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FRONT COVER: Pair of great crested newts *Triturus cristatus* (B. Lewis)

INTRASPECIFIC VARIATION IN THE AVOIDANCE RESPONSE OF STREAM FROG (*MANNOPHRYNE TRINITATIS*) TADPOLES TO FISH AND PRAWN PREDATORS

M. J. JOWERS, R. CAMPELL-PALMER, P. T. WALSH AND J. R. DOWNIE

Division of Environmental and Evolutionary Biology, Institute of Biomedical and Life Sciences, Graham Kerr Building, University of Glasgow, Glasgow, Scotland, UK

The stream frog, *Mannophryne trinitatis*, lives in and beside steep mountain streams of Trinidad's Northern and Central ranges. Male frogs have strong anti-predator behaviour and prefer to deposit tadpoles in pools that lack predators (particularly the fish *Rivulus hartii* and the freshwater prawn *Macrobrachium carcinus*). The two predators are rarely found in the same streams and different *M. trinitatis* populations may show specific anti-predator behaviour to the predators they encounter in the wild. To assess tadpole spatial avoidance of predators, we presented small and larger tadpoles from four *M. trinitatis* populations to each predator. Three tadpole sources were from the Northern Range: Mount Saint Benedict, Lopinot (where *R. hartii* is abundant), and the Maracas Bay area (where *M. carcinus* is present); the fourth was from Tamana cave, Central Range, where neither predator occurs. To determine predator detection mechanisms employed by the tadpoles, we presented the predators in three container types: a mesh cage (for chemical and visual detection), an opaque container with holes (chemical but no visual detection), and a transparent container (visual but no chemical detection). Different sized tadpoles (large and small) showed the same response to predators, and tadpoles principally used chemical cues to detect predators. All populations showed a stronger response to the presence of *R. hartii* than to *M. carcinus*. We attribute this latter difference to the restricted distribution of *M. carcinus* and to the few sympatric zones between the tadpoles and these predators. Thus tadpoles lacked a specific anti-predator response to *M. carcinus*. Naïve tadpoles from Mount Saint Benedict and Tamana that had never previously encountered either of the predators showed strong anti-predator responses, suggesting that the anti-predator response is likely to be inherited.

Key words: anti-predator behaviour. *Macrobrachium*, *Rivulus*, Trinidad

INTRODUCTION

Amphibian larvae under strong predation pressures have evolved a variety of anti-predator strategies such as changes in levels of activity, release of alarm substances, schooling, shifts in microhabitat, chemical secretion and spatial avoidance, to increase their chances of survival (Huey, 1980; Hews, 1988; Petranka *et al.*, 1987; Lawler, 1989; Magnusson & Hero, 1991; Watt *et al.*, 1997; Laurila, 2000; Thiemann & Wassersug, 2000; Pearl *et al.*, 2003). Chemoreception has been well documented in amphibian larvae and is thought to be the primary mechanism by which they detect predators (Petranka *et al.*, 1987; Kats *et al.*, 1988; Skelly & Werner, 1990; Bridges & Gutzke, 1997; Brönmark & Hansson, 2000; Eklöv & Werner, 2000; Van Buskirk, 2001). Tadpoles that have co-evolved with predators or adapted to continuous predator presence react to chemical cues from actual predators (Kats *et al.*, 1988; Petranka *et al.*, 1994; Laurila *et al.*, 1997; Petranka & Hayes, 1998; Relyea & Werner, 1999; Relyea, 2001) and those that do not nor-

mally encounter predators have a minimal response if any to the chemical cues released by predators in experimental conditions (Semlitsch & Reyer, 1992; Lefcort, 1996; Kiesecker *et al.*, 1996; Schmidt & Amézquita, 2001; Pearl *et al.*, 2003).

In addition to the anti-predator responses exhibited by larvae, adult amphibians can assess the presence of predators so as to avoid oviposition or deposition of tadpoles in predator-containing environments (Reserfats & Wilbur, 1989; Bradford, 1989; Kats & Sih, 1992; Petranka *et al.*, 1994; Downie *et al.*, 2001). A lack of adult anti-predator behaviour has a significant negative impact on amphibian larval success (Laurila & Aho, 1997). Habitat selection or choice for tadpole deposition is predator density dependent and influences tadpole densities in pools, which in turn has immediate consequences for other physiological, developmental and behavioural traits of larvae, hence shaping prey communities and habitat choice for breeding (Smith, 1983; Alford, 1986; Skelly, 1992; Hopey & Petranka, 1994; Lardner, 2000).

As with many dendrobatid species, male *Mannophryne trinitatis* (Garman, 1888) (see Murphy, 1997 for species nomenclature) transport tadpoles on their backs after hatching and deposit them in predator-free pools, which are part of or near a stream. They show

Correspondence: J. R. Downie, Division of Environmental and Evolutionary Biology, Institute of Biomedical and Life Sciences, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK.
E-mail: J.R.Downie@bio.gla.ac.uk

strong anti-predator selective behaviour when releasing larvae into pools, with tadpoles commonly deposited in pools which lack the freshwater fish *Rivulus hartii* (Boulenger, 1890) or the freshwater prawn *Macrobrachium carcinus* (Linnaeus, 1758) (Cummins & Swan, 1995; Downie *et al.*, 2001). Finding a suitable deposition site may take several days and may be costly either to the transporting male or the tadpoles (Downie *et al.*, 2005). Therefore, tadpoles should not encounter predators (except for opportunistic species) during development and might therefore be expected to show weak predator spatial avoidance responses in the presence of *R. hartii* or *M. carcinus* (Hopey & Petranks, 1994). On the other hand, if the predators are able to migrate extensively, deposition-selectivity may not result in predator avoidance for the lifetime of the larvae, so predator-avoidance behaviour may still be adaptive.

This study had six aims: to determine whether: (1) *M. trinitatis* tadpoles show anti-predator behaviour when presented with either *Rivulus hartii* or *Macrobrachium carcinus*; (2) *M. trinitatis* tadpoles detect predators primarily by chemical or visual cues or by both; (3) *M. trinitatis* tadpoles from different regions show stronger spatial predator avoidance to predators that are found in the same region; (4) tadpoles have different levels of spatial avoidance to different predators; (5) larval anti-predator behaviour is inherited or acquired through conditioning or experience; and (6) the predator avoidance response is dependent on tadpole size and/or age.

MATERIAL AND METHODS

STUDY SITES AND TAXA

Tadpole and predator collection (collected using handnets) and experimental trials took place during the 2002 rainy season, July - August in Trinidad, West Indies. Four populations of *M. trinitatis* tadpoles were collected from three sites in the Northern Range and one in the Central Range. The Northern Range sites were: (1) Lopinot (61° 20'W–10° 40'N), which has few *R. hartii* and no *M. carcinus*; (2) Mount Saint Benedict (61° 23'W–10° 41'N), which has many *R. hartii*, and no *M. carcinus*. The collection of small tadpoles at this site was from direct depositions by male frogs into tubs containing stream water positioned near the stream; (3) East Maracas Bay, three streams (61° 27'W–10° 46'N), one of which has many *M. carcinus* but no *R. hartii*; the others lack both predators. See Downie *et al.* (2001) for site

descriptions. The Central Range site was Tamana cave (61° 11'W–10° 29'N) whose stream has neither predator. See Kenny (1978–79) for site description. *R. hartii* were collected from Mount Saint Benedict and *M. carcinus* from East Maracas Bay.

TADPOLE AND PREDATOR MAINTENANCE

After collection, tadpoles were separated by eye into two size classes, small and large, and then maintained in separate tanks with constantly aerated, dechlorinated tap water. A random sample from each size class and site was measured: total body length to 0.1 mm using callipers; wet weight after removing surface water, measured using an electronic balance to 0.001 g. The ambient temperature in the laboratory varied little and kept the water at 27.5°C. Because *R. hartii* are capable of jumping, their tank was covered with muslin. Tadpoles were fed daily with tropical fish food flakes. *R. hartii* were fed fish food flakes daily and both predators were fed (non experimental) *M. trinitatis* tadpoles every other day. Tadpoles were kept in stock tanks for up to ten days, then released and a new tadpole stock was captured.

EXPERIMENTAL DESIGN

Batches of fifteen tadpoles from each of the four *M. trinitatis* populations were observed in glass tanks (90.5 cm × 35 cm × 35 cm) in the presence of each of the predators (*R. hartii* or *M. carcinus*), or in a control situation (no predator) using three types of containers. To test whether tadpoles reacted to visual or chemical cues, the three different predator containers were: a cage of green plastic mesh (8 cm × 8 cm × 5 cm; tadpoles could see the predator and detect it by any chemicals released); a white plastic opaque container with randomly perforated holes (6.5 cm in height × 13 cm in diameter; tadpoles could not see the predator but any chemicals released could be detected); a transparent plastic container with no openings (5 cm in diameter × 9 cm in height; tadpoles could see the predator but no predator chemicals could reach the tadpoles). Because *M. trinitatis* tadpole Gosner (1960) stages are difficult to assess, tadpoles were separated by size measurements (Table 1). Each treatment was repeated with small and large tadpoles from each population. To avoid conditioning of tadpoles to any one predator or container, the order of trials and controls was randomized with a maximum of four trials per day for each population. In

TABLE 1. Small and large *M. trinitatis* tadpole sizes: mean wet weight (g) and total body length (cm) (±SD) of all four populations (Mount Saint Benedict, Tamana, Maracas and Lopinot).

Populations	Large		Small	
	Mean weight	Mean length	Mean weight	Mean length
Benedict (<i>n</i> =20)	0.201±0.051	2.615±0.304	0.072±0.012	1.843±0.258
Maracas (<i>n</i> =20)	0.258±0.065	2.922±0.295	0.085±0.021	1.946±0.182
Tamana (<i>n</i> =20)	0.391±0.098	3.420±0.240	0.074±0.025	1.930±0.228
Lopinot (<i>n</i> =20)	0.201±0.033	2.74±0.197	0.075±0.015	1.831±0.277

TABLE 2. Summary of univariate analysis of variance (ANOVA) for all factors: containers, populations, predators (without controls), tadpole sizes (large and small) and interaction between the significant variables.

Source	SOS	Error	df	F	P
Predator	1.518	2.908 (df 288)	1	137.3	$P<0.001$
Container	1.781	2.908 (df 288)	2	80.5	$P<0.001$
Population	0.153	2.908 (df 288)	3	4.6	$P<0.01$
Size	0.009	2.908 (df 288)	1	0.8	NS
Predator × Container	0.289	2.908 (df 288)	2	13.0	$P<0.001$
Container × Population	0.447	2.908 (df 288)	6	6.7	$P<0.001$
Population × Predator	0.092	2.908 (df 288)	3	2.8	$P<0.05$
Predator × Container × Population	0.142	2.908 (df 288)	6	2.1	$P<0.05$

addition, the stock population from which each trial group was randomly selected was around 100 tadpoles, minimising the chance that any one tadpole would repeatedly take part in similar trials. The tank rear glass was divided into nine 10 cm sections by white tape stuck to the outside and marked A, 1, 2, 3, 4, 5, 6, 7, 8, where A marked the position of the predator container. To decrease tadpole disturbance, the tank sides were screened with muslin except at one side for observations. The tanks contained approximately 17 litres of de-chlorinated tap water which was aerated prior to the experiments, but not during them.

For each trial, we selected 15 tadpoles randomly from the required stock tank, using a handnet to introduce them all at once into section 4. They were left for 30 min to habituate with an empty predator container at A. One predator (either *Rivulus* or *Macrobrachium*) was placed in the container after the habituation period. To avoid differences in the experimental manipulation between predator and predator-free (control) trials, the containers were similarly opened and closed for control trials. Tadpole distribution in each tank section was recorded every 10 min for one hour following introduction of the predator. After each trial, the water was changed, containers were washed and predators and tadpoles were returned to stock before preparing the next trial. Each trial was repeated three times, giving a total of 216 trials.

DATA ANALYSIS

The number of tadpoles recorded in each tank section after each 10 min period was multiplied by the distance (in cm) from the predator container (beginning of section A) and divided by the total number of tadpoles in all three trials ($n=45$ tadpoles, three repeats of each trial with 15 tadpoles in each trial). This generated a data point representing the mean distance between tadpoles and predator container after each 10 minute period, with a minimum value of 10 (all tadpoles in section A) and a maximum value of 90 (all tadpoles in section 8). Because normal probability plots revealed highly right skewed data, we transformed each data point using the fourth root to correct for deviations from normality (Kittlitz, 1999), giving a possible range between 1.77 and 3.08.

We used parametric univariate analysis of variance (ANOVA) and Bonferroni corrections to compare differences between populations, tadpole sizes, predators, and type of predator container. All analyses were performed with the statistical package SPSS v10.

RESULTS

The size distributions of the tadpoles from the four sites are shown in Table 1. There were no significant differences in positional distribution between the two tadpole size classes (data not shown for individual experiments: Table 2 shows the lack of significance of size as a factor explaining tadpole positional distribution in all experiments). Because of this, all analyses were performed using the data from the two size classes combined.

The positional distribution of tadpoles did not change significantly with time in control experiments (with mesh container, ANOVA: $F_{5,39}=2.02$, $P=0.98$; with opaque container, ANOVA: $F_{5,39}=0.96$, $P=0.455$; with transparent container, ANOVA: $F_{5,39}=0.45$, $P=0.447$). However, the positional distribution of tadpoles did change significantly with time in some experimental situations. When any chemicals from *R. hartii* could be detected by tadpoles, tadpoles moved further away from the predator with time (predator in mesh container, ANOVA: $F_{5,39}=5.24$, $P<0.001$; predator in opaque container, ANOVA: $F_{5,39}=3.84$, $P=0.006$), suggesting that the tadpoles sensed and attempted to avoid *R. hartii*. When *R. hartii* could only be detected visually by the tadpoles, they did not move significantly further from the predator with time (predator in transparent container, ANOVA: $F_{5,39}=1.80$, $P=0.137$). In experiments with *M. carcinus*, there were no significant changes in tadpole distribution with time in any of the experimental situations (predator in mesh container, ANOVA: $F_{5,39}=0.39$, $P=0.855$; predator in opaque container, ANOVA: $F_{5,39}=0.13$, $P=0.984$; predator in transparent container, ANOVA: $F_{5,39}=0.65$, $P=0.666$).

The predator and the type of container used in the trials were the two most significant variables in explaining the positional distribution of the tadpoles (Tables 2-4, Fig. 1). All *M. trinitatis* tadpole populations showed significant movement away from the two predators when the tadpoles used visual and chemical cues com-

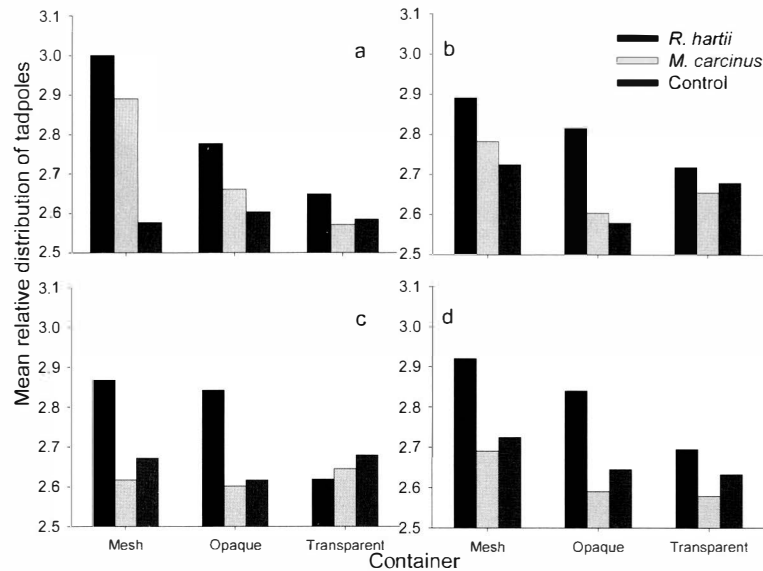


FIG. 1. *M. trinitatis* tadpole avoidance response to predators and in control experiments using the three predator containers for each population. A: Mount Saint Benedict; B: Tamana Cave; C: Maracas Bay; D: Lopinot. Calculation of the avoidance index is explained in Materials and Methods – Data Analysis. High values indicate that tadpoles were distributed further away from the container than experiments with low value results.

TABLE 3. Analysis of differences in tadpole distributions: comparisons between container types (mesh, opaque, transparent) for each population and each predator-control pairing. Where the mean value is positive, tadpoles moved further from the first factor in the comparison with time; when negative, they moved closer to the first factor. Non-significant values shown as NS.

(A) UNIVARIATE ANOVAS

Population	Container	SOS	Error	df	F	P
Benedict	Mesh	1.163	0.292 (33 df)	2	65.8	P<0.001
	Opaque	0.189	0.505 (33 df)	2	6.2	P<0.01
	Transparent	0.041	0.177 (33 df)	2	3.8	P<0.05
Tamana	Mesh	0.17	0.181 (33 df)	2	15.4	P<0.001
	Opaque	0.403	0.416 (33 df)	2	16.0	P<0.001
	Transparent	0.023	0.185 (33 df)	2	2.1	NS
Maracas	Mesh	0.415	0.355 (33 df)	2	19.3	P<0.001
	Opaque	0.433	0.386 (33 df)	2	18.5	P<0.001
	Transparent	0.018	0.278 (33 df)	2	1.1	NS
Lopinot	Mesh	0.356	0.308 (33 df)	2	19.1	P<0.001
	Opaque	0.417	0.301 (33 df)	2	22.9	P<0.001
	Transparent	0.08	0.426 (33 df)	2	3.1	NS

(B) PAIRED COMPARISONS WITH BONFERRONI CORRECTIONS

Population	Predator comparison	Mesh		Opaque		Transparent	
		Mean	P	Mean	P	Mean	P
Benedict	<i>R. hartii</i> vs <i>M. carcinus</i>	0.11	P<0.05	0.117	NS	0.077	P<0.05
	<i>R. hartii</i> vs Control	0.424	P<0.001	0.174	P=0.005	0.064	NS
	<i>M. carcinus</i> vs Control	0.314	P<0.001	0.057	NS	-0.013	NS
Tamana	<i>R. hartii</i> vs <i>M. carcinus</i>	0.108	P<0.01	0.211	P<0.001	0.061	NS
	<i>R. hartii</i> vs Control	0.166	P<0.001	0.236	P<0.001	0.037	NS
	<i>M. carcinus</i> vs Control	0.058	NS	0.025	NS	-0.024	NS
Maracas	<i>R. hartii</i> vs <i>M. carcinus</i>	0.25	P<0.001	0.24	P<0.001	-0.025	NS
	<i>R. hartii</i> vs Control	0.196	P<0.001	0.225	P<0.001	-0.055	NS
	<i>M. carcinus</i> vs Control	0.054	NS	-0.015	NS	-0.029	NS
Lopinot	<i>R. hartii</i> vs <i>M. carcinus</i>	0.229	P<0.001	0.249	P<0.001	0.116	NS
	<i>R. hartii</i> vs Control	0.186	P<0.001	0.197	P<0.001	0.062	NS
	<i>M. carcinus</i> vs Control	-0.042	NS	-0.052	NS	-0.053	NS

TABLE 4. Analysis of differences in tadpole distributions: comparisons between predator types (*R. hartii*, *M. carcinus*, control) for each population and each container type. Where the mean value is positive, tadpoles moved further from the first factor in the comparison with time. When negative, they moved closer to the first factor. Non-significant values shown as NS.

(A) UNIVARIATE ANOVAS

Population	Predator	SOS	Error	df	F	P
Benedict	<i>R. hartii</i>	0.76	0.366 (33 df)	2	34.3	$P<0.001$
	<i>M. carcinus</i>	0.651	0.326 (33 df)	2	32.9	$P<0.001$
	Control	0.004	0.282 (33 df)	2	0.3	NS
Tamana	<i>R. hartii</i>	0.183	0.200 (33 df)	2	15.1	$P<0.001$
	<i>M. carcinus</i>	0.205	0.256 (33 df)	2	13.2	$P<0.001$
	Control	0.133	0.326 (33 df)	2	6.8	$P<0.01$
Maracas	<i>R. hartii</i>	0.446	0.476 (33 df)	2	15.5	$P<0.001$
	<i>M. carcinus</i>	0.011	0.325 (33 df)	2	0.6	NS
	Control	0.046	0.219 (33 df)	2	1.9	NS
Lopinot	<i>R. hartii</i>	0.312	0.508 (33 df)	2	10.1	$P<0.001$
	<i>M. carcinus</i>	0.094	0.460 (33 df)	2	3.2	NS
	Control	0.074	0.066 (33 df)	2	18.4	$P<0.001$

(B) PAIRED COMPARISONS WITH BONFERRONI CORRECTIONS

Population	Container comparison	<i>R. hartii</i>		<i>M. carcinus</i>		Control	
		Mean	P	Mean	P	Mean	P
Benedict	Mesh vs Opaque	0.223	$P<0.001$	0.23	$P<0.001$	-0.026	NS
	Mesh vs Transparent	0.352	$P<0.001$	0.319	$P<0.001$	-0.008	NS
	Opaque vs Transparent	0.128	$P<0.05$	0.089	NS	0.0183	NS
Tamana	Mesh vs Opaque	0.075	NS	0.179	$P<0.001$	0.146	$P<0.01$
	Mesh vs Transparent	0.174	$P<0.001$	0.128	$P<0.01$	0.045	NS
	Opaque vs Transparent	0.098	$P<0.05$	-0.05	NS	-0.099	NS
Maracas	Mesh vs Opaque	0.025	NS	0.015	NS	0.053	NS
	Mesh vs Transparent	0.248	$P<0.001$	-0.028	NS	-0.003	NS
	Opaque vs Transparent	0.223	$P<0.001$	-0.043	NS	-0.057	NS
Lopinot	Mesh vs Opaque	0.08	NS	0.1	NS	0.09	$P<0.001$
	Mesh vs Transparent	0.225	$P<0.001$	0.112	NS	0.101	$P<0.001$
	Opaque vs Transparent	0.145	$P<0.05$	0.011	NS	0.01	NS

bined or independently to detect the predators (predators in mesh and in opaque containers (Tables 3-4; Fig. 1). Tadpoles that were able to use these cues to detect the predators avoided *R. hartii* significantly more than *M. carcinus* (Table 3, Fig. 1). Overall, when chemicals could not be detected (predators in transparent containers), tadpoles showed non-significant spatial avoidance differences between the two types of predator (Table 3) or between populations (Table 5). All tested *M. trinitatis* populations reacted significantly differently to the presence of predators and controls in mesh and transparent containers (Table 5). However, there were no significant differences in distribution between the four different *M. trinitatis* populations to the two predators when using only chemical detection to detect the predators (opaque container, Tables 5, Fig. 1). The Mount Saint Benedict tadpoles showed the strongest avoidance to *R. hartii* when visual and chemical cues were employed, being significantly greater than all other *M. trinitatis* populations (Table 5, Fig. 1).

DISCUSSION

TADPOLE SIZE EFFECTS

Responses of tadpoles of different size classes (representing different times since deposition) to predator cues might have been expected for two reasons. First, many predators hunt selectively on prey according to their size. However, both the predators used in this study range considerably in size, so it is likely to be adaptive for tadpoles to be able to detect predators as soon as they enter the stream. Second, predator detection could be partly a learned response, improving with time. The lack of any significant difference in response to predator cues between the two tadpole size classes suggests that predator detection is essentially an inherent ability of tadpoles.

PREDATOR DETECTION MECHANISM

Our observations support the earlier results showing that amphibian larvae detect predators primarily by

TABLE 5. Analysis of differences in tadpole distributions. Comparisons between populations (Benedict, Maracas, Lopinot, Tamana) for each container type and each predator. Where the mean value is positive, tadpoles moved further from the first factor in the comparison with time. When negative, they moved closer to the first factor. Non-significant values shown as NS.

(A) UNIVARIATE ANOVAS

Predator	Container	SOS	Error	df	<i>F</i>	<i>P</i>
<i>R. hartii</i>	Mesh	0.123	0.202 (44 df)	3	8.9	<i>P</i> <0.001
	Opaque	0.032	0.878 (44 df)	3	0.5	NS
	Transparent	0.069	0.470 (44 df)	3	2.2	NS
<i>M. carcinus</i>	Mesh	0.506	0.631 (44 df)	3	11.8	<i>P</i> <0.001
	Opaque	0.035	0.388 (44 df)	3	1.4	NS
	Transparent	0.067	0.348 (44 df)	3	2.8	<i>P</i> <0.05
Control	Mesh	0.187	0.304 (44 df)	3	9.1	<i>P</i> <0.001
	Opaque	0.023	0.342 (44 df)	3	1.0	NS
	Transparent	0.069	0.247 (44 df)	3	4.1	<i>P</i> <0.05

(B) PAIRED COMPARISONS WITH BONFERRONI CORRECTIONS

Predator	Population comparison	Mesh		Opaque		Transparent	
		Mean	<i>P</i>	Mean	<i>P</i>	Mean	<i>P</i>
<i>R. hartii</i>	Benedict vs Tamana	0.11	<i>P</i> <0.01	-0.037	NS	-0.067	NS
	Benedict vs Maracas	0.134	<i>P</i> <0.001	-0.064	NS	0.03	NS
	Benedict vs Lopinot	0.08	<i>P</i> <0.05	-0.062	NS	-0.045	NS
	Tamana vs Maracas	0.024	NS	-0.026	NS	0.097	NS
	Maracas vs Lopinot	-0.053	NS	0.001	NS	-0.075	NS
	Lopinot vs Tamana	0.029	NS	-0.024	NS	0.021	NS
<i>M. carcinus</i>	Benedict vs Tamana	0.108	NS	0.056	NS	-0.083	NS
	Benedict vs Maracas	0.274	<i>P</i> <0.001	0.059	NS	-0.073	NS
	Benedict vs Lopinot	0.2	<i>P</i> =0.001	0.07	NS	-0.007	NS
	Tamana vs Maracas	0.167	<i>P</i> <0.01	0.002	NS	0.01	NS
	Maracas vs Lopinot	-0.074	NS	0.01	NS	0.065	NS
	Lopinot vs Tamana	-0.092	NS	-0.018	NS	-0.075	NS
Control	Benedict vs Tamana	-0.148	<i>P</i> <0.001	0.024	NS	-0.094	<i>P</i> <0.05
	Benedict vs Maracas	-0.094	<i>P</i> <0.05	-0.01	NS	-0.089	<i>P</i> <0.05
	Benedict vs Lopinot	-0.157	<i>P</i> <0.001	-0.039	NS	-0.047	NS
	Tamana vs Maracas	-0.054	NS	-0.037	NS	0.004	NS
	Maracas vs Lopinot	-0.062	NS	-0.025	NS	0.041	NS
	Lopinot vs Tamana	0.008	NS	0.063	NS	-0.046	NS

chemicals released in the water. Stauffer & Semlitsch (1997), testing the responses of *Rana esculenta* and *Rana lessonae* tadpoles to fish by three possible mechanisms of predator detection (visual, tactile and chemical), determined that chemical cues produced the strongest response, while a combination of chemical and tactile cues resulted in a significantly stronger response than visual and tactile. They suggested that predator movements produced directional waves of chemical cues that could alert tadpoles to predator distance. When visual information is not available (at night, in turbid waters, with dense aquatic vegetation, with conspicuous or ambush predators) chemical and

tactile cues may be critical to the assessment of predation risks (Kiesecker *et al.*, 1996). Small underwater currents produced by predators in mesh cages may explain the strong response to predators in these containers and why the tadpoles' responses were stronger to *R. hartii* than to *M. carcinus* (the former predator moving more often than the latter one: personal observations).

SPECIFIC ANTI-PREDATOR RESPONSE

All tadpole populations reacted to *R. hartii* significantly more than to *M. carcinus*. These findings are similar to other studies where responses to crayfish

were not as strong as to predatory fish (Lefcort, 1996; Bridges & Gutzke, 1997; Pearl *et al.*, 2003). Kurzava & Morin (1998) suggested that fish are functionally distinct from other kinds of aquatic predators, being more efficient and exerting greater pressure on anuran prey populations. However, Gascon (1992) showed that dragonfly larvae chemicals were more of a deterrent to anuran larvae (*Osteocephalus taurinus*, *Epipedobates femoralis*, *Phyllomedusa tomoptera*) than chemicals from *Rivulus* species.

Tadpoles at high densities are known to react more strongly to predation threat, increasing the accuracy of predation risk assessment (MacNamara & Houston, 1992). *M. trinitatis* tadpoles are often found in large numbers (even thousands: Kenny, 1969) in small pools, and therefore the spatial avoidance response we observed when tadpoles were presented to predators may under-represent the possible response, due to the small number of tadpoles used in our trials. The two populations (Benedict and Lopinot) that naturally share habitat with *R. hartii* exhibited the greatest avoidance to this predator. Surprisingly, tadpoles from Maracas showed a significantly higher response to *M. carcinus* when chemical detection was impeded (transparent container) and Tamana tadpoles showed a greater response when this predator was in a transparent container than when enclosed in an opaque container, indicating that visual cues may also be used for predator detection.

Tadpoles are known to assess predation risk and respond to multiple predators (Semlitsch & Reyer, 1992). This behaviour is likely to be dependent on the amount of single cues emitted from a predator, the greater the amount emitted, the greater the tadpole response (Semlitsch & Reyer, 1992; Lefcort, 1996; Manteifel & Zhushhev, 1998; Van Buskirk, 2001). This may suggest that the strong avoidance response behaviour to *R. hartii* is a consequence of the larger amount of cues emitted by this predator. Differences in predator avoidance may reflect local environmental adaptations to predators but may be a disadvantage for survival when both predators are present in the same habitat. Kurzava & Morin (1988) showed that very few metamorphs of several anuran species survived when two predators (*Notophthalmus viridescens* and *Enneacanthus obesus*) were combined in artificial ponds, suggesting that different anuran species had specific anti-predator behaviours to different predators. In our experiments, lack of strong anti-predator response to *M. carcinus* could imply that if both predators were present in the same stream, *M. trinitatis* tadpoles would detect *R. hartii* cues but not *M. carcinus* cues.

