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FRONT COVER: Male *Mannophryne trinitatus* carrying larvae (S. Watt)

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INTER-POPULATION VARIATION IN LIFE-HISTORY TRAITS OF A CHINESE LIZARD (*TAKYDROMUS SEPTENTRIONALIS*, LACERTIDAE)

WEI-GUO DU[1], XIANG JI[1] AND YONG-PU ZHANG[2]

[1]*Hangzhou Key Laboratory for Animal Sciences, School of Life Sciences, Hangzhou Normal College, Zhejiang, People's Republic of China*

[2]*School of Life and Environmental Sciences, Wenzhou Normal College, Wenzhou, Zhejiang, People's Republic of China*

Detecting inter-population differences in life-history traits is the first step in exploring the proximate and ultimate causes of such variation. We measured maternal body size and reproductive output of the lacertid lizard *Takydromus septentrionalis* from two island populations in eastern China to quantify inter-population variation. We captured female *T. septentrionalis* from the field and conducted a "common garden" experiment in the laboratory to measure their reproductive output. The study revealed major divergences in female body sizes, clutch mass and egg mass, but no significant difference in these traits was found between the first clutch and the later clutches. This suggests that the inter-population divergences persisted when the same groups of females were maintained in identical conditions in captivity. In contrast, there were no inter-population differences in size-adjusted fecundities, clutch size and relative clutch masses. Therefore, maternal body size plays an important role in determining female reproductive output in this species, but it does not account for all variation in reproductive traits. The egg size is less variable than the clutch size in each population, which gives support to the optimal egg size theory.

Key words: body size, fecundity, inter-population variation, offspring size, reproductive output

INTRODUCTION

Life-history traits are directly related to organismal fitness and hence are major targets of natural selection. Species differ substantially in life-history traits, reflecting both genetic and environmental effects. Even geographically separate populations of a single species can evolve different life histories depending on local ecological conditions (Roff, 2002). As ectotherms, squamates are highly dependent upon climatic conditions and have thus attracted considerable research on the contribution of the environment to life-history variation (Dunham *et al.*, 1988; Adolph & Porter, 1993; Niewiarowski, 1994; Angilletta *et al.*, 2004). Whereas earlier studies on this topic focused on interspecific variation in life histories (e.g. Tinkle *et al.*, 1970; Dunham *et al.*, 1988), inter-population variation in life histories has been emphasized more recently (e.g. Forsman & Shine, 1995; Niewiarowski *et al.*, 2004). The inter-population comparison may lend considerable insight toward our understanding of genetic and environmental causes of life-history variation and the evolution of life histories (Niewiarowski, 1994; Angilletta *et al.*, 2004; Niewiarowski *et al.*, 2004). To achieve this end, we need a broad collection of data sets describing inter-population variation in life histories of squamates. However, such studies mainly focus on North American and European species (e.g. Dunham *et al.*, 1988; Castilla & Bauwens, 1989; Niewiarowski, 1994), whereas the information on Asian taxa is quite limited (but see Hasegawa, 1994). Therefore, life-history data on Asian species, even descriptive data, should be very useful in completely understanding life-history evolution in squamates.

For inter-population studies of life histories, while the geographic pattern of life-history variation such as latitudinal and altitudinal variations has attracted a great number of studies (Ballinger, 1983; Dunham *et al.*, 1988; James & Shine, 1988; Grant & Dunham, 1990), islands have been of special interest to ecologists because of the rapid adaptive shifts possible in island taxa with small and discrete populations, living under different conditions and selective pressures (Case, 1982). Compared with parallel studies on geographically separated populations in the continent – for example, elevational comparisons (e.g. Ballinger, 1977; Grant & Dunham, 1990) – inter-island comparisons can reveal microgeographic variation in life histories for populations with relatively low gene flow or migration among them. Several authors have quantified inter-island variation in morphology and some ecological traits of snakes and lizards (e.g. Shine, 1987; Case & Schwaner, 1993; Hasegawa, 1994; King, 1997; Thorpe & Malhotra, 1998); these studies suggested that the inter-island variation might stem from both genetic and environmental factors.

