation in these three traits. Given such small α values, the ancestral estimates from OU models were similar to those obtained from BM models. In adult size and relative litter mass the estimated α values were somewhat larger (1.4 and 1.8, respectively), but still small enough to result in similar BM and OU ancestral reconstructions.

Rates of evolution

The BM model provided a substantially better fit than the OU model when estimating evolutionary rates (Δ AlCc = 9.0). We show the estimated rates in Table 3. According to simulations and a Kruskal-Wallis test, these evolutionary rates differed significantly among life-history traits (χ^2 = 420.1, *df* = 4, *P* < 0.001). The highest rate was observed in relative litter mass (0.170) and the lowest in adult size (0.007). Post-hoc comparisons revealed significant differences among most traits (Table 3), excepting between neonate size (0.009) and adult size (0.007) and between litter size (0.126) and relative litter mass (0.170).

Phylogenetic signal

Size at maturity, adult size, and relative litter mass did not exhibit significant phylogenetic signal according to both λ and K (Table 3). In fact, the estimated λ values for these three life-history traits were quite close to zero. In contrast, litter size exhibited strong phylogenetic signal according to λ (1.01, P = 0.003). Furthermore, according to K (1.35, P = 0.004), the litter sizes of these lizards exhibit stronger resemblance between related species than expected from their phylogenetic relationships. The estimated λ value for neonate size was considerably high (0.83), but not statistically different than zero (P = 0.34). However, according to K (0.74) neonate size exhibits marginally significant phylogenetic signal (P = 0.06; Table 3).

The 'equal-rates' model provided substantially better fit to the observed changes among taxa in the type of environment, compared to both 'symmetric' (Δ AlCc = 17.7) and 'all-rates-different' (Δ AlCc = 328.8) models. The estimate of phylogenetic signal for type of environment was remarkably low (λ < 0.001) and not significantly different than zero (P = 0.64).

Differences among environments

The model that estimated differences among environments in the optimal value of litter size provided a better fit compared to the model that estimated a single optimum for all environments (Δ AICc = 6.5). According to 95% confidence intervals around the estimated optimal values of this trait (Fig. 4), lizards that have evolved in cloud forests produce more neonates per litter (backtransformed estimates to the original scale: 5.2 neonates) compared to those evolving in all other environments. In addition, the optimal litter size for lizards evolving in tropical forests (3.1 neonates) was larger than that for lizards evolving in oak-pine forests (2.1 neonates; Fig. 4). The phylogenetic half-life revealed a relatively fast adaptation to these optimal values ($t_{1/2} = 0.19$).

In contrast, the single-optimum model provided substantially better fit than the model that estimated differences among environments for adult size, neonate size, and relative litter mass (Δ AICc = 7.1, 2.8, and 15.3, respectively). For size at maturity, the single-optimum model also provided the best fit, but the different-optima model differed in less than two AICc units from this top model (Δ AICc = 1.6). However, regardless of the similar fit between these two alternative models, we considered stronger evidence for a single optimum because estimating a different optimal size at maturity for each type of environment did not substantially improve the model fit.

DISCUSSION

Our results clearly demonstrate several important differences among life-history traits in the way they have evolved in xenosaurid lizards. Larger species mature at larger body sizes and there is strong evidence for an evolutionary trade-off between the number and size of offspring. Adult size and neonate size have both evolved slowly in *Xenosaurus*. In contrast, relative litter mass and litter size have evolved quickly. However, despite **Table 2.** Results from phylogenetic generalised least squares (PGLS-BM and PGLS-OU) and ordinary least squares (OLS) regression models fitted to life-history traits of lizards of the genus *Xenosaurus*. PGLS was implemented using two evolutionary models: Brownian motion (BM) and Ornstein-Uhlenbeck (OU). The fit of each model to the data was evaluated using the Akaike Information Criterion (AICc), with the smallest value indicating the best-fitting model. Differences in AICc values between each model and the best-fitting model (Δ AICc) are also shown. For each pair of life-history traits, models are listed according to their AICc values (from lowest to highest, from best to worst). Standard errors of the regression slopes are shown within parentheses. PGLS-OU estimates an additional parameter, α , which measures the strength of stabilising selection. In two cases (regressions between size at maturity and adult size, and between litter size and adult size) the PGLS-OU model did not provide parameter estimates because the optimisation algorithm failed to converge.

Life-history traits	Model	AICc	∆AICc	Regression slope	Р	α
Adult size (Y) ~ Size at maturity (X)	OLS	-40.3	0	0.5 (0.08)	< 0.001	
	PGLS-BM	-32.0	8.3	0.4 (0.11)	0.002	
Litter size (Y) ~ Size at maturity (X)	PGLS-BM	10.3	0	-0.002 (0.73)	0.99	
	PGLS-OU	14.7	4.4	-0.002 (0.73)	0.99	1x10 ⁻⁸
	OLS	17.9	7.6	1.4 (1.14)	0.23	
Neonate size (Y) ~ Size at maturity (X)	PGLS-BM	-18.6	0	-0.1 (0.20)	0.51	
	OLS	-16.3	2.3	-0.2 (0.24)	0.51	
	PGLS-OU	-14.5	4.1	-0.1 (0.20)	0.52	0.6
Relative litter mass (Y) ~ Size at maturity (X)	OLS	5.8	0	-0.5 (0.66)	0.44	
	PGLS-OU	10.0	4.2	-0.7 (0.66)	0.34	8.4
	PGLS-BM	11.2	5.4	-1.3 (0.76)	0.11	
Litter size (Y) ~ Adult size (X)	PGLS-BM	10.0	0	-0.4 (1.24)	0.75	
	OLS	19.3	9.3	-0.01 (1.85)	0.99	
Neonate size (Y) ~ Adult size (X)	PGLS-BM	-19.6	0	0.2 (0.35)	0.54	
	OLS	-17.1	2.5	0.2 (0.40)	0.67	
	PGLS-OU	-15.5	4.1	0.2 (0.35)	0.56	0.5
Relative litter mass (Y) ~ Adult size (X)	OLS	4.5	0	-1.0 (1.07)	0.35	
	PGLS-OU	8.8	4.3	-1.1 (1.08)	0.34	13.7
	PGLS-BM	12.1	7.6	-1.3 (1.46)	0.39	
Neonate size (Y) ~ Litter size (X)	OLS	-22.8	0	-0.2 (0.04)	0.002	
	PGLS-OU	-20.9	1.9	-0.2 (0.04)	0.001	4.4
	PGLS-BM	-20.8	2.0	-0.2 (0.07)	0.04	
Litter size (Y) ~ Relative litter mass(X)	PGLS-BM	10.4	0	0.3 (0.23)	0.16	
	PGLS-OU	14.7	4.3	0.3 (0.23)	0.16	1x10 ⁻¹⁰
	OLS	19.6	9.2	0.6 (0.51)	0.25	
Neonate size (Y) ~ Relative litter mass (X)	PGLS-BM	-16.0	0	0.002 (0.07)	0.98	
	OLS	-14.5	1.5	-0.1 (0.11)	0.61	
	PGLS-OU	-12.0	4.0	-0.01 (0.08)	0.94	0.8

of the high evolutionary rate in litter size, this trait has retained strong phylogenetic signal. We also found some resemblance between closely related species in neonate size, whereas all other traits diverged throughout time without a clear phylogenetic pattern. Additional studies are needed to determine the causes of these differences in the tempo and mode of evolution among life-history traits, and below are some hypotheses that could guide future research.

The first evolutionary relationship that we detected was between average body size and minimum size at maturity. This relationship was positive: larger species mature at larger sizes. This pattern was clear in the raw data (in both a significant non-phylogenetic correlation and a significant slope from the OLS regression) and when taking into account the phylogeny (a significant correlation between FIC and a significant slope from the PGLS-BM regression). The non-phylogenetic approach provided a better fit which indicated that phylogenetic relationships add little to our understanding of the correlated evolution between mean body size and size at maturity. The evolution of larger sizes both as adults and at sexual maturity may be the result of local responses to selective pressures. For example, larger body sizes could be the result of predation on smaller lizards (i.e. low extrinsic adult mortality; Day et al., 2002). If the chances of death are low, delaying maturity can provide an additional advantage because larger females would produce more or larger offspring (Olsson et al., 2002). In fact, Ballinger et al. (2000) and Rojas-González et al. (2008a) demonstrated that larger females produce more newborns per litter in both X. grandis grandis and X. platyceps, respectively. Further increases in adult size are then favoured because even greater reproductive output can be attained and the risk of mortality may decrease further as body size increases. In addition, both



Figure 3. Phenograms depicting ancestral states for (a) size at maturity, (b) adult size, (c) litter size, (d) neonate size, and (e) relative litter mass of lizards of the genus *Xenosaurus*. Taxon codes are as given in Table 1.

adult size and size at maturity may be positively affected by factors that promote fast body growth, such as warm temperatures and high food availability (Adolph & Porter, 1993; 1996; Zúñiga-Vega et al., 2005). In fact, body growth rates and, consequently, adult size and size at maturity may exhibit plastic responses to temporal and spatial variation in both temperature and food availability (Madsen & Shine, 2000; Angilletta, 2001). Also, selection on age at first reproduction indirectly affects adult size and size at maturity, such that environments that promote early reproduction, indirectly promote small size at maturity and small adult size (Beckerman et al., 2010). Several taxa exhibit a similar pattern in which the evolution of delayed maturity is also associated with the evolution of a larger body size (Blueweiss et al., 1978; Kozlowski, 1996; Morand, 1996).

The second clear pattern that we detected was an evolutionary trade-off between number and size

Table 3. Rates of evolution and estimates of phylogenetic signal (λ and K) for life-history traits of lizards of the genus *Xenosaurus*. We also show post-hoc pairwise comparisons from a Kruskal-Wallis analysis of variance. NS indicates non-significant differences; * indicates *P* < 0.001.