PREDATOR DISTRIBUTION

Schmidt & Amézquita (2001) found that *Phyllomedusa tarsius* tadpoles showed an anti-predator behavioural response to a widely-distributed aeshnid dragonfly nymph species, but not to a more dangerous belostomatid bug. They concluded that the tadpoles encountered bugs in their natural environment too

rarely for an anti-predator response to have evolved. Different levels of spatial avoidance response to the two predators in our experiments may be explained by the current distribution of *R. hartii* and *M. carcinus* in Trinidad. *M. carcinus* are much less widespread than *R. hartii*, in streams utilised by *M. trinitatis*. Of all the streams surveyed at Maracas Bay, only one had *M. carcinus*. *M. carcinus* distribution is heavily constrained by abiotic factors and its need for a larval stage amphidromous migration (March *et al.*, 1998; Chung, 2001). Many of the north coast streams which support *M. trinitatis* populations are too steep to allow such migrations and the southern slopes of the Northern Range lack immediate access to the sea, hence have no *Macrobrachium* populations. *R. hartii* is an efficient colonizer and adapts to a variety of habitats, with the ability to leave streams and search over several metres to locate more suitable pools (even up steep stream slopes: Seghers, 1978). The distribution patterns of the two predators suggest that *M. trinitatis* tadpoles are unlikely to encounter *M. carcinus* as often as *R. hartii* and therefore may not have evolved specific anti-predator responses to these shrimps.

ADAPTATION OR INHERITANCE?

Tadpoles from the Mount Saint Benedict site were collected from containers placed at the site. These tadpoles were naïve (they had never been exposed to chemical cues from, or presence of *R. hartii* or *M. carcinus*). However, this population showed the strongest response to predators. Therefore this behaviour cannot reflect experience in the natural environment and must be inherited rather than acquired. This conclusion is also supported by the behaviour of tadpoles from Tamana cave, which showed anti-predator response to both predators, despite the lack of these predators in the cave. Although *R. hartii* is absent from Tamana cave, it is common throughout the Central Range. Because the Tamana cave population is part of the Central Range meta-population (Jowers & Downie, 2004, and unpublished data) this may explain why *M. trinitatis* tadpoles from this population show anti-predator behaviour to *R. hartii*.

At some sites, *M. trinitatis* tadpoles were found in high numbers (hundreds or even thousands) in predator-free pools (Tamana cave). At other sites, tadpoles were hard to find and were deposited in lower numbers in small pools (Mount Saint Benedict and Lopinot). Predator colonization could have significant consequences for successive generations at these breeding sites. Therefore anti-predator behaviour is of extreme importance for these palatable tadpoles and an inherited response to predators is likely to be advantageous to their survival.

ADULT AND LARVAL DETECTION MECHANISM

Male *M. trinitatis* depositing tadpoles seem highly efficient at assessing predation pressure in pools. Therefore, larvae should not normally face predators

and might have been expected to lack anti-predator responses. Downie *et al.* (2001) demonstrated that males preferred predator-free pools to pools containing either normally encountered or not normally encountered predators. When males were presented with pools containing either *M. carcinus* or *R. hartii*, males from Lopinot, Mount Saint Benedict and Maracas Waterfall (preyed on by *R. hartii* in the wild) avoided *R. hartii* but deposited in containers with *M. carcinus*. The males from Maracas (preyed on by *M. carcinus* in the wild) deposited few tadpoles in the *R. hartii* containers and none in *M. carcinus* containers. It should be noted, however, that Downie *et al.* (2001) were unable to be certain whether deposition selectivity was due to pool selection by transporting males or detachment selection by tadpoles.

However, the ability of predators to migrate within the stream environment means that deposition selectivity may not be a complete anti-predator protection. It is, therefore, not so surprising that we were able to demonstrate predator avoidance behaviour by tadpoles.

The strengths of the tadpole anti-predator responses in tadpoles from different populations and to the two predators were somewhat different to the deposition selectivity differences found by Downie *et al.* (2001), but the overall difference, that the response was greater to *R. hartii* than to *M. carcinus*, fits with their differential distribution and migration abilities.

LIMITATIONS OF THE STUDY

In the field, *M. trinitatis* tadpole anti-predator behaviour strategies are likely to be affected by abiotic factors which may influence different populations to exhibit anti-predator behaviours that depend on the locality they inhabit. For example, in the Mount Saint Benedict stream and pools, the substrate is composed primarily of leaf litter and tadpoles use it as refuge, reducing their inactivity levels. In contrast, *M. trinitatis* tadpoles in Tamana cave, where leaf litter is absent, show high activity levels and although limited refuge is available under rock crevices, tadpoles rarely hide under them. Thus, our laboratory-based experimental design did not allow tadpoles to exhibit the variety of anti-predator behaviours that otherwise may have been observed, such as refuge use, differences in activity levels and diurnal rhythms (Sih *et al.*, 1992).

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GREAT CRESTED NEWTS (*TRITURUS CRISTATUS*) AS INDICATORS OF AQUATIC PLANT DIVERSITY

DANIEL H. GUSTAFSON¹, CECILIA JOURNATH PETTERSSON^{1,2} AND JAN C. MALMGREN^{1,2}

¹Section of Biology, Department of Natural Sciences, Örebro University, Sweden

²County Administration Board, Conservation and Monitoring of Biological Diversity, Sweden

In a field study in south central Sweden, we analysed the diversity of macrophytes in paired samples of ponds in a total of five geographically separated sites. Each pair of ponds involved one pond with presence of great crested newts (*Triturus cristatus*) and one pond in which newts were absent. Ponds with presence of great crested newts had a significantly higher mean number of plant species than ponds without newts. Newts occurred in ponds that tended to have a lower amount of pond area covered by surface vegetation, although this difference was not statistically significant. Macrophyte diversity also tended to increase more steeply in ponds with *T. cristatus*, compared with ponds without newts. Broad-leaved pond weed (*Potamogeton natans*) and square-leaved liverwort (*Chiloscyphus pallescens*) were among the plants that were most associated with presence of great crested newts. Plant diversity had a slightly more nested structure for ponds with great crested newts than for those without, which indicates a more homogeneous plant species assemblage in the former group of ponds. Overall, the results indicate that the great crested newt may be a reliable and useful indicator species for high plant species richness in ponds and small wetlands, which may be valuable for environmental monitoring and conservation in pond landscapes.

Key words: Amphibia, habitat selection, pond succession, species distribution patterns

INTRODUCTION

A common assumption in modern conservation is that some species can be used as reliable indicators of biological diversity. Such 'indicator species' are assumed to mirror changes in population processes, species distributions and viability in other taxa, at both local and regional scales, thus providing a tool for measuring and monitoring effects on biodiversity (Pearson, 1995). Selecting suitable indicator species is both difficult and controversial, and is often made with incomplete background information (Simberloff, 1998; Caro & O'Doherty, 1999; Noss, 1999; Simberloff, 1999; Hess & King, 2002). However, a primary requirement must be that the species really has the ability to indicate the attributes of concern for conservation (Lindenmayer, 1999). Because few species have been tested or validated empirically for their value as indicators, studies of co-occurrence patterns and of the strength of correlations in diversity patterns among taxa are needed (Simberloff, 1998; Lindenmayer, 1999; Noss, 1999; Simberloff, 1999).

The great crested newt, *Triturus cristatus*, is a caudate amphibian (family Salamandridae) with a biphasic life-cycle including both terrestrial and aquatic habitats. As such, it appears to fulfil all criteria for indicator species suggested by Caro & O'Doherty (1999), namely: (1) it is

widely distributed (Griffiths, 1996); (2) it has high demands for habitat quality and is potentially restricted to mature and stable environments (Oldham *et al.*, 2000; Malmgren, 2001); (3) it has a well-known biology (Thiesmeier & Kupfer, 2000; Arntzen, 2003); and (4) it is easy to sample and observe, at least in its aquatic phase (Langton *et al.*, 2001). Although the aquatic habitat requirements of this species have been examined in detail (Beebee, 1985; Pavignano *et al.*, 1990), we have not been able to find any published report where its potential value as a biodiversity indicator has been tested. Of particular interest is the functional relationship between the great crested newt and its environment, especially since distribution and abundance of aquatic vegetation appear important for the species (e.g., Griffiths *et al.*, 1996; Oldham *et al.*, 2000; Langton *et al.*, 2001). Aquatic plants control the productivity of invertebrate prey (e.g., Oertli *et al.*, 2002), provide safe egg-laying sites (e.g., Miaud, 1993, 1994; Marco *et al.*, 2001), and offer protection from predators (Griffiths *et al.*, 1996; Oldham *et al.*, 2000). Thus, plant diversity has both structural and functional value in pond ecosystems, and the hypothesis that there is a relationship between distribution patterns of great crested newts and aquatic plant species richness merits further investigation.

We conducted a pilot study to examine if the great crested newt has any value as an indicator of plant diversity in ponds. Specifically, we tested if patterns of plant species richness are different in ponds where great crested newts are present compared with ponds where they are absent.

Correspondence: D. Gustafson, Section of Biology, Department of Natural Sciences, Örebro University, SE-701 82 Örebro, Sweden.
E-mail: daniel.gustafson@nat.oru.se

MATERIALS AND METHODS

We examined potential breeding ponds for great crested newts as part of a species survey in Örebro county, south-central Sweden, in the summer of 2002. Ten larger sites with several ponds were surveyed, using standardized visual observation and funnel traps in parallel for three consecutive nights at each pond in April–June, complemented with a survey for larvae by dip-netting two times at each pond in August (Gustafson *et al.*, 2004). We randomly selected five of the sites for a detailed study of aquatic plant communities (Table 1). The sites were situated several kilometers from each other (range 22.2–73.5 km, mean 33.3 km ± 4.4 SE). In each of the selected sites, we randomly selected one pond with confirmed occurrence (observations of breeding adults) of great crested newts and one where the species was found to be absent during all survey attempts. The two sites in each pair were separated by a maximum of 480 m and a minimum of 60 m (Table 1), which is within normal dispersal distance of the great crested newt (Jehle, 2000; Jehle & Arntzen, 2000; Schabetsberger *et al.*, 2004; Jehle *et al.* 2005a; Jehle *et al.* 2005b). This design provided us with ten ponds comprising a set of five matched presence/absence pairs.

During the last two weeks of July, at the peak of the vegetation period, aquatic macrophytes were sampled by establishing transects across the ponds with a width of 0.5 m. The transects were laid out to cover the centre of the ponds where there was a maximum of visible vegetation, and reaching 1 m up on the shore from the

water’s edge. Along each transect, and down to a maximum depth of 1.5 m, all vascular plants and mosses were collected and identified. In cases where grazing damage or other factors made identification to species impossible, plants were at least identified to genus or family. The surface vegetation cover was estimated visually as the area covered by emerging plants (i.e. those with floating leaves and those with leaves protruding above the water surface, combined) as a proportion of the total pond area.

The number of identified plant species was used as a measure of plant species richness in each of the ponds. We used a paired two-sample *t*-test, assuming equal variance, to test if great crested newt occurrence reliably indicated a high diversity of plants. To test the hypothesis that plant diversity increases more steeply in ponds occupied by great crested newts compared with non-inhabited ponds, we performed a Type II regression analysis according to Sokal & Rohlf (1995). Thus, the relationship was estimated by Pearson’s product-moment correlation and a reduced major axis regression, since both variables were measured with the same unit and with equal error. We also performed a Spearman correlation analysis on the relationship between plant diversity and surface vegetation cover in ponds to see if these factors correlated with presence and absence data.

Potential associations between plant species and occurrence of great crested newts were examined with a hypergeometric probability test. We also used the nestedness temperature calculator (Atmar & Patterson,

TABLE 1. Description of surveyed ponds at five aquatic plant community study sites. Pond data are presented with coordinates from the Swedish national grid (RT90), a short habitat description, presence or absence of great crested newts (*T. cristatus*), depth of pond (m), area of pond surface (m²), the amount of surface vegetation cover (%), and the number of identified plant species. Distances between the two ponds in every area are also presented. Pond names are given for reference, with short designations in parentheses.

Ponds	Coordinates	Description	<i>T. cristatus</i>	Pond depth (m)	Pond surface area (m ²)	Surface veg. cover (%)	No. plant species	Pond distance (m)
Grönelid (A1)	6513459 / 1426238	Pond in grazed pasture	Absent	1.5	40	50	22	60
Grönelid (A2)	6513483 / 1426148	Pond in grazed pasture	Present	3	300	25	36	
Lekeberga (B1)	6567645 / 1447242	Oxbow pond in grazed pasture	Absent	0.5	100	95	20	480
Lekeberga (B2)	6568176 / 1447304	Oxbow pond in grazed pasture	Present	1.5	200	80	42	
Kortorp (C1)	6539777 / 1468172	Marsh in coniferous forest	Absent	0.5	100	90	20	270
Kortorp (C2)	6539926 / 1468444	Pond in grazed pasture	Present	0.75	50	60	28	
Rockebro (D1)	6533124 / 1436718	Tarn in coniferous forest	Absent	0.5	30	90	12	270
Rockebro (D2)	6533024 / 1436992	Tarn in coniferous forest	Present	0.5	70	70	11	
Oset (E1)	6573005 / 1469627	Pond in grazed marsh	Absent	1.5	350	3	33	170
Oset (E2)	6572983 / 1469438	Pond in grazed marsh	Present	1.5	400	30	37	

1993), which describes species distribution patterns by calculating the degree of nestedness in the data. To simplify, with this method, a perfectly nested system has a temperature of 0° and lacks all randomness, whereas a system lacking all order has a temperature of 100°. The calculations were based on the observation matrices, separately for ponds with and without *T. cristatus*.

RESULTS

In total, we identified 117 plant species during the study (99 vascular plants, 17 mosses, 1 charophyte). The number of species found per pond is presented in Table 1. The five most frequently recorded species were floating sweet-grass (*Glyceria fluitans*; represented in 8 of the 10 ponds), marsh bedstraw (*Galium palustre*; 8 of 10), soft rush (*Juncus effusus*; 7 of 10), bottle sedge (*Carex rostrata*; 7 of 10) and common sedge (*Carex nigra*; 7 of 10). No single plant species was found in all ten ponds, and 50 species (43 %) were found in only one pond. The variation in number of plant species per pond was high (range 11–42, mean 26.1 ± 3.4 SE). Ponds with and without great crested newts had a cumulative plant species richness of 93 and 68 species, respectively. In general, ponds with great crested newts had a significantly higher mean plant species richness (on average 30.8 ± 5.4 SE) than ponds in which the species was absent (on average 21.4 ± 3.4 SE) (paired *t*-test: $t=2.35$, $df=4$, $P<0.05$). Although ponds with *T. cristatus* had a significantly higher plant species richness compared with the absence-ponds, there was no significant linear relationship (Pearson's product-moment correlation; $r=0.68$, $P>0.05$). Only one study site (D) went against the general pattern of higher plant species richness in *T. cristatus* ponds and this site also had the lowest overall species richness. Although ponds with great crested newts had a lower amount and less variation in area covered by surface vegetation (mean cover $53.0 \pm 10.91\%$

SE, range 25–80%) than ponds where the species was absent (mean cover $65.6 \pm 17.63\%$ SE, range 3–95%), the difference was not statistically significant (paired *t*-test, $t=1.23$, $df=4$, $P>0.05$). Plant diversity declined as the amount of pond surface vegetation cover increased (Fig. 1), but this tendency was not significant either (Spearman's correlation test; $r=-0.55$, $P>0.05$).

In the hypergeometric test six plant species were significantly associated with the occurrence of great crested newts. Only two of these species were associated on a higher level of significance, namely broad-leaved pondweed (*Potamogeton natans*, $P<0.05$) and square-leaved liverwort (*Chiloscyphus pallescens*, $P<0.05$), both being found in four ponds with *T. cristatus*, but in none of the absence-ponds. Four additional species were associated at a lower level of significance (common sedge, *Carex nigra*, $P<0.10$; marsh willowherb, *Epilobium palustre*, $P<0.10$; meadowsweet, *Filipendula ulmaria*, $P<0.10$; bladder sedge, *Carex vesicaria*, $P<0.10$). Plant diversity had a slightly more nested structure for ponds with great crested newts (matrix temperature=46°) than for those without (matrix temperature=51°), which indicates a more homogeneous plant species assemblage in the former group of ponds.

DISCUSSION

Although this study is based on a limited sample, the results demonstrate that great crested newts occur in ponds with a significantly higher mean number of plant species compared to ponds where they are absent. Further, newts occurred in ponds that tended to have a lower amount and less variation in pond area covered by surface vegetation, compared to absence-ponds, although the difference was not statistically significant. A few plant species were associated with the presence of *T. cristatus*, most notably broad-leaved pondweed (*Potamogeton natans*) and square-leaved liverwort (*Chiloscyphus pallescens*). None of these species belonged to the group of species most frequently observed. Ponds with great crested newts were slightly more homogeneous than ponds in which the newt did not occur, at least from the perspective of aquatic plant species composition and nestedness.

The productivity of pond ecosystems is ultimately controlled by sun insolation, temperature and nutrient availability. In early stages of pond community succession the general openness of the pond favours phytoplankton as primary producers. In later stages macrophytes establish and contribute to a higher structural complexity (Friday, 1987). With further succession, especially in eutrophic ponds where nutrient availability is rarely limiting, diversity generally decreases and only a few macrophyte species dominate (Engelhardt & Ritchie, 2001, 2002; Loreau *et al.*, 2001). In line with the latter pattern we observed a (non-significant) tendency for plant species richness to decrease with increasing vegetation cover. However, we could not find any obvious correlations between sur-

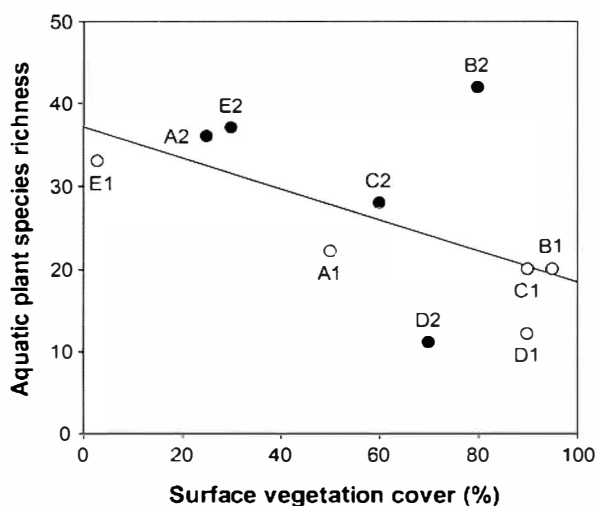


FIG. 1. Relationship between aquatic plant species richness and pond vegetation cover in the ten studied ponds. Filled and open circles represent, respectively, ponds where great crested newts were present and absent. Pond designations follow Table 1.

face vegetation cover and newt abundance, although earlier studies have shown positive relationships (Joly *et al.*, 2001).

A possible scenario explaining some of the variation in occurrence is that the great crested newt has a preference for ponds in a certain range of pond succession stages, correlated with high macrophyte diversity. Our nestedness analysis show that there is a tendency for ponds with newts to be more nested, which implies that the plant species occur in a more equilibrated coexistence in ponds with newts than in ponds without newts. This could be due to the fact that a particular pond, in its peak macrophyte diversity stage, may provide very favourable conditions for newts in terms of prey productivity, temperature regimes and availability of cover and egg-laying facilities. In later stages when the plant community is overtaken and dominated by only a few macrophyte species, and especially surface covering species, the situation for newts may quickly deteriorate. In such cases the depletion in sun insolation may cause a collapse in temperature regimes, which can cause deterioration of plant and food productivity in the pelagic zone, all of which can be negative to the reproductive success of great crested newts. Succession inevitably leads to overgrowth, which Oldham (1994) described as one of the most common threats to great crested newt populations. Earlier studies have shown that the great crested newt has its highest occurrence in ponds with a submerged plant cover of 50-75% and an emergent vegetation cover of 25-50% (Oldham, 1994; Langton *et al.*, 2001), which corresponds to ponds with a well established macrophyte flora in a mid-succession stage (Oldham *et al.*, 2000; Langton *et al.*, 2001). The higher nestedness matrix temperature for presence-ponds than absence-ponds in our study could support the hypothesis that great crested newts prefer stable and mature habitats or ponds in a favourable stage of succession (discussed in Malmgren, 2001, 2002a,b). Due to the low sample size however, it would be too speculative to infer too much from the present results. Pond succession and its effects on the native ecosystems is an issue that needs more attention in future studies.

The diversity and structure of the macrophyte flora in ponds is important for the diversity of several other groups of organisms, especially among macroinvertebrates (e.g. Friday, 1987; Oertli *et al.*, 2002) and zooplankton (Cottenie & De Meester, 2004). Heino (2000) showed that total species diversity among macroinvertebrates increased with habitat heterogeneity. Habitat structure seemed to be more important than factors related to water chemistry in determining the structure of littoral macroinvertebrate assemblages. The macrophytic flora constitutes an important part of the habitat, which implies that a higher diversity among plants would also bring a greater heterogeneity and a higher diversity among other groups of organisms. Higher plant diversity may also by itself indicate higher water quality, longer continuity or greater productivity. Although for example Oertli *et al.* (2002) found only a

weak relationship between floral diversity and amphibian species richness, there might be an indirect relationship between amphibians and macrophyte diversity. Our results suggest that higher macrophyte species diversity is positively associated with the presence of great crested newts, and other ecosystem functions may possibly be revealed by its presence.

We observed a significant association between broad-leaved pond weed (*Potamogeton natans*) and great crested newts. This plant species has a wide distribution and is common in many different water bodies. Nevertheless, we find it likely that *P. natans* is a species with a structure of importance for great crested newts, as it has big floating leaves that can serve as protection against predators and long, thin underwater-leaves that can function as an egg-laying substrate. Presence of both *P. natans* and great crested newts may also be related to ponds with long hydroperiods or permanent water. For egg laying, plants with thin and easily folded leaves are clearly preferred by great crested newt females. Sweet or flote grasses (*Glyceria* spp.), water mint (*Mentha aquatica*) and water forget-me-not (*Myosotis scorpioides*) are plants that have been demonstrated to serve this purpose (Langton *et al.*, 2001). The results of this study support that finding, as those species occurred frequently in great crested newt presence-ponds. Also, square-leaved liverwort (*C. pallescens*) was significantly associated with great crested newts, but similar to *P. natans* this species is common in wet areas and has a wide distribution. We suggest that there are no important correlations between the great crested newt and specific plant species. Instead, it is probable that particular structures, provided by certain plants, are more important, and that these macrophytes may be good indicators of a certain pond succession stage.

The results acquired from this study are particularly interesting since each pair of ponds were situated in the same area, well within dispersal distance for newts (Jehle, 2000; Jehle & Arntzen, 2000; Joly *et al.*, 2001; Malmgren, 2001) and plants (Moller & Rordam, 1985; Linton & Goulder, 2003), but geographically isolated from the next pair by several kilometres. The null hypothesis of no differences between pairs of ponds would imply that two adjacent ponds would have more similar species compositions compared with ponds in other areas, irrespective of the presence or absence of newts. Due to limited data we were unable to fully resolve this issue. It would be useful to conduct similar studies in a greater number of locations, also taking into account both the relative abundance of the different plant species and the population densities of great crested newts.

Amphibians may be useful as indicators of biodiversity changes in pond and wetland landscapes, since they have life cycles often including both terrestrial and aquatic phases, making them particularly vulnerable to habitat alterations and environmental stress (Houlahan *et al.*, 2000). One of the main reasons for declining great crested newt populations is land-

scape fragmentation (Griffiths *et al.*, 1996; Langton *et al.*, 2001). In Sweden, agricultural landscapes have changed dramatically during the last 50 years, resulting in a much lower amount of mosaic landscapes (Ihse, 1995). As great crested newts and many plant species are sensitive to habitat fragmentation this change implies a general decrease in diversity for amphibians as well as for the flora. In this sense this study suggests that the great crested newt may be a useful indicator of high plant species richness and perhaps also of other sensitive species in the face of habitat disturbance. Further, it emphasizes how complex amphibians are in terms of habitat selection, and this in itself deserves more investigation (e.g., Joly *et al.*, 2001; Beja & Alcazar, 2003; Jakob *et al.*, 2003).

The conservation of ponds and pond landscapes is a challenge since they constitute complex habitats with multifaceted layers of interest, both regarding the various stages of succession and the diversity of organisms (Guest, 1997). Future studies that explore interactions between occurrence patterns and diversity of different taxa and ecosystem function can therefore contribute not only to a greater understanding of species such as the great crested newt, but also aid in determining priority areas for new surveys, management plans and conservation measures, as well as provide insights in broader ecological connections. In such studies, we find that the great crested newt is a likely candidate for being an effective umbrella species for pond landscape conservation and restoration.

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INTERPOPULATIONAL VARIATION IN REPRODUCTIVE CYCLES AND ACTIVITY OF THE WATER SNAKE *LIOPHIS MILIARIS* (COLUBRIDAE) IN BRAZIL

LÍGIA PIZZATTO^{1,3} AND OTAVIO A. V. MARQUES²

¹Pós-Graduação em Ecologia, Departamento de Zoologia, Universidade Estadual de Campinas, Campinas, SP, Brazil

²Laboratório de Herpetologia, Instituto Butantan, São Paulo, SP, Brazil

This study reports on aspects of reproduction in the water snake *Liophis miliaris* from four regions in Brazil: (1) northern coastal Atlantic forest, (2) southern coastal Atlantic forest, (3) northern inland Atlantic forest; and (4) southern inland Atlantic forest. In the northern coastal Atlantic forest, where there is little climate variation, the reproductive cycle of this species is continuous, with vitellogenesis and oviposition occurring throughout the year. Newly hatched snakes are found mainly in January. In other regions the cycle is seasonal and related to warmer and rainy periods, with vitellogenesis and oviposition occurring mainly from September to February. Hatchlings are more abundant from February to April, at the end of the rainy season. In the northern Atlantic forest newly hatched snakes have smaller body sizes than in the other regions. Sperm production seems to occur throughout the year in all regions, and where reproduction is seasonal, mating seems to be disassociated from vitellogenesis, suggesting that sperm may be stored by females over the winter. Females with oviductal eggs did not feed, whereas those ones with secondary vitellogenic follicles fed more frequently than non-reproductive females. In all regions, the activity pattern of adult *Liophis miliaris* seems to be related to reproductive cycles and climate variation.

Key words: geographic variation, reproduction, seasonal activity, Serpentes

INTRODUCTION

Snakes can reproduce seasonally even in tropical areas (Shine, 2003). In most Neotropical species vitellogenesis starts in the early rainy season (September-October), egg-laying occurs throughout the latter part of this season (January-February), and hatching occurs mostly at the end of the rainy season and onset of the dry season (March-May) (*cf.* Marques, 1996a; Marques & Puerto, 1998; Fowler *et al.*, 1998; Hartmann *et al.*, 2002; Marques, 2002; Pinto & Fernandes, 2004). In viviparous snakes, female reproductive cycles are usually seasonal but there is not a single pattern in the timing of vitellogenesis and parturition (Sazima, 1992; Almeida-Santos & Salomão, 1997; Bizerra *et al.*, 2005; Aguiar, 2002; Almeida-Santos & Orsi, 2002; Oliveira *et al.*, 2003). On the other hand, some oviparous species (e.g. *Erythrolamprus aesculapii*, *Xenodon neuwiedii*, *Oxyrhopus guibei*) display continuous cycles (Marques, 1996b; Jordão, 1996; Pizzatto & Marques, 2002), but reproductive peaks can occur in the rainy season (*cf.* Pizzatto & Marques, 2002). Spermatogenic cycles are less well known but apparently both seasonal and continuous patterns can occur (Janeiro-Cinquini *et al.*, 1993; Pizzatto & Marques, 2002; Shine, 2003).

As in other reptiles, reproductive cycles in snakes can be related to climatic variation (*cf.* Seigel & Ford, 1987).

In temperate areas, reproduction is non-continuous and limited by seasonal temperature cycles, whereas in the tropics it can be related to both temperature and rainfall (Seigel & Ford, 1987). In tropical habitats where variation in temperature throughout the year is minimal but rainfall is usually seasonal, snakes tend to reproduce in the rainy season (e.g. Vitt & Vangilder, 1983; James & Shine, 1985; Shine *et al.*, 1998). Therefore, reproductive patterns are high variable in the tropics mostly due to climatic complexity (Greene, 1997). As a result, widespread tropical species are good models for studying the effect of climatic parameters on reproduction.

Liophis miliaris belongs to the colubrid tribe Xenodontini and occurs mainly in forested areas from southern Guiana to Argentina (Dixon, 1989). It is associated with aquatic habitats and feeds mainly on anurans and fishes (Vitt & Vangilder, 1983; Michaud & Dixon, 1989; Sazima & Haddad, 1992; Marques & Souza, 1993). Previous studies of *Liophis* have suggested the probability of continuous reproductive cycles in species from the Brazilian caatinga (northern Brazil – Vitt & Vangilder, 1983) and also in the Amazon region (Martins & Oliveira, 1998). However, *Liophis miliaris* from southeastern Brazil is suspected to have a seasonal cycle (Albolea, 1998; Marques 1998; Marques & Sazima, 2004). The aim of this study was to obtain data for the purpose of testing two main hypotheses: (1) that *L. miliaris* may reproduce continuously and reproductive patterns can differ among populations in different geographic areas, and (2) that

Correspondence: L. Pizzatto, Pós-Graduação em Ecologia, Departamento de Zoologia, Universidade Estadual de Campinas, CP 6109, 13083-970, Campinas, SP, Brazil. E-mail: ligia_oceanica@yahoo.com

reproductive patterns in *L. miliaris* are related to climatic parameters such as temperature and rainfall.

MATERIAL AND METHODS

POPULATIONS STUDIED

We studied four distinct populations of the water snake *Liophis miliaris* (Fig. 1) from the following areas:

1. *Northern Coastal Atlantic Forest (NCAF)*: this population occurs in south of Bahia state (13°48'N, 18°04'S, 30°08'E and 40°43'W, Fig. 1). Currently this area is managed for the cultivation of cacao, which is grown inside the rainforest. The climate is aseasonal with high temperatures and rainfall throughout the year (Fig. 2).

2. *Southern Coastal Atlantic Forest (SCAF)*: located in São Paulo-Paraná states, (23°26'N, 25°52'S, 45°04'E and 48°50'W), on the east of Serra do Mar. This region is mostly covered by rainforest. Spring and summer (September to March) are usually rainy with high temperatures, and autumn-winter (April to August) is warm and dry (Fig. 2).

3. *Northern Inland Atlantic Forest (NIAF)*: located in São Paulo state, (20°12'N, 24°41'S, 45°06'E and 51°06'W), in the west of Serra do Mar. This area is mainly covered by semi-deciduous seasonal forest. Climatic variation is similar to SCAF but monthly fluctuations are more pronounced (Fig. 2).