The northern grass lizard *Takydromus septentrionalis* is a small (up to 80 mm snout–vent length [SVL]) slender-bodied, long-tailed (up to 270 mm) lacertid. Among the 16 or 17 species of grass lizards from the genus *Takydromus* in the oriental and palearctic regions (Arnold, 1997), *T. septentrionalis* is a later evolved species (Lin *et al.*, 2002), and is mainly distributed over a large area of eastern and northern

Correspondence: Wei-Guo Du, Hangzhou Key Laboratory for Animal Sciences, School of Life Sciences, Hangzhou Normal College, 310036, Hangzhou, Zhejiang, People's Republic of China. *E-mail*: duweiguo@mail.hz.zj.cn

TABLE 1. Inter-population variation in seasonal reproductive output of the northern grass lizard, *Takydromus septentrionalis*. One-way ANOVA as well as ANCOVA with maternal SVL as a covariate were used to detect between-island differences in reproductive traits. Symbols immediately after *F* values represent significant level: NS=non-significant, ** = $P<0.01$.

	Beiji island (n=40)		Dongtou island (n=27)			
	Mean ± SE	Adjusted mean±SE	Mean±SE	Adjusted mean±SE	ANOVA $F_{1,74}$	ANCOVA $F_{1,73}$
Number of clutches	2.0±0.2	1.9±0.2	1.9±0.2	1.9±0.2	0.46^{NS}	0.004^{NS}
Seasonal fecundity	5.2±0.4	4.9±0.4	4.7±0.5	5.1±0.5	0.71^{NS}	0.05^{NS}
Seasonal total egg mass (g)	1.60±0.11	1.49±0.11	1.14±0.13	1.24±0.13	6.42^{**}	0.19^{NS}

China (Zhao & Adler, 1993). These insectivorous lizards are primarily diurnal and terrestrial; females produce clutches of 1–5 elongate eggs from April to July (Ji *et al.*, 1998; Du, 2003). *T. septentrionalis* has relatively small energy reserves and therefore the energy to produce a clutch of eggs mainly come from the current food intake (Du et al., 2003). The lizards are locally abundant on two offshore islands, Dongtou and Beiji, 13 km apart off the eastern coast of China. These two islands have similar annual average air temperature and annual total precipitation (Zhejiang Bureau of Meteorology). Microhabitats for lizards are grassy areas. However, the grassy habitat is partly covered by pine trees (*Pinus massoniana*) on Dongtou island, but is quite open on Beiji island. Therefore, this system provides us with an excellent model to explore life-history variation in lizards between islands with similar climate conditions but different microhabitat features. Here we investigate life-history characteristics of adult northern grass lizards from the two islands. To determine whether or not local populations diverged from each other in these life-history traits, we recorded body size of the lizards and conducted a "common-garden" experiment to detect their reproductive traits.

MATERIALS AND METHODS

On 10 April 1999, we collected 117 adult *Takydromus septentrionalis* (76 females and 41 males) from two offshore islands, Beiji island (27°35' N, 120°10' E) and Dongtou island (27°50' N, 121°28' E), in Zhejiang province, eastern China. All animals were caught by hand or noose and transported to Hangzhou Normal College. Immediately after arrival, the animals were weighed (±0.001g), measured SVL (±0.01mm) and individually marked (toe-clipped). The lizards were randomly allocated to terraria (60×40×30cm, each containing 9-10 females plus 5-6 males) with sand and grass to mimic natural habitats where these lizards are found. A 60W light bulb suspended 15 cm above the floor provided opportunities for behavioural thermoregulation from 0700 h to 1700 h. Food (larvae of *Tenebrio molitor*) and water (containing mixed vitamins and minerals) were provided ad libitum. We palpated the abdomens of each female every five days, and any animal with oviductal eggs was transferred to a small glass terrarium (20×15×20 cm) filled with 2 cm-deep moist sand. Each small terrarium was checked at least three times a day for freshly laid eggs. All eggs were weighed (± 0.001g) promptly so as to minimize potential changes in mass due to water exchange. Postpartum females were returned to their original terraria. The experiment was carried out between 10 April and 10 July.