Life-history trait	Evolutionary rate	Adult size	Litter size	Neonate size	Relative litter mass	λ	P-value	К	P-value
Size at maturity	0.021	*	*	*	*	7×10 ⁻⁵	0.99	0.48	0.39
Adult size	0.007	-	*	NS	*	7×10 ⁻⁵	0.99	0.59	0.23
Litter size	0.126		-	*	NS	1.01	0.003	1.35	0.004
Neonate size	0.009			-	*	0.83	0.34	0.74	0.06
Relative litter mass	0.170				-	7×10 ⁻⁵	0.99	0.29	0.77



Figure 4. Estimated optimal litter sizes of xenosaurid lizards for different environments according to the adaptation-inertia method. Error bars represent 95% confidence intervals. We also show the estimated phylogenetic half-life $(t_{1/2})$ for litter size.

of offspring. Regardless of the assumed evolutionary model, a negative relationship between these two traits was always evident. This trade-off between number and size of offspring was also evident from our ancestral state reconstructions because some of the clades (including some hypothesised ancestors) that evolved large litters also evolved small offspring and vice versa. Thus, when selection favoured the evolution of more offspring in a particular species, a likely consequence was the evolution of a reduction in the size of each individual offspring (or vice versa). This trade-off is likely the result of a limited space within the reproductive tract (i.e. a biomechanical trade-off). All the lizard species from this genus are strict crevice dwellers, which means that they rarely go out of their crevices (Lemos-Espinal et al., 1998; 2004). These crevices are usually narrow, and the space within is barely enough to fit the body of these lizards. This has resulted in a flattened morphology that clearly restricts the space that these viviparous females can devote to offspring production (Lemos-Espinal et al., 2012). Similar constraints on reproductive allocation imposed by a flattened morphology have been observed in other crevice-dwelling lizards (e.g., Dalmatolacerta oxycephala and Tropidurus semitaeniatus; Vitt, 1981; Bejakovic et al., 1996; Ribeiro et al., 2012). Other viviparous reptiles also exhibit this trade-off between number and size of offspring (King, 1993; Doughty & Shine, 1997; Gignac & Gregory, 2005; Uller & Olsson, 2005). A similar evolutionary trade-off has been documented in diverse taxa such as butterflies (García-Barros, 2000), fishes (Elgar, 1990), turtles (Elgar & Heaphy, 1989), and birds (Figuerola & Green, 2006).

In addition to providing estimates of the phenotypes of the common ancestor of the genus, our ancestral reconstructions also revealed an interesting difference among traits in the relative timing of diversification. Litter size and neonate size began diversification early in the history of the genus. In contrast, size at maturity and relative litter mass remained phenotypically invariant for a long time period before diverging into distinct phenotypic values. These differences might be related to how the selective environment differentially affected these life-history traits during the early diversification of the genus. A biogeographic hypothesis would suggest that in the initial geographic distribution of the oldest ancestors, the environment was relatively constant, exerting stabilising selection on size at maturity and relative litter mass. However, litter size and offspring size were relatively more sensitive to slight changes in the environment. Then, as these xenosaurid lizards colonised new regions, they experienced a wider array of selective environments, which in turn began promoting changes in size at maturity and relative litter mass in parallel to further changes in litter size, neonate size, and average adult size. Interestingly, the genus Xenosaurus is apparently of Nearctic origin (Gauthier, 1982), whereas most extant taxa have a Neotropical distribution (Zamora-Abrego, 2009; Lemos-Espinal et al., 2012; Nieto-Montes de Oca et al., 2017). The late diversification of size at maturity and relative litter mass could have coincided with the colonisation of more tropical regions. Interestingly, we found no phylogenetic signal in the types of environments that these lizards currently inhabit. This result indicates that closely related species were able to colonise drastically different ecological conditions (Losos, 2008), which is now reflected in the relatively wide diversity of environments where these 14 taxa of xenosaurid lizards can be found (cloud forests, tropical forests, oak-pine forests, and xeric scrubs).

The rates of evolution differed drastically among lifehistory traits. Adult size and neonate size have evolved at a slower pace, as indicated by the slowest observed rates. This is also evident in the relatively small amount

of interspecific variation observed in both traits (less than 0.24 in the log scale between the largest and smallest observed values; Figs. 3b and 3d). In contrast, relative litter mass and litter size exhibited the fastest evolutionary rates, which is also consistent with greater amount of interspecific variation (1 and 0.8 in the log scale between the largest and smallest observed values of litter size and relative litter mass, respectively; Figs. 3c and 3e). The difference in the rate of evolution between relative litter mass (fastest rate) and adult size (slowest rate) is about 24-fold (relative litter mass: 0.170, adult size: 0.007). Therefore, in this genus both adult size and neonate size are relatively conserved life-history traits, likely as a result of strong stabilising selection, whereas relative litter mass and litter size are labile traits. However, even though adult size has evolved at a slow rate, the changes that have occurred throughout the history of the genus have not retained trace of the phylogeny as indicated by negligible phylogenetic signal in this trait. Hence, such moderate and slow phenotypic changes in adult size likely occurred as particular responses to local conditions. These changes may represent either local adaptations or plastic responses. In contrast, neonate size, which also has evolved at a relatively slow rate, has retained some trace of the phylogenetic relationships, a pattern that is partially consistent with gradual evolution through genetic drift rather than with adaptive or plastic responses to particular ecological conditions.

Notably, the relatively large evolutionary changes observed in litter size have closely tracked the phylogeny and related species have similar litter sizes. This high phylogenetic signal in litter size was evidenced by the high and significant estimates of λ and K as well as by the reconstruction of ancestral states (Fig. 3c). However, despite this high phylogenetic signal, an environmental effect on litter size could be possible if some of the species and common ancestors that share either large or small litters (see these two clearly distinct groups in Fig. 3c), also shared a common feature of the environment with a selective effect on this life-history trait. Our results of the adaptation-inertia method indicate that this is in fact the case. We found an evident effect of the type of environment that these lizards inhabit on litter size: the largest optimal value of this trait corresponded to cloud forests. All the four taxa that inhabit this type of environment (X. tzacualtipantecus, X. sp. [Zoquitlán], X. sp. [Sierra de Juárez], and X. sp. [San Lucas Camotlán]) have evolved relatively large litters (Fig. 3c). In contrast, all four taxa that inhabit oak-pine forests (X. phalaroanthereon, X. platyceps, X. mendozai, and X. sp. [Concepción Pápalo]) have evolved relatively small litters (Fig. 3c). In fact, the smallest optimal value of litter size corresponded to oak-pine forests (Fig. 4).

What is the potential benefit of large litters in cloud forests? Cloud forests represent a unique ecosystem that receives large amounts of humidity from rain, clouds, and fog and which contains a mixture of tropical and temperate flora and fauna (Sánchez-González et al., 2008). In Mexico, cloud forests have the highest biodiversity per unit area, harbouring approximately 10% of all Mexican flora and 12% of the terrestrial vertebrates (Pineda & Halffter, 2004; Ornelas et al., 2013). Thus, the diversity and abundance of potential predators of xenosaurid lizards (e.g., birds and snakes) might be higher in cloud forests compared to other environments. Therefore, a potential selective factor that could promote large litters is a high mortality risk in these environments (Pérez-Mendoza & Zúñiga-Vega, 2014). High mortality selects for larger litters because this increases the probability of at least one offspring surviving to reproduce (Promislow & Harvey, 1990; Roff, 2002). Testing this hypothesis would require mortality estimates for all these species and, until present, mortality data are only available for X. g. grandis, X. platyceps, and X. mendozai (Zúñiga-Vega et al., 2007; Rojas-González et al., 2008b; Zamora-Abrego et al., 2010; Zúñiga-Vega, 2011; Molina-Zuluaga et al., 2013). In contrast, temperate environments such as oakpine forests (usually located at higher elevations; Table 1) may constrain reproductive output. In this type of forest, temperatures are generally colder and food availability (diversity and abundance of arthropods) may be relatively low. Thus, lizards could not afford to produce larger litters and, hence, the smallest litters that we observed in oak-pine forests may represent a plastic response to restrictive conditions (Rohr, 1997; Zeng et al., 2013). A common garden experiment would help to understand whether the observed differences among taxa in litter size represent adaptive genetic differentiation or plastic responses.

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FULL PAPER



The trade of Kinosternon scorpioides on Marajó island, Brazilian Amazon: from hunting to consumption.

Samuel Silva de Cristo¹, Pedro Chaves Baía Júnior², Joilson Silva da Silva¹, José Ribamar Felipe Marques³ & Diva Anelie de Araújo Guimarães¹

¹ Institute of Biological Sciences, Federal University of Pará. Belém, Pará, Brazil

² Federal Institute of Education, Science and Technology of Pará. Abaetetuba, Pará, Brazil

³ Brazilian Agricultural Research Corporation - Embrapa Eastern Amazon Region. Belém, Pará, Brazil

We studied the trade network of the Scorpion Mud Turtle, Kinosternon scorpioides, on Marajó Island, Brazil, from hunting to the final product consumption. We conducted semi-structured interviews at the urban centers of the cities of Soure, Salvaterra and Cachoeira do Arari, where we investigated: A) the socioeconomic profile of the merchants and their activity; B) the origin, form and the frequency of marketing K. scorpioides; and C) the demand for the product. Scorpion Mud Turtle hunting was carried out using the following methods: hand-collection (100%), probing (62%) and burning (54%). They were hunted for two reasons: personal consumption and sale, which was conducted in urban centers. The hunters knew the empirical aspects of the species biology in the wild: habitat, trophic ecology, and reproduction, among others. The trade of K. scorpioides is still a strong activity on Marajó Island, and the legal regulations are not enough to inhibit this practice, which poses a threat to the future of this species in this area. The establishment of educational efforts and captive breeding programs of this species will contribute to employment, to local family income, and to the conservation of this genetic resource. These efforts also be important for that the local population could produce and consume this species in a more sustainable way.