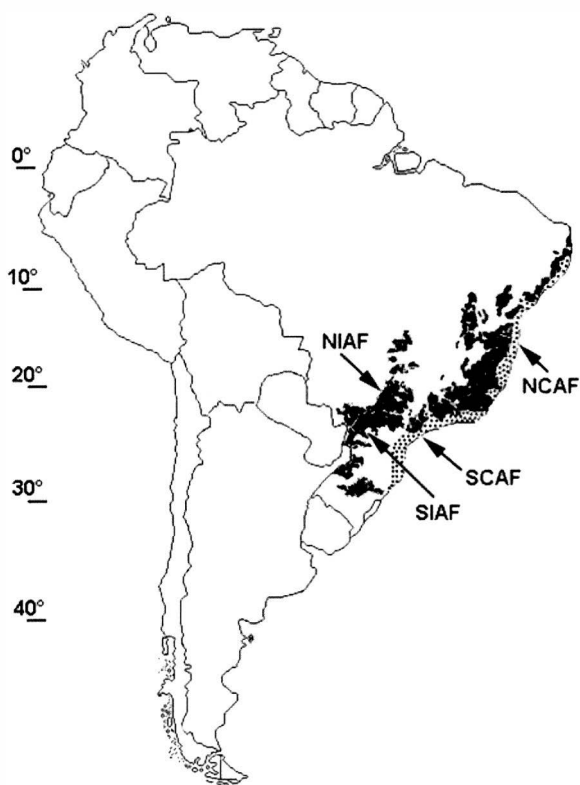


FIG. 1. Distribution of the *Liophis miliaris* populations studied in Brazil. NCAF = Northern Coastal Atlantic Forest, SCAF = Southern Coastal Atlantic Forest, NIAF = Northern Inland Atlantic Forest, SIAF = Southern Inland Atlantic Forest. Dark areas = Inland Atlantic Forest domain, dotted areas = Coastal Atlantic Forest domain.

4. *Southern Inland Atlantic Forest (SIAF)*: located in Paraná state, (22°51'N, 27°17'S to 45°18'E and 54°35'W). This area is covered by the semi-deciduous seasonal forests and by the Araucaria forest. Spring-summer (September to March) is usually hot but temperatures are lower than in the other areas. Autumn-winter (April to August) tends to be cold with abundant rainfall throughout the year and no clear dry season (Fig. 2).

MORPHOLOGICAL MEASUREMENTS

Liophis miliaris exhibits marked variation in colour pattern and according to the most recent review (Dixon, 1989) it is represented by seven subspecies. Following this classification, the population from NCAF is representative of *L. m. merremii*, whereas populations from the remaining areas studied (SCAF, NIAF, and SIAF) are *L. m. orinus*. Moreover, individuals from SCAF differ in colour pattern from NIAF and SIAF and possibly belong to distinct taxa (see Gans, 1964; Marques *et al.*, 2004). Thus, these populations may represent three distinct subspecies (and not to two, as previously proposed by Dixon, 1983; 1989). Alternatively, these individuals may belong to two or three different species of the genus *Liophis*, a possibility which could be confirmed only after a taxonomic revision of the group. In light of these unresolved taxonomic issues, we use the most recent classification as proposed by Dixon (1983, 1989), and consider the four populations of *L. miliaris* defined in this study which at least are representative of a single monophyletic group.

We examined a total of 289 preserved specimens of *Liophis miliaris* (127 adult females, 140 young and 22 adult males) from NCAF; 249 (84 adult females, 106 young and 59 adult males) from SCAF; 167 (49 adult females, 80 young and 38 adult males) from NIAF and 201 (84 adult females, 64 young and 53 adult males) from SIAF. These specimens are held in the collections of Museu de Zoologia da Universidade de Santa Cruz (MZUESC), Comissão Executiva do Plano da Lavoura Cacaueira (CEPLAC), Museu de História Natural da Universidade Estadual de Campinas (ZUEC), Instituto Butantan (IB) and Museu de História Natural do Capão da Imbuia (MHNCI). We measured snakes SVL to the nearest 1 mm, and after dissection recorded the following additional data: (1) reproductive condition - mature or immature (females were considered mature if they had follicles in secondary vitellogenesis (>10 mm in diameter), oviductal eggs or folded oviducts (Shine, 1977a); males were judged to be mature if the testes were enlarged and turgid, or if the deferent ducts were opaque and convoluted, indicating the presence of sperm (see Shine, 1977b); (2) diameter of the largest ovarian follicles or eggs in females (measured with a vernier calliper to the nearest 0.1 mm); (3) oviductal condition: folded or not (much folded oviducts indicate recent egg-laying); (4) length, width and thickness of the testes (measured with a vernier calliper to the nearest 0.1 mm), testicular volume (TV), as estimated using

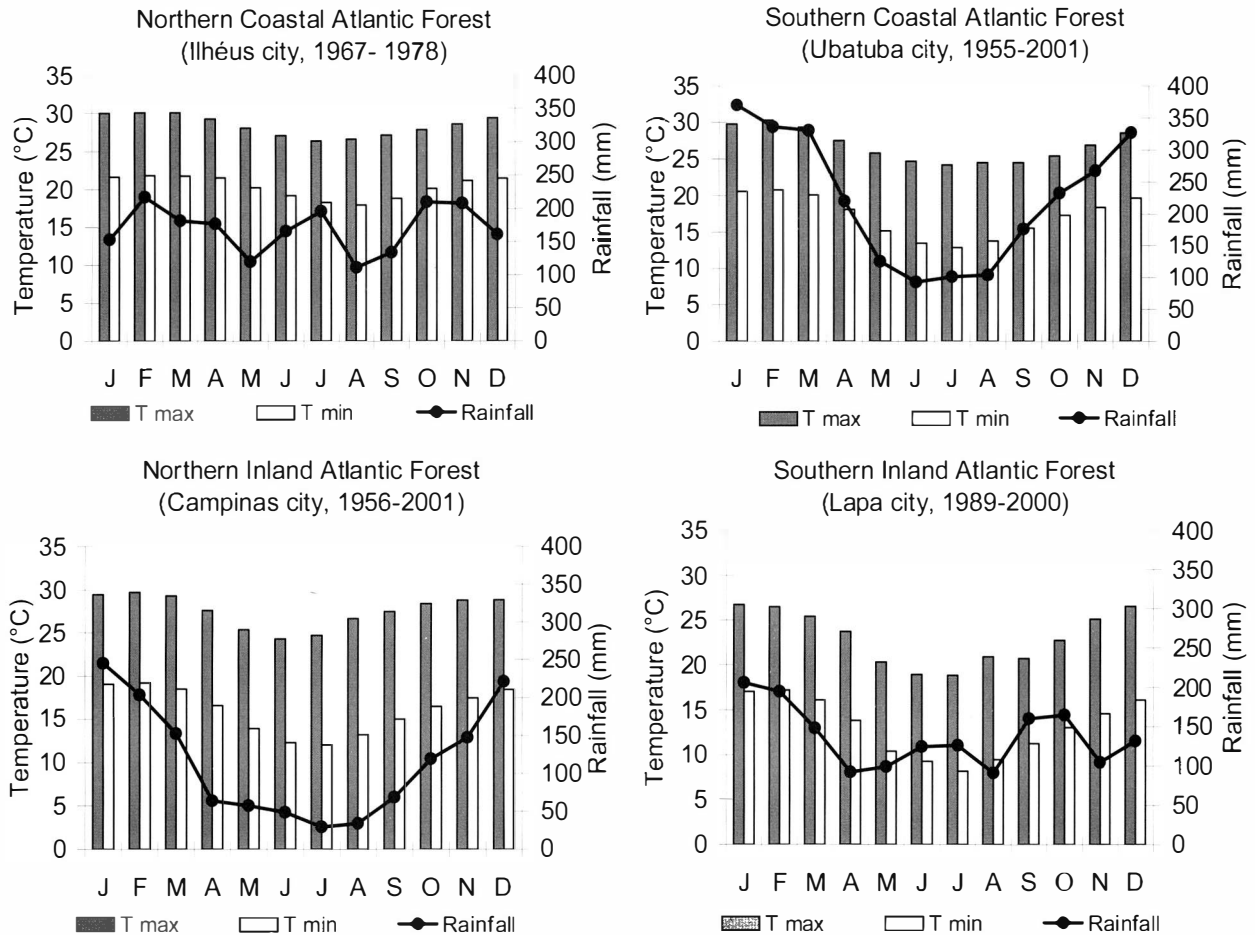


FIG. 2. Climatic patterns in the northeastern and southeastern Brazilian areas where the studied populations of *Liophis miliaris* were collected.

the ellipsoid volume formula $TV = 4/3abc$ where a = half of length, b = half of width and c = half of thickness (see Pleguezuelos & Feriche, 1999); (5) deferent duct diameter close to the cloacae (measured with a vernier calliper to the nearest 0.1 mm), and (6) presence of prey in the stomach

Testis volume and deferent duct diameter indicate spermatogenic activity and sperm release and storage (Volsøe, 1944; Fox, 1952; Shine, 1977b; Marques *et al.*, 2004). Snake testicular volume and deferent duct diameter were related to SVL and these allometric relations vary throughout the reproductive cycles (i.e. throughout the year – Shine, *et al.* 1998). Thus, we calculated the residuals of the regressions of these measures by SVL in the four seasons: January-March, April-June, July-September and October-December. Residuals were then compared in a Kruskal-Wallis test to identify differences on these variables within the seasons (Zar, 1999).

OVIPOSITION AND ACTIVITY PERIODS

We gathered data on the period of egg-laying from seven females which were collected while they were gravid (detected by palpation) and maintained in captivity (without males) until oviposition. Captive individuals were maintained at room temperature

(around 19–31 °C), with water provided *ad libitum* and food (fishes and frogs) offered every ten days.

The number of neonates from SCAF and NIAF received monthly at Instituto Butantan were used as estimative of recruitment in these regions (*cf.* Marques *et al.*, 2001, but see also Shine, 1980a). In the remaining areas the number used was the number of neonates collected during each month.

Climatic diagrams were supplemented with data collected from localities in the areas studied. These data were obtained from CEPLAC, Instituto Agrônomo de Campinas and Instituto Agrônomo do Paraná. We inferred snake activity by comparing the number of individuals collected in each of the four seasons (see Shine, 1980a and Marques *et al.*, 2001 for use and comments on this method) using a Chi-square test (Zar, 1999). Relationships between number of snakes collected per month (dependent variable), minimum temperature and mean rainfall were investigated by multiple correlations (Zar, 1999).

RESULTS

FEMALE REPRODUCTIVE CYCLE

Females with vitellogenic follicles, oviductal eggs or much folded oviducts were found throughout the year in

TABLE 1. Percentage of fed females, their reproductive status and evidence of multiple clutches in *Liophis miliaris* from Brazil. Pizzatto & Marques, Reproduction and activity in *Liophis miliaris*. NCAF = Northern Coastal Atlantic Forest, SCAF = Southern Coastal Atlantic Forest, NIAF = Northern Inland Atlantic Forest, SIAF = Southern Inland Atlantic Forest.

	NCAF	SCAF	NIAF	SIAF
Fed females	17% (n=22)	9.5% (n=8)	12.2% (n=6)	10% (n=8)
Fed females in secondary vitellogenesis	36.4% (n=8)	50% (n=4)	83.3% (n=5)	87.5% (n=7)
Fed females pregnant	0	0	0	12.5% (n=1)
Multiple clutches		one captive female laid eggs in January 2000, December 2000 and February 2001; one preserved female with oviductal eggs and vitellogenic follicles (18.5 mm).	two captive females laid eggs in intervals between two months and 11 months; one preserved female with oviductal eggs and vitellogenic follicles (16.3 mm).	four females with oviductal eggs and vitellogenic follicles (10.7; 10.9; 11.1 and 12 mm respectively).

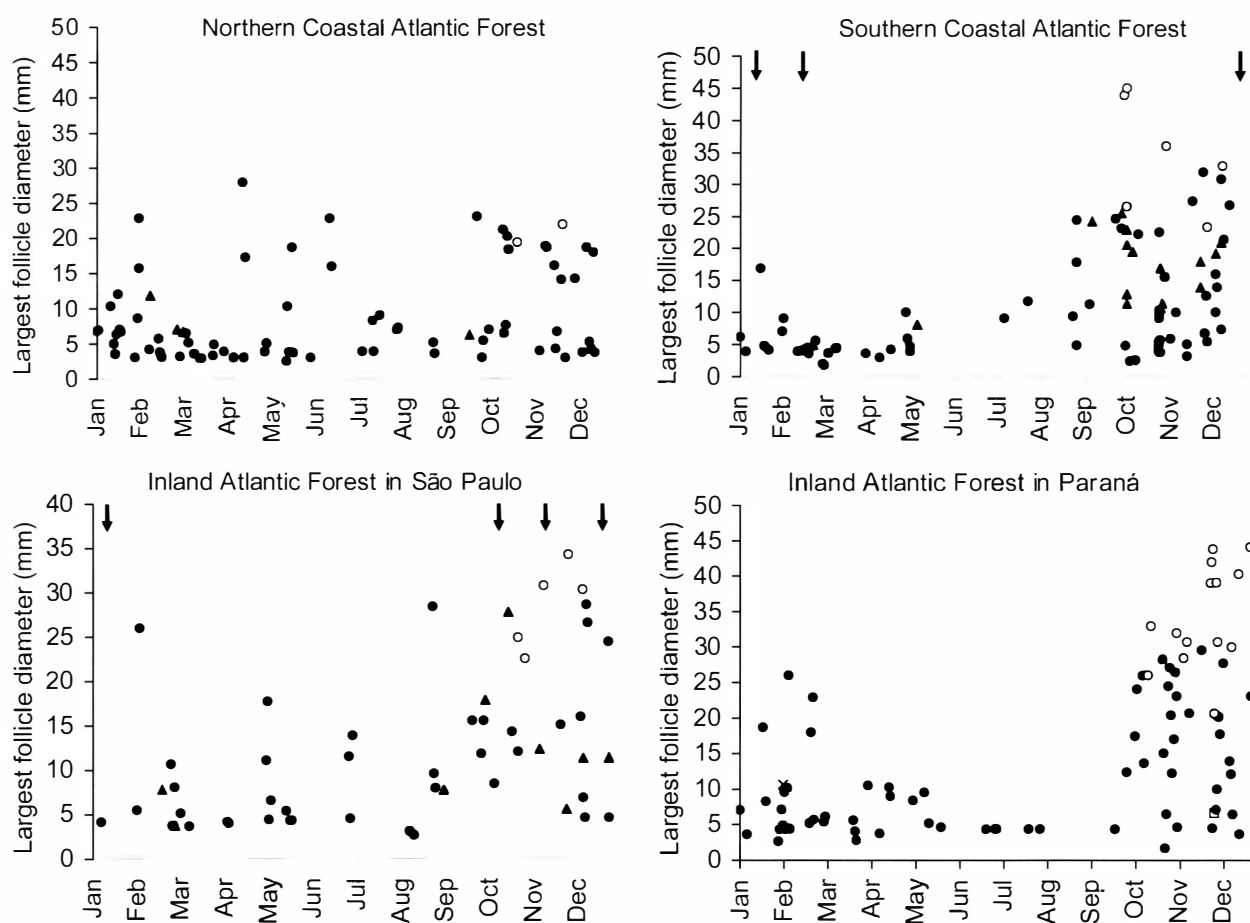


FIG. 3. Seasonal variation in the diameter (in mm) of the largest follicle (full circles) in *Liophis miliaris*. Empty circles = oviductal eggs, triangles = follicle and folded oviducts, cross = follicle and *corpora lutea*, rows = oviposition.

NCAF but only from September to January in SCAF and NIAF, and from October to February in SIAF (Fig. 3). Evidence of multiple clutches was found in all populations except in NIAF (Table 1). In general, pregnant females do not feed (Table 1) but females in secondary vitellogenesis had prey items in stomach more frequently than non-reproductive females (Table 1).

In NCAF, newly hatched snakes (SVL < 160 mm – see Pizzatto & Marques, 2005 for hatchling sizes) were recorded throughout the year but mainly in January (Fig. 4). In the other three areas, newly-hatched snakes (SVL < 230 mm) were more abundant between February and April (Fig. 4).

TESTICULAR CYCLE

None of studied populations showed significant variation, among the four seasons, in the residual volume of testis (Kruskal-Wallis test - SCAF: HL. Pizzatto and O. A. V. Marques $H=0.079$, $P=0.994$, $n=52$; NIAF: 0.432 , $P=0.933$, $n=37$; SIAF: $H=0.010$, $P=0.999$, $n=48$) or deferent duct diameter (Kruskal-Wallis test - SCAF: $H=0.343$, $P=0.952$, $n=55$; NIAF: 0.113 , $P=0.990$, $n=37$; SIAF: $H=0.414$, $P=0.916$, $n=48$), indicating a continuous spermatogenic cycle. The sample of

males ($n=22$) from NCAF was of insufficient size to permit analysis.

ACTIVITY

Adult females were most commonly collected from January to March in NCAF ($\chi^2=11.1$, $df=3$, $P=0.011$, Fig. 4), from October to February in SCAF ($\chi^2=33.4$, $df=3$, $P<0.0001$; Fig. 4), from October to December in NIAF ($\chi^2=15.1$, $df=3$, $P<0.0017$; Fig. 4) and also in SIAF ($\chi^2=45.0$, $df=3$, $P<0.0001$; Fig. 4). Number of females collected per month is positively correlated to minimum temperature in NCAF ($R^2=0.60$, $P=0.016$, $\beta_{(\text{min temp})}=1.79$, $t=3.50$, $P=0.007$; $\beta_{(\text{mean rainfall})}=-0.01$, $t=-0.52$, $P=0.615$) and also in SIAF ($R^2=0.41$, $P=0.089$, $\beta_{(\text{min temp})}=1.24$, $t=2.32$, $P=0.045$; $\beta_{(\text{mean rainfall})}=-0.02$, $t=-0.47$, $P=0.652$), but not in SCAF ($R^2=0.33$, $P=0.920$) or NIAF ($R^2=0.15$, $P=0.527$).

Adult males were most commonly collected in SCAF from October to February ($\chi^2=19.8$, $df=3$, $P=0.0002$; Fig. 4) and from January to March in SIAF ($\chi^2=10.5$, $df=3$, $P=0.0149$; Fig. 4). The number of collected males did not vary in NIAF ($\chi^2=1.6$, $df=3$, $P=0.676$; Fig. 4). Number of males collected per month was not correlated with minimum temperature or mean rainfall in any of the regions (SCAF: $R^2=0.57$, $P=0.193$; NIAF:

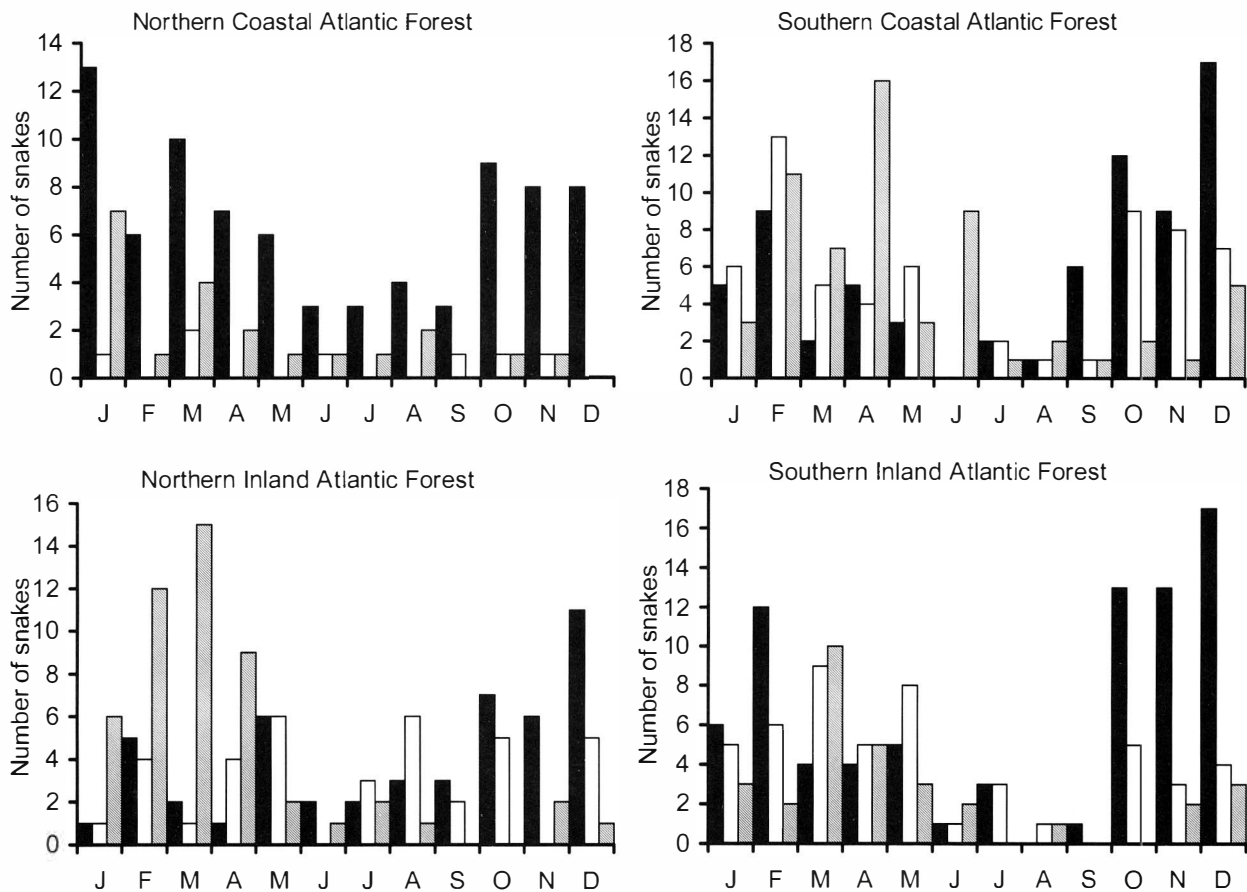


FIG. 4. Seasonal collection of *Liophis miliaris* from the studied populations in Brazil. White bars = adult males, black bars = adult females, striped bars = hatchlings.

$R^2=0.12$, $P=0.555$; SIAF: $R^2=0.28$, $P=0.221$). The sample of males from NCAF was too small to analyse.

DISCUSSION

FEMALE REPRODUCTIVE CYCLES

In the NCAF, the climate is homogeneous throughout the year, which may allow constant food availability (Begon *et al.*, 1990). Such a condition may result in continuous reproduction in *Liophis miliaris* from this region, as evidenced by the year-round presence of vitellogenic follicles. However, even continuous cycles show reproductive peaks in the warmer and rainier periods (*cf.* Seigel & Ford, 1987; Pizzatto & Marques, 2002). In this region, the small increase in temperature from October to March can cause a reproductive peak at the end of the year (suggested in Fig. 3) and a recruitment peak in January (see Fig. 4). There is no evidence of multiple clutch production in *L. miliaris* from the NCAF (Pizzatto & Marques, 2006), thus a continuous cycle within this population may be due to asynchronous reproduction (*cf.* Seigel & Ford, 1987).

In the remaining populations, where climatic variation is more marked, reproduction seems to be seasonal with oviposition taking place during the rainy season. In SCAF and NIAF, temperature variation is tenuous but with a marked dry season (from April to September). *Liophis miliaris* feeds upon aquatic prey in aquatic habitats (Marques & Souza, 1993; Marques & Sazima, 2004) and in southeastern Atlantic forest (SCAF and NIAF) a decrease in the number of temporary ponds in the dry season restricts prey availability (Marques *et al.*, 2001). Thus, rainfall may be the principle factor that restricts the reproductive cycle in female *L. miliaris* in these regions. Reproductive seasonality has also been observed in other snakes and lizards from tropical areas where monthly temperature variation is minimal but seasonality of rainfall is marked (*cf.* Saint-Girons, 1982; James & Shine, 1985; Shine *et al.*, 1998; Brown & Shine, 2006). On the other hand rainfall is homogeneous throughout year in SIAF but the reproductive cycles of snakes are seasonal. Female abundance is related to minimum temperature in this area. *Liophis miliaris* tends to remain coiled and inactive at temperatures between 12 and 18°C (Abe, 1977) and, in SIAF, mean temperatures are about 13°C from June to August and minimum temperatures are under 10°C. The reproductive season in SIAF starts only in October when minimum temperatures are close to 15 - 20°C and extends until February, while in the other seasonal areas it is from late August to February. Thus, in SIAF the beginning and end of the reproductive period may be delayed because of a delay in the arrival of warmer weather compared to SCAF and NIAF. This pattern is similar to those described for other tropical (Madsen & Shine, 1996) and temperate snakes (Naulleau *et al.*, 1998).

Reproductive seasonality in *Liophis miliaris* from SCAF, NIAF and SIAF is probably due to reproductive

synchrony within these populations, and multiple clutches can also occur (Pizzatto & Marques, 2006). Egg-laying during warmer and rainier periods tend to benefit egg incubation during more favourable climatic conditions for embryonic development (Vinegar, 1977), diminishing egg incubation time, increasing hatching success, and generating larger neonates (Qualls & Shine, 1998; Ji & Dou, 2001; Shine & Elphick 2001).

Multiple clutches in captive females maintained in the absence of males is indicative of long-term sperm storage (from a particular reproductive season to the next), which in some colubrids has been shown to occur in the female infundibulum (see Fox, 1956; Halpert *et al.*, 1982; Aldridge, 1992; Pizzatto & Marques, 2002).

TESTICULAR CYCLES

The relationship between testis volume and body size is a good indication of part of male reproductive effort (Begon *et al.*, 1990) and it also permits the description of testicular cycles in snakes (Volsøe, 1944; James & Shine, 1985; Pleguezuelos & Feriche, 1999; Shine *et al.*, 1999). Continuous sperm production is suggested by at least three factors. The first one is the absence of variation in residual testis volume in all populations. The second is the absence of variation in residual diameter of the deferent ducts, suggesting that sperm are not stored by males. In some snakes species, sperm storage by males in the deferent duct (which increases its diameter) generally occurs when spermatogenesis is seasonal (or continuous with a peak) and mating is dissociated from sperm production (Shine, 1977b; Almeida-Santos *et al.*, 2004). The third one is that climatic variation is not related to male abundance (and consequently male activity), in most populations, presumably it has little influence on sperm production. As mating is simultaneous with sperm production, male reproductive cycles may be classified as pre-nuptial, at least in NIAF and SCAF (see Saint-Girons, 1982; Seigel & Ford, 1987). In any case, small variations in sperm production are only detected by histological analyses.

ACTIVITY

In the areas where reproductive cycles are seasonal, snake activity can be directly related to both reproduction and feeding. Female abundance (and consequently activity – see Marques *et al.*, 2001) increases from October to December, when they are reproductively active. As demonstrated for other species, reproductive females often spend more time basking and are slower in their movements than non-reproductive ones (Shine, 1979; 1980b; Seigel *et al.*, 1987). Females also become more active during egg-laying periods as they search for suitable oviposition sites. In addition, female *Liophis miliaris* did not stop feeding in the early stage of reproduction (*i.e.* secondary vitellogenesis), when they need energy to invest in egg production. A high percentage of females with prey in the stomach had fol-

licles in secondary vitellogenesis. This suggests that females must increase feeding activity during early reproductive time. Thus, females may be more commonly collected during the reproductive season both due to reproductive and feeding activity.

Activity in *Liophis miliaris* may be related to certain climatic factors (see Gibbons & Semmlitsch, 1987). In SIAF, temperature decrease is more pronounced in autumn-winter than in the other areas and probably causes a decrease in female activity, reflected in the number of individuals collected. Even in NCAF where climate is more homogeneous minimum temperature has a significant influence on female activity. In the other areas there are no obvious relationships between the number of snakes and the environmental variables measured.

In southern areas, a decrease in male activity occurs only in the middle of the coolest and driest season (July to September) and the number of males from April to June (early cold/dry season) did not differ from the expected distribution. This probably happens because during this period males are engaged in searching for females (*cf.* Shine, 1980a) and do not limit their activity even with the decrease in temperature/rainfall.

In conclusion, female reproductive pattern in *Liophis miliaris* from Brazil differs among populations, mainly between those from northward (NCAF) to southward (SCAF, NIAF and SIAF) and these differences must be primarily due to the influence of temperature.

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DISCRIMINATION OF MOOR FROG (*RANA ARVALIS*) AND COMMON FROG (*RANA TEMPORARIA*) INDIVIDUALS USING A RAPD TECHNIQUE

CHARLES SNELL AND I. H. EVANS

Medway School of Science, University of Greenwich at Medway, Chatham Maritime, UK

A method has been developed for discriminating between the common frog (*Rana temporaria*) and the moor frog (*Rana arvalis*) using either of two primers in RAPD analysis of DNA samples extracted from larval tail tips. These two frog species can be extremely difficult to distinguish morphologically at the egg clump and larval stages, which are very convenient stages for monitoring populations when there are conservation concerns. The adults need capture and detailed morphological examination to effect certain identification, this being particularly true for edge-of-range populations. The two primers also distinguished DNA samples of common toad (*Bufo bufo*), natterjack toad (*Bufo calamita*), pool frog (*Rana lessonae*) and the marsh frog (*Rana ridibunda*). Additionally, findings are reported for a third primer which distinguished, intraspecifically, between relatively closely located common frog (*Rana temporaria*) populations in southern England.

Key words: anura, frog identification, molecular genetics, population assessment

INTRODUCTION

Palearctic frogs of the genus *Rana* have been categorised into two groups: the “water” (or “green”) frogs and brown (or “grass”) frogs. Water frogs are predominantly aquatic, often green in colour, and are typically found in freshwater shallows, or basking near the waterside. Brown frogs are, conversely, predominantly terrestrial, normally brown coloured, and spend most of their time concealed in herbage (hence “grass” frogs), often at considerable distances from open water. The north-west European water frogs are the marsh frog *Rana ridibunda*, the pool frog *Rana lessonae* and the edible frog *Rana esculenta*, while the brown frogs in the same region are the common frog *Rana temporaria*, the moor frog *Rana arvalis* and the agile frog *Rana dalmatina* (Matz & Weber, 1983; Nöllert & Nöllert, 1992; Arnold, 1995; Gasc, *et al.* 1997; Arnold & Ovenden, 2002).