We calculated relative clutch mass (RCM) as the ratio of clutch mass to maternal postoviposition mass (Shine, 1980). The difference in RCM between populations was tested using an analysis of covariance with clutch mass as the variable and body mass as the covariate. Linear regression was used to detect the relationship between maternal body size and reproductive traits. To detect divergence between populations and among clutches, we conducted analyses of variance (ANOVA) for reproductive traits that were independent of maternal SVL and analysis of covariance (ANCOVA) for variables correlated with maternal SVL.

RESULTS

TIMING OF OVIPOSITION AND TOTAL SEASONAL FECUNDITY

Females from the Beiji population laid their eggs after being captured for 27.2±1.4 days (n=49), which was earlier than for Dongtou females (38.0±2.5, n=27; $F_{1,74}$=16.04, $P<0.001$). Total seasonal reproductive output of Beiji females was higher than that of Dongtou females in terms of total egg mass, but no differences in number of clutches or seasonal fecundity were found between the two populations (Table 1). Because larger females produced more eggs, and a greater total egg mass (SVL vs total seasonal fecundity, r^2=0.084, $F_{1,74}$=6.82, $P<0.01$; vs total seasonal clutch mass, r^2=0.135, $F_{1,74}$=11.59, $P<0.01$), we then reanalysed inter-population variation in fecundity after including maternal SVL as a covariate. This analysis indicated that total clutch size and total seasonal clutch mass at the mean SVL of 66.68 mm did not differ significantly between the two island populations (Table 1).

FEMALE BODY SIZE AND RELATIVE CLUTCH MASS

Female body size at maturity differed significantly between the two populations. The SVLs of minimal re-

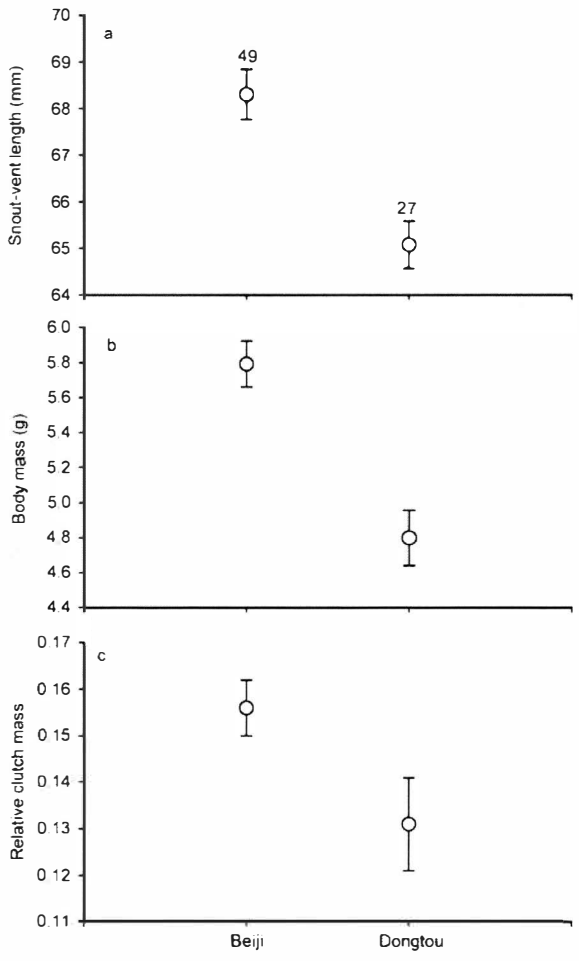

FIG. 1. Variation in maternal body size (A), mass (B) and relative clutch mass (C) in the northern grass lizard *Takydromus septentrionalis* between Beiji and Dongtou island populations. Graphs show mean values and associated standard errors. Numbers above the error bars in the upper graph are sample sizes, and apply to all graphs within this figure.