Key words: biodiversity; ethnobiology; scorpion mud turtle; chelonians

INTRODUCTION

he use of chelonians in the Amazon is not only for meat consumption, but is rooted in the local culture of the people, who for decades learned from their ancestors to use turtles for food, medicine and an alternative source of income (Pezzuti et al., 2010). Such uses of turtles have been socially, culturally and economically important since it originated in pre-Columbian communities, and this persists today (Schaan, 2010). Chelonians are not currently used in a sustainable way, leading to population declines of many Amazonian species (Schaan, 2010; Alcântara, 2014). In addition, due to socio-environmental conditions in the region (Fearnside, 2015) and the permanent demand for consumption of these animals (Pantoja-Lima et al., 2014), species of chelonians are increasingly threatened in the Amazon.

There are few investigations in to the actual uses of this resource, from capture to the final consumer, although there have been some studies in the last decade focusing on the use of chelonians in the Brazilian Amazon (Fachín-Terán et al., 2004; Rebelo et al., 2005; Pantoja-Lima et al., 2014; Morcattye & Valsecchi, 2015). These studies are extremely important for the establishment of viable conservation alternatives for hunting species. To the authors knowledge, there is no information in the literature on the Kinosternon scorpioides trade network on Marajó Island, although this animal has been exploited in this region for decades on a large scale in a non-sustainable way (Alho, 1985).

Due to the demand for their meat and its by-products, as an alternative to the harvesting from the wild, Brazilian law allows some species of chelonians to be bred in captivity subject to regulation by environmental control bodies (Podocnemis expansa, Podocnemis unifilis, Podocnemis sextuberculata and K. scorpioides; Araújo et al., 2013).

This study takes into account that existing legal prohibitions do not, in fact, impede the trade of K. scorpioides in the Arari microregion (Marajó, Brazil), which may lead to critical loss of natural stock. Therefore, the understanding of the dynamics of the use of this resource will contribute to the development of programs directed toward local sustainability, as well as conservation of this species. The aim of this study was to describe the trade of K. scorpioides and the knowledge associated with its biology in the wild.

METHODS

This research was conducted in the Arari microregion, Marajó Island, north of the State of Pará, Brazilian Amazon, in the municipalities of Salvaterra (n = 12



Figure 1. Map of the study area, highlighting the municipalities of the east coast of Marajó Island, Brazil (Soure, Salvaterra, Cachoeira do Arari and Santa Cruz do Arari).

consumers; and n = 4 traders) (00°45'21" S; 48°45'54" W), Soure (n = 13 consumers; and n = 2 traders) (00°43'48" S; 48°30'24" W), Cachoeira do Arari (n = 14 consumers; and n = 4 traders) (01°00'36"S; 48°57'36" W) and Santa Cruz do Arari (n = 13 hunters in the village of Jenipapo; and n =17 consumers) (00°39'45" S; 49°10'40" W) (Fig. 1).

Free and semi-structured interviews were conducted (Albuquerque et al., 2014) with hunters, middlemen, traders and consumers of K. scorpioides, whose selection was based on the snowball technique (Albuquerque et al., 2014). The interviews covered: A) the socioeconomic profile of the traders and of their activity; B) the origin, form and frequency of the marketing of K. scorpioides and its by-products; C) the demand for the purchase of these products. Informal conversations, participant observation, and free and semi-structured interviews were carried out (Albuquerque et al., 2014), with topics for discussion including use, hunting practices, and knowledge about K. scorpioides. A questionnaire was provided to each hunter in each home visited (Viertler, 2002). Data triangulation was used through: a) crossinformation, which consisted of subjecting others to the information of a given interlocutor; b) repeated information, which according to Albuquerque et al. (2014) is a method of control, taking into account the period in which the questions were asked, and which after a time were formulated again; c) lack of contradiction with the interlocutor, based on the model of the union of diverse competences, in which the information provided by only one subject was considered (Hays, 1976).

RESULTS

The commercial network of *K. scorpioides* in the micro region of Arari, from hunting to the final consumer, involved a group of about five social levels (Figure 2), each with characteristics and defined roles.

The hunters of *K. scorpioides* are artisanal fishermen. Hunting of *K. scorpioides* is practiced exclusively by men, both for their own consumption and for commercialisation. Of the 13 artisanal fishermen living in the village of Jenipapo (Santa Cruz do Arari) interviewed, 69% (n = 9) stated that they hunted *K. scorpioides*. Of these nine, 78% had performed this activity for more than 10 years, and about 90% had practiced it alone without the help of third parties. It was an activity performed only at a time when it was more difficult to obtain other protein sources (e.g., fish, beef or chicken) or when



Fig. 2. Commercial network established for the extraction of *K. scorpioides* in Marajó Island, Brazil.

there was a need to diversify the diets. According to the interviewees, during the 1970s and 1980s, *K. scorpioides*, as well as other wild animals, were the only sources of animal protein available for the village of Jenipapo when there was a shortage of other food sources. However, comments on the impacts of hunting *K. scorpioides* were ambivalent, since for some it was a scarce resource due to its long history of use; for others it was an abundant resource.

Traders of *K. scorpioides* were mostly (90%; n=9) adult males with a low level of schooling (80% did not complete elementary school). Trade was conducted over 10 years ago, by 100% (n = 10) of them. Traders residing in the urban area were generally former fishermen (n=4) or cowboys (n=2), retired and self-employed (n=4). The trade of animals was diffuse, being more frequent in peripheral neighbourhoods, near municipal ports.

The monthly income of the activity for 50% of respondents averages US \$377.14. The value of the product varied according to the time of year. The turtles



Fig. 3. *K. scorpioides* marketed alive in 2015 for the amount of US \$17.14 (*n* = 12 animals). Source: Cristo (2015).

were marketed alive in groups of twelve animals (bunch), with values ranging from US \$12.86 to US \$28.57 in the driest period (Fig. 3). During the rainy season (December to March), when there was less availability of turtles, supposedly due to the difficulty in collecting, the sale value increased, to around US \$40.00 (12 animals).

K. scorpioides was traded in Santa Cruz do Arari to owners of boats from other neighbouring municipalities (e.g., Anajás, Ponta de Pedras and Cachoeira do Arari). In general, the middlemen who purchased the animals from the hunters in Jenipapo village (Santa Cruz do Arari) resold them to the owners of restaurants in the capital, Belém-Pará. This occurred during the season of greater availability of the animal in nature, that is, in the least rainy period (months of April to November).

The purchase of *K. scorpioides* meat was primarily for consumption (96%, n=54), especially by distant relatives and/or friends. Occasionally the product was resold (3%) or sent to neighboring municipalities (5%). In all cases, the animals came from hunting and/or trade, and 55% of the purchases were obtained directly from the hunter by order. All of the consumers interviewed (n = 56) stated that they had consumed *K. scorpioides* at least once in the last year; 98% with a frequency of three or more times in the year. The main reasons (n = 56) for *K. scorpioides* hunting were: food flavour (72%), cultural habit (14%), diet diversification (13%), and obtaining income (1%).

The restaurants of Cachoeira do Arari (n = 4), Salvaterra (n = 4) and Soure (n = 2) commercialised the meat of *K. scorpioides* in the form of a delicacy known as "casquinha de muçuã", which contained meat of two animals, on average, accompanied by manioc flour (*Manihot esculenta*). The value of this delicacy was between \$4.30 and \$10.00 USD, but it was not on the menu and instead was verbally communicated to the customer. The main consumers were urban dwellers in the region studied (80%), but also tourists. It was observed that this product was in high demand, even in the months of the rainy season, outside the season of greater supply. The average monthly income earned by traders was between \$ 251.43 and \$ 502.86, during the time when there was greater supply.

From the hunting reports, three techniques were described: a) opportunistic hunting by hand-collection; b) burning; and c) probing. These activities were cited by 100%, 54% and 62% of respondents, respectively. The opportunistic hunting by hand-collection involved the manual capture of *K. scorpioides* without the use of any extra utensils tools. It was used at the beginning of the period of fish reproduction, since hunters believed that *K. scorpioides* was "a fish at the beginning of spawning". It also happened when these animals were located in shallow aquatic environments, or during the tide change, when they were feeding under the surface and/or at the edge of the water. Hunting performance was maximised by searching within the flood fields for places where *K. scorpioides* were feeding.

Burning consisted of setting fire to the grasses of the fields during the dry season, which caused *K. scorpioides* to move in to the open to escape from smoke, where they were easily caught by hand. In this way, all animals

in the field were captured regardless of size or sex, and in large quantities. These animals were used for both consumption and sale. The application of this technique demanded empirical knowledge about the seasonal climatic variation in the area of the fields, which included the direction of the wind, the probability of rainfall, and microhabitats for turtle hibernation, estivation and/or thermoregulation. Burning was practiced with the help of other people to control the fire.

Probing was carried out with the use of a long rod of wood or metal, whose tip was protected by a plastic wrap, to locate animals burrowed in mud both in the dry and rainy season. The hunter walks in flooded environments, especially on the edge of watercourses during the dry season, probing the ground until hearing the sound of the metal or wood probe on the turtles' carapace, which are then captured by hand. The application of this technique demands empirical knowledge of the seasonal variation of the lakes and turtle behaviour.