Accurate species identification is essential in ecological studies and in population monitoring programmes responding to concerns about global amphibian decline. The European ranges of *Rana temporaria* and *Rana arvalis* overlap substantially, and adult individuals of the two species are not always easy to distinguish morphologically (Fig. 1). While useful morphological indicators such as the size and shape of the metatarsal tubercle on the inner rear toe are available (but only after capture) for adults, species identification in earlier developmental stages – which can be particularly valuable in population monitoring – is much more problematic. One effective brown frog population census method is the counting of spawn clumps (Griffiths & Raper, 1994; Loman, 1996). In brown frog census work in southern Sweden, Loman (2001) found that up to 15 percent of spawn clumps belonging to either *Rana temporaria* or *Rana arvalis* could not be discriminated on morphological grounds. Again,

larvae of the two species are difficult to distinguish, the identification problems being compounded by the phenotypic plasticity of anuran larvae (Vences *et al.*, 2002). Indeed, recent claims for new *Rana* species in the Pyrenees based on substantial geographical variation shown by apparent *R. temporaria* populations (Vences, 1992; Arano *et al.*, 1993; Vences *et al.*, 1998; Veith *et al.*, 2002) may just reflect morphological variability in *R. temporaria* tadpoles (Vences *et al.*, 2002). However, it may indeed be the case that unrecognised and new species occur in some European brown frog populations. These considerations prompted us to develop an unambiguous molecular method for identifying *R. temporaria* and *R. arvalis* individuals, applicable to developing eggs, larvae and adults.

The methodology we selected was the DNA-based RAPD technique (Williams *et al.*, 1990, 1993), which has proved useful in genetic studies of rare and endangered amphibian populations (Kimberling *et al.*, 1996),



FIG. 1. An illustration of identification difficulties that can present with the two brown frogs *Rana temporaria* (left) and *Rana arvalis* (right). Handbook descriptions usually describe the moor frog as normally being stripe-backed and with a more pointed snout than the common frog. In this illustration the randomly caught common frog (from a London garden) on the left has a bolder stripe and a more pointed snout than the moor frog (from southern Sweden) on the right.

and which we have recently used to clarify the genetic affinities of different populations of the pool frog *R. lessonae* (Snell *et al.*, 2005). More pertinently, RAPD has been shown to be effective in discriminating amphibia at the species level in the cases of green frogs of the species *R. esculenta*, *R. lessonae* and *R. ridibunda* (Zeisset & Beebee, 1998), and larval toads of the species *Bufo bufo* and *B. calamita* (Bardsley, *et al.*, 1998).

We report here a RAPD method in which either of two primers clearly and correctly identified individuals from a large sample group as either *Rana temporaria* or *Rana arvalis*; the two primers also gave distinct, species-specific band patterns for *R. esculenta*, *R. lessonae*, *B. bufo* and, in the case of primer OT-A3, *B. calamita*. It had been hoped to include *R. dalmatina* in the study, but no tissue samples were available. RAPD data for a third primer are also reported, because, though less useful in species diagnosis, this primer discriminated intra-specifically, between different southern English populations of *R. temporaria*.

MATERIALS AND METHODS

TISSUE SAMPLES

Egg samplings (ca. 30 ova from each site) from *Rana arvalis* were obtained from frogs from southern Sweden, Denmark and Poland. The adults were clearly identified by their metatarsal tubercles and the spawning was at least ten days later than is normal for *Rana temporaria*. *Rana temporaria* eggs were collected in late February from ponds in S.E. London, Bromley, Bexley, Suffolk and Dorset (all UK sites). In order to produce tissue containing enough DNA for extraction and recovery, the eggs were placed in separate tanks (labelled according to species and population), where they were allowed to develop. The water used was filtered, conditioned ("ReptiSafe" treated – see below) mains tapwater, which was allowed to stand for 10 days, then seeded generously with *Daphnia* as natural water-filtering agents. When the larvae had reached ca. 15–20 mm in length, small sections of tail fin tip (stored in absolute alcohol after removal) were used as a source of DNA and the larvae returned to the tanks to allow the tail tips to begin to part-regenerate naturally. The larvae were finally released back into their ponds of origin. The loss of tail tips in anuran larvae has been shown to cause little loss of ecological fitness and may in fact be a mechanism to reduce predation (Wilbur & Semlitsch, 1990; Vences *et al.*, 2002) somewhat analogous to tail loss in lizards.

SAMPLE SIZES

Primer OT-A3: Rana arvalis: Denmark, 26; Sweden, 21; Poland, 15. *Rana temporaria*: Suffolk, 24; Bexley, 19; Bromley, 17; S.E. London, 18; Dorset 17.

Primer CS-L1: Rana arvalis: Denmark, 21; Sweden, 24; Poland, 24. *Rana temporaria*: Suffolk, 26; Bexley, 17; Bromley, 16; S.E. London, 18; Dorset 17.

Primer OT-C6: Rana arvalis: Denmark, 12; Sweden, 14; Poland, 14. *Rana temporaria*: Suffolk, 28; Bexley, 13; Bromley, 17; S.E. London, 17; Dorset, 15. *R. arvalis* numbers were substantially lower for primer OT-C6 than those used for primer OT-A3 & CS-L1 as, with this primer, the main focus was on *Rana temporaria*.

REAGENTS

Agarose, deoxyribonucleotides and 100 base-pair DNA marker ladders were obtained from Gibco-BRL, UK. Chelex 100 resin was from Bio-Rad (CA., USA). DNA polymerase derived from the organism *Thermus islandicus* ("Thermoprime Plus") was obtained from Advanced Biotechnologies, Epsom, UK. PCR buffers were supplied with the enzymes. Primers were synthesized by Operon Technologies (Gosforth, UK.), Cruachem (Glasgow), and Microzone (Lewes, E. Sussex). All other chemicals were from Sigma Chemical Co., Poole, Dorset, UK, and solutions were made using sterile distilled water. Aquarium water conditioning agent was "ReptiSafe" (Zoo Med Inc., CA. or, in the UK, from Livefood UK online).

DNA EXTRACTION

Tissue fragments (c. 4 mg) were statically incubated in 160 µl of sterile distilled water and 40 µl Chelex-100 resin overnight in a water bath at 55°C (Walsh *et al.*, 1991; Zeisset & Beebee, 1998). The samples were then briefly vortexed, boiled for eight minutes in a water-bath, re-vortexed and centrifuged at 5000 × g for three minutes at room temperature. The resulting supernatant (stored at –20°) was used as the DNA source for subsequent PCR amplifications. DNA concentrations were determined by measuring absorbance at 260 nm, and were adjusted to approximately 50 µg/ml by dilution or freeze-drying (100 µg/ml and 25 µg/ml gave the same PCR amplification results as 50 µg/ml). All procedures were carried out in designated pre- and post-PCR areas. All solutions and apparatus were also confined to pre- or post-PCR areas and rigorously sterilized where appropriate. Freshly autoclaved pipette tips and PCR-dedicated, thin-walled microfuge tubes (Advanced Biotechnologies, Epsom, Surrey, UK.) were used throughout.

RAPD ANALYSIS

RAPD-PCR was essentially that described by Williams *et al.* (1993) with some modifications. Each PCR assay contained 2 µl of extracted supernatant (with DNA) in a final volume of 20 µl with 100 µM each of dATP, dCTP, dGTP, dTTP, 0.2 µM 10-mer oligonucleotide primer and 0.8 units of DNA polymerase. Addition of the enzyme supplier's buffer to each reaction resulted in final reaction concentrations of 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM (NH₄)₂SO₄, 3.5 mM MgCl₂ and 0.01% (v/v) Tween 20. Glycerol was added to a final concentration of 5% (v/v).

TABLE 1. Primer OT-A3 band patterns in other anuran species. Sizes in average base pair numbers. Heavy bands shown in bold. Provenance of samples: *Bufo bufo*, larvae from southeast London, *Bufo calamita*, larvae from captive colony, original provenance Norfolk, UK, *Rana ridibunda*, larvae from North Kent Marshes and *Rana lessonae* larvae from a captive colony of mixed origin.

Species	Sample no.	Band sizes (bp): heavy bands are shown in bold.							
<i>Bufo bufo</i>	4		719	678		522	499		
<i>Bufo calamita</i>	3	948	839	719		572			
<i>Rana ridibunda</i>	4			653		560	500	428	341
<i>Rana lessonae</i>	6		722	643	575		497	438	350

Using either of two thermocyclers (Techne PHC3 and a Techne "Genius"), a thermocycling protocol was used which started with a denaturation cycle of 94°C for four minutes followed by 40 cycles of three segments (with a ramp rate of 60%) consisting of: 94°C x 1 minute, 36°C x 1 minute, and 72°C x 2 minutes, with a final extension cycle of 72°C for six minutes. On completion of the reaction, 5 µl of loading buffer (containing 2.5 mg/ml bromophenol blue) were added and the mixture electrophoresed at 4 V/cm through 1.5 % w/v agarose in TBE (67.5 mM Tris-borate, 1.5 mM EDTA (pH 8.0)) running buffer, allowed to run for 5 to 6 cm, then stained in a bath of sterile distilled water containing 1 mg/l ethidium bromide.

Typically, three wells of each gel were loaded with DNA molecular weight standards, and one well contained, as a control, the products of a PCR reaction using all of the reagents except DNA. Gel images were captured by a video camera linked to a computer and digitised (GDS-7600 Gel Documentation System, UVP Ltd., Cambridge, UK).

RESULTS

PRIMER SELECTION

One hundred and five 10-mer primers were tested in preliminary experiments (not reported here) and two of these, OT-A3 and CS-L1, were chosen as being the most discriminating between *Rana temporaria* and *Rana arvalis* DNA on the basis of polymorphic band resolution and repeatability: these were used in later analyses. A third primer, OT-C6, which separated the two species less effectively, but seemed capable of discriminating quite closely situated *R. temporaria* populations, was also chosen for further work. To test reproducibility, replicate experiments, seven for each of the three primers, were made. Each successive gel run used an increasing number of samples, which included DNA from the individuals used in the previous run plus DNA from new individuals and populations: all gave the same results except in the case of primer 3 where it became increasingly obvious that different *Rana temporaria* populations were producing varied banding patterns (see below). The results from the two different thermocyclers were entirely comparable.

RESULTS FOR SELECTED PRIMERS

Primer OT-A3 (AGTCAGCCAC). (NB Primer sequences all given 5' to 3'). This primer gave band

patterns that were clearly different for *Rana temporaria* (95 individuals tested) and *Rana arvalis* (62 individuals tested; Fig. 2). Two bands were highly diagnostic for *R. temporaria* (407 bp band in 97% of individuals tested, 910 bp band in 90%) and one band was completely diagnostic for *R. arvalis* (560 bp band in 100%). As a further check on the species specificity and utility of this primer, it was tested with DNA samples from four other NW European anuran species (Table 1): the band patterns were identical for all individuals of a species (*R. lessonae*, *R. ridibunda*, *Bufo bufo* and *B. calamita*), and were species specific, differing clearly from the patterns for *R. temporaria* and *R. arvalis* (Fig. 2).

Primer CS-L1 (TCCCTTCCTC). This also yielded highly distinctive band patterns for *R. temporaria* (94 individuals tested) and *R. arvalis* (69 individuals tested; Fig. 3). Again, for *R. arvalis*, one, possibly complex, band (380 bp) occurred in all the *R. arvalis* individuals, and in none of the *R. temporaria* individuals tested with this primer. Also, 68% of *R. temporaria* individuals gave a strong band at 540 bp, with no matching band in *R. arvalis*. This primer was tested for species specificity with DNA samples from three other NW European anuran species (Table 2), and again, the band patterns were uniform within species, and species-specific.

Primer OT-C6 (GAACGGACTC). This primer was interspecifically discriminating, though less so than primers OT-A3 and CS-L1, but it showed an interesting ability to distinguish four different southern English populations of *Rana temporaria* (Fig. 4). Compared to the rural populations in Dorset and Suffolk, the urban S.E. London population lacked a heavy band at about the 445 bp position (see Fig. 4, arrow 2). The more suburban populations of Bromley and Bexley lacked a band at about the 680 bp position (Fig. 4, arrow 1) when compared with the Dorset and Suffolk populations. Larvae of *Rana temporaria* separated by only around 5 km / 3 miles (Bexley or Bromley to the S.E. London collection site) were distinct in two regions (the two positions indicated by arrows 1 and 2 in Fig. 4).

DISCUSSION

In this study we have described a RAPD method which, using either of two primers, unambiguously identified larvae of varied geographical origin as either *Rana temporaria* or *Rana arvalis*. Reproducibility is sometimes a concern in RAPD experiments, however, high reproducibility of band patterns was seen in the replicate amplifications carried out here, and it can also

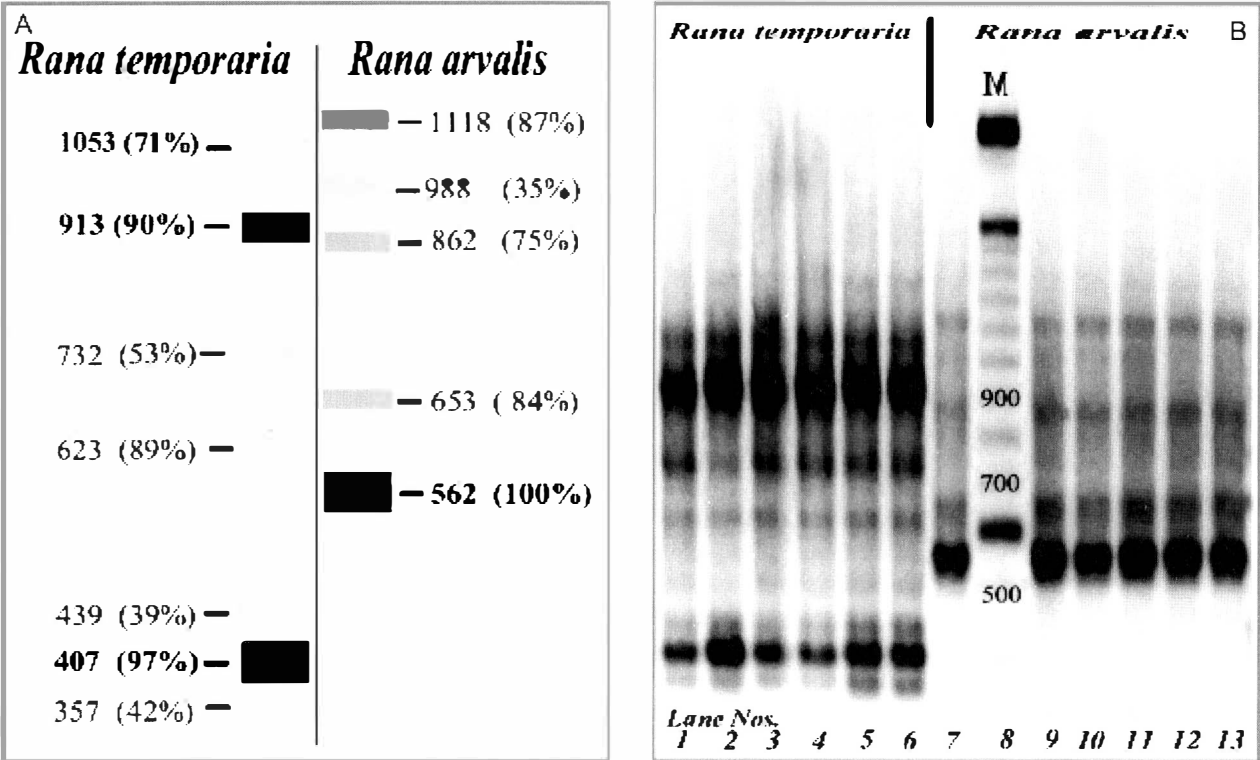


FIG. 2. RAPD results for *Rana temporaria* and *Rana arvalis* using primer OT-A3. A. graphical synopsis of primer OT-A3 results based on 157 lanes (=individuals). It is evident that many bands are inter-specifically discriminating. Rectangles shown in bold represent bands which gave strong amplification results as well as being interspecifically discriminating. B. part of one of the primer OT-A3 gels, representative of this primer's results overall, and demonstrating the clarity of species separation. Lanes 1-6, *R. temporaria*; lanes 7 and 9-13, *R. arvalis*; lane 8, molecular weight markers (100 bp DNA ladder).

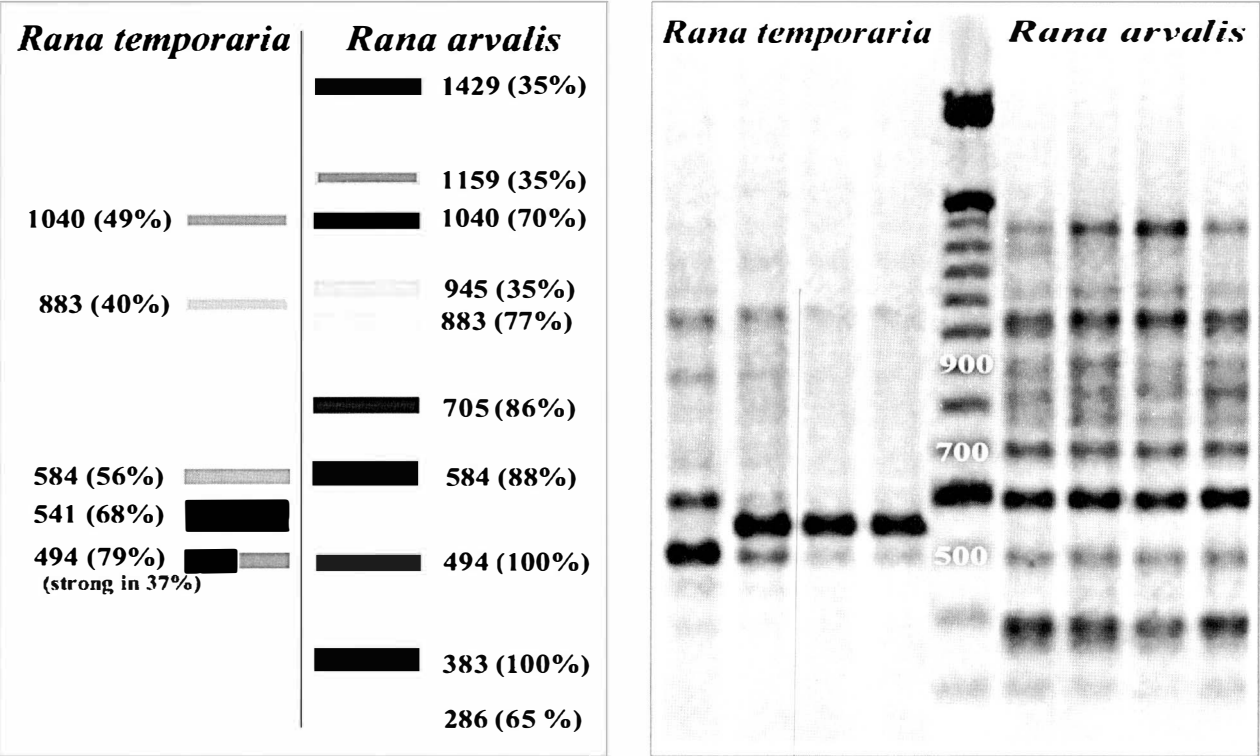


FIG. 3. RAPD results for *R. temporaria* and *R. arvalis* using primer CS-L1. A. Graphical synopsis of primer CS-L1 results based on 163 lanes (= individuals). Initial numbers are average band lengths in base pairs. Parenthesised numbers represent percentage of that species with that band. Rectangles shown in bold represent bands which gave strong amplification results and contributed to the species specificity of the band patterns. A. Part of one of the primer CS-L1 gels, representative of this primer's results overall, and indicating the distinctiveness of the band patterns for the two species. Lanes 1-4, *R. temporaria*; lanes 6-9, *R. arvalis*; lane 5, molecular weight markers (100 bp DNA ladder).

TABLE 2. Primer CS-L1 band patterns in other anuran species. Sizes in average base pair numbers. Heavy bands shown in bold. Provenance of samples: *Bufo bufo*, larvae from S.E. London, *Rana ridibunda*, larvae from north Kent marshes and *Rana lessonae* larvae from captive colony of mixed origin.

Species	Sample no.	Band sizes (bp). Strong bands shown bold						
<i>Bufo bufo</i>	4	827	661	560	481	424	352	
<i>Rana ridibunda</i>	3		738	654	495			303
<i>Rana lessonae</i>	8		693	522	499			



FIG. 4. A section of a primer OT-C6 gel where all samples were *Rana temporaria*. The gel contains two internal 100 base pair marker lanes (the fifth and last lanes), the 600 base pair position of which has been marked with a white spot. The two main points of dissimilarity are marked with directional circles numbered 1 and 2.

be pointed out that results reported in our recent pool frog study (Snell *et al.*, 2005) have been replicated by different workers in the Greenwich laboratory, in an extension of that investigation.

Primer OT-A3 gave a particularly clear contrast in banding patterns for the two species, with a 560 bp band being a strong, unique marker for *R. arvalis* (Fig. 2), whereas with primer CS-L1 the *R. arvalis* patterns were more complex (see Fig. 3), though there was a distinct and unique *R. arvalis* band at 380 bp. It is also helpful that the primers discriminated individuals of other anuran species (see Tables 1 and 2). The technique is relatively inexpensive and not too time-consuming: DNA extraction needs up to one hour's manipulation followed by incubation overnight. Using fast thermocyclers the work can then be completed within a working day, furthermore, a single large format electrophoresis tank running several rows of wells on large gels can analyse upwards of 120 samples. Tail tips can be collected quickly from large numbers of larvae with low risk of larval mortality, even for very small tadpoles (Gosner stage 26 and above: Gosner, 1960).

We anticipate that our identification method will be most useful in population monitoring in relation to con-

servation concerns using samples from egg clumps in the later stages of egg development and from egg samplings allowed to develop in "captive" conditions (as here) or with wild-caught larvae, however, the method could have wider applications. Adult brown frogs can be very similar in appearance and may need expert guidance to distinguish, especially in the case of northwest European fringe populations of common and moor frogs. Fig. 1 gives an example of this confusing similarity, where a common frog (randomly caught in a London garden) is shown alongside a moor frog (on the right) from a southern Swedish population: the common frog actually resembles most handbook descriptions of the moor frog (with its central dorsal stripe and more pointed snout), and the moor frog is more in agreement with common frog descriptions. Fortunately, and reliably in the case of moor and common frogs, the metatarsal tubercle size can be used. These features are not immediately obvious without close examination and therefore require the capture of animals in order to be useful. Our method could be applied to buccal swabs (Pidancier *et al.*, 2003) or to toe clippings from adults in cases where the metatarsal tubercle size may not have great value (e.g. when there is a possibility of unrecog-

nised sibling or sub-species in the same geographic area). The possible occurrence of cryptic species in European brown frog populations has been previously raised by experienced and well-travelled herpetologists (Bentley, J., Harrison, C., pers. com., 1997). The availability of two species-specific primers could be useful and informative in tests with samples from geographical areas where "cryptic" or sub-species are suspected and the results yield unexpected band patterns. This application may be particularly relevant to the Mediterranean peninsulae where brown frog diversity tends to be the highest. Even as recently as 1993 a new brown frog species from the Pyrenees area (*Rana pyrenaica*) has been described (Serra-Cobo, 1993).

Interestingly, sub-fossil remains of the moor frog (*Rana arvalis*) and the agile frog (*Rana dalmatina*) have recently and unexpectedly been found in Middle Saxon (c. 600-950 AD) archaeological digs in the fenland districts of England (Gleed-Owen, 1999, 2000), indicating that these species could have been native to Britain, and may have persisted into modern times. Indeed, the close similarity of fringe populations of moor frogs to common frogs could mean that there is a chance of unrecognised remnant populations of the moor frog, especially in more remote areas, which our technique could offer confirmatory identification.

The results reported for primer OT-C6 indicate that even quite closely adjacent populations of *R. temporaria* in southern England are genetically distinguishable, using our RAPD method, though it should be acknowledged that the numbers of individuals sampled are not large, and that codominant markers are more useful for population studies (Brede & Beebee, 2004). In the case of the S.E. London and Bexley/Bromley populations, separated by only around 5 km, our findings are consistent with those of Hitchings and Beebee (1996), which showed that, in urban situations, man-made features such as busy roads can act as efficient barriers to dispersal and migration in common frog populations, and so encourage genetic divergence.

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INTRA-SEX SYNCHRONY AND INTER-SEX COORDINATION IN THE REPRODUCTIVE TIMING OF THE ATLANTIC CORAL SNAKE *MICRURUS CORALLINUS* (ELAPIDAE) IN BRAZIL

SELMA M. ALMEIDA-SANTOS¹, LÍGIA PIZZATTO² AND OTAVIO A. V. MARQUES¹

¹Laboratório de Herpetologia, Instituto Butantan, Av. Dr. Vital Brazil, 1500, 005503-900, São Paulo, SP, Brazil

²Pós-Graduação em Ecologia, Universidade Estadual de Campinas, IB, Zoologia, CP 6109, 13083-970, Campinas, SP, Brazil

Dissection of preserved Atlantic coral snakes *Micrurus corallinus*, plus field data and histological analysis, provided information on male reproductive cycles. Testes are larger during autumn, when sperm production occurs, and smaller in spring, when spermatogenesis stops. The diameter of the distal deferent ducts is small in summer–autumn, when sperm are hardly found in the lumen, and it increases in winter–spring, when sperm is abundant, just prior to the mating season. Thus, the male cycle of *M. corallinus* is post-nuptial, whereas the female cycle is pre-nuptial. Although gametogenesis is not simultaneous in both sexes, the coordination of their cycles is guaranteed by sperm storage by males. Our data indicate that the diameter of the deferent duct is a good indication of the mating season, mainly when reproductive cycles are post-nuptial. Mate searching and aggregation occurs in the spring, and activity in both sexes may be highly related to their reproductive cycles.

Key words: aggregation, reproduction, snake, spermatogenic cycle, sperm storage

INTRODUCTION

Continuous reproductive cycles in snakes are expected in tropical regions without a well-marked dry season (Fitch, 1982; Saint-Girons, 1982; Vitt, 1983; Seigel & Ford, 1987), although several tropical species show seasonal reproduction and intra-sex synchrony (Fitch, 1982; Fowler *et al.*, 1998; Marques, 1996, 1998, 2002; Marques & Puerto, 1998). This synchrony may be a strategy by which individuals maximize their reproductive success (see Ims, 1990). Mating periods are unknown for most snake species and male reproductive cycles are less understood than those of females (Saint-Girons, 1982). Moreover, there is very little information on either the sperm storage organs of the male or the seasonal location of the sperm (Schuett, 1992). Although mating and fertilization in snakes may occur in the same period (e.g., Marques, 1996), in several species mating time does not coincide with fertilization (Fox 1956; Darevsky, 1971; Halpert *et al.*, 1982; Schuett, 1992; Almeida-Santos & Salomão, 1997; Almeida-Santos *et al.*, 2004). In addition, when spermatogenesis is not coincident with vitellogenesis (see Jordão, 1996; Bizerra, 1998) it is very difficult to determine mating periods.

The Atlantic coral snake, *Micrurus corallinus*, is a common snake in the Atlantic forest domain in south-eastern Brazil (Marques *et al.*, 2004). It occurs in dense ombrophilous and semi-deciduous seasonal forests in Brazil, Paraguay, Uruguay and Argentina (Campbell & Lamar, 2004). The female reproductive cycle is sea-

sonal, with vitellogenesis and mating occurring in the early rainy season, oviposition in mid-rainy season and hatching at the end of the rainy season and in the early dry season (Marques, 1996). However, nothing is known on the reproductive cycles of males. Here we present data about testicular activity, sperm storage in the deferent ducts, reproductive aggregation, and relation of activity to reproductive cycles of males and females.

MATERIAL AND METHODS

A total of 187 *M. corallinus* males were examined from the collections of the Instituto Butantan and Museu de História Natural da Universidade Estadual de Campinas. The sample included only adults (larger than 440 mm in snout-vent length – see Marques 1996) from São Paulo State, south-eastern Brazil (between 19.7°N, 25.3°S, 53.2°W and 44.2°E). The following data were taken from each specimen: (1) snout-vent length (SVL); and (2) testis length and diameter of a deferent duct at its distal end (see Fig. 1), both recorded on the right side. Spermatogenic cycles were determined relating the testicular length to spermatogenic activity (Volsøe, 1944; Shine, 1977; Seigel & Ford, 1987). Similar correlation has been shown between morphology of the deferent duct and sperm storage (Yokoyama & Yoshida, 1993; Sever *et al.*, 2002; Almeida-Santos, 2005).