Hunters also had knowledge of the trophic, reproductive, and behavioral biology of K. scorpioides life history. They identified a variety of environments in which animals could be found. In the dry season, they would be present in large numbers in fields below or in the middle of the foliage. In the rainy season, when the rivers overflowed and flooded the fields, K. scorpioides could be found in shallow flooded areas, pools, ponds, and puddles formed between pastures. They could also be found along rivers and in the archaeological earth mounds on Marajo Island (tesos). As for the diet of K. scorpioides, hunters reported that it is omnivorous, and consumes a wide variety of food items including algae, vegetation (Commelina longicaulis), small invertebrates (beetles, Coleoptera: Chrysomelidae), and detritus. Hunters believed that the feeding sites of the species are natural floodplains, dry environments, floodplains at low water depth, in open land and/or under various foliage.

The hunters (92%, n=12) claimed to be able to distinguish the sex of K. scorpioides by observing certain morphological characteristics: specimen size (23%), tail length (15%), and shape of the shell (54%). According to most hunters, the first two characteristics could be observed at any age, but the differences in the shell could only be verified only in adult animals. Mating was believed to occur at the beginning of the rainy season (December), but could continue for five months through the rainy season. Copulation likely occurs underwater in shallow areas, and/or under shrub vegetation. Nesting was reported to begin in April, with increasing intensity in the months of June and July, in diverse microhabitats (mainly between the roots of the trees, in soft and clean earth, or under fallen foliage). Three to eight eggs were deposited with an incubation period of two to five months. After the mating and nesting period, K. scorpioides generally showed a pattern of inactivity during the driest period, from July to December, where it would remain burrowed for six months until the next rainy season.

DISCUSSION

The *K. scorpioides* is threatened by illegal trade on Marajó Island. The hunting of this species in this area showed that this activity serves both for consumption and for sale. The empirical biological aspects of this species in the wild is known by the hunters.

The trade of K. scorpioides in Arari, Marajó Island was carried out exclusively with live animals extracted from the natural populations, not from legalised breeding facilities, and destined for consumption both locally and in towns. According to our observations, the animals were not used as a medicinal product as identified by Alves et al. (2008), who described the biotherapeutic use of this species in Mexico and elsewhere in Brazil. Unlike the observation of Shiping et al. (2006), the sale of K. scorpioides was not carried out only in one place, but occurred in different areas of the cities surveyed. K. scorpioides was marketed in a bundle of twelve animals, similar to reports by (Delducque, 2000). Based on the value of a monthly income and the value of the dozen turtles, it is estimated that up to 17 dozen of K. scorpioides/month/trader could be traded; that is, 204 animals/month or 2,448 animals/year.

The origin of animals detected for the market indicates that this phenomenon is typically intra-regional (e.g., on Marajó Island) but can reach more distant places, such as large urban centers (e.g., Belém, Pará) and other states. For that to happen, the networks that are established until the final sale must involve several players: *K. scorpioides* hunters who sell to middlemen, traders, restaurants and also directly to consumers.

The frequency of consumption of *K. scorpioides* by the vast majority (98%, n=55) of interviewees indicated a high demand. This fact was highlighted by Renctas (2002), Baía-Júnior et al. (2010), Morcatty & Valsecchi (2015), Morsello et al. (2015), and Van Vliet et al. (2016) as a potential threat to wild animals, in this case the sustainability of populations of *K. scorpioides*.

The taste preference for wild meat, according to Koster et al. (2010), influences the choice of which animals are consumed. There are customs linked to the cultural tradition in the local population that the marajoaras that reside in other regions usually request the shipment of these products. According to Nasi et al. (2011), this practice persists because many residents of urban areas in tropical countries still have strong links to rural culture, preferring game meat rather than other sources of protein. This was also observed by Morcatty and Valsecchi (2015) when studying the factors that influenced the hunting and trade of the tortoise *Chelonoidis denticulatus*.

Based on the income of restaurants with dishes based on *K. scorpioides*, at the time of greatest supply, a profit of approximately 50% is estimated on the average sale of 36 to 72 meals of *K. scorpioides*/month. This represents the purchase of six to 12 dozen live animals. According to Morcatty & Valsecchi (2015), middlemen profit more than hunters, and final traders always more than everybody else. The latter, with better purchasing power than the others, control the hunting trade (Van Vliet et al., 2016). In studies carried out in Belém, Pará, it was observed that the average demand in restaurants was 260 animals/ month, or 1,248,000 animals/year to fulfil the demand of the entire market, assuming 400 restaurants (Palha et al., 2006). However, the supply to the market is always based on the extraction from natural populations, since there is no legalised commercial breeding of the species in the country. In this sense, legalised breeding in captivity would be able to supply the market, as well as contribute to the reduction of pressure on natural stocks (Silva et al., 2004, Silva et al., 2014).

Hunting of *K. scorpioides* was affected by water cycles. During the rainy season, opportunistic capture was conducted via hand-capture and probing, and in the dry season, burning was used. Pezzuti et al. (2004) and Barboza et al. (2013) observed the use of artifacts for hunting other species of turtles of the Amazon, such as dart, reeds, mallets, jaticá harpoons, arrows, rifles, baits and longlines, which were not identified in the present study. Barboza et al. (2013) analyzed the hunting methods of the chelonians in the floodplain of the Amazon River in Santarém and identified a wide combination of these techniques based on local ecological knowledge.

Hand collection of *K. scorpioides* occurred mainly due to the reproductive behaviour of the females during the transition between the rainy for dry season, at the tide change. At that time, females could easily be found nesting, which facilitated their encounter and capture. This was also a strategy frequently used for the collection of *P. expansa*, *P. unifilis* and *P. sextuberculata* (Pritchard & Trebbau, 1984; Fachin-Terán et al., 2004; Barboza et al., 2013). Probing has been described in the capture of tracajás *Podocnemis unifilis* (Fachín-Terán et al., 2004; Barboza et al., 2013). Moll & Moll (2004) also verified the use of probing in Asia, Australia and the U.S.A.

The information obtained from the hunters on the diet of K. scorpioides corroborated the literature. According to Pritchard and Trebbau (1984), the species is mainly carnivorous, and according to Marques et al. (2008), also an opportunistic omnivore. The sexual dimorphism reported in the empirical reports of this work is in agreement with studies carried out in captivity with K. scorpioides, where differences were observed in relation to animal size, shape of the shell and tail length (Marquez, 1995; Berry & Iverson 2001, 2011). Regarding reproduction, mating occurs during the rainy season and is related to the greater availability of resources (Sexton, 1960), followed by a period of nesting, subject to seasonality, during the less rainy period. This pattern was also observed in captivity (Castro, 2006). These facts indicate that the physiological conditions observed in nature are reached in an ex situ environment, which is a positive factor for the captive production of the species and facilitates its conservation.

The so-called "ultimate period", in which *K. scorpioides* burrows for months in a state of estivation during the dry season was also reported by Delduque (2000), Souza (2004), Pereira et al. (2007), Vogt (2008) and Berry & Iverson (2011). This behaviour was described by these authors as an adaptation for the regulation of the heat water exchange under adverse environmental conditions.

CONCLUSION

Here we show that the commercial network for K. scorpioides in the Marajó Island complements the income of hunters and produces profitability for the other actors involved from the extraction of the animal from nature, up to the sale to the consumer. It is difficult to quantify the impact of this trade, although it is crucial to assess the status of K. scorpioides populations and to construct appropriate conservation strategies, but the legal status does not prevent the sale of this animal. Further study is needed to establish the connection of the Marajó Island trade to that of large urban centers, and the creation of a productive chain or just a diffuse and seasonal trade. In addition, research on biological data in nature, mainly on reproduction and population density for this species, is necessary. Consumption and high demand of K. scorpioides are associated with regional cultural factors, but human overcrowding and lack of in situ management would, over time, reduce the access and availability of this animal. It is necessary to organise efforts to apply environmental education to this activity. It may be possible to meet the market demand for this turtle through the implantation of captive breeding programs.

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FULL PAPER



Frogs in pre-industrial Britain

Lee Raye

Swansea University, Singleton Park, Swansea, SA2 8PP, U.K.

This paper examines the pre-industrial historical record of Britain's anuran species. The records examined include especially the writings of naturalists and physicians, most notably Gerald of Wales (1188), John of Gaddesden (c.1314), Edward Wotton (1552), Timothie Bright (1580), Thomas Brown (1646), Robert Lovell (1660), Christopher Merrett (1667), Robert Sibbald (1684) and John Morton (1712). The common frog is attested as present throughout the period. Several reliable historical records are located that describe the presence or absence of a water frog species (*Peloyphylax* spp.: two records of presence, two of absence) and the tree frog (*Hyla arborea*: five records of presence, three of absence). The moor frog (*Rana arvalis*) and agile frog (*Rana dalmatina*) are not described separately – if present in the time period, they may have been considered varieties of the common frog. The evidence of presence comes exclusively from England. The records taken together confirm the presence of populations of water frogs between (at least) the fifteenth and eighteenth century, and provide new evidence attesting to populations of tree frogs between the sixteenth century (when the species may have been introduced) and the eighteenth century (when the species seems to have become locally extinct/locally distributed).

Key words: pool frog, tree frog, native status, species history, historical analysis

INTRODUCTION

n the nineteenth century, there were six common research methods used to investigate species history. We can rank these through their use in Harting's (1880, pp.115-205) influential discussion of wolves in British animals extinct within historic times. These are, in order of importance: historical (the analysis of contemporary written sources), archaeological (which was called 'geological', and was seen as the only method that gave reliable evidence from the distant past), folkloric and linguistic (which could give evidence from pre-historical period), toponymic (place-name evidence), and art historical (generally only used to illustrate arguments). By the time Ritchie (1920, pp.3–4) wrote, some of these methods had already declined in importance. Ritchie considered historical, art historical and archaeological to be the proper methods of study for species historians. Species historians no longer considered folklore collected in modern times to be good evidence. We now recognise that folklore is not a repository of ancient wisdom, perfectly preserved by an ignorant peasantry, but a contemporary set of beliefs and practices received and adapted successively by each new generation of participant (Gazin-Schwartz & Holtorf 1999, pp.9–13). Linguistic evidence was also rejected because words change meaning unpredictably over time, and the existence of a word for a species in a language is often unrelated to that species' presence/absence in the local area anyway (Dent 1974, pp.23–7). Place-name evidence was rejected by Ritchie, but has since been recovered by species historians, especially due to the efforts of Yalden and his co-authors (Aybes & Yalden, 1995; Yalden, 2007; Boisseau & Yalden 1998). The idea that we can draw conclusions about the presence/absence of species from naturalistic artwork has also since been criticised by some scholars (D'Aronco & Cameron, 1998, p.41; Backhouse, 1981, pp.165–174; Raye, 2013), although its use as championed by Yapp (1981) remains current.

At the same time, whilst historical source analysis has continued to be relevant, analysis of historical records has become much more complicated. The dates of some key sources have been challenged (e.g. Drout et al., 2014; Linnard 1984), 'forgeries' have been detected and separated from authentic historical source traditions (e.g. Morgan, 1983; Thomson, 1952) and historians now have a more sophisticated model of authorship as a process rather than as a single event; texts have authors, editors, scribes, translators and readers who all add additional layers of meaning (e.g. Charles-Edwards, 1978).

Perhaps because of these set-backs, the methods of species historians have become increasingly scientific. Modern zooarchaeological analysis has been developed scientifically, particularly by the use of absolute dating methods like carbon-14 dating (Bayliss, 2009) that allows archaeologists to be more confident about the context of organic remains, as well as the use of wet-sieving and flotation methods that allow the remains of much smaller species to be detected (McKenna et al., 1994).

Correspondence: Lee Raye (lee.raye@gmx.co.uk)

However, even scientific zooarchaeology has limitations. In the case of the pool frog, the archaeological record has been skilfully and carefully consulted (Gleed-Owen, 2000; Snell, 2015). However, in other cases, archaeological remains can be deceptive. The natterjack toad (Bufo calamita) is likely to be a native of Ireland, but natterjacks are not found in the archaeological record (Beebee, 2002), perhaps because the size of the bones mitigates against their survival and retrieval by archaeologists, and archaeological work is focused on human habitation (where natterjacks would be unlikely to occur). At the same time, the remains of a land tortoise have been found at Stafford Castle in Britain (Thomas, 2010), which clearly do not indicate the presence of a wild population of Testudinae. The presence of burrowing species can be especially complicated as they are capable of burrowing their way into the archaeological strata of older periods and either mixing the strata or leaving their remains in an anachronistic stratum (Bocek, 1986). In terms of species history therefore, where the historical and the archaeological methods can corroborate each other, this provides the most secure conclusions.

In modern times, species historians have also added an additional method of inquiry. Genetic satellite (phylogeographic) analysis provides historical evidence from the genetic material of modern species (Snell et al., 2005; Teacher et al., 2009). Genetic methods are especially exciting because they do not rely on the survival of contemporary material, and can therefore produce new data relatively quickly. This same advantage is also true of the call analysis methodology (Wycherley et al., 2002).

The methods of researching species history that remain frequently used today include genetic research, zooarchaeology, historical analysis, place-name evidence and art history. This paper is focused on historical analysis, and in the Conclusion, we will evaluate how useful the approach has been in answering questions of presence/ absence, native status and introduction routes compared to zooarchaeology and genetic analysis in particular.

Amphibians in pre-industrial Britain

Over the last twenty-five years there has been a growing scholarly consensus that at least one species of amphibian became extinct in the historical period (i.e. the last two thousand years). Until the end of the twentieth century, it was commonly believed that Britain possessed six native amphibian species (Buckley & Foster, 2005): the common toad (*Bufo bufo*), natterjack toad (*B. calamita*), common frog (*Rana temporaria*), smooth newt (*Lissotriton vulgaris*), palmate newt (Lissotriton helveticus) and great crested newt (*Triturus cristatus*). We now commonly add at least the pool frog to this list (*Peloyphylax lessonae*), and sometimes also the moor frog (*Rana arvalis*), the agile frog (*Rana dalmatina*) and the European tree frog (*Hyla arborea*).

The evidence for these species being native is variable. The tree frog is considered to be possibly native on the basis of two previously long established populations and a study of the historical record (Snell, 2006; Snell, 1991), but this study provided only two sources from before the twentieth century, and there are known to have been a series of introductions in the nineteenth and twentieth centuries (Lever, 2009, pp.217-8). The moor and agile frogs are thought to be native based on early medieval archaeological evidence, but the evidence comes from a single site (Gleed-Owen, 2000). The species that has been most investigated is the pool frog, which occurred in Britain in the twentieth century and was the subject of an interdisciplinary study by English Nature and others from 2000-2004. Scholars pursued several different avenues of research to investigate the species. Zooarchaeological surveys produced remains of the frog from late Saxon Gosberton, Lincolnshire (Gleed-Owen, 2000), and late Saxon Ely, Cambridgeshire (Beebee et al., 2005; Gleed-Owen, 2001, pp.5, 25–26). Genetic analysis (Snell et al., 2005) and call analysis (Wycherley et al., 2002) confirmed that the population present in 20th century Britain was closely related to populations found in Norway and Sweden, and therefore should be seen as part of the northern clade, with an introduction pathway via Scandinavia. Finally a search of historical and archival sources from the industrial period (Kelly, 2004) proved continuity of population from the eighteenth to the twentieth century. Based on this evidence, conspecific northern clade pool frogs have been reintroduced to the wild and are currently being introduced to a second site in East Anglia (Buckley & Foster, 2005; ARC, 2015).

Previously the pool frog reintroduction project was subjected to criticism (Burton & Langton, 2005; Langton & Burton, 2005). From the perspective of a critic of the introduction, an undocumented introduction from Norway or Sweden prior to 1750 would produce a population of British pool frogs that might also be consistent with the genetic data, call analysis data and archival records (although see contra: Buckley & Foster, 2005, p.5). This possibility is especially concerning because water frogs are invasive in some areas, and have, for example been known to displace common midwife toads and yellow bellied toads in central Europe (Roth et al., 2016). From the perspective of Anthropocene scholarship, the native fauna of a country constitutes a safe operating zone for biodiversity (Rockström et al., 2009), and removing and adding species is potentially disruptive.

However, the archaeological evidence argues against this possibility. In addition to the two late Saxon sites (Gleed-Owen, 2000; Gleed-Owen, 2001), further archaeological remains of water frogs have been identified, proving that the pool frog was also present at a religious site in late medieval London (Snell, 2015). This evidence came midway through the thousand-year gap between Gleed-Owen's (2000) study of the archaeological record from the end of the first millennium and Kelly's (2004) study of the archival records from the end of the second millennium. Based on this evidence the conclusion that the pool frog is a native is a secure one.

METHODS

This paper describes a directed search for references to frog species other than the common frog in the pre-

industrial historical record of Britain.

It is not possible to explore every pre-industrial reference to the frog in a paper of this length. Therefore, for the purposes of our study, I have identified two promising genres of evidence from the pre-industrial period:

1. The earliest British natural histories from before Linnaeus (1735) as described at the time by Scheuchzer (1716), in Miall (1912), and with respect to herpetologists in particular, in Adler (1989). The natural history genre was selected because naturalists are expected to be the most reliable surviving witnesses of the presence/absence and native status of various frog species in their area within their lifetimes.

2. The work of British physicians describing materia medica (natural pharmaceutical ingredients), where they mention frogs. These have been indexed by Early English Books Online, and The Malaga Corpus of Late Middle English Scientific Prose. This genre was chosen because physicians are also likely to be comparatively reliable informants. Before the popularisation of chemical medicine, physicians were considered to be students of the natural world (physis) and particularly the plants and animals harvested for medicine (Cooper, 2007, pp.88–90).

Supplementary references were also found by recourse to the available searchable national corpus projects (i.e. The Oxford English Dictionary, The Middle English Dictionary, The Dictionary of Old English, Geiriadur Prifysgol Cymru, The Dictionary of the Scots Language, The Dictionary of Latin from British Sources, Welsh Prose 1300-1425). Corpuses in Anglo-Norman, Norse and Gaelic have been considered outside of the scope of this study, since with these languages it is difficult to separate out material that geographically describes the native British fauna rather than an Irish or continental fauna.

Most of these sources have been either scanned with Optical Character Recognition (OCR) or have been edited into facsimiles and editions. These can generally be searched through online database-specific search engines. Most of the databases collect orthographical variations of words together, but where necessary I searched for a range of terms (e.g. 'rana', 'rane' 'ranuncul*', 'padock', 'pudoke', 'pode'). The natural history texts (1) were also searched manually. The resulting references were then sorted with the aim of producing references to the presence/absence of frog species other than Rana temporaria in pre-industrial Britain.

RESULTS

Medieval naturalists

We begin with references from the pre-industrial naturalists since these are generally clearer and more indepth than the medical references and will allow us to identify the most common terminology in use.

Medieval scholarship generally emphasised by-rote learning of a set corpus of material. Medieval naturalists also generally excluded the local knowledge that ordinary people must have possessed about their local environments (Etheridge, 2007; Holmes, 1936). There are, nevertheless, two medieval British naturalists' accounts that we might consider. The first is De Proprietatibus Rerum by Bartholomeus Anglicus (c.1240 CE). This was translated in 1397 by John Trevisa into English. Book 18 of these texts discusses animals, and the frog is discussed in chapter 91 of this book (Anglicus, 1492; the frog section is not included in Steele, 1905). Both author and translator distinguish five species of frog: *Rana aquatice* (water frogs); *Rana palustres* (Trevisa: 'moor & mire frogs', moor frogs); *Rana rubete* (common toad); *Rana calamite* (natterjack toad); and *Rana agredule* (common frog?). This text is significant as the only pre-industrial historical text I have found to mention the moor frog.