As testis length was related with SVL ($R^2=0.19$, $P<0.0001$), we used the residuals from the linear regression of testis length and SVL as measures of relative testis length (see Shine, 1992). Deferent duct diameter was not related to SVL ($R^2=0.03$, $P=0.300$), then, the residuals were not used in this case. Variation in relative length of testis and deferent duct diameter was analyzed

Correspondence: S. M. Almeida-Santos, Laboratório de Herpetologia, Instituto Butantan, Av. Vital Brazil, 1500. 05535-900 São Paulo, SP, Brazil. E-mail: almeidasantos@butantan.gov.br

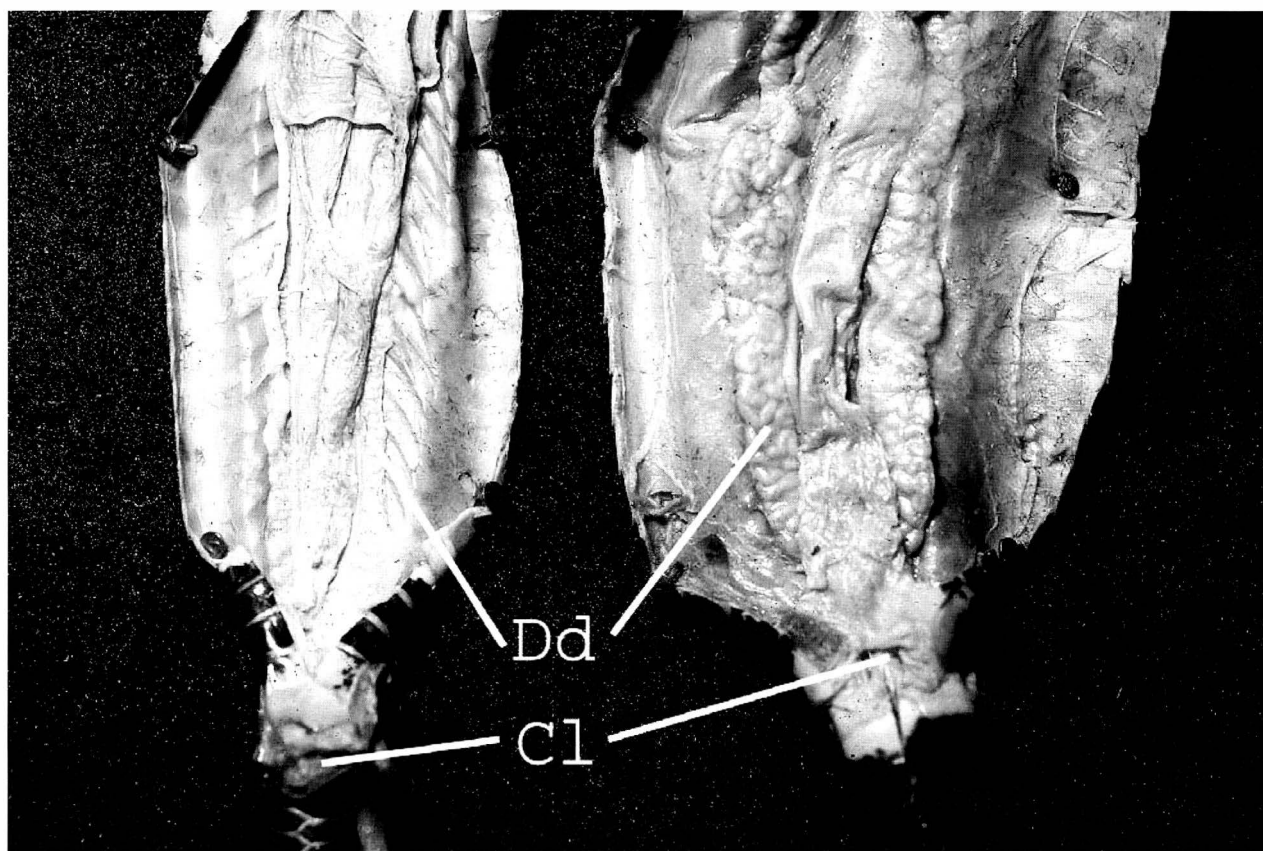


FIG. 1. Distal end of deferent duct in *Micrurus corallinus* without sperm storage (left); IB 43864, São Roque, SP, Brazil, 24.IV.1981) and showing sperm storage (right); IB 4541, São José do Rio Pardo, SP, Brazil, 17.X.1998). Deferent duct (Dd), cloaca (Cl). Scale: 2 cm = 4.2 mm.

by ANOVA and a post hoc Tukey test (Sokal and Rohlf 1995), to infer spermatogenic cycle (see Volsøe, 1944; Shine, 1977; Seigel & Ford, 1987) and sperm storage (Yokoyama & Yoshida, 1993; Sever *et al.*, 2002; Almeida-Santos *et al.*, 2004), respectively.

Fifteen specimens from the sample used for morphological measurements (four from spring, three from autumn, five from winter and three from summer) were selected randomly and the right testis and distal region deferent duct were removed, dehydrated in ethanol, and embedded in paraffin. Histological sections were cut at 5 μ m and stained in hematoxylin/eosin. Sections of the testes and deferent duct were examined to determine the stage of the testicular cycle and the presence or absence of spermatozoa, respectively.

Data on the female reproductive cycle and snake activity were obtained from Marques (1996), where seasonal activity was inferred from collection data (see Marques *et al.*, 2001 for discussion about this method). In the present study these data on seasonal activity are combined in different seasons which represent the major climatic variations in the study area (Nimer, 1989). In south-eastern Brazil the rainy season comprises austral spring (October – December) and summer (January – March), whereas the dry season comprises autumn (April – June) and winter (July – September). The number of males and females per season were compared by Chi-squared test to infer variation in activity (H_0 = there is no variation in number of snakes per season; Zar

1999). Records on mating aggregation in nature were also used in the present study.

RESULTS

The adult males examined averaged 523.5 mm SVL (SD=78.9 mm, range 462–743 mm, $n=187$). Testes reach their maximum relative length during autumn and their minimum in spring ($F=3.206$, $P=0.025$, $df=3$; Fig. 2), whereas distal deferent ducts are at their smallest diameters in summer–autumn and increase in winter–spring ($F=14.61$, $P<0.0001$, $df=3$; Figs. 1,3). In this study, we distinguished two main stages of testicular cycle in *M. corallinus*, regression and spermiogenesis. In the spring, the epithelium was exhausted and highly disorganized, with little spermatogonia and sperm in the lumen, characterizing the regression phase. During the summer, the epithelia were starting to be reorganized, there were more spermatogonia than in the previous season, but no sperm were found in the lumen. During the autumn and winter, we recorded the spermiogenesis phase, when seminiferous tubes were highly organized and the lumina were lined by rows of metamorphosing spermatids and spermatozooids. Mature spermatozoa were the predominant cells in the seminiferous tubules. The deferent duct was straight and no sperm were observed in its lumen during the autumn. During the winter, the deferent duct was slightly convoluted and little sperm was found,

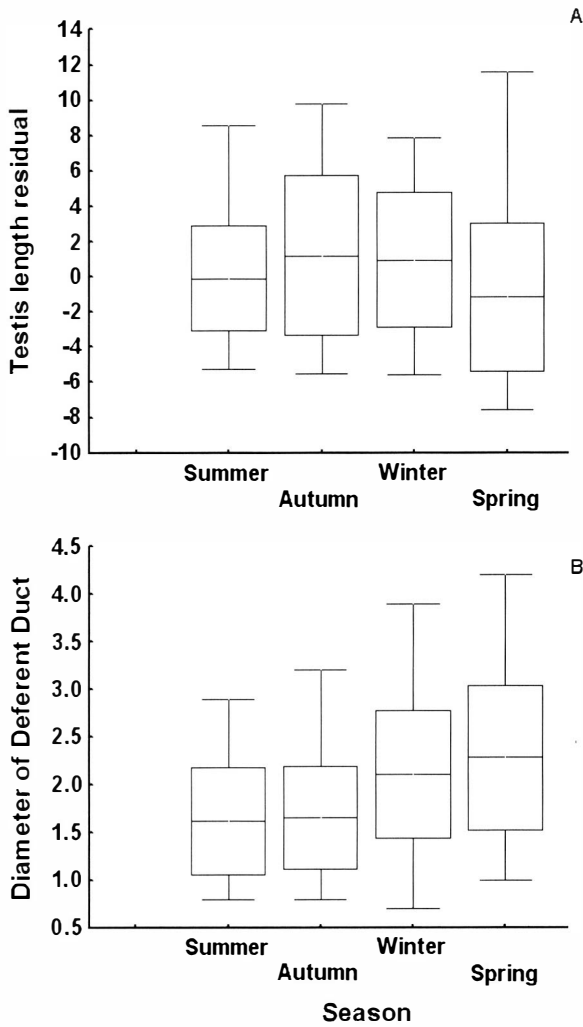


FIG. 2. Seasonal variation in the residual of testis length (A) and deferent duct diameter (B) in *Micrurus corallinus*. Lines indicate ranges, boxes indicate standard deviation, and small squares indicate mean values.

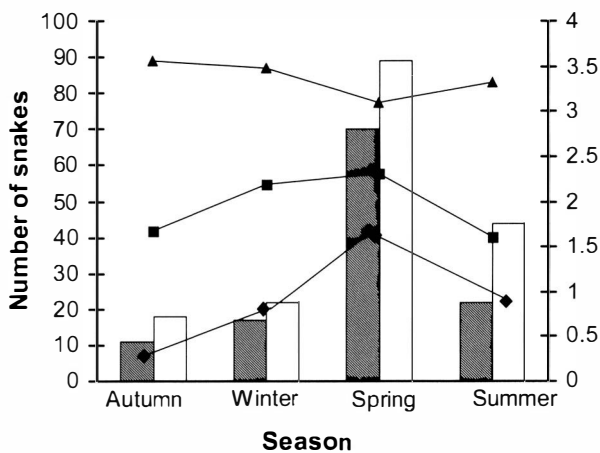


FIG. 3. Seasonal activity of adult males (closed bars) and females (open bars) of *Micrurus corallinus* in south-eastern Brazil (data from Marques 1996) and its relation to reproductive cycles of sperm storage (squares), sperm production (triangles), and vitellogenesis (diamonds). The right axis is diameter of deferent duct in mm (representing sperm storage), relative testis length 10^2 (representing sperm production), and diameter of the largest follicle or oviductal egg in mm (representing vitellogenesis).

whereas in the spring, the deferent duct was convoluted and completely full of sperm, and then, in the summer, it was slightly convoluted again and the amount of sperm started to decrease.

The activity in both males and females peaks in spring (males: $\chi^2=73.1$, $df=3$, $P<0.0001$; females: $\chi^2=73.6$, $df=3$, $P<0.0001$). The number of females collected outnumbered males throughout year, but differed significantly only in summer ($\chi^2=3.7$, $df=1$, $P=0.055$).

A mating aggregation of *M. corallinus* was observed in the field on 28 October 1999. The group consisted of a male with an everted hemipenis and two females (P. B. de Souza, pers. comm.). Another group composed of two males and one female was observed on 17 December 1999 (G. Ferranti, pers. comm.). In both cases females had vitellogenic follicles, and deferent ducts of males had large diameters.

DISCUSSION

In post-nuptial or aestival spermatogenesis (Type I, according to Schuett, 1992) many snakes exhibit maximum testes sizes in autumn – reflecting maximal spermatogenic activity – and mating occurs early in the following spring, utilizing sperm stored over winter in the deferent ducts (Saint-Girons, 1982; Seigel & Ford, 1987). Some authors suggest that sperm storage in the male reproductive tract indicates prolonged mating time (Quinn, 1979; Jackson & Franz, 1981; Johnson *et al.*, 1982; Bull *et al.*, 1997) or an adaptation to the timing of the mating season (Shine, 1977; Garstka *et al.*, 1982; Saint-Girons, 1982; Mitchell & Zug, 1984). The latter seems to be the case in *M. corallinus*: sperm produced largely in autumn is stored in the distal end of deferent ducts (as in the viperid *Protobothrops flavoviridis* – see Yokoyama & Yoshida, 1993) until the mating season (spring), when ovarian follicles in females are in secondary vitellogenesis (see Marques, 1996), and testicular size is minimal. Therefore, diameter of the distal deferent duct is a good indication of the mating season, especially when reproductive cycles are post-nuptial. The reproductive cycle of *M. corallinus* males is post-nuptial (or Type I, see Schuett, 1992), whereas the female cycle is pre-nuptial because vitellogenesis coincides with mating (Garstka *et al.*, 1982; Saint-Girons, 1982; Seigel & Ford, 1987). Although gametogenesis in both males and females is not simultaneous, the co-ordination of their reproductive cycles is guaranteed by sperm storage in males.

Our results are similar to those recorded for *M. tener* from Texas, in which vitellogenesis and oviposition occur during spring (Quinn, 1979) and testicular recrudescence peaked in autumn. Quinn (1979) suggests that sperm is stored by females in the oviducts, although the mating time is still uncertain. In Florida, *M. fulvius* has secondary vitellogenesis in late winter–spring and egg-laying in late spring–summer, whereas testes size is maximum during autumn and decrease in spring (Jackson & Franz, 1981). Thus, the same pattern of intra-sex synchrony and inter-sex co-ordination is

seen in other species and populations of *Micrurus* (pers. obs.). Species of *Micrurus* seem to have both vitellogenesis and spermatogenesis adjusted to the same season of the year in different areas of the same latitude (see Werler, 1951; Campbell, 1973; Quinn, 1979; Jackson & Franz, 1981; this study). However, in equatorial areas the reproductive pattern can differ. *Micrurus nigrocinctus* in Costa Rica presents a more extensive female cycle, from five to seven months of vitellogenesis and oviposition (see Solórzano & Cerdas, 1988; Goldberg, 2004). Moreover, Goldberg (2004) also recorded sperm production throughout the year (despite the lack of data in some months), for this species. Detailed investigation on spermatogenesis and vitellogenesis in other species of *Micrurus* are essential to characterize the reproductive patterns and understand the climatic influence on the reproduction of *Micrurus*.

Field observations of *M. corallinus* mating were recorded in October and November (Marques, 1996), and during the mating season, ritual combat was not observed. Ritual combat among males occurs in several snake species during the mating season (Gillingham, 1987; Greene, 1997) and was recently described for some tropical species (Almeida-Santos *et al.*, 1999; Almeida-Santos & Marques, 2002), including *Micrurus* (Almeida-Santos *et al.*, 1998). This behaviour seems to be common in species in which males are larger than females (Shine, 1978, 1994). However, in some species, both from tropical or temperate areas, more than one male may court a female without combat (see Slip & Shine, 1988; Greene, 1997; Feio *et al.*, 1999; Rivas, 1999), although some agonistic interaction generally occurs (Capula & Luiselli, 1997). Our findings indicate that *M. corallinus* aggregate for mating and it is possible that there is no combat among males, as females are larger than males (Marques, 1996). In the genus *Micrurus*, combat ritual is recorded only for *M. frontalis* (Almeida-Santos *et al.*, 1998), in which sexual dimorphism is apparently absent (Roze, 1996; O. A. V. Marques, pers. obs.). Mating aggregations in tropical snakes probably are more common than generally thought and observations on aggregations such as that reported for *Imantodes cenchoa* (Doan & Arriaga, 1999) may actually be mating aggregations.

Seasonal activity of *M. corallinus* seems to be strongly influenced by the reproductive cycle, although other factors may also have an impact on activity patterns (see Marques, 1996; Marques *et al.*, 2001). The increase of activity in females during spring is probably due to vitellogenesis and mating when thermoregulation time is longer (see Marques, 1996; Shine, 1979). The high number of females in summer occurs just after oviposition when they may forage for food to replace energy lost in egg reproduction. In males, spermatogenesis is uncoupled from mating and testicular recrudescence occurs in summer, after sexual activity ceases. Thus, male activity decreases in summer and autumn (Fig. 3) when energy is needed for sperm production, which may be costly (Olsson *et al.*, 1997).

Sperm are released from the testes during autumn and stored in deferent ducts over winter until spring when they mate. Thus, male activity increases in spring probably due to their searching for females (Duvall *et al.*, 1992), which could originate aggregation and perhaps competition during mating. Aggregation and female reproductive synchrony could favour polygyny rather than monogamy in *M. corallinus*, probably the most common snake mating system (Duvall *et al.*, 1992).

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PHYLOGENETIC RELATIONSHIPS AMONG POISON FROGS OF THE GENUS *DENDROBATES* (DENDROBATIDAE): A MOLECULAR PERSPECTIVE FROM INCREASED TAXON SAMPLING

J. L. ROBERTS¹, J. L. BROWN¹, R. VON MAY^{2,3}, W. ARIZABAL⁴, A. PRESAR¹, R. SYMULA⁵,
R. SCHULTE⁶ AND K. SUMMERS¹

¹Dept. of Biology, East Carolina University, Greenville, North Carolina, USA

²Florida International University, Miami, Florida, USA

³Asociación para la Conservación de la Cuenca Amazónica, Puerto Maldonado, Peru

⁴Museum of Natural History, University of San Antonio de Abad, Cuzco, Peru

⁵Dept. of Integrative Biology, University of Texas, Austin, Texas, USA

⁶Instituto de Investigaciones de las Cordilleras Orientales, Tarapoto, Peru

Despite many taxonomic revisions, systematic relationships among members of the genus *Dendrobates* remain poorly understood, particularly the connections between taxa in Amazonia and those in northern South America and Central America. We combine new mitochondrial sequence data with data from previous analyses in order to investigate the relationships among *Dendrobates* from each major biogeographic region. We address the phylogenetic position of taxon not included in previous molecular systematic analyses, including *Dendrobates flavovittatus*, *D. duellmani*, *D. galactonotus*, *D. mysteriosus*, and a new *Dendrobates* species from Brazil. We attempt to resolve relationships among former members of the genus "*Minyobates*," and we consider the biogeographic and behavioural implications of the overall tree topology.

Key words: Amazonia, *Minyobates*, Neotropics, systematics

INTRODUCTION

Neotropical poison frogs of the genus *Dendrobates* are well known for their bright coloration and potent skin toxins (e.g. Myers & Daly, 1983). Despite many taxonomic revisions (e.g. Silverstone, 1975; Myers, 1982; Caldwell & Myers, 1990), systematic relationships among the members of this genus remain poorly understood. Recent studies employing molecular characters (Summers *et al.*, 1999; Vences *et al.*, 2000, 2003; Symula *et al.*, 2001, 2003; Santos *et al.* 2003) have resolved relationships among species living in Central America and northern South America, as well as among the majority of species from western and central Amazonia. However, the connections between the taxa in Amazonia and those in northern South America and Central America remain poorly resolved. In this paper we combine mitochondrial DNA (mtDNA) sequences from previous analyses with sequences from species within the genus *Dendrobates* that previously have not been sampled in order to provide a more complete analysis of systematic relationships within the genus. Thorough taxon sampling enhances the probability of accurately reconstructing phylogenetic relationships among the members of a clade (Zwickl & Hillis, 2002). In this analysis we have included the majority of taxa from each of the three major biogeographic regions in

which members of the genus *Dendrobates* occur: Central America, northern South America, and Amazonia.

The major goals of this study are: (1) to carry out a comprehensive molecular systematic study of the genus *Dendrobates*; (2) to investigate the relationships among members of the genus *Dendrobates* in Amazonia, northern South America, and Central America; (3) to investigate the biogeographic implications of the evolutionary relationships within *Dendrobates*; and (4) to resolve relationships among former members of the genus *Minyobates*, some of which are now considered members of the genus *Dendrobates* (Vences *et al.* 2003).

Myers (1987), suspecting that *Dendrobates* was not monophyletic, defined the genus *Minyobates* to include eight species of miniature dendrobatids, most of which formerly belonged to Silverstone's (1975) *D. minutus* species group (*M. abditus*, *M. altobueyensis*, *M. bombetes*, *M. fulguritus*, *M. minutus*, *M. opisthomelas*, *M. steyermarki*, and *M. viridis*). Clough & Summers (2000) and Vences *et al.* (2000) showed that at least some members of the genus *Minyobates* (*M. minutus* and *M. fulguritus*, respectively) fall within the clade formed by the members of the genus *Dendrobates* and suggested that *Minyobates* may be synonymous with *Dendrobates*. Vences *et al.* (2003) and Santos *et al.* (2003) corroborated the placement of *D. minutus* and *D. fulguritus* within *Dendrobates*, but Vences *et al.* (2003) noted the isolated position of *M. steyermarki*, the type species of *Minyobates*, at the base of the

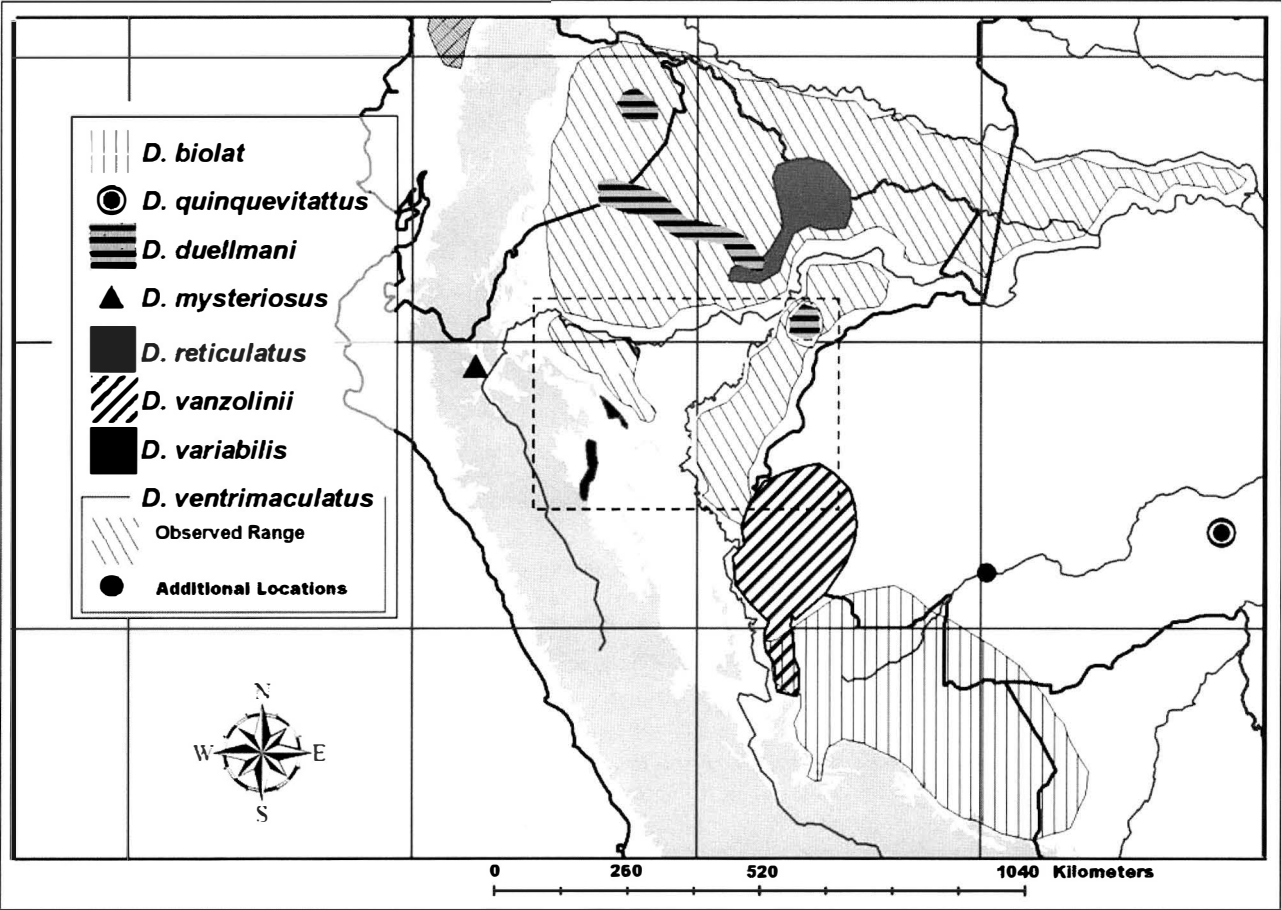


FIG. 1. Distribution of western Amazonian *Dendrobates*. Areas above 1000 m elevation shaded. The dashed box depicts the area covered in Fig. 2.

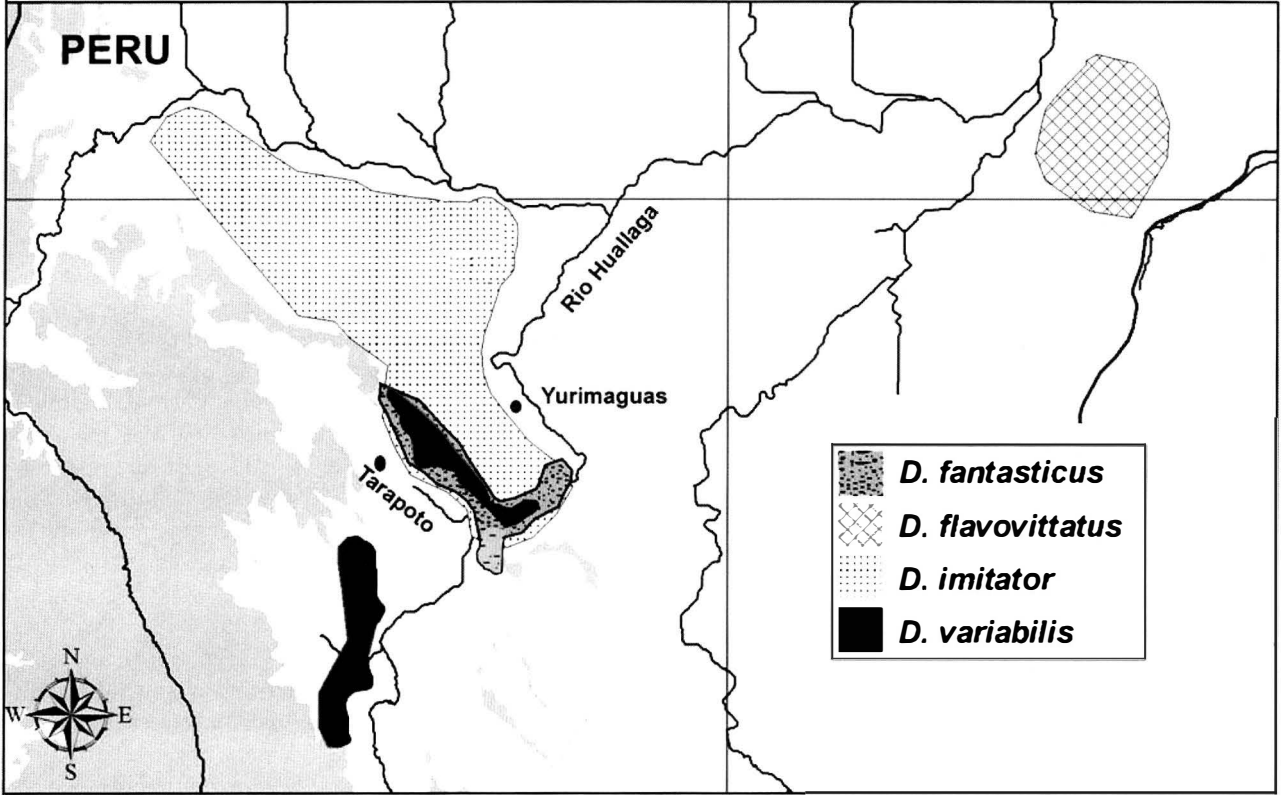


FIG. 2. Distribution of north central Peruvian *Dendrobates* (detail from Fig. 1 to illustrate ranges of *D. imitator*, *D. fantasticus*, and *D. flavovittatus*). Areas above 1000 m elevation shaded.

Dendrobates clade and suggested that *Minyobates* may be a monotypic genus. In an analysis of toxin sequestration in dendrobatids, Daly *et al.* (2003) suggested that further molecular analysis is needed to resolve the taxonomic validity of *Minyobates*. To address this question we included in our analysis three members of the *Dendrobates minutus* group, from which the genus *Minyobates* was described (Myers, 1987): *Dendrobates claudiae* Jungfer *et al.* 2000, from the northern limit of the range (Bocas del Toro Archipelago, Panama), *Dendrobates minutus* from southeastern Panama and northern Colombia, at the center of the range, and *Minyobates steyermarki* from Cerro Yapacana in southern Venezuela.

MATERIALS AND METHODS

SAMPLE COLLECTION

The majority of sequences used in this study are derived from previous studies (e.g. Summers *et al.*, 1999; Clough & Summers, 2000; Symula *et al.*, 2003), although some were sequenced for this study. Collection localities and sequence origins for all samples are listed in Table 1. Tissues samples sequenced for this study were taken as toe clips from each frog. Collecting and export permits from Peru were obtained from the Ministry of Natural Resources (INRENA) in Lima, Peru (Authorization No. 061-2003-INRENA-IFFS-DCB, Permit No. 002765-AG-INRENA and CITES Permit No. 4326). Voucher specimens for each species collected in Peru were deposited at the Museo de Historia Natural, Universidad Mayor de San Marcos, Lima, Peru.

Samples from Brazil were collected by J. P. Caldwell and were obtained via a tissue grant to the corresponding author from the Louisiana State University Museum of Natural Sciences Collection of Genetic Resources. Tissues obtained by J. P. Caldwell were collected dur-

ing expeditions funded by the National Science Foundation (DEB-9200779 and DEB-9505518 to L. J. Vitt and J. P. Caldwell). Samples of *Dendrobates* sp. from Mato Grosso were obtained from J. Frenkel. The general distributions of each species analyzed in this study are shown in Figs. 1-3.

DNA EXTRACTION, DNA AMPLIFICATION, SEQUENCING

Genomic DNA was extracted from tissue samples preserved in high concentration salt buffer (DMSO/NaCl/EDTA) using the Qiagen DNeasy Tissue Kit. Samples collected by J. P. Caldwell were originally stored in 70% ethanol and then transferred to high concentration salt buffer for storage prior to extraction. The 16S ribosomal RNA (rRNA), 12S rRNA, cytochrome *b*, and cytochrome oxidase I mitochondrial gene regions were amplified using DNA primers and protocols described in Summers *et al.* (1999), Clough & Summers (2000), and Symula *et al.* (2001) for a total of 1591 base pairs in the final dataset. We used the following primer sets: 16S: LGL 381, LGL 286 (Palumbi *et al.*, 1991); 12S: 12SA-L, 12Sb-H (Kocher *et al.*, 1989), Df12SA, Df12SB (Symula *et al.*, 2001); cytochrome *b*: CB1-L, CB2-H (Palumbi *et al.*, 1991), KSCYB1(A)-L, KSCYB(C)L, KSCYB1-H (Clough & Summers, 2000); cytochrome oxidase I: CO1A, CO1F (Palumbi *et al.*, 1991), DfCO1A, DfCO1B, DiCO1A, DiCO1B (Symula *et al.*, 2001). We were unable to sequence cytochrome oxidase I for *Dendrobates duellmani* Schulte, 1999 from Ecuador, *D. galactonotus* Steindachner, 1864, *D. quinquevittatus* Steindachner, 1864, *D. sylvaticus* Funkhouser, 1956, *D. vanzolinii* Myers, 1982, *D. ventrimaculatus* Shreve, 1935 from Ecuador, *D. ventrimaculatus* from French Guiana, or *D. sp.*, the undescribed species from Mato Grosso, Brazil.