Unfortunately, this work needs to be rejected for our purposes. De Proprietatibus Rerum is a complete encyclopaedia, and Book 18 is not dedicated solely to native species. The very next entry after Rana (the frog) is a semi-mythical account of Salamandra (the salamander), and there are also sections on exotic species like elephants (*Loxodonta africana*) and lions (*Panthera leo*). The work is therefore of very limited use for establishing the presence/absence of British anuran species. It draws heavily on the international bestiary tradition (compare placement in: Barber, 2006, p.116; see: Steele, 1905, p.138). The bestiary tradition is obviously not considered for the same reason (see: George, 1981).

By contrast, Gerald of Wales is generally one of the few medieval authors to make reliable observations of local fauna (Holmes, 1936). In Gerald's (1188) Topography of Ireland, it is noted:

In France and Spain, frogs are loud and talkative, in Britain they are mute, in Ireland there are none.

(Dimock 1867, p.62 (I:28))

As Beebee et al. (2005) have noted, the mating call of the common frog is low frequency, and not usually heard any distance from the breeding pool. This is not the case for the pool frog or the tree frog, meaning that the calls can serve as species indicators (see also: Beebee & Griffiths, 2000, p.87; Dutt 1906, pp.165-174). This evidence suggests that twelfth century Britain lacked water and tree frogs, and the Ireland lacked all frogs. But it is not clear how far we can trust Gerald's remarks. Other preindustrial naturalists agreed with Gerald that Ireland did not have a native population of frogs. The most reliable are Bartholomew of England's De Proprietibus Rerum (XV:80) and George Owen's Description of Pembrokeshire (Miles, 1994, pp.225-6). The claim is also made by O'Suillivan in The Natural History of Ireland, written in 1626. One of O'Suillivan's main reasons for writing was to correct and criticise Gerald of Wales (O'Suillivan, 2009, pp.19-20), and this would have presented an ideal opportunity, but in fact O'Suillivan exactly follows Gerald:

[Ireland] does not produce the frog, the scorpion, the toad, the viper, the serpent or any poisonous animal or insect. (O'Suillivan 2009, pp.166–7)

However, it is clear from the historical evidence that common frogs were known in Ireland before the best known introduction to Dublin in 1699 (Scharff, 1893), and the genetic evidence suggests that whilst a good number of Irish frogs are descended from the standard western European stock (and were probably introduced) some seem to have been present throughout the last Glacial Period (and are native) (Teacher et al., 2009).

Further, Gerald's note was also only added to later versions of the Topography of Ireland. In these versions, Gerald concentrates on adding material of classical and religious allegorical significance and quotations from earlier authorities rather than naturalistic observations (Bartlett, 1982, pp.143–4). The idea that Ireland lacked poisonous creatures (especially snakes) was central to Gerald's claim of its holiness as the most western isle, and the Isle of the Saints. The claim is of course not original to Gerald, and can be found in Bede's Ecclesiastical History of the English People, written in 731 CE (Sherley-Price et al., 1955, p.46 (I.1)). This comment therefore might need to be understood as a moral and religious one (as suggested in Scharff 1893) rather than one based on naturalistic observation.

It is worth noting the large amount of corroborating but generally unreliable evidence that pre-industrial Britain was known to have loud-calling frogs. One of Aldhelm of Sherborne's letters uses the metaphor of a loud pool of frogs croaking (Lapidge & Herren, 1979, p.154), but the use of metaphor is not a reliable indicator of species presence (Raye, 2016). Additionally, the earliest Old English glossaries give frog as a translation for the Latin word luscinius (nightingale). This might be a reference to the calling of the water frog, just like Kelly noted a thousand years later in archival references to East Anglian water frogs (Kelly, 2004) but is more likely evidence of the earlier confusion between acredula (small bird) and agredula (frog) (see: Dictionary of Old English). The loud calling of 'water frogs in fenlands', is also noted as a model of lack of discretion in early eleventh century expanded Old English Rule of Chrodegang, (Napier 1916, pp.94–6) but this example is translated directly from the ninth century continental European Latin version, so does not provide evidence of native status. From the early modern period we might also cite Lupton's Thousand Notable Things of Sundry Sortes, which suggests leaving candles burning on the banks of the pond to stop frogs calling at night (1579, p.130). Like the other thousand things, this remedy is almost certainly borrowed by Lupton from elsewhere, although presumably Lupton including the remedy might suggest it was considered to be useful for Britain as well. An early modern Welsh almanac style manuscript called Llyfr Ffortun Bangor (NLW Film 1067 (Bangor 4)) from 1685-8 in the National Library of Wales contains amongst its signs foretelling rain: 'when frogs call, this foretells rain in a short time' (Williams, 1927, p.107). Frogs croaking before rain is mentioned by naturalists as the particular property of the tree frog (Topsell, 1658, p.724; Gesner, 1554, p.61), although the reference here is probably originally based on the observations of Aratus of Soli in the third century BCE (Poochigian 2010, I.991). There is a reference in John Evelyn's (1664, p.297) Sylva to frogs wailing in the leaves of alder and willow trees beside rivers, but this is part of a direct translation from René Rapin's (1665, pp.47-48) French Hortorum. This association appears to be folkloric. A modern study of the tree frogs in Zurich found the probability of calling activity is not usually affected by rainfall (Blankenhorn, 1972). Kelly's (2004) clearer evidence that frogs in the fens were known to call loudly, and were known as nightingales comes from the eighteenth century, especially Morton (1712, pp.440–2).

Renaissance naturalists

Among the renaissance natural histories, the most useful sources for those interested in biodiversity and native status are those that are exclusively interested in species found locally or nationally. Therefore, I do not include naturalists' encyclopaedias that fail to distinguish native and non-native species, (Topsell, 1658; Ray, 1693; Wilkins, 1668; Walton, 1653). The most reliable accounts are: Wotton (1552) De differentalis animalium libri decem, Merrett (1666) Pinax rerum naturalium Britannica, Sibbald (1684) Scotia Illustrata, Morton (1712) The Natural History of Northampton-shire and Pennant (1776) British Zoology vol. 3: Reptiles and Fish. To these accounts we can add two further sources that discuss native frogs (Harrison (1577) The Chronicles of England, Scotland and Ireland, and Brown (1646) Pseudodoxia Epidemica). These authors are not naturalists, and therefore their opinions are less reliable, but they are scholars and their opinions seem informed by observation of the natural world.

There is some difficulty in identifying the species intended by these authors. Among the early naturalists between Gessner (1551) and Linnaeus (1735), binomial taxonomy was the standard, but the terms themselves were not yet standardised (Cooper, 2007, p.169). Some species had several terms, and authors might make up their own. However, we can generally be confident attributing modern species to each piece of terminology for four reasons: (i) the naturalists were aware their terminology was vague and often gave detailed descriptions of the species they included, (ii) although the terminology was not standardised, the most popular terms are used in multiple texts, and differentiated from each other, (iii) this is the era when naturalistic scientific illustration began (see: Etheridge, 2007), and some contemporary European authors provide useful pictures when they described these species (see Fig. 1 & 2), (iv) Linnaeus made a collection of species described in Systema Naturae. Specimens of the species of herpetofauna named by Linnaeus have been examined and identified by Dundee (1994). Table 1 gives the results.

Two of these identifications may surprise readers. (i) The term green frog in this period refers to the tree frog (*Hyla arborea*) not to the water frogs (*Pelophylax* spp.), while (ii) the term water frog becomes ambiguous after 1700. I will discuss the evidence for these identifications in the section below. As explained above, these identifications are as secure as possible, based on where the authors differentiate between species, where they identify multiple names together, where there are pictures available of the species, and where a Linnaean specimen survives. However, when reading this table, it should be cautioned that historical evidence can be difficult to interpret with a high degree of confidence and it is important to read this table alongside the relevant text so that the limitations of each source is understood.

It is also important to note that I have only included here names used multiple times, as most authors in this **Table 1.** Linnaean identification of Latin terms used by renaissance naturalists.

Term	Attributed by	Probable species
Rana aquatile Rana aquatica Rana aquatica innoxia Water frog	Brown Walton Merrett Pennant	Pelophylax spp.& (later) Rana temporaria
Rana Frog	Harrison Merrett Sibbald Pennant	Rana temporaria? Rana spp.
Rana viridis Ranunculus viridis Green frog	Brown Walton Merrett	Hyla arborea
Rana temporariae Rana temporaria 'Short-lived frog'	Brown Pennant	Rana temporaria
Rana arborea Ranununculus arboreus Dryopetes Tree frog	Brown Merrett	Hyla arborea
Rana fluviatilis Rana fluviorum River frog	Wotton Pennant	Rana temporaria? Rana sp.?
Rana terrestris Land frog	Walton Merrett	Hyla arborea & Rana temporaria?

period invent or use a wide variety of names. For example, only including the Latin names, Pennant gives:

Rana fluviorum Rana aquatica innoxia Rana aquatica Rana temporaria Rana (Pennant 1776, p.9)

All these names are attributed to the common frog, to try and make it as clear as possible which frog Pennant is referring to.

Altogether, of the authorities presented here, Harrison (1577), Sibbald (1684) and Morton (1712) describe only the common frog as a native; Wotton (1552) describes the common frog and tree frog as native; Brown (1646) and Merrett (1666) describe the common frog, tree frog and water frog, and Pennant (1776) describes the common frog, water frog and the 'great frog' (Pennant, 1776, p.20), which is only found in a single Scottish loch and therefore sounds like either a piece of folklore or an introduced species. There is clearly some disagreement between our sources here.