PCR amplifications were purified with the Qiagen QIAquick PCR Purification Kit. Products were sequenced using Applied Biosystems' (ABI) PRISM

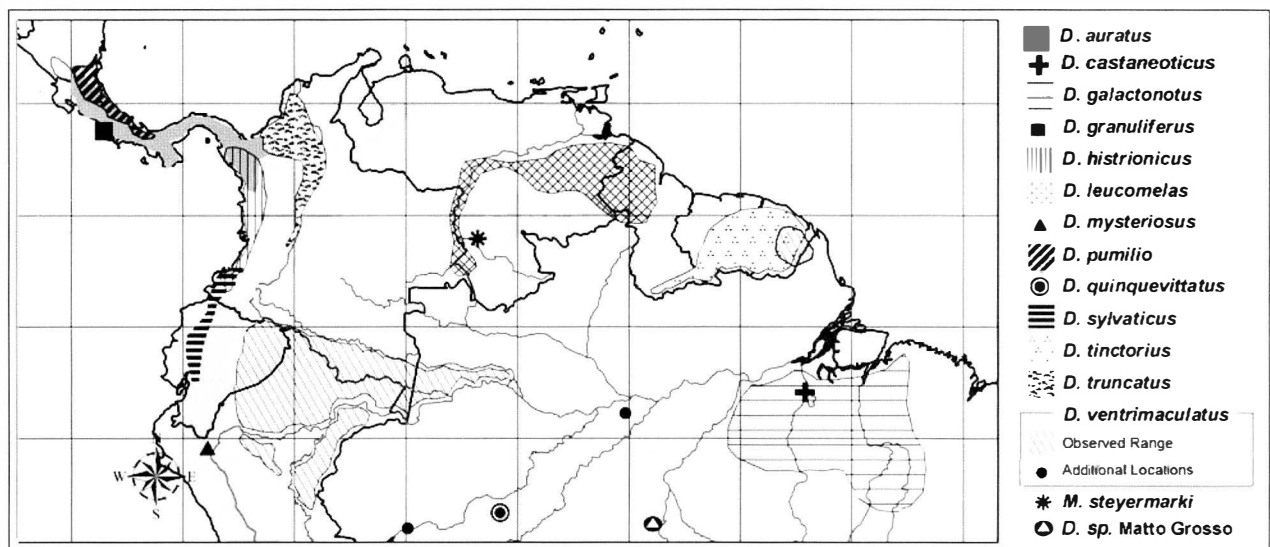


FIG. 3. Distribution of Central American and eastern Amazonian *Dendrobates*. Areas above 1000 m elevation shaded.

TABLE 1. Species names, collection localities, and GenBank accession numbers for taxa included in the analyses.

Species	Location	12S	16S	COI	CytB
<i>Colostethus marchesianus</i>	Peru	AF128584	AF128583	AF128585	NA
<i>Colostethus talamancae</i>	Costa Rica	AF128587	AF128586	AF097496	AF128588
<i>Epipedobates trivittatus</i>	Peru	AF128570	AF128569	AF128571	NA
<i>Dendrobates arboreus</i>	Panama	AF128611	AF128610	AF097504	AF128612
<i>D. amazonicus</i>	Iquitos, Loreto, Peru	AF482770	AF482785	AF482815	AF482800
<i>D. auratus</i>	Panama	AF128602	AF098745	AF097501	AF128603
<i>D. biolat</i>	S. Peru	AF482779	AF482794	AF482823	AF482809
<i>D. castaneoticus 1</i>	E. Brazil	AF482774	AF482789	AF482818	AF482804
<i>D. castaneoticus 2</i>	E. Brazil	AF482775	AF482790	AF482819	AF482805
<i>D. claudiae</i>	Colombia?	DQ371304	DQ371315	DQ371324	DQ371334
<i>D. duellmani E</i>	Napo, Ecuador	AY364566	AY263246	NA	NA
<i>D. duellmani P</i>	Tahuayo, Loreto, Peru	DQ371305	DQ371316	DQ371325	DQ371335
<i>D. fantasticus 1</i>	N. Sauce, San Martin, Peru	AF412444	AF412472	AF412416	AF412500
<i>D. fantasticus 2</i>	Cainarachi, San Martin, Peru	AF412447	AF412475	AF412419	AF412503
<i>D. flavovittatus</i>	Tahuayo, Loreto, Peru	DQ371306	DQ371317	DQ371326	DQ371336
<i>D. galactonotus</i>	E. Brazil	DQ371300	DQ371311	NA	DQ371330
<i>D. granuliferus</i>	Costa Rica	AF128608	AF098749	AF097505	AF128609
<i>D. histrionicus 1</i>	Ecuador	AF128617	AF128616	AF097498	U70154
<i>D. histrionicus 2</i>	Ecuador	AF124098	AF124117	NA	AF173766
<i>D. imitator 1</i>	Huallaga, San Martin, Peru	AF412448	AF412476	AF412420	AF412504
<i>D. imitator 2</i>	Pongo, San Martin, Peru	AF412459	AF412487	AF412431	AF412515
<i>D. lamasi</i>	Tingo Maria, Huanuco, Peru	AF482778	AF482793	AF482822	AF482808
<i>D. leucomelas</i>	Venezuela	AF128593	AF124119	AF097499	AF128594
<i>D. minutus</i>	Panama	AF128590	AF128589	AF128591	MMU70163
<i>D. mysteriosus</i>	N. Peru	DQ371303	DQ371314	DQ371323	DQ371333
<i>D. pumilio</i>	Bocas del Toro, Panama	AF128614	AF128613	AF097500	U70147
<i>D. quinquevittatus</i>	E. Brazil	AF482773	AY263253	NA	AF482803
<i>D. reticulatus 1</i>	Punta Itaya, Loreto, Peru	AF482772	AF482787	AF482817	AF482802
<i>D. reticulatus 2</i>	B. Achille, Loreto, Peru	AF482771	AF482786	AF482816	AF482801
<i>D. sp.</i>	Mato Grosso, Brazil	DQ371309	DQ371320	NA	DQ371339
<i>D. speciosus</i>	Panama	AF128596	AF098747	AF097503	AF128597
<i>D. sylvaticus</i>	Ecuador	AY364569	AY364569	NA	AF324041
<i>D. tinctorius</i>	French Guiana	AF128605	AF128604	NA	AF128606
<i>D. vanzolinii</i>	Peru	AF128599	AF128598	NA	AF128600
<i>D. variabilis</i>	Cainarachi, San Martin, Peru	AF412463	AF412491	AF412435	AF412519
<i>D. ventrimaculatus B1</i>	Solimoes, Amazonas, Brazil	DQ371307	DQ371318	DQ371327	DQ371337
<i>D. ventrimaculatus B2</i>	Porto Walter, Acre, Brazil	DQ371301	DQ371312	DQ371322	DQ371331
<i>D. ventrimaculatus B3</i>	Solimoes, Amazonas, Brazil	DQ371308	DQ371319	DQ371328	DQ371338
<i>D. ventrimaculatus E1</i>	Ecuador	AF482780	AF482795	AF482824	AF482810
<i>D. ventrimaculatus E2</i>	Ecuador	AF128620	AF128619	AF097502	AF120013
<i>D. ventrimaculatus FG</i>	French Guiana	DQ371302	DQ371313	NA	DQ371332
<i>D. ventrimaculatus P1</i>	N. Bonilla, San Martin, Peru	AF412466	AF412494	AF412438	AF412522
<i>D. ventrimaculatus P2</i>	Near Rio Napo, Loreto, Peru	AF482781	AF482796	AF482825	AF482811
<i>Minyobates steyermarki</i>	Venezuela	DQ371310	DQ371321	DQ371329	DQ371340
<i>Phyllobates bicolor</i>	Choco, Colombia	AF128578	AF128577		AF128579

(Perkin-Elmer Corporation, Foster City, CA, USA) Sequencing Kit. Samples were then prepared for sequencing as in Clough & Summers (2000).

SEQUENCE ANALYSIS

Each sample was sequenced in both directions and complimentary sequences were aligned using Autoassembler version 1.4.0 (ABI, 1995). Consensus

sequences were transferred to Gene Jockey (Taylor, 1990) for alignment with a sequence of the same region from a different individual. We translated the protein coding sequences to confirm that they were in the proper reading frame and did not contain stop codons. We aligned the DNA sequences using Clustal X (Thompson *et al.*, 1997). For the cytochrome oxidase I and cytochrome *b* gene regions, alignments were unam-

biguous and contained no gaps. For the 16S rRNA and 12S rRNA gene regions, regions of ambiguous alignment were removed from the analysis. The resulting dataset included 1591 unambiguous base pairs.

PHYLOGENETIC ANALYSIS

Phylogenetic analyses were carried out using Bayesian inference in MrBayes (Version 3.0b4, Huelsenbeck & Ronquist, 2001) and Maximum Likelihood (ML) in PAUP* version 4.0b10 (Swofford, 2002). We included three species from taxa closely related to *Dendrobates* as outgroups in the analysis: *Epipedobates trivittatus* (Spix, 1824), *Colostethus talamancae* (Cope, 1875), and *Colostethus marchesianus* (Melin, 1941) (Table 1).

We partitioned the dataset into seven partitions as follows: non-coding gene regions (12S + 16S ribosomal RNA), cytochrome oxidase I (COI) 1st position codons, COI 2nd position codons, COI 3rd position codons, cytochrome *b* (cyt *b*) 1st position codons, cyt *b* 2nd position codons, and cyt *b* 3rd position codons, and used MrModeltest version 2.0 (Nylander, 2004) to determine which model of DNA substitution best fit each partition. Data may better be explained by partitioning a dataset than by applying an average model across genes and codon positions, as indicated by higher model likelihood scores in partitioned analyses (Mueller *et al.*, 2004).

We applied the models indicated by MrModeltest and used MrBayes version 3.0b4 (Huelsenbeck & Ronquist, 2001) to infer a tree topology including only those taxa for which a full set of sequence data (12S rRNA, 16S rRNA, cytochrome *b* and cytochrome oxidase I) was available. We ran four simultaneous Markov Chain Monte Carlo (MCMC) chains for one million generations, saving trees every 100 generations. We examined a plot of $-\ln$ likelihood scores and discarded all trees before $-\ln$ stabilization (burn-in phase). We created a 50% majority rule consensus tree from the remaining trees in PAUP*, then repeated the Bayesian analysis to ensure consistency of topology and posterior clade probabilities for the consensus tree.

The consensus tree derived from the Bayesian analysis was loaded as a backbone constraint topology in PAUP*. We used Modeltest version 3.0.6 (Posada & Crandall, 1998) to determine the appropriate model of DNA substitution for the unpartitioned dataset, implemented the specified model parameters, and conducted a Maximum Likelihood search in PAUP* that included the taxa with incomplete datasets (i.e. those lacking COI sequence data).

Wiens (1998) suggested that adding characters, despite incomplete taxon sampling, usually increases phylogenetic accuracy, but may be misleading. We compared the tree topology recovered using a backbone constraint of taxa with complete datasets (described above) to a topology recovered by a second Bayesian run of 5 million generations, including taxa with and

without complete character sets, using MrBayes version 3.1.2. The tree topologies obtained by the two different methods were consistent; however the inclusion of taxa with incomplete datasets lowered the posterior probabilities at many branches between taxa with complete datasets. This decrease may be a result of the equivocal placement of taxa with incomplete datasets within the phylogeny. Finally, we used Shimodaira-Hasegawa (1999) tests to assess the validity of certain relationships among taxa by comparing our tree topology to alternative topologies.

RESULTS AND DISCUSSION

The complete dataset included a total of 1591 base pairs, 305 from 12S rRNA, 540 from 16S rRNA, 196 from cytochrome *b*, and 550 from cytochrome oxidase I. Of the 1591 base pairs, 625 were variable, 471 of which were parsimony informative. Fig. 4 shows the tree that resulted from the ML search that added those taxa with incomplete sequence data to the backbone constraint tree derived from those taxa with complete sequence data.

Symula *et al.* (2003) found a division between eastern Amazonian (mainly Brazilian) *Dendrobates* (e.g. *D. castaneoticus* Caldwell & Myers, 1990 and *D. quinquevittatus*) and western Amazonian (mainly Peruvian) *Dendrobates*. Within the western clade there was a well-supported division between southern (i.e. *D. lamasi* Morales, 1992, *D. biolat* Morales, 1992, *D. vanzolinii*, and *D. imitator* Schulte, 1986) and northern (i.e. *D. ventrimaculatus*, *D. variabilis* Zimmermann & Zimmermann, 1988, *D. amazonicus* Schulte, 1999, *D. reticulatus* Boulenger, 1884, and *D. fantasticus* Boulenger, 1884) taxa, roughly corresponding to the Inambari and Napo refuge regions, respectively (Symula *et al.*, 2003). This division within the western Amazonian clade was also recovered by Santos *et al.* (2003). We recovered a tree topology in overall accordance with the findings of Symula *et al.* (2003) and Santos *et al.* (2003), but our analysis included several new taxa. We consider the placement of these taxa in terms of general biogeography and trends in parental care where notable.

Dendrobates flavovittatus Schulte, 1999 falls within the "southwestern" clade (roughly corresponding to the Inambari refuge region) described by Symula *et al.* (2003), including *D. biolat*, *D. lamasi*, *D. vanzolinii*, and *D. imitator*, and further supports the hypothesis (Symula *et al.*, 2001, 2003) of a northward radiation by southern ancestors in this clade (Fig. 2). All members of the *D. vanzolinii* group (*D. biolat*, *D. flavovittatus*, *D. imitator*, *D. lamasi*, and *D. vanzolinii*) are believed to demonstrate biparental care, though this has not been confirmed in *D. flavovittatus*.

Although their placement within the "northwestern" clade (roughly corresponding to the Napo refuge region) described by Symula *et al.* (2003) supports the findings of Santos *et al.* (2003), two *Dendrobates*

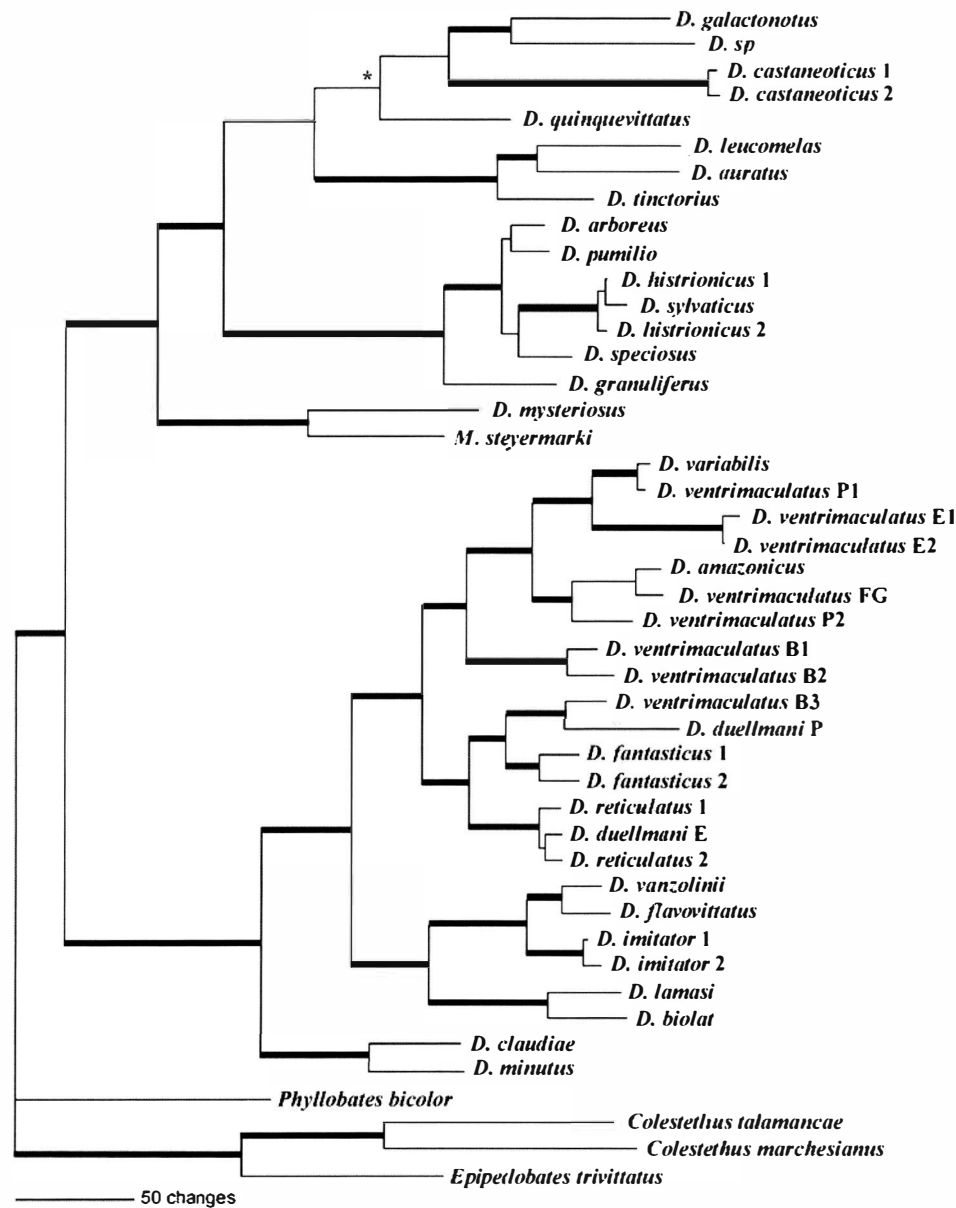


FIG. 4. Maximum Likelihood phylogram derived from a Bayesian backbone constraint consensus tree constructed using only taxa for which 12S, 16S, cytochrome *b* and cytochrome oxidase I sequence data were available (1591 bp). Thick lines indicate Bayesian posterior probabilities greater than 75.

duellmani Schulte, 1999 individuals from populations on either side of the Amazon River in northeastern Peru and eastern Ecuador did not fall out together. The individual from the Napo River in eastern Ecuador fell out with two *D. reticulatus* individuals from the same geographic region while a *D. ventrimaculatus* individual from eastern Brazil was sister to the *D. duellmani* individual from the Tahuayo River. Jukes-Cantor genetic distances between the Napo River *D. duellmani* and the two *D. reticulatus* individuals ranged from 2.09% to 2.71% (compared to 1.32% between the two *D. reticulatus* individuals). The genetic distance between the Tahuayo River *D. duellmani* and its sister, *D. ventrimaculatus* from Amazonas, Brazil, was 5.54%, still closer than the distance of 6.18% between the two *D. duellmani* individuals. Hence, *D. duellmani* may need revision with respect to the specific populations

that should be considered members of this species. Given geographic location and morphology, the *D. duellmani* samples from Yasuni, Ecuador are most likely the nominal form.

With respect to the *D. ventrimaculatus* species group, our results support the findings of Symula *et al.* (2003); *D. ventrimaculatus* itself did not form a monophyletic group. These findings further support the suggestion by Caldwell & Myers (1990) that *D. ventrimaculatus* comprises a complex of species that are distinguishable from formerly synonymous *D. quinquevittatus*, but which share several morphological characters. An individual *D. ventrimaculatus* from western Peru along the Andean slope was sister to *D. variabilis* from the same geographic area; this pair grouped with two other western Amazonian *D. ventrimaculatus* from Ecuador. A second Peruvian in-

dividual, from the Rio Napo in eastern Peru, grouped with *D. amazonicus* (also from eastern Peru) and its sister, a *D. ventrimaculatus* from French Guiana. Two Brazilian *D. ventrimaculatus*, one from Porto Walter in the west and one from Amazonas in the east, formed the base of this *D. ventrimaculatus*/*D. variabilis*/*D. amazonicus* clade. The third Brazilian *D. ventrimaculatus*, also from Amazonas, was most closely related to *D. duellmani* from Peru, as discussed above; both of those individuals are part of a larger clade that also includes *D. fantasticus* and *D. reticulatus*. These relationships, which generally were supported by high Bayesian posterior clade probabilities (see Fig. 4), suggest that *D. ventrimaculatus* may need taxonomic revision in order to maintain reciprocally monophyletic species names in *Dendrobates*. Caldwell & Myers (1990) suggest that a species from eastern Ecuador may represent *D. ventrimaculatus* sensu stricto, while other populations may belong to undiagnosed members of a *D. ventrimaculatus* species complex.

Dendrobates sp. from Mato Grosso, Brazil, appears to be the sister taxon to *Dendrobates galactonotus*, with *Dendrobates castaneoticus* sister to the pair. This phylogenetic relationship is supported by morphology. *Dendrobates* sp. from Mato Grosso is similar in appearance to *D. galactonotus*, with a yellow-orange dorsum and legs mottled by irregular, barbell- to kidney-shaped blotchy spots, and a black venter. This group of Brazilian species forms a larger clade that includes the eastern Amazonian species *D. leucomelas* Steindachner 1864 and *D. tinctorius* Wagler, 1830, as well as the southern Central American *D. auratus* Dunn, 1931. This topology agrees with the findings of Vences *et al.* (2003), contrary to Silverstone's (1975) suggestion that *D. galactonotus* may be more closely related to the morphologically similar *D. tinctorius* than to the sympatric *D. castaneoticus* or *D. quinquevittatus*. All of the species that have been studied in this group have male parental care (Weygoldt, 1987; Summers & McKeon, 2004). Sister to the male care clade is the southern Central American/northern South American *D. histrionicus* Berthold, 1845 clade, all of which express female or asymmetric biparental care (Weygoldt, 1987; Summers & McKeon 2004). The topology of the female care clade suggests that this trait evolved in Central America and then spread to northern South America (with *D. arboreus* Myers, Daly & Martínez 1984 and *D. pumilio* Schmidt, 1857 from Central America as sister taxa to *D. sylvaticus* from Ecuador).

Our phylogenetic analysis indicates that the clade from central and eastern Amazonia (*D. castaneoticus*, *D. galactonotus*, *D. sp.* and *D. quinquevittatus*) is the sister taxon to the male care clade from northern South America and Central America (including *D. auratus*, *D. leucomelas*, and *D. tinctorius* in this analysis, as well as *D. truncatus* Cope, 1861) (Fig. 3). This arrangement is plausible biogeographically; the range of *D. tinctorius*,

which extends to the Guyana Shield, approaches the range of *D. galactonotus* in northeastern Brazil (Fig. 2). Hence, it seems likely that divergence of a perhaps widespread ancestral population gave rise to the *D. galactonotus* clade, in central and eastern Amazonia, and the *D. auratus* clade, which spread northward and westward from Amazonia. The sister taxon of these two clades is the female care clade from Central America and northern South America, which includes *D. arboreus*, *D. speciosus*, *D. pumilio*, *D. sylvaticus*, and *D. histrionicus* in this analysis, as well as *D. granuliferus* Taylor, 1958, *D. lehmanni* Myers & Daly 1976, *D. vicentei* Jungfer, Weygoldt & Juraske, 1996 and *D. occultator* Myers & Daly, 1976. The simplest biogeographic scenario would involve the divergence of the ancestor of the female care clade from an ancestral species within the northern male care clade (*D. auratus*, *D. leucomelas*, and *D. tinctorius*). However, it appears instead that the ancestral species that eventually gave rise to the female care clade diverged from Amazonian stock before the divergence of the *D. auratus* clade and the *D. galactonotus* clade (Fig. 1). We used a Shimodaira-Hasegawa (1999) test to determine that a topology that placed the female care clade as sister to *D. auratus* was significantly less likely than the topology we recovered ($P < 0.01$). As an alternative, we also tested (Shimodaira & Hasegawa, 1999) the *D. galactonotus* clade as sister to the female care clade. While the test was not significant, the *D. galactonotus* clade and the female care clade occurred as sister taxa in only 24 of 9502 (0.25%) post burn-in Bayesian trees (a Bayesian analysis including all taxa was conducted in order to examine this percentage).

Dendrobates mysteriosus Myers, 1982 consistently fell out as sister to *Minyobates steyermarki*, which may be the result of long branch attraction. Both species occupy limited, isolated ranges (*D. mysteriosus* in northern Peru and *M. steyermarki* in southern Venezuela) (Fig. 3) and may represent relicts of ancient lineages (Schulte, 1990). Vences *et al.* (2003) noted the position of *M. steyermarki*, sister to *Dendrobates*, and suggested the validity of *Minyobates* as a potentially monotypic genus; however, this suggestion was based on the results of analysis of a single gene (16S). In our analyses, based on analysis of multiple gene regions, *D. mysteriosus* and *M. steyermarki* nearly always fell within *Dendrobates*, leading us once again to question the validity of the genus *Minyobates*. Shimodaira-Hasegawa tests forcing *M. steyermarki* and *D. mysteriosus* outside of the rest of the *Dendrobates*, both separately and together, were not significant, though the test of *D. mysteriosus* alone outside *Dendrobates* yielded a nearly significant p-value of 0.06. Of 9,502 post burn-in Bayesian trees, 30 placed *D. mysteriosus* alone outside *Dendrobates*, none placed *M. steyermarki* alone outside *Dendrobates*, and 92 placed *D. mysteriosus* and *M. steyermarki* together outside *Dendrobates*. We have no reason to suspect that *D.*

mysteriosus and *M. steyermarki* are evolutionarily closely related (i.e. as sister taxa), so we do not advocate retaining *Minyobates* and including *D. mysteriosus* in that genus, however we were not able to accurately resolve the relationships among *M. steyermarki*, *D. mysteriosus*, and the rest of the *Dendrobates* with the data available to us.

The position of *Dendrobates quinquevittatus* was also poorly resolved by our ML search using the Bayesian backbone constraint tree. Symula *et al.* (2003) and Vences *et al.* (2003) found *D. quinquevittatus* to be closely related to *D. castaneoticus* and *D. galactonotus*. This relationship was recovered in some of our analyses, but at times we also found *D. quinquevittatus* as sister to *D. mysteriosus* and *M. steyermarki*. More sequence data (we were lacking COI data for *D. quinquevittatus*) may help resolve the position of *D. quinquevittatus* within *Dendrobates*.

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TROPHIC, REPRODUCTIVE AND PARASITOLOGICAL ASPECTS OF THE ECOLOGY OF *LEPTODACTYLUS CHAQUENSIS* (ANURA: LEPTODACTYLIDAE) IN ARGENTINA

EDUARDO F. SCHAEFER, MONIKA I. HAMANN, ARTURO I. KEHR, CYNTHYA E. GONZÁLEZ AND MARTA I. DURÉ

CECOAL-CONICET, C.C. 140, (3400) Corrientes, Argentina

We studied the trophic and reproductive ecology and document the helminth fauna of the Ceí's white-lipped frog, *Leptodactylus chaquensis*, from north-eastern Argentina. This frog is a generalist predator, using an intermediate strategy between active foraging and sit and wait predation. The diet consisted of 17 types of prey and was dominated numerically and volumetrically by coleopterans. The number of mature ova per female (ovarian complement) ranged from 31 13 to 16234, and the ovum diameter varied from 0.4 to 1.2 mm. The testes mass ranged from 0.32 to 1.54 g, and the species has an explosive reproductive pattern. The parasite fauna was rich, consisting of 20 species of helminths (twelve trematodes, one cestodes, six nematodes and one acanthocephalan), the kidneys, lungs and large intestine being the organs most infected. The trophic niche breadth and the habitats where this species is living structured the parasite community.

Key words: diet, frog, helminths, parasites, reproduction

INTRODUCTION

The Argentinean members of the *Leptodactylus ocellatus* group are represented by *L. ocellatus* (Linnaeus, 1758) and *L. chaquensis* Ceí, 1950. Both species are sympatric in the Argentinean provinces of Corrientes, Entre Ríos and Santa Fé (Ceí, 1980). In Corrientes Province, both species are syntopic. The distribution area of *L. chaquensis* in South America encompasses northern Argentina, eastern Bolivia, Paraguay, northern Uruguay, and Brazil (Mato Grosso do Sul) (Frost, 2004). *Leptodactylus ocellatus* has been studied mainly because of its complex reproductive behaviour (Vaz-Ferreira & Gerhau, 1975, 1986), while Gallardo (1964) and Basso (1990) gave data about the adult diet for several Argentinean populations.

Until now, the diet and trophic patterns of Argentinean *L. chaquensis* have never been studied. Some reproductive characteristics of populations from north-western Argentina were analyzed by Perotti (1994, 1997). The parasitic helminths of this frog have been poorly studied with some parasitic nematodes recorded for Paraguayan populations (Baker, 1987). The helminths of the Argentinian populations are unknown.

The main goals of this study of an Argentinian population of *Leptodactylus chaquensis*, were: (1) to describe its diet, the width of its trophic niche and its foraging pattern; (2) to analyse its reproductive characteristics (ovum diameter and ovum numbers); and (3) to determine the number of helminth taxa infecting this frog under natural conditions.

MATERIALS AND METHODS

The study area was demarcated by a maximum distance of approximately 40 km towards the east and south of the city of Corrientes (27° 30' S, 58° 45' W), while the Paraná River defined its western and northern limits. Data were collected monthly from 1996 to 2004. Adults of *L. chaquensis* were hand-captured preferentially between 1800 to 2300 hours, using the sampling technique defined as "visual encounters survey" (Crump & Scott Jr., 1994). Data were collected monthly from 1996 to 2004, with a minimum of two samples per month in the field. The study area is characterized by its wide variety of habitats, including numerous temporary, semipermanent and permanent ponds. The predominant vegetation is the forest, with herbaceous strata composed of grasses, numerous cacti and terrestrial bromeliads.

Specimens for diet and reproductive study were captured, humanely euthanased and fixed in 10% formalin and deposited in the Centro de Ecología del Litoral (CECOAL-CONICET) collection. For the parasitological study, a separate sample of frogs was kept alive until needed for later analysis.

TROPHIC STUDY

Sex (determined by examination of gonads and external nuptial features), body length (mm), and maximum mouth width (mm) were recorded for each individual. Diets were analyzed by removing the complete alimentary canal, as recommended by Schoener (1989). We only included prey that had at least 70% of their body undigested. Prey items were identified to the order level using the keys of Brewer & Arguello (1980) and Coronado Padilla & Márquez Delgado (1978). The number of prey items per stomach for each prey cat-

Correspondence: E. F. Schafer, CECOAL-CONICET, C.C. 140, (3400) Corrientes, Argentina. E-mail: eclschaefer247@yahoo.com.ar

egory and the individual volume of each prey item were recorded. All measurements were taken with caliper to the nearest 0.1 mm. The volume of each prey item was estimated using the formula for an ellipsoid (Dunham, 1983; Duré & Kehr, 2004). The diversity index used was the Shannon index (H') (Shannon & Weaver, 1949) using decimal logarithm ($\log_{10}$). The niche breadth was calculated using Levins' index (Levins, 1968). We also calculated the standardized niche breadth by expressing it on a scale from 0 to 1. Hurlbert (1978) suggest the following measure for standardized niche breadth, where B_A = Levin's standardized niche breadth; B = Levins's measure of niche breadth; and n = number of food items.

Parametric and non-parametric tests were used in order to establish the relationship between the predator morphology and the prey volume (Kehr, 1994; Zar, 1996).

REPRODUCTIVE STUDY

The morphometric variables considered for both sexes were: snout-vent length (SVL) (mm), body mass (g) and net weight (g) (total body mass without gonad mass) (for females only; Prado *et al.*, 2000). The reproductive variables recorded for each individual were: ovary mass, mature ova number (ovarian complement), mature ovum diameter, mature ovum coloration and testes weight, coloration and form. All variables were registered on individuals fixed in formaldehyde (10.0%). Body length and ovum diameter (estimated from 100 randomly selected mature ova/female) were determined to the nearest 0.1 mm by caliper. Body and testes mass was measured in the laboratory after the individuals were blotted to remove excess liquid. For that an electronic balance to the nearest 0.01 g was used. Male maturity was determined by testes size and presence of nuptial excrescences. The maturity of the ova was determined by degree of pigmentation (Crump, 1974; Basso, 1990; Perotti, 1994, 1997). Once the ovarian complement for each female had been recorded, 100 mature ova were selected randomly to obtain the mean ovum diameter. The reproductive effort (RE) for both sexes was measured as a percentage of mature gonad mass relative to body mass (Kuramoto, 1978; Perotti, 1994, 1997; Prado *et al.*, 2000). Net weight of female and male body (total body mass without gonad mass) was used for correlation and comparative analysis.

PARASITOLOGICAL STUDY

Frogs were transported to the laboratory, humanely euthanased and their snout-vent length (SVL) and weight recorded. At necropsy, hosts were sexed and the alimentary canal, lungs, liver, gall bladder, kidneys, body cavity, musculature, integument and brain examined for parasites by dissection. Helminths were observed *in vivo*, counted and killed in hot distilled water before being fixed in 70% ethyl alcohol. Digeneans, cestodes and acanthocephalans were stained with carmine hydrochloride, cleared in creosote and mounted in Canada

balsam. Nematodes were cleared in glycerin or lactophenol and examined as temporary mounts. The systematic determination of the helminth was carried out following the approaches given by Anderson (2000), Anderson *et al.* (1974), Baker (1987), Gibson *et al.* (2002) and Yamaguti (1961; 1963; 1971; 1973). The infection prevalence, intensity and abundance were calculated according to Bush *et al.* (1997). Parasite community analysis was determined from richness, abundance, diversity (Shannon index, H') (Shannon & Weaver, 1949) and evenness ($J' = H' / H'_{\max}$) (Pielou, 1966; Zar, 1996). All indices were used with decimal logarithms ($\log_{10}$). Chi-square test with Yates' correction for continuity was used for sex proportion comparisons.

RESULTS

TROPHIC STUDY

Fifty-seven individuals with identifiable stomach content (37 males and 20 females; 56.0% of all animals captured) were collected during two periods, January 1998 to May 1999 and January 2002 to August 2003.

The diet consisted of 17 types of prey (Table 1) and was dominated numerically (24.9%) and volumetrically (24.80%) by coleopterans. The orthopterans were also important volumetrically (22.0%). Coleopterans were the most frequently represented prey in 30 individuals (53.0% of adults). Other numerically important items were Formicidae, Araneida and Orthoptera. Prey diversity was 0.94. Niche breadth was 6.63 and the standardized niche breadth was 0.35.

Leptodactylus chaquensis showed a positive and significant relationship between body length and mouth width ($\ln y = -1.33 + 1.03 \ln x$, $n=57$, $r=0.91$, $F_{1,55}=274.0$; $P<0.0001$). Another positive and significant relationship was observed between mouth width and the mean prey volume by stomach ($\ln y = -6.41 + 3.71 \ln x$, $n=57$, $r=0.40$; $F_{1,55}=10.3$, $P=0.002$).

No relationship was found between the body length and the number of prey found in stomachs ($\ln y = -0.25 + 0.28 \ln x$, $n=57$, $r=0.06$, $F_{1,55}=0.20$, $P=0.65$).

REPRODUCTIVE STUDY

Reproductive characteristics of *L. chaquensis* were determined from thirty five individuals captured during the breeding season between 1996 and 2003 (fourteen females and twenty one males). Thirteen gravid females were collected during the following seasons and year: seven during spring (October and November), five in summer (January and February), and one in autumn (April). Twenty one mature males were collected in the following seasons and year: sixteen during spring (September-December), and five in summer (January and February). All meristic and reproductive data are summarized in Table 2. For females, no significant correlation was found between body length and ovarian complement ($r_s=0.41$, $n=13$, $P=0.17$), ovary mass

TABLE 1. Types of prey in the diet of *Leptodactylus chaquensis* (n=57) from Corrientes, Argentina. Volume in mm³; Freq.: number of frogs eaten each prey.

Prey type	Number	%	Volume (mm ³)	%	Frequency
INSECTA					
Coleoptera	46	24.9	4806.9	24.8	30
Hemiptera	13	7.0	3276.5	16.9	11
Hymenoptera (Formicidae)	30	16.2	302.5	1.6	20
Hymenoptera (No Formicidae)	3	1.6	72.6	0.4	3
Diptera	2	1.1	6.3	0.03	2
Homoptera	9	4.9	346.1	1.8	7
Orthoptera	12	6.5	4271.8	22.0	9
Odonata	2	1.1	250.3	1.3	2
Phasmantodea	3	1.6	774.0	4.0	3
Mantodea	1	0.5	5.1	0.03	1
Dictioptera	2	1.1	2478.9	12.8	2
Insect larvae	22	11.9	1363.3	7.0	14
ARACHNIDA					
Araneida	35	18.9	1015.1	5.2	16
Phalangida	1	0.5	24.2	0.1	1
MOLLUSCA					
Gastropoda	1	0.5	20.6	0.1	1
MYRIAPODA					
Diplopoda	2	1.1	194.0	1.0	2
CRUSTACEAE					
Decapoda	1	0.5	171.6	0.9	1
TOTAL	185	100.0	19375.1	100.0	

($r_s=0.32$, $n=13$, $P=0.30$) or reproductive effort ($r_s=0.02$, $n=13$, $P=0.94$). For males, a significant correlation was observed between body mass (net weight) and testis mass ($r_s=0.64$, $n=21$, $P<0.01$), but not between body mass (net weight) and reproductive effort (RE) ($r_s=-0.19$, $n=21$, $P>0.05$). A significant positive correlation was also found between body length and testes mass ($r_s=0.55$, $n=21$, $P<0.05$), but not between body length and reproductive effort ($r_s=-0.26$, $n=21$, $P>0.05$). No significant differences were observed between sexes for body length and body mass (net weight) (body length: Mann-Whitney U -test = 117, $P=0.31$, $n_1=14$,

$n_2=21$); (body mass: Mann-Whitney U -test = 163, $P=0.59$, $n_1=14$, $n_2=21$). Each ovum was half dark grey or black and half white. The testes were white and bean-shaped. The adults showed preferences for either humid or dry earth, and were also found in mud, near the shore of temporary, semipermanent and permanent ponds, and in flooded high grass (approximately 1m high). Foam nests containing eggs, were observed partially hidden among the flooded vegetation, in areas with water deeper than 20 cm.

TABLE 2. Mean $\pm$ SD body length (SVL); body mass (BM); net body mass (total body mass - gonad mass) (NBM); ovarian complement (OC, total mature ova count number per female); gonad mass (GM); reproductive effort (RE, percentage of gonad mass relative to net body mass) and ova diameter (OD), for females and males of *Leptodactylus chaquensis* from Corrientes, Argentina. Range and sample size in parentheses.

Variables	Females	Males
SVL (mm)	65.3 $\pm$ 7.82 (54.80-81.50; $n=14$)	62.9 $\pm$ 5.43 (54.50-75.17; $n=21$)
BM (g)	33.9 $\pm$ 11.31 (19.53-62.11; $n=14$)	33.9 $\pm$ 10.43 (16.01-54.17; $n=21$)
NBM (g)	30.6 $\pm$ 9.62 (18.41-52.24; $n=13$)	33.0 $\pm$ 10.16 (15.56-52.63; $n=21$)
OC	4401.2 $\pm$ 2231.10 (750-7812; $n=13$)	—
GM (g)	3.4 $\pm$ 2.47 (0.90-9.87; $n=13$)	0.90 $\pm$ 0.39 (0.32-1.94; $n=21$)
RE (%)	10.7 $\pm$ 5.74 (3.35-20.62; $n=13$)	2.8 $\pm$ 0.86 (1.00-4.41; $n=21$)
OD (mm)	0.8 $\pm$ 0.14 (0.40-1.20; $n=1100$)	—

TABLE 3. Summary of helminth taxa, number of parasites prevalence (%), mean abundance, mean intensity, stage and site of infection in *Leptodactylus chaquensis* from Corrientes, Argentina. Number of collection is cited below each taxa.

Helminths	No.	%	Mean abundance	Mean intensity (min-max)	Stage in frog	Site of infection
TREMATODA						
<i>Haematoloechus longiplexus</i> CECOAL 03111804	92	40.0	2.04±3.77	6.38 (1-23)	Adult	Lung
<i>Gorgoderina parvicava</i> CECOAL 03032702	29	20.0	0.64±1.99	3.22 (1-12)	Adult	Urinary bladder
<i>Glythelmins repandum</i> CECOAL 03032704	49	27.0	1.08±2.39	4.08 (1-9)	Adult	Small intestine
<i>Glythelmins palmipedis</i> CECOAL 03042807	70	62.0	1.55±2.22	2.50 (1-10)	Adult	Small intestine
<i>Catadiscus</i> sp. CECOAL 01032802	160	58.0	3.55±6.98	6.15 (1-39)	Adult	Large intestine
<i>Travtrema</i> sp. CECOAL 03012905	34	24.0	0.75±2.24	3.09 (1-13)	Metacerc.	Muscle, mesenteries, body cavity , pharyngeal zone
<i>Bursotrema</i> sp. CECOAL 03051607	3331	69.0	74.02±297.60	107.45 (1-2000)	Metacerc.	Kidney
Strigeidae gen. sp. 1 CECOAL 03052702	15	9.0	0.33±1.55	3.75 (1-10)	Metacerc.	Body cavity
Strigeidae gen. sp. 2 CECOAL 03042806	13	2.0	0.02±0.14	13	Metacerc.	Liver
Diplostomidae gen. sp. CECOAL 03042804	3	4.0	5.15±33.97	1.50 (1-2)	Metacerc.	Body cavity
Plagiorchiata gen. sp. 1 CECOAL 03012105	41	13.0	0.91±3.85	6.83 (1-24)	Metacerc.	Muscle mesenteries, body cavity pharyngeal zone
Plagiorchiata gen. sp. 2 CECOAL 03051605	53	7.0	1.17±6.51	17.66 (1-43)	Metacerc.	Kidney, muscle
NEMATODA						
<i>Cosmocerca podicipinus</i> CECOAL 03012903	81	62.0	1.80±2.58	2.89 (1-12)	Adult	Lung, large intestine
<i>Cosmocerca parva</i> CECOAL 03031001	10	7.0	0.22±0.95	3.33 (1-5)	Adult	Large intestine
<i>Aplectana delirae</i> CECOAL 03092418	2	2.0	0.04±0.29	2	Adult	Large intestine
<i>Aplectana</i> sp. CECOAL 02112902	5	2.0	0.11±0.74	5	Adult	Large intestine
<i>Porrocoecum</i> sp. CECOAL 03092418	1	2.0	0.02±0.14	1	Larvae	Serous of stomach
<i>Camallanus</i> sp. CECOAL 03042804	1	2.0	0.02±0.14	1	Larvae	Small intestine
ACANTHOCEPHALA						
<i>Centrorhynchus</i> sp. CECOAL 03012105	11	16.0	0.24±0.64	1.57 (1-3)	Larvae	Serous of stomach, mesenteries
CESTODA						
Unidentified metacestodes CECOAL 03041001	13	2.0	0.28±1.93	13		Larvae mesenteries

PARASITOLOGICAL STUDY

A total of 45 frogs were captured between 2001 and 2003. Five were collected between March and June 2001; four between September and November 2002 and 36 between January and November 2003. Parasite prevalence was 100% in both sexes and there was no significant difference between females (26) and males (19) (χ^2 with Yates correction for continuity = 1.11, $df=1$, $P>0.05$).

The component community consisted of twenty helminth parasite taxa (larvae and adults), including twelve trematodes, one cestode, six nematodes and one acanthocephalan. The prevalence, mean abundance and intensity, minimum and maximum parasite numbers, stage and localization are detailed in Table 3. Helminth species diversity ($H' = 0.38$) and evenness ($J' = 0.29$) were low with few species being well represented. The mean helminth species richness was 4.56 ± 1.85 (maximum = 8) species per frog infected. Multiple species infections were common with 1, 2, 3, 4, 5, 6, 7 and 8 species occurring in 4, 3, 4, 10, 10, 7, 5 and 2 individuals respectively of *L. chaquensis*.

Of all metacercaria found in different organs, the most common taxon (with a prevalence > 50.0%) was *Bursotrema* sp. (located in the kidney). The adult trematodes recorded in the small intestine (*Glythelmins palmipedis*), in the large intestine (*Catadiscus* sp.) and in the lungs (*Haematoloechus longiplexus*) presented an infection prevalence >40%. The nematodes (*Cosmocerca podicipinus*) found in the large intestine and in the lungs presented an infection prevalence >50%. Cestodes and acanthocephalans presented infection prevalence <20%.

DISCUSSION

TROPIC CHARACTERISTICS

According to the type and prey proportion, *L. chaquensis* appears to be a generalist with a foraging strategy considered as intermediate between a pure sit-and-wait and an actively foraging predator. The prey of a conventional sit-and-wait predator are active, the encounter rate with prey is low, niche breadth is wide, and the sensory mode is visual (Perry & Pianka, 1997; Duré & Kehr, 2004). Coleopterans and hymenopterans (ants) were important prey for this species. They are relatively mobile prey. Nevertheless, *L. chaquensis* also selected relatively sedentary prey (insect larvae and spiders), which suggests a change from sit-and-wait behavior to actively foraging. The same behaviour was observed in *Leptodactylus latinasus* and *L. bufonius* (Duré & Kehr, 2004). Our results indicate that as the mouth size of *L. chaquensis* increases (proportionally to body size) they consume larger prey items (> volume). On the other hand, the largest frogs did not show an increase in the number of prey items ingested. Similar results on diet composition have been reported by Duré (1999).

Basso (1990) also recorded coleopterans (mobile prey) and insect larvae (sedentary prey) as the most im-

portant prey items for the sister species *L. ocellatus* in an Argentinian population, and Maneyro *et al.* (2004) also recorded coleopterans, arachnids and larvae as important items in the diet of *L. ocellatus* in Uruguay. The number of the prey items was also similar between the two species. Presumably, the foraging strategy of *L. ocellatus* is similar to that observed in *L. chaquensis* in being intermediate between sit and wait and an actively foraging, depending of availability of prey.

Leptodactylus chaquensis behaves as a non-selective predator, which optimizes the ingestion of nutrients by consuming increasingly larger volume prey as their mouth width increases. Nevertheless, when only small prey are available, they are ingested in large numbers, independently of frog body size.

REPRODUCTIVE ECOLOGY

Reproduction in *L. chaquensis* occurs between October and February. Within this period, reproduction can be considered to be explosive and dependent on the amount of rainfall. This means that breeding activity is intense for one or more days, with the synchronous arrival of both sexes at the breeding sites (Wells, 1977). Our results are consistent with those reported by Prado *et al.* (2005), who classified the reproductive mode of *L. chaquensis* from the southern Pantanal (Brazil) as number 8 (i.e., with foam nest and exotrophic tadpoles in lenitic waters) and the reproductive activity pattern as explosive. Prado *et al.* (2002) also classified the reproductive mode of this species as mode 1 (similar definition to mode 8 described above).

The mean reproductive effort of females ($RE = 10.70 \pm 5.74\%$) was lower than that recorded by Prado *et al.* (2000) and Prado & Haddad (2005) in populations from the Pantanal (Brazil). The reproductive effort of males ($RE = 2.78 \pm 0.86\%$) was also lower than in the Pantanal (4.13% - Prado & Haddad, 2003). The testis mass relative to body mass in species of the family Leptodactylidae ranges from 0.04 to 4.13%, with the testes of *L. chaquensis* (4.13%) and *L. podicipinus* (0.75%) being larger than those of other leptodactylids from the Pantanal (Prado & Haddad, 2003).

Leptodactylus chaquensis, *Chiromantis xerampelina* and *Rhacophorus arboreus* have much larger testes than other anuran species, including rhacophorids and leptodactylids (Prado & Haddad, 2003). *Leptodactylus chaquensis*, *L. macrosternum* and *L. ocellatus*, all belong to the "ocellatus" group (Heyer, 1969), and deposit their eggs in foam nests on the water surface. Testis size does not appear to be related to the species group, at least in the "ocellatus" group, with *L. chaquensis* exhibiting a much greater testis mass than the other two species from the same group.

Recently, Prado & Haddad (2003) reported multimale spawning in *L. chaquensis*. In species having external fertilization, the strategies available to males to increase their fertilization success include: (1) increasing the number of sperms released and (2) maintaining their proximity to females (Gross, 1985; Jennions &

Passmore, 1993). Therefore, selection would favour males with high sperm production, and hence with large testes (Jennions & Passmore, 1993). Large testes size in frogs with multimale spawning supports the sperm competition hypothesis.

PARASITES

Of all the helminth groups, trematodes with an aquatic life cycle presented a high species richness, both as adult and larval stages. They were primarily represented by the metacercariae of *Bursotrema* sp., which had the greatest infection intensity and prevalence. Our results are similar to those reported by Duré *et al.* (2004) for *Pseudopaludicola boliviana*, captured in the same area. It must be pointed out that both hosts (*L. chaquensis* and *P. boliviana*) use the same microhabitat, along the edge of water bodies, which favours their infection with these metacercariae. The highest occurrence (> 40.0%) among adult trematodes corresponded to species found in the lungs (*H. longiplexus*), small intestine (*G. palmipedis*), and in the large intestine (*Catadiscus* sp.), all of them with indirect life cycles. For *H. longiplexus*, hosts acquire infection by feeding on aquatic and terrestrial insects. These possible intermediate hosts (e. g. Odonata and other insect larvae) were part of the diet of *L. chaquensis*. For *G. palmipedis* and *Catadiscus* sp., amphibians become infected when they ingest their own skin or eat other frogs (Grabda-Kazubska, 1976; Smyth & Smyth, 1980). The life cycle of *Catadiscus* species are unknown, but in general it may resemble that of other amphibian paramphistomes (e.g. genus *Megalodiscus*), their metacercariae encyst on the frog's skin (Smyth & Smyth, 1980). *Glythelmins palmipedis* may resemble the life cycle of *G. quieta*, their metacercariae encysting in the skin of tadpoles and frogs (Leigh, 1946).

Nematodes with terrestrial and direct life cycles were the second most abundant group of helminths, *C. podicipinus* being the dominant species. Their larvae penetrate the skin of the host and, after migrating to the lungs to complete their development, they are located in the large intestine (Anderson, 2000). On the other hand, *L. chaquensis* was found to be the paratenic host of *Centrorhynchus* sp. (cystacanths), birds being their definitive host (e.g., falconiformes, strigiformes). The possible intermediary hosts of this taxon (i.e., coleopterans and orthopterans) were predominant food items of *L. chaquensis* (infection prevalence 16.0 %).

As a generalisation, terrestrial frogs are more infected with nematodes (Bolek & Coggins, 2000; 2003) and aquatic amphibians are more commonly infected with trematodes (Hamann & Kehr, 1998; 1999; McAlpine & Burt, 1998; Kehr *et al.*, 2000; Bolek & Coggins, 2001; Muzzall *et al.*, 2001; Kehr & Hamann, 2003; Hamann, 2004). The data suggest that *L. chaquensis* show a wide variation in the helminths they harbour, acquiring helminths characteristic of both aquatic and terrestrial frogs, due to habitat variability or feeding strategy, or to both factors.

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A CHYTRIDIOMYCOSIS EPIDEMIC AND A SEVERE DRY SEASON PRECEDE THE DISAPPEARANCE OF *ATELOPUS* SPECIES FROM THE VENEZUELAN ANDES

MARGARITA LAMPO¹, ARGELIA RODRÍGUEZ-CONTRERAS^{2,5} ENRIQUE LA MARCA³
AND PETER DASZAK⁴

¹Centro de Ecología, Instituto Venezolano de Investigaciones Científicas, Apartado 21827, Caracas, Venezuela

²Facultad de Ciencias, Núcleo La Hechicera, Universidad de los Andes, Mérida, Venezuela

³Laboratorio de Biogeografía, Facultad de Ciencias Forestales y Ambientales, Universidad de Los Andes, Apartado 116, Mérida, Venezuela

⁴Consortium for Conservation Medicine, 460 West 34th Street, New York, NY 1001, USA

⁵Present address: Museo de Historia Natural La Salle. Apartado 1930, Caracas, Venezuela

Chytridiomycosis has been identified as one of the major forces driving global amphibian declines. Between 1988 and 1994, five *Atelopus* species endemic to the Venezuelan Andes disappeared. We examined histological samples of Andean *Atelopus* species available in Venezuelan museum collections for the presence of the chytrid fungus *Batrachochytrium dendrobatidis*. When infection was detected, sympatric species were examined to investigate the occurrence of the pathogen and how widespread it was. Infection with *B. dendrobatidis* is reported for the first time in *Atelopus carbonensis*, *A. mucubajensis* and *A. soriano*, *Mannophryne cordilleriana* and an undescribed *Leptodactylus* species. The spatio-temporal patterns of prevalence of this pathogen in *Atelopus* individuals, with all infections concentrated in one year but spread over distant locations, suggest that synchronized epidemic outbreaks occurred in populations of these *Atelopus* species in the years prior to their disappearances. Local climate data indicate that one of the most severe dry seasons recorded in the region since 1970 coincided with these epidemic events. The climatic-linked epidemic hypothesis seems a plausible explanation for the coincidence between the observed amphibian declines, the chytridiomycosis outbreaks and the droughts recorded in that area.

Key words: amphibian declines, *Batrachochytrium dendrobatidis*, climate change, emerging diseases

INTRODUCTION

Batrachochytrium dendrobatidis, a pathogenic agent responsible for the fungal disease chytridiomycosis, has been implicated in population crashes and extinctions of many amphibian species around the world. Among the anuran genera endemic to South America and the Caribbean, *Atelopus* appears to be one of the most affected by this disease (La Marca *et al.*, 2005). Declines have affected 61 species in the taxon, a previously unknown degree of biodiversity loss for a single genus (IUCN *et al.*, 2004; Lötters *et al.*, 2004a; La Marca *et al.*, 2005). Mass mortalities and declines of *Atelopus chiriquiensis* (Panama and Costa Rica) and *Atelopus varius* (Panama) have been attributed to chytridiomycosis (Lips, 1998; Berger *et al.*, 1998). In Venezuela, *B. dendrobatidis* has been reported only in a museum specimen of *Atelopus cruciger*, a critically endangered endemic species from lowland rainforests and cloud forests of the Cordillera de la Costa, collected in 1986 (Bonaccorso *et al.*, 2003) and live populations of bullfrogs (*Lithobates catesbeianus*, Ranidae), an exotic species introduced

recently in disturbed Andean cloud forests, well after the *Atelopus* declines were first observed (Hanselmann *et al.*, 2004).

The Venezuelan Andes hosts eight *Atelopus* species; with the possible exception of *A. tamaense*, all of them are endemic to the country. Six of these species have suffered declines: *A. carbonensis*, *A. chrysocollaris*, *A. mucubajensis*, *A. oxyrhynchus*, *A. pinangoi* and *A. soriano* (La Marca & Reinthaler, 1991; La Marca, 1995a,b; La Marca & Lötters, 1997; Manzanilla & La Marca, 2004). All were abundant from 1920, the earliest herpetological records available in the country, until the late 1980s. However, regardless of the numerous efforts to find these species in their former habitats, none of them have been recorded after 1994 (La Marca, 2004; La Marca *et al.*, 2005), except for the recent record of a single individual of *A. mucubajensis* (Barrio-Amorós, 2004). As a result, all of these species are currently critically endangered except for *A. vogli*, which is most probably extinct (IUCN *et al.*, 2004; Lötters *et al.*, 2004b).

Habitat destruction, fragmentation and over collection were suggested previously as probable causes for the declines of the Andean *Atelopus* populations (La Marca & Lötters, 1997), but this conclusion was

Correspondence: M. Lampo, Centro de Ecología, Instituto Venezolano de Investigaciones Científicas, Apartado 21827, Caracas 1020-A, Venezuela. E-mail: mlampo@gmail.com

reached prior to the discovery of chytridiomycosis in other *Atelopus* species. Although their restricted distribution and small home ranges (Dole & Durant, 2006) makes these species vulnerable to local disturbances, the apparent synchrony between decline events on several countries suggests a more complex interaction of factors (Daszak *et al.*, 1999; Daszak *et al.*, 2003). Pounds *et al.* (1999, 2006), Pounds & Crump (2004) and Pounds & Puschendorf (2004) suggested large scale climate change as the key factor driving epidemic outbreaks of chytridiomycosis and amphibian extinctions. To test whether *B. dendrobatidis* was present prior to the declines reported for the Andean

Venezuelan *Atelopus* species between 1988 and 1994, we examined histological samples of most *Atelopus* species, and a selected sample of other amphibian species, available at the major Venezuelan museum collections. In addition, we analyzed local climate data to determine whether unusual climate events in the Andean region coincided with these declines.

METHODS

CHYTRIDIOMYCOSIS DETECTION

Samples were taken from four museum collections: Laboratorio de Biogeografía de la Universidad de Los Andes (ULABG) and Colección de Vertebrados de La

TABLE 1. Number of specimens sampled of each species in each of the four herpetological collections used: Colección de Vertebrados de La Universidad de Los Andes (CVULA), Museo de Biología de la Universidad Central de Venezuela (MBUCV), Museo de Historia Natural La Salle (MHNLS) and Laboratorio de Biogeografía de la Universidad de Los Andes (ULABG).

Species	CVULA	MBUCV	MHNLS	ULABG	Total
<i>Aromobates alboguttatus</i>	13	0	11	0	24
<i>Aromobates leopardalis</i>	31	0	0	0	31
<i>Aromobates molinarii</i>	0	0	0	4	4
<i>Aromobates</i> sp.1	0	0	0	2	2
<i>Aromobates</i> sp.2	0	0	0	2	2
<i>Atelopus carbonerensis</i>	12	0	3	3	18
<i>Atelopus chrysocorallus</i>	0	0	0	4	4
<i>Atelopus mucubajensis</i>	3	0	0	11	14
<i>Atelopus oxyrhynchus</i>	11	4	2	17	34
<i>Atelopus pinangoi</i>	0	0	0	3	3
<i>Atelopus soriano</i>	11	0	0	6	17
<i>Atelopus</i> sp.	0	0	0	2	2
<i>Atelopus tamaense</i>	0	0	0	3	3
<i>Centrolene andinum</i>	7	0	7	0	14
<i>Centrolene venezuelense</i>	0	0	8	0	8
<i>Chaumus marinus</i>	0	4	0	3	7
<i>Dendropsophus meridensis</i>	3	0	0	1	4
<i>Dendropsophus microcephalus</i>	0	0	0	1	1
<i>Eleutherodactylus briceni</i>	0	0	0	1	1
<i>Eleutherodactylus ginesi</i>	0	0	0	2	2
<i>Eleutherodactylus lancinii</i>	0	0	0	2	2
<i>Eleutherodactylus prolixodiscus</i>	0	0	0	1	1
<i>Eleutherodactylus</i> sp.1	0	0	0	3	3
<i>Eleutherodactylus</i> sp.2	0	0	0	3	3
<i>Eleutherodactylus</i> sp.3	0	0	0	1	1
<i>Eleutherodactylus</i> sp.4	0	0	0	1	1
<i>Flectonotus pygmaeus</i>	0	0	0	1	1
<i>Gastrotheca nicefori</i>	0	0	0	2	2
<i>Hyalinobatrachium duranti</i>	0	0	21	0	21
<i>Hyloscirtus jahni</i>	0	0	0	3	3
<i>Hyloscirtus lascinius</i>	0	0	0	1	1
<i>Hyloscirtus platydactylus</i>	0	0	0	2	2
<i>Hypsiboas</i> cf. <i>crepitans</i>	22	0	0	24	46
<i>Mannophryne collaris</i>	27	0	3	2	32
<i>Mannophryne cordilleriana</i>	0	0	0	1	1
<i>Mannophryne</i> sp.	0	0	0	12	12
<i>Rhinella</i> sp. ("typhonius" group)	0	0	0	1	1
<i>Scarthyla vigilans</i>	0	0	0	1	1

Universidad de Los Andes (CVULA) in Mérida, and Museo de Historia Natural La Salle (MHNLS) and Museo de Biología de la Universidad Central de Venezuela (MBUCV) in Caracas (Table 1). These collections include most of the *Atelopus* specimens from the Andean region available in Venezuela. Sampling was carried out in two phases. First, we screened a large sample of *Atelopus* specimens from the Andean region housed in these collections. Secondly, at locations and dates where infections were detected in *Atelopus*, other Andean species were also sampled to examine the spatial and temporal distribution of the disease.

Skin tissue was removed from the ventral abdominal and pelvic regions of specimens using a scalpel blade or a tissue biopsy punch (Baker’s dermal punch 4 and 6 mm, J. A. Webster, Inc.). In addition, toe clips including interdigital membranes were obtained from some specimens. Tissue samples were washed with phosphate buffer, dehydrated through a graded ethanol series, embedded in paraffin, sectioned at 5 µm and stained with haematoxylin and eosin (Humason *et al.*, 1997) and were examined under a light microscope for the presence of *B. dendrobatidis* or lesions consistent with chytridiomycosis. Lesions were classified as mild, moderate or severe depending on the percentage of skin section with zoosporangia, and the number of epithelial layers showing infected keratinaceous cells (Berger *et al.*, 1999; Pessier *et al.*, 1999; Nichols *et al.*, 2001).

CLIMATE PATTERNS

Temperature and rainfall data were obtained from the Venezuelan Ministry of Environment and Natural Resources (MARN) for four climatic stations in the Andean region: Santo Domingo (8°52’27’’N; 70°40’27’’W; 2155 m), La Cuchilla (8°38’00’’N, 71°21’10’’W; 2280 m), Tovar (8°20’30’’N, 71°44’40’’W; 952 m) and Mérida-Airport (8°35’21’’N, 71°09’38’’W; 1500 m). We analyzed rainfall data from the first three stations, as these were the closest ones to the sites where chytridiomycosis was detected. Temperature data, however, were obtained from the Mérida-Airport station because this was the only one in the area with a complete temperature series that covered all years between 1970 and 1990. Pearson correlation analyses were per-

formed to assess whether the mean, maximum and minimum monthly air temperature recorded at the Mérida-Airport station showed similar patterns to those from the other three stations (Sokal & Rohlf, 1995).