Much of this dispute can be cleared up by examination of the scope of the authors. Harrison, Sibbald and Morton are significant not only in distinguishing only a single native species of frog, but also in considering only part of Britain. Harrison and Sibbald are both describing the fauna of Scotland and Morton is describing the fauna of Northamptonshire. The records can thus be reconciled by the suggestion that the water frog and tree frog were not widely distributed in these parts of Britain when the authors wrote, although Northamptonshire's situation on the edge of the fens, where the pool frog was known to exist in later times (Kelly, 2004) makes this suggestion more difficult. Despite Morton's talents as a county naturalist, it is difficult to take him seriously as an authority on anurans. He admits he "never yet had the hardiness of meddling with them [toads] so far" (i.e. he never studied them in the field) and even indulges the theory that toads are just dried up frogs (1712, p.440). However, it is only fair to suggest that, given Morton's late date of writing, it is equally possible that any water and tree frogs that may have been formerly present had become locally distributed and uncommon. When he wrote, John Jonston (1657, p.185), usually one of the most reliable continental naturalists, gives a confused statement in his section on water frogs that 'Rana viridis' ('the green frog' - a term he otherwise reserves for the tree frog, meaning that it is not entirely clear which species the term is being used for here) is not found in England. Later, Gilbert White, an English author also attests that absence of the tree frog in a letter to Pennant in The Natural History of Selborne (Mabey, 1977, p.50). Pennant appears to have been convinced by White's testimony (presumably it agreed with his own experience) and he leaves the tree frog out of his account of the British species (Snell, 2006). White's view on the subject continued to be the established one in the twentieth century (Mabey, 1977, p.273).

The overall pattern here is that every renaissance British account interested in native species across the whole of Britain before 1700 identifies at least two and sometimes three species of frog as native but accounts from Scotland, and from southern Britain after 1700 only distinguish one species. None of the accounts record the moor frog (*Rana arvalis*) or agile frog (*Rana dalmatina*). It is possible that the naturalists could not distinguish them from the common frog (*Rana temporaria*).

Terminology used for the tree frog and water frog

As explained above, there are two instances on our list of names changing in meaning. The first is a simple one. Although we often call the water frogs (i.e. the pool frog, edible frog (*Pelophylax* kl. *esculentus*), and marsh frog (*Pelophylax ribundus*)) 'green frogs' today, in the sixteenth and seventeenth century the terms *Rana viridis* and green frog originally described the tree frog. We can be certain about this from contemporary labelled specimens of the species (Dundee, 1994), and from pictures. We have a British authority for this. Topsell calls the tree frog the 'green frog' in his translation of Gessner. He distinguishes it from the common frog and the water frog and depicts it on a leaf as we see in Fig. 1.



Figure 1. Illustration of a tree frog on an Acer leaf from Topsell (1658, p.724), where it was copied from Gessner (1554, p.60). Heading moved into the frame from higher on the page. Image is in the public domain.

We can also be clear about the identity of the green frog based on other contemporary textual accounts (Aldrovandus 1637, pp.589–624). An example from Britain is in Ray:

Rana arborea or *Rannunculus viridis*; The small Treefrog or Green frog. It is very small, with a green colour which bathes it on all sides. It is easily distinguished because it settles in the leaves of trees. (Ray 1693, p.251)

An example from our texts is in Merrett:

Ranunculus viridis {the green frog} or Dryopetes {the tree frog}, see Gessner Historia Animalium, vol. 2, p.60. (Merrett, 1666, p.169)

The second instance of a name change is that of *Rana aquatica*, the water frog. This term is not used by Linnaeus or Gessner, but there is an excellent picture of the species by the later German naturalist Roesel von Rosenhof (1758), see Fig. 2. Von Rosenhof calls the frog *Rana viridis aquaticae*, and depicts what we would call in modern times a water frog, and distinguishes it from the *Rana fuscae terrestris* (common frog) and *Rana arborae* (the tree frog).

The term is also distinguished from the common frog by two of our authors, most clearly by Brown:

By Frogs I understand not such as arising from putrefaction, are bred without copulation, and because they subsist not long, are called Temporariæ; nor do I mean the little Frog of an excellent Parrat green, that usually sits on Trees and Bushes, and is therefore called Ranunculus viridis, or arboreus; but hereby I understand the aquatile or Water-Frog, whereof in ditches and standing plashes we may behold many millions every Spring in England... (Brown 1646, pp.172–3)

Note: in medieval and early modern Europe, common frogs were thought to be produced spontaneously from mould and rot (as in Gerald of Wales: O'Meara, 1982, p.52 (I:25); Topsell, 1658, p.720; Walton, 1653, chap.8; Seymour, 1975, p.130). This was also believed of most small invertebrates. Species that were produced spontaneously rather than through sexual reproduction were thought to be demonic rather than divine. Common frogs in particular were supposed to live a short time (hence: temporariae) before returning into mud (see: Sleigh 2012b; Sleigh 2012a, pp.67–8).

The distinction of the term water frog also exactly follows Merrett:

Rana, a frog (J. 187, plate 75; A. 591). This is either a *Rana terrestris* {the land frog}, which is bigger and yellow, or smaller and black. The second is called by ordinary people *Rana aquatica* {the water frog}, or *Rana maculata* {the spotted frog}. (Merrett 1666, p.169)

As Merrett notes in the quotation above, this distinction between water frogs and land frogs is also followed by two of the most influential continental authors, Aldrovandus (1637, p.591) and Jonston (1657, p.187). The term 'land frog' later comes to refer to the toad, but Merrett describes 'Bufo, a toad' separately on the same page.

However, by around 1700 the term becomes generic,



Figure 2. High quality scanned illustration page showing a water frog at rest, and two water frogs in amplexus from von Rosenhof (1758, fig.13). This scan licensed for publication by Heidelberg University Library under CC-BY-SA 3.0.

and *Rana aquatica* is one of the nine synonyms listed for the common frog by Pennant (1776, p.9), possibly following Ray (1693, p.251) or Topsell (1658, p.718), who use the term as the main Latin name for the common frog.

Medieval Physicians

In addition to the naturalists accounts, accounts by physicians and particularly pharmaceutical texts often contain references to amphibians. (Getz 1992). The toad is ubiquitous throughout medical history, its use inspired by the strange properties of the bufotoxins found in its parotoid glands and other chemical compounds (DeGraaff, 1991, pp.71–76), but the medicinal use of frogs in Britain seems to have lasted 500 years, c.1200-1700 CE. In this period, frogs, especially tree frogs, were commonly prescribed as medicine. They were useful for their moist and cooling properties under the Galenic humour system (see for example: Ettmüller 1699, pp.73, 299; Lovell, 1660, pp.52–3), but also prescribed based on experience of symptom alleviation.

An early example is found in Gilbertus Anglicus' Compendium of Medicine, (ed. 1510, fol.205v), one of the first medical handbooks, originally compiled in c.1240. Here, the treatment is for anal fistulas. Gilbertus Anglicus, the compiler, suggests taking the heart of *ranunculus parvus et viridis* [i.e. a tree frog] washed in wine with juice extracted from wormwood, and feeding it to the patient with morsels of bread. This treatment is not translated in the Middle English version of Compendium Medicinae,

System of Physic. There is however, a reference to using the fat of 'grene froggis, bat lyuen among trees' to treat deafness in the Middle English version of Lanfrank's 'Science of Cirurgie' (see: von Fleischhacker 1894, p.257). These medical references are of questionable value. Gilbertus Anglicus should be seen as a compiler of medicinal recipes rather than an author in his own right (Esteban-Segura 2013, p.19; 23), and Lanfrank was born on the continent, meaning that there was no reason that either should be discussing specifically British cures. Our texts do suggest that tree frogs were sometimes thought to have medicinal properties, however, we are not justified in suggesting they were universally lauded. The general perception of frogs in the period was negative, and some physicians even believed they could be internal parasites like worms (Hunt & Benskin 2001, pp.120, 174).

The most original discussion of tree frogs as a remedy from the medieval period can be found in the Rosa Anglica, a medical handbook from c.1314. The author, Iohannis Anglicus, or John of Gaddesden once again compiles descriptions of diseases and symptoms from elsewhere, but the treatments advised here are unique. John's work is unusual in that he created many original (perhaps dubious) medicines, and sold his 'secrets' at high price to rich patrons. These are shared freely for the use of other doctors in the handbook (Capener, 1972). One such is a recipe using tree frog fat as an ointment to help rotten teeth fall out. The description of the frog is very specific.

This is the secret cure for which I have received good money from the Barbers. Take a green frog which is arboreal in its habitat. It climbs from tree to tree, and many of them may be found in Provence. Take its fat and anoint the tooth with it. The tooth will fall out straight away. (trans. of: Anglicus 1502, p.120)

The text here seems like an introduction to the tree frog, as if John does not believe that his readers will be aware of the species. As well as being described like this, they are also mentioned as native to Provence, in modern day south-east France -- the text I am translating gives Puincia, which is an ambiguous shortening, but an earlier version of the text gives Provincia (Cholmeley, 1912, p.40). On balance, this reference implicitly suggests John of Gaddesden did not believe the tree frog to be native to Britain, since he introduces the species as a novelty, and suggests its ordinary home is abroad. The popularity of these medical recipe books stems from the increasing demand for fast-acting pharmaceutical cures over medical regimens in the second half of the medieval period. The apothecaries at the time were incorporated in the Company of Grocers, and commonly imported popular and exotic remedies to meet demands (Getz, 1992).

Renaissance Physicians

Tree frogs continue to be popular medicinal simples in the sixteenth and seventeenth centuries. The most common use of the species is for putting out teeth as in the Rosa Anglica, there are references to this use in Levens' Pathway to Health (1596, p.20). A mixed medicine of tree frog and water frog together is recommended in the Treasure of pore men (Anonymous 1526, fol.16 v.) and in Moulton's Compleat Bone-Setter (1657, p.120). Medicine from tree

frog and water frog is also suggested for the ague and for hair-loss in The Treasury of Healthe (Lloyde, 1553), and for hair loss in the Homish apothecarye (Hollybush 1561, fol.2 r.). These references must all be treated with caution for our purposes since they might still just reflect the importation of exotic references.