We examined trends for the mean, maximum and minimum monthly air temperatures for the 1951-1990 period (*n*=480). Fourier analyses were used to detect periodicities and the series were fitted to linear models with harmonic terms using regression analyses. The series were transformed to remove the location by subtracting each value to the mean temperature for the complete series. Linear trends were removed when present and 95% confidence intervals were estimated. Atypical values were detected by examining the outliers in the mean annual values with respect to the 95% confidence intervals of the transformed series.

Trends in the mean monthly precipitation were also determined for the 1970-1996 period. We used accumulated precipitation for the first five months (January-May) of each year, as these are better estimates of humidity and availability of water during the local dry season. A Fourier analysis was used to detect annual trends and 95% confidence intervals were estimated for the mean accumulated precipitation for the first five months to identify extreme values.

RESULTS

Skin sections from 335 specimens from six families (Bufonidae, Centrolenidae, Dendrobatidae, Hylidae, Leptodactylidae, Plethodontidae) belonging to 13 genera and 39 species of amphibians were examined (Table 1). These included animals collected between 1952-2002. *Batrachochytrium dendrobatidis* was detected in seven of the 95 *Atelopus* specimens examined (Table 2). All seven specimens showed zoosporangia, in different developmental stages, localized in less than 30% of the epithelial surface examined. Many of these zoosporangia were found empty, and some contained septa characteristic of *B. dendrobatidis*. Skin sections showed hyperkeratosis with up to three layers of infected keratinaceous cells present in some regions (Fig. 1).

The museum catalogue information associated with each infected *Atelopus* individuals indicated that the pathogen was present in populations located at three lo-

TABLE 2. Catalogue data for Venezuelan Andean specimens found positive for chytridiomycosis.

Catalog ID	Species	Coordinates	Year of collection
ULABG 2107	<i>Atelopus carbonerensis</i>	8°38’42’’N y 71°23’24’’W	1988
ULABG 2108	<i>Atelopus mucubajensis</i>	8°51’00’’N y 70°42’50’’W	1988
ULABG 2109	<i>Atelopus mucubajensis</i>	8°51’00’’N y 70°42’50’’W	1988
ULABG 2005	<i>Atelopus soriano</i>	08°15’28’’N y 71°43’08’’W	1988
ULABG 2006	<i>Atelopus soriano</i>	08°15’28’’N y 71°43’08’’W	1988
ULABG 2103	<i>Atelopus soriano</i>	08°15’28’’N y 71°43’08’’W	1988
ULABG 2104	<i>Atelopus soriano</i>	08°15’28’’N y 71°43’08’’W	1988
ULABG 4269	<i>Leptodactylus</i> sp.	08°29’27’’N y 71°31’30’’W	1996
ULABG 4886	<i>Mannophryne cordilleriana</i>	8°52’53’’N y 70°38’10’’W	2002

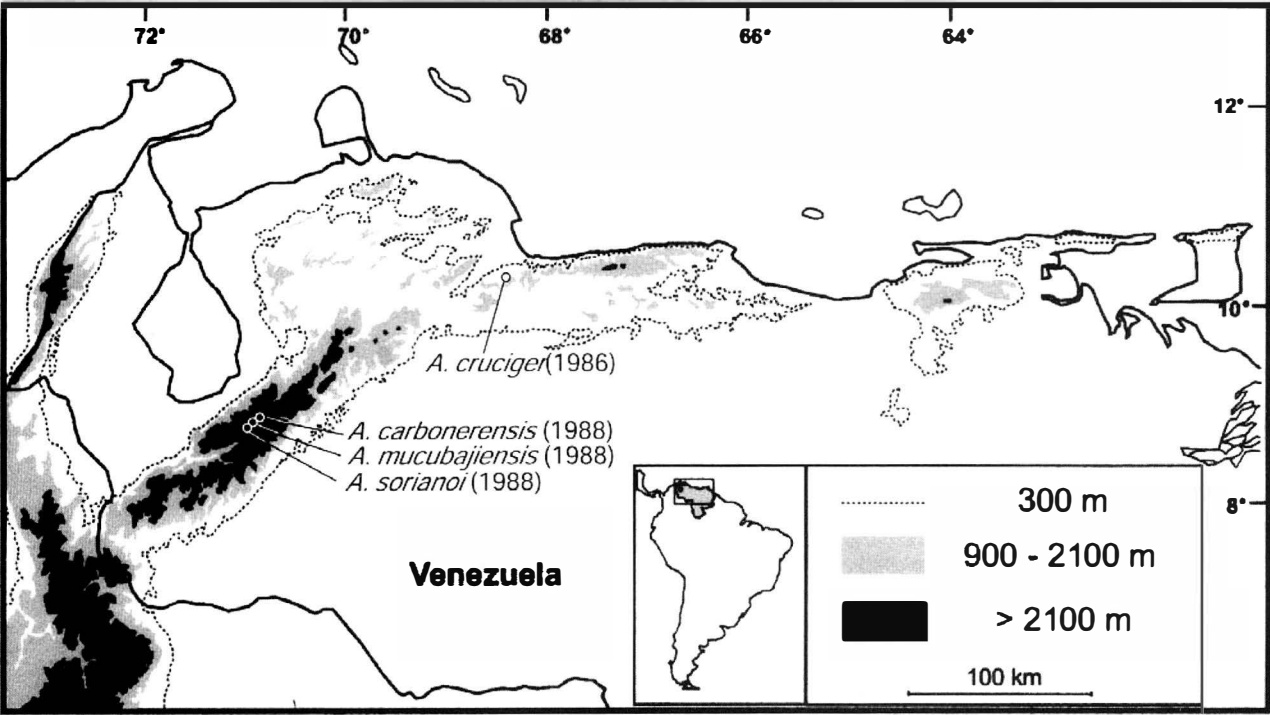


FIG.1. Known locations and dates of infection with *Batrachochytrium dendrobatidis* in *Atelopus* species from Venezuela. The *A. cruciger* record corresponds to Bonaccorso *et al.* (2003).

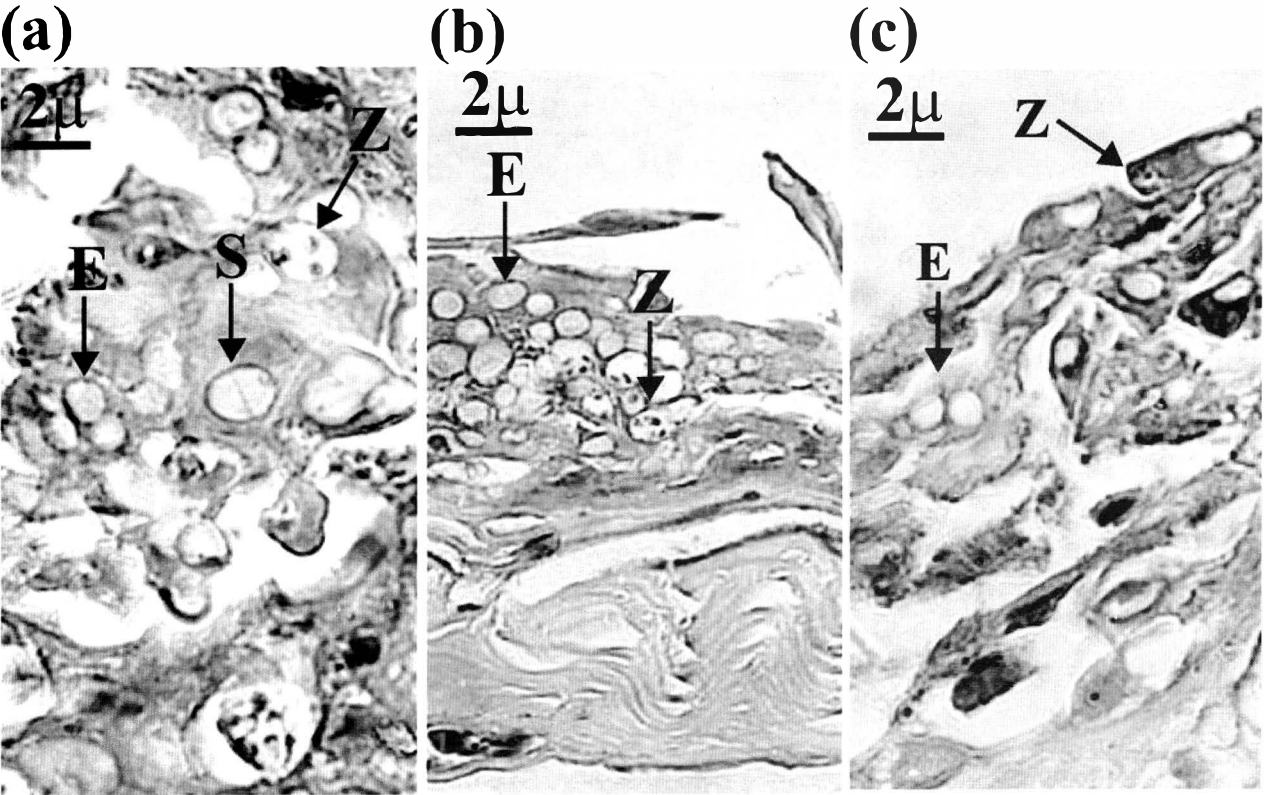


FIG. 2. Infection with *Batrachochytrium dendrobatidis* in native *Atelopus* species from Venezuela:(a) developing sporangia (Z) and empty zoosporangia with (S) and without (E) septa are visible in the stratum corneum of the epithelium of *Atelopus soriano*; (b) marked hyperkeratosis and some hyperplasia and developing and empty zoosporangia in a skin section of *Atelopus mucubajensis*; and (c) *Atelopus carbonerensis*.

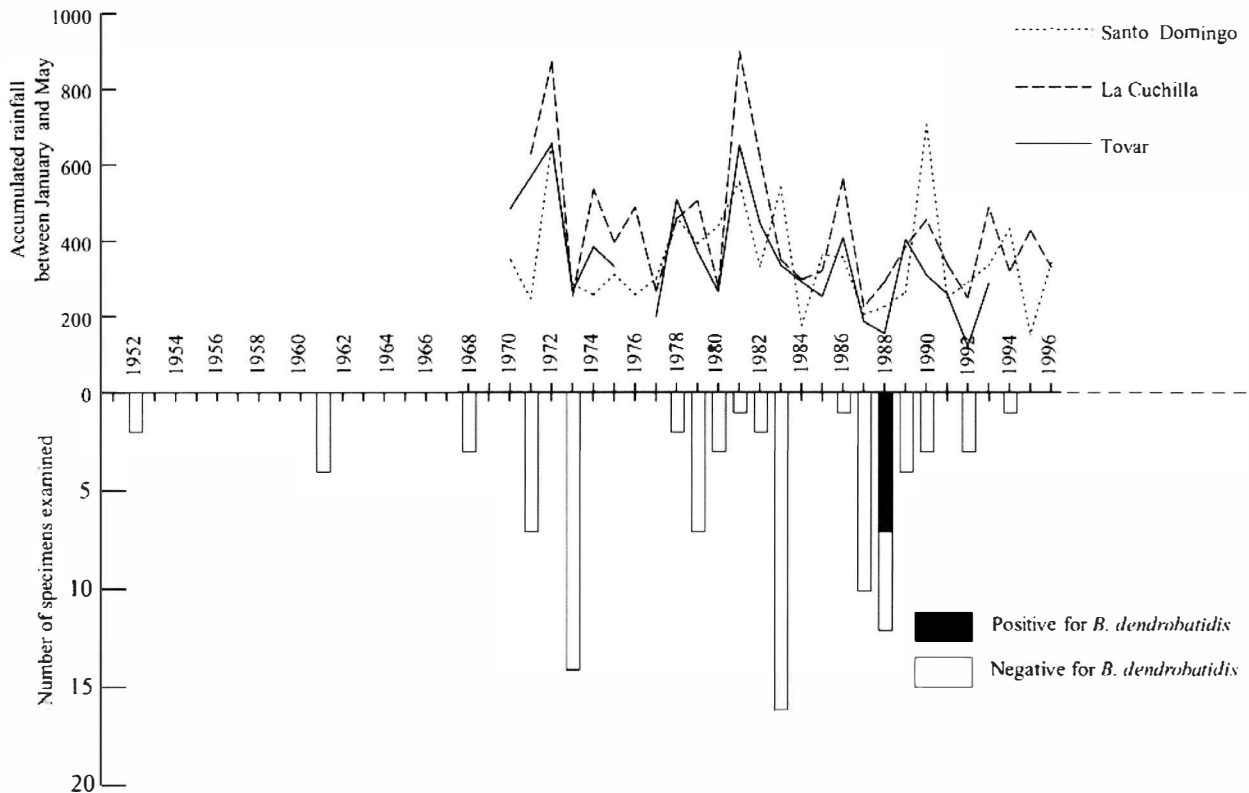


FIG. 3. Annual rainfall trends and distribution of infection with chytridiomycosis in *Atelopus* specimens available from the Venezuelan Andean region. The sample includes *A. carbonerensis*, *A. chrysocorallus*, *A. mucubajensis*, *A. oxyrhynchus*, *A. pinangoi*, *A. soriano*, *A. sp.* and *A. tamaense*.

calities (Table 2, Fig. 2). These sites, separated by more than 60 km, belong to different river basins. Despite the geographic isolation between these sites, all infections occurred in individuals collected only during 1988. The fact that these frogs, collected by different persons on several dates, were kept apart and fixed separately rules out the possibility of cross contamination prior to fixation. The prevalence of infection during that year appeared to be high; 58 % (7/12) of all *Atelopus* specimens examined had evidence of *B. dendrobatidis* (Fig. 3). In contrast, no infection was detected in any of the 72 *Atelopus* frogs collected between 1952 and 1987, or the 11 frogs collected after 1988. In addition, one *Mannophryne cordilleriana* and one *Leptodactylus* sp. currently under description by one of us (ELM) collected in 2002 and 1996 respectively, were also found to be infected (Table 2). Both of these samples showed less than 5% of the epithelial surface infected, without signs of hyperkeratosis.

CLIMATE ANALYSIS

Mean, maximum and minimum monthly air temperatures from La Cuchilla and Tovar were correlated with those recorded at the Mérida-Airport station (Pearson $R^2 = 0.83$ and 0.95 , respectively). All these stations, located on the western slope of the Cordillera de Mérida, are under the influence of a similar climatic regime. In general, the latter station showed greater air temperatures than the Tovar station but lower than La Cuchilla

as the result of differences in altitude. In contrast, the mean monthly air temperature data from the Santo Domingo station, nearest to the location where *A. mucubajensis* was found infected, showed little correlation with their equivalents from the Mérida-Airport station (Pearson $R^2 = 0.2249$). This station is located on the eastern slope of the Cordillera de Mérida and most influenced by a different climatic regime (Vivas, 1992).

Fourier analyses suggested that, besides the annual and biannual cycles associated with the dry and wet seasons, the maximum and mean monthly air temperatures showed periodic cycles about every 20 years. A least square fit of these variables to a linear model with the corresponding harmonic terms showed that the mean and maximum monthly air temperature increased at a rate of 0.02°C per year between 1951 and 1990 ($F=56.4$, $df=472$, $P<0.001$ and $F=30.8$, $df=472$, $P<0.001$, respectively). Also, due to the apparent 20 year cycling, maximum and mean air temperatures during the 1980s were, on average, higher than during the previous decade. However, no significant deviations in the mean, maximum or minimum monthly air temperatures with respect to the complete series mean were observed for 1988, when the infections were detected.

The annual rainfall trends showed no relationship with the number of infected specimens detected. However, two patterns appeared when annual trends in the January-May accumulated rainfall were examined. Before 1988, when the chytridiomycosis epizootic was

observed, three distinct peaks in accumulated rainfall (1972, 1981 and 1986) were followed by the three highest numbers of *Atelopus* specimens deposited in the museums (1973, 1983 and 1988; Fig. 3). Since 1988, however, only a fourth peak (1990) in the accumulated rainfall was recorded, but only three *Atelopus* specimens were deposited thereafter. Second, between 1987 and 1988 one of the lowest values in the January-May accumulated rainfall recorded in the region coincided with the 1988 epizootic. At Santo Domingo and Tovar, lower values were recorded during 1984 and 1992, respectively, but no specimens from nearby locations were deposited in the collections during those years.

DISCUSSION

The presence of zoospores of *B. dendrobatidis* in museum specimens of *A. carbonerensis*, *A. mucubajensis* and *A. soriano* show that these species were infected with the causative agent of chytridiomycosis, in the years preceding their local disappearances. Possible explanations for the relationship between the presence of *B. dendrobatidis* and the subsequent declines of the host populations include: *B. dendrobatidis* has been endemic to the region with little effect on *Atelopus* species and other amphibians, and local populations disappeared from the region for reasons different than the presence of this pathogen; alternatively, epidemics of this chytrid were the cause, alone or in combination with other factors, of the declines of these three Andean *Atelopus* species and possibly that of *A. cruciger*. The temporal distribution of prevalence in museum specimens, with all infections occurring in one year, cannot be attributed to random variation, or to a selective sampling of infected individuals as none of them were morbid at the time of collection (see McCallum, 2005). This suggests that *B. dendrobatidis* was either rare or absent prior to 1984, and then increased its prevalence or infected naïve populations thereafter, reaching high levels of infection in 1988. This pattern is more consistent with a significant increase in the prevalence of the pathogen, characteristic of an epidemic event.

The epidemiological and clinical patterns suggest a possible link between the epizootic in 1988 and the severe declines of these three *Atelopus* species from the Cordillera de Mérida. First, the epidemic die-off after 1988 was followed by the abrupt disappearance of two species, *A. carbonerensis* and *A. soriano*. There were only two records of *A. carbonerensis* and none of *A. soriano* after 1988, despite significant efforts to find these species in the habitats where they used to live (La Marca & Lötters, 1997). For *A. mucubajensis*, nine specimens were found between 1989 and 1994, but these were collected in sites distant to those where infected individuals were detected in 1988. None of these species were observed in the last ten years, except for a single record of *A. mucubajensis* in 2004 (Barrio-Amorós, 2004). Second, chytridiomycosis has been linked to mortality events in some bufonid species

(Muths *et al.*, 2003) including two other *Atelopus* species (Lips, 1998; Berger *et al.*, 1998). The absence of infected specimens found dead at the time of collection constitutes an important gap in linking the presence of *B. dendrobatidis* with declines (Daszak *et al.*, 1999; McCallum, 2005). However, some of the pathological evidence found in this study appears consistent with fatal chytridiomycosis. Some lesions observed showed a pathology consistent with severe disease (Berger *et al.*, 1998; Pessier *et al.*, 1999). Although it is not possible to determine whether the disease would have progressed and eventually caused mortality in these animals, this evidence shows that some *Atelopus* frogs had clinical signs of the disease prior to the declines of three of these species.

The spatial distribution of prevalence of chytridiomycosis in *Atelopus* species suggests that the increase in prevalence detected during 1988 was not local, but occurred simultaneously in populations separated by more than 60 km in different river basins. The synchronization of epizootics in distant locations can occur if the pathogen spreads rapidly through naïve populations upon its introduction, or if epidemics are driven by regional climatic changes, as suggested by the climate-linked epidemic hypothesis. Severe droughts could drive epidemic outbreaks of chytridiomycosis by increasing the transmission rates of the *B. dendrobatidis* or predisposing the host populations to heightened impact of pathogens (Daszak *et al.*, 2003). Most amphibians control the evaporative water loss by restricting their activities to habitats in which dehydration may be avoided easily (Shoemaker *et al.*, 1992). A contraction in the availability of humid microhabitats as a result of prolonged dry periods could lead to population crowding where transmission rates of *B. dendrobatidis* may increase above the required threshold for an epidemic. Dry periods can also act directly as a stressor in anurans; individuals exposed to dehydration may suffer from hyperosmotic stress that could affect the function of all organ systems, especially the cardiovascular (Shoemaker *et al.*, 1992). Local climatic data indicated that one of the most severe dry seasons recorded in the Cordillera de Mérida between 1972 and 1991 occurred during 1988, coincident with our observed high prevalence of *B. dendrobatidis* in Andean *Atelopus* species. The extinction of *A. ignescens* also coincided with warm and dry periods although no evidence of *B. dendrobatidis* was found in this species (Ron *et al.*, 2003). In the absence of evidence of other epidemic events, it is impossible to draw inference about drought and chytridiomycosis from these data. Climatic data from other regions where chytridiomycosis has been reported is needed to test whether droughts have played a role in driving epidemic outbreaks of this disease.

The rediscovery of one *A. mucubajensis* (Barrio-Amorós, 2004) and two populations of *A. cruciger* (Eliot, 2003), after many years of intensive search, is heartening. Despite the fact that *A. carbonerensis*, *A. oxyrhynchus* and *A. soriano* have not been sighted in

the last ten years, we do not consider them to be extinct. The possibility of finding remnant populations of these andean *Atelopus* species remains open, but search effort must be intensified.

The data presented in this paper highlight the need to understand the role of climate as a cause of amphibian declines, whether this is directly (e.g. Daszak *et al.*, 2005), or via changes in the dynamics of a host-pathogen system (e.g. Pounds *et al.*, 2006). Finally, our study shows that a comprehensive understanding of how climatic events interact with chytridiomycosis epidemics will be fundamental to designing effective conservation programs to ensure the long term persistence of *Atelopus* species.

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SHORT NOTES

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**BIOLOGY OF THE BLINDSNAKE
TYPHLOPS BRONGERSMIANUS
 (TYPHLOPIDAE) IN A
 SEMIDECIDUOUS FOREST FROM
 CENTRAL BRAZIL**

ROBSON W. AVILA, VANDA L. FERREIRA AND
 VANESSA B. SOUZA

*Departamento de Ciências do Ambiente, Laboratório de
 Zoologia, Campus de Corumbá, Universidade Federal de
 Mato Grosso do Sul, Brazil*

The biology of blindsnakes is virtually unknown. Herein, we present data on the biology of the blindsnake *Typhlops brongersmianus* from a semideciduous forest in Central Brazil. Males had longer tails and matured at smaller sizes than did conspecific females. Reproduction was highly seasonal, with clutch size of 4-5 eggs and oviposition in the early wet season. *T. brongersmianus* fed mainly on the pupae and larvae of ants, with ingestion of large number of prey items. Our findings agree with other studies of diet and reproduction of scolecophidians from other parts of the world.

Key words: diet, Neotropics, reproduction, snake

Although scolecophidian snakes are diverse (up to 300 species) and abundant throughout most of the world's land masses, they have attracted far less scientific attention than have the larger and more spectacular henophidian and cenophidian species (Shine & Webb, 1990). However, understanding their biology is crucial for interpreting the overall evolution of squamates, particularly the distinctive ecological attributes of higher snakes, because they represent the base of ophidian phylogeny (Greene, 1997; Webb *et al.*, 2000; Webb *et al.*, 2001).

Among the three Scolecophidia families, Typhlopidae is the more diverse, with 215 species confined to warmer parts of the world, with more than 70% belonging of the genus *Typhlops* (Greene, 1997). Despite their high diversity, the knowledge of the blindsnakes genus *Typhlops* is based entirely on African species (Webb *et al.*, 2001). In South America, *Typhlops brongersmianus* Vanzolini (1972) is the most widespread species, and occurs from Uruguay to Venezuela (Lema, 1982; Lema, 1987). However, virtually nothing is known about their biology.

In Brazilian's Pantanal, *T. brongersmianus* is relatively abundant and occurs in elevated parts of the region, including deforested areas (Strüssmann & Sazima, 1993). In this paper, we describe some ecological aspects of *T. brongersmianus* from a semideciduous forest in Pantanal, Central Brazil.

The study was conducted in Santa Cruz Hill (19°24'49" S, 57°22'47" W), Urucum massif in a semideciduous forest, Municipality of Corumbá, Mato Grosso do Sul State, Brazil. The region is characterized by Aw climate (Köppen classification), with an average temperature of 25°C and a mean precipitation of 1483 mm. The climate is highly seasonal with two well defined seasons: dry (April-September) and rainy (October-March) (Brasil, 1992).

Snakes were caught monthly in pitfall traps with drift-fences (three sampling units with 24 buckets) from July 2000 to December 2002, humanely euthanased, fixed in 10% formalin, preserved in 70%GL ethanol and deposited in Zoological Reference Collection, Laboratory of Zoology, Campus of Corumbá, Universidade Federal de Mato Grosso do Sul (UFMS), under the acronym CEUCH.

For each *T. brongersmianus* captured we recorded the following morphometric variables in mm: snout-vent length (SVL), head length (HL), head width (HW) and tail length (TL). To evaluate the sexual size dimorphism we utilized a Mann-Whitney Z test for SVL and an ANCOVA for HL, HW and TL, with SVL as covariate.

Males were considered reproductively active if they possessed enlarged testes or opaque efferent ducts. We utilized only the length and width of the right testicle for analysis, because testicular volume of both sides was not different (t -test=0.72, df=22, $P=0.479$). Females were considered reproductively active if they possessed vitellogenic follicles or oviductal eggs, and the simultaneous presence of both follicles and eggs was considered evidence of production of more than one clutch per season.

We dissected each specimen and analyzed the stomach contents. Prey items were identified to order and the number of each life cycle stage (larvae, pupae, adults) was recorded. We measured prey length and width and calculated the volume of prey items using the formula for a prolate spheroid.

All statistical tests appear with alpha set at 0.05 and, throughout the text means are followed by ± 1 SD.

A total of 43 individuals (18 females and 24 males) were examined. The majority of the specimens were captured from September to December (81.4%), which corresponds to the end of the dry season until the middle of the rainy season.

Adult females reached 275 mm SVL (238 $\pm$ 20.77 mm, $n=11$) and males 262 mm SVL (231.32 $\pm$ 22.49 mm, $n=22$), but there was no significant sex difference in SVL. However, adult males had longer tails than adult females of the same body length (Table 1). Males

Correspondence: R. W. Ávila. Departamento de Ciências do Ambiente, Laboratório de Zoologia, Campus de Corumbá, Universidade Federal de Mato Grosso do Sul. Avenida Rio Branco, 1270. Caixa Postal 252. CEP 79301-970. Corumbá, Mato Grosso do Sul, Brazil. E-mail: robsonavila@gmail.com

TABLE 1. Descriptive statistics of morphometric variables and analysis results of sexual size dimorphism in *T. brongersmianus* from Central Brazil. Values shows means $\pm$ standard deviations.

	Males	(n=22)	Females (n=11)	Results
SVL	227.18	± 24.83	241.91	± 20.88 $U=159.50, P=0.14$
HL	8.41	± 0.69	8.75	± 0.87 $F_{1,42}=0.37, P=0.546$
HW	5.72	± 0.74	6.01	± 0.61 $F_{1,42}=1.63, P=0.211$
TL	6.50	± 0.88	6.23	± 1.58 $F_{1,42}=8.803, P<0.001$

and females attained sexual maturity at 180 mm SVL and 211 mm SVL respectively.

Reproduction of *T. brongersmianus* was highly seasonal and began during the late dry season. Females commenced vitellogenesis in September (one female, probably initiated in July-August) and contained eggs in October (early wet season). Clutch sizes of four females averaged 4.67 ± 0.58 eggs (range 4-5). The size of four well developed eggs was 8.27 ± 2.46 mm long and 4.11 ± 0.85 mm wide, with a mean volume of 78.05 ± 48.09 mm³. We did not register the simultaneous occurrence of both eggs and follicles. Males collected in August-September had large testes, whereas males collected during July and October-December had small testes. Testes volume was significantly greater In August-September than in other months ($t=3.032$, $df=9$, $P=0.014$).

Stomachs of *T. brongersmianus* ($n=14$) contained various stages of ant development (of two unidentified species), mostly larvae and pupae in volume (90.84%, see Table 2). The mean number of prey per stomach was 15.82 ± 13.27 (range 1-49). The average size of prey was 4.18 ± 1.86 mm long and 1.55 ± 0.57 mm wide, while the mean prey volume was 7.56 ± 10.90 mm³ ($n=209$ prey items).

The seasonal reproduction of *T. brongersmianus*, with reproduction in the wet season, is very similar to others species of *Typhlops*, such as *T. bibronii* (Webb *et al.*, 2001), as well as others typhlopids (Webb *et al.*, 2001; Shine & Webb, 1990) and leptotyphlopids (Webb *et al.*, 2000). Moreover, this features fits with the general pattern shown by most Australian (Shine, 1985) and South American snakes (Fitch, 1970; Vitt & Vangilder, 1983). This can be due to seasonal variation in resource levels, hatchling survival rates and/or the costs of reproduction (Shine, 2003). This reproductive cycle may also explain the seasonality of captures of *T. brongersmianus* (most snakes were captured during the rainy season); however, food availability, tolerance to

climatic conditions and phylogenetic constraints could also be responsible for the observed patterns (Marques *et al.* 2001).

Previous scientific reports of the biology of *T. brongersmianus* stated that this species had an insectivorous diet, but did not provide any qualitative or quantitative data (Strüssmann & Sazima 1993). Strüssmann (1992) found ant pupae and adult termites in stomachs of specimens from the northern part of the Pantanal. The absence of termites in diet of *T. brongersmianus* in southern Pantanal possibly reflects the prey availability in the two regions. Overall, the diet of *T. brongersmianus* found in the present study is similar to that of many other scolecophidian species (Webb & Shine, 1993; Shine & Webb, 1990; Greene, 1997). The infrequent ingestion of huge numbers of tiny prey is considered the most distinctive characteristic of the scolecophidians (Webb & Shine, 1993) and evolved to minimize the time spent inside ant nests and, thus, to reduce the risk of prey-inflicted injuries (Webb *et al.*, 2001). Most typhlopids studied to date feed on invertebrates and appear to locate these prey items by following pheromonal trails (Watkins *et al.*, 1969; Webb & Shine, 1992).

In conclusion, our study shows that the diet and reproductive habits of *T. brongersmianus* are remarkably similar to those of typhlopids snakes from other parts of the world. Nonetheless, the biology of most South American blindsnakes species is virtually unknown, and future studies of this poorly studied snake fauna are necessary for elucidating general patterns in the ecology of blind snakes.

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TABLE 2. Ant life cycle stages in diet of *Typhlops brongersmianus* ($n=14$) from semideciduous forest from Central Brazil. Frequency= proportion of snakes that contained each prey type.

Prey	Frequency (%)	Number	% Num	Volume	% Vol
Adults	28.57	11	5.26	26.01	2.86
Eggs	42.86	61	29.19	100.95	6.29
Larvae	85.71	85	40.67	682.30	42.53
Pupae	42.86	52	24.88	775.01	48.31
Total		209		1604.27	

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