However, this reservation cannot be made of the Treatise on English medicines attributed to Timothie Bright. In the early modern period a debate arose between physicians who advocated the use of the most successful and exotic remedies as the best (although these were often imported in at great cost, and sometimes adulterated) and those who advocated local medicines to be mystically more effective for the people living in an area (see esp. Cooper, 2007, pp.29–45). Bright was firmly on the side of indigenous medicine, and drew up a list of native ingredients to assist. 'Greene frogges' (=tree frogs, see above) were included near the top of the list:

And heerein (gentle reader) thou art not to looke I should set downe all medicines which our natiue soyle is knowne to bestow vpon vs for cure of these diseases... And first to begin with Cankers, which being not exulcerated but remayning humors, are cured (if with any medicine) by the iuyce of Nightshade, all the sortes of Endiue and Succorie, with Agrimonie, with Saint Iohns wort, wilde Clarie, called Oculus Christi, the flesh of Snayles boyled, Crayfishes, greene Frogges, and to conclude, with all kinde of metalls and mineralls; and among them Leade, how so euer it be vsed, is most souereigne (Bright 1580, pp.44–45).

Bright was hostile to exotic remedies, so the inclusion of tree frogs on this list is possible evidence of the species' presence. However, since Bright was not a naturalist, this evidence may not be reliable.

From the seventeenth century there is a reference in A Friend to the Sick to the use of tree frogs against plague carbuncles (Sermon, 1673, p.223) and an introduction to the use of 'green frogs' in Pechey's (1697, p.213) Plain Introduction. They continue to be referred to in direct translations of foreign texts (e.g. Barbette, 1687, p.99; Ettmüller 1699, p.69; Surflet & Markham 1616, pp.39, 614). The last of these references also refers to the use of water frogs in medicine, which is also introduced in considerable detail in Schröder's Zoologia (1659, pp. 127– 130). However, this century also sees the end of the use of frogs in British medicine. Neither species is mentioned in the official national dispensatories and pharmacopoeias.

The strongest medical reference from this century comes from another British author, Robert Lovell, and his (1660, p.53) Panzooryktologia. This medical text discusses all natural simples commonly in use, including a series of exotic species like the crocodile and chameleon. At the beginning of every species' account are three bullet points on P[lace found], M[eat/Diet], and N[ames]. The Place found is usually specific, e.g. the chameleon is found 'in Asia, Africa, and India, or the Indies' (1660, p.30). The green frog is said to be found 'Almost everywhere, in woods, and among reeds'. This makes it likely that the species was present in Britain, but since Lovell is not explicit, the evidence is not certain.

Table 2. Evidence for water frogs and tree frogs from Britain. "Pres." = Attested present, "Ab." = Attested absent, "-" = Not mentioned) This table also lists pre-Linnaean names given in the historical sources.

Text	Date	Rana temporaria	Peloyphylax spp.	Hyla arborea
Ely archaeological remains (Beebee et al. 2005)	850-1050	Pres.	Pres.	-
Gosberton archaeological remains (Gleed-Owen 2000)	880-1040	Pres.	Pres.	-
Topography of Ireland (Dimock 1867)	1188	Pres. 'Rana'	Ab. Ranae in Gallia et Ita	alia clamosae et garrulae
Rosa Anglica (Anglicus 1502)	1314	-	-	Ab. 'Rana viridis'
Greyfriars archaeological remains (Snell 2015)	15th c.	Pres.	Pres.	-
De differentalis animalium libri decem (Wotton 1552)	1552	Pres. 'Rana fluviatilis'	-	Pres. 'Rana parva'
A Treatise (Bright 1580)	1580	-	-	Pres. 'greene Frogges'
Pseudodoxia Epidemica (Brown 1646)	1646	Pres. 'Rana temporariae'	Pres. Rana aquatile	Pres. 'Ranunculus viridis' / 'Rana arboreus'
De Quadrupedibus (Jonston 1657)	1657	-	Ab. Rana viridis	
Panzooryktologia (Lovell 1660)	1660	Pres. 'Those belonging to rivers'	-	Pres. 'Ranunculus vir.' / 'Frog of the land', / 'green frog'
Pinax rerum naturalium Britannica (Merrett 1666)	1667	Pres. 'Rana terrestris'	Pres. 'Rana aquatica'	Pres. 'Ranunculus viridis' / 'Dropetes'
The Natural History of Selbourne (Mabey 1977)	1788	Pres. 'frog'	-	
Records from East Anglia (Kelly 2004)	1770-1995	Pres.	Pres.	-

CONCLUSIONS

Table 2 lists the most reliable historical evidence for the presence/absence of water frogs and tree frogs in the pre-industrial period. It does not include the agile frog or moor frog, since these are not mentioned by any reliable historical source. If these species were still present in Britain at the time (as Gleed-Owen (2000) suspects for the moor frog) they may have not been distinguished from the common frog.

For each of the contemporary sources, I have given the terms used by the author. Generally, the common frog is called the 'temporary', or 'river' frog. The tree frog is called the 'tree', 'little', or 'green' frog (the last term only comes to refer to the water frog later on). The water frog is generally the water frog, except in the ambiguous reference by Jonston. Prior to this paper, there were three main temporal points of evidence for the existence of water frogs in Britain. Gleed-Owen (2000) found remains from the late-Saxon period; Snell (2015) found remains from the fifteenth century, and Kelly (2004) found continuous historical evidence dating from the end of the eighteenth century to the twentieth, when the pool frog became extinct. There are gaps of four centuries between Kelly's evidence and Snell's and five centuries between Snell's evidence and Gleed-Owen's. There is also of course no evidence for the presence of water frogs in Britain before the late-Saxon remains, but there is very little evidence for the history of small species before this, so the lack of evidence there need not surprise us. Our study has been able to contribute two additional pieces of reliable evidence to fill the gap between the fifteenth and eighteenth century. Our findings therefore confirm that pool frogs were formerly present in Britain's wetlands for at least five centuries. This strongly suggests that reintroduction of the species will do no harm, even if its native species were not certain. Unfortunately, this study has not been able to fill the gap between the eleventh and fifteenth century, except for a single piece of less reliable evidence suggesting that pool frogs were not present. The testimony of Gerald of Wales is most probably to be understood as moralistic rather than naturalistic, and is not enough to overturn our understanding of the species as a native (as represented in: Beebee et al., 2005), but further research on this species is recommended to explain the historical evidence.

Our paper has been able to provide more useful evidence on the presence of the tree frog in the historical period. It is clear that the tree frog was present in Britain from at least the sixteenth century, when it is attested by an early renaissance naturalist and a localist physician, Timothie Bright. Bright, like other early modern localist physicians was interested in locally-occurring materia medica, but not exclusively native ones (Wear, 2000, pp.74–5). There are two earlier sources that suggest that the tree frog may not be a native, most importantly John of Gaddesden's assertion that the tree frog should be sought in Provence (implying it cannot be found locally). The simplest explanation of this evidence is to suggest that the tree frog was imported in the sixteenth century, and live populations were purposefully or accidentally established. Species were commonly introduced to

new habitats in the pre-modern period. For example, carp were commonly transported in wet rushes, straw or barrels of water and were introduced across Europe to be bred in fish ponds (Hoffmann, 1996; Landsberg, 2003, pp.68–9). Various species of plant and animal were shipped internationally, most commonly dead and preserved as medical simples for pharmaceutical science (Getz, 1992). This probably included tree frogs as we have seen. Monastery physic gardens imported living herbs to grow for use in medicine, possibly including aquatic plants (Landsberg, 2003, pp.38–9; Harvey, 1992). Frogs were also frequently transported for use as fish food (Currie, 1988, p.274; Amherst, 1896, p.39). Before 1660, most gardens had still-water ponds (servatoria), and apparently frogs were commonly seen in them, since Francis Bacon praises the popularisation of fountains for the new lack of annoying flies and frogs (Montagu, 1844, p.52). Species can also be introduced unintentionally (McDevitt, 2016), and Gerald of Wales (I.22) records one example of the inadvertent transportation of common toads to Ireland (O'Meara, 1982, p.51). At the other end of our time period, we also have the testimony of one of Britain's foremost naturalists, Gilbert White, that the tree frog was no longer present in the eighteenth century, by which point populations may have become extinct, or purely localised, if we accept Snell's (2006) suggestion of a historical relict population in the New Forest. The reason for decline is difficult to ascertain. Since there was clearly a great demand for medicine made of the species, we might speculate that the tree frog became extinct from Britain due to overexploitation for medical purposes, just like the medicinal leech (Hirudo medicinalis) that declined around the same period (Elliott & Kutschera, 2011).

From the perspective of our wider study, it is possible to make some observations about the analysis of historical sources from our findings. In this case, the historical record seems to more useful than the archaeological record for discussions of the early modern presence/ absence of the tree frog at least, and possibly also the pool frog. This is because archaeological evidence is not presented with an explanation. For example, there are several ways that the remains of a frog might end up in the archaeological record, whereas in the historical record we occasionally have existing range data included alongside species records. However, the historical record in this case was less useful than the archaeological record in ascertaining native status, because of the failure of renaissance naturalists to distinguish between 'local' and 'native' (Cooper, 2007, pp.32-3). The archaeological record also gives earlier records, from a period when very few texts provide useful information about natural history. Finally, it is worth noting that both the historical and the archaeological records provide only a few hints for us to speculate about possible introduction pathways. This issue can be much better answered by genetic analysis and, in the case of frog species, call-analysis studies.

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