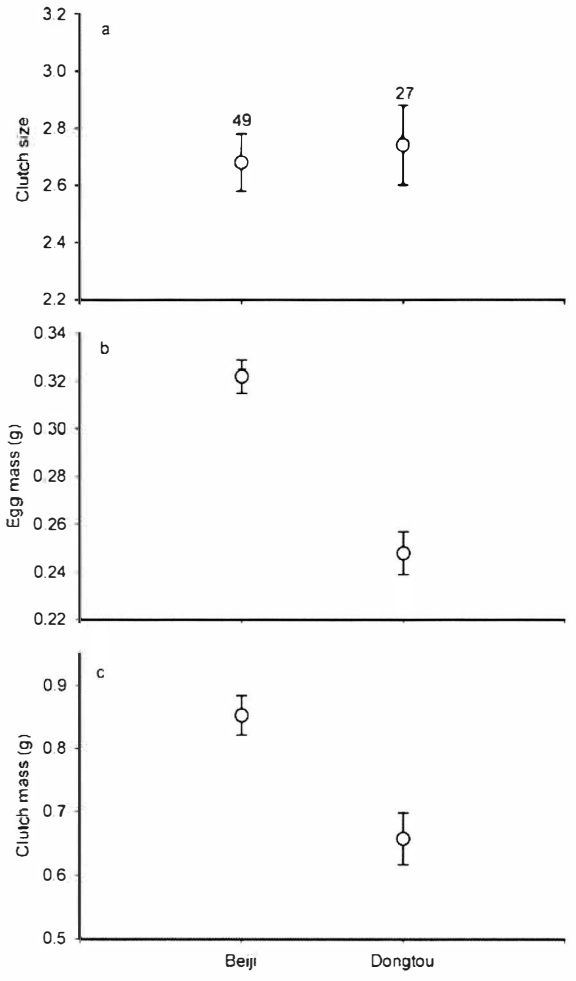

FIG. 2. Variation in clutch size (A), egg mass (B) and clutch mass (C) of the northern grass lizard *Takydromus septentrionalis* between Beiji and Dongtou island populations. Analyses of covariance were performed to detect the inter-population variation. Maternal snout–vent length was used as the covariate, which was set at 66.68 mm. Graphs show adjusted mean values and associated standard errors. Numbers above the error bars in the upper graph are sample sizes, and apply to all graphs within this Figure.

productive females on Beiji and Dongtou islands were 57.5 mm and 54.5 mm, respectively. Sexually mature females from the Beiji population were larger than those of the Dongtou population both for mean SVLs ($F_{1,74}$=15.38, P<0.001; Fig. 1A) and body masses ($F_{1,74}$=22.15, P<0.001; Fig. 1B). Relative clutch mass (RCM) also varied between populations, with the RCM of Beiji females being higher than that of Dongtou females ($F_{1,73}$=10.74, P<0.01; Fig. 1C).

CLUTCH SIZE, CLUTCH MASS AND EGG MASS

Clutch size, clutch mass and egg mass were positively correlated with female SVL (clutch size, r^2=0.347, $F_{1,74}$=10.13, P<0.01; clutch mass, r^2=0.529, $F_{1,74}$=28.80, P<0.00001; egg mass, r^2=0.114, $F_{1,74}$=9.52, P<0.01). We thus used two-way ANCOVA with SVL as a covariate to detect variations in reproductive traits arising from inter-population and clutch effects. The analysis indicated that there was a significant difference in reproductive traits between the two populations ($F_{3,111}$=18.77, P<0.00001), but not between the clutches ($F_{3,111}$=1.04, P=0.38). To identify the source of inter-population differences in reproductive traits, we further performed individual analyses of covariance on the three traits. Whereas females from the two populations produced clutches with similar numbers of eggs ($F_{1,73}$=0.11, P=0.75; Fig. 2A), females from Beiji island produced larger eggs ($F_{1,73}$=43.01, P<0.00001; Fig. 2B) and thereby heavier clutch mass ($F_{1,73}$=12.92, P<0.001; Fig. 2C) than did those from Dongtou island. Because the effects of maternal body size have been removed using ANCOVA in our analysis, the significant inter-population difference in egg size was not entirely attributable to maternal body-size variation, though egg mass was highly correlated with maternal SVL. The coefficients of variation of egg size for the Beiji and Dongtou populations were 12.5% and 16.0%, respectively, which was less variable than clutch size in both

populations (26.4% for the Beiji population and 26.2% for the Dongtou population).

DISCUSSION

As reported previously for other reptile species (e.g. *Eumeces okadae*, Hasegawa, 1994), the northern grass lizard showed significant inter-island variation in a wide range of life-history traits. These variations were correlated with maternal body size both between and within populations; such correlation is common in lizards (Fitch, 1985; Dunham *et al.*, 1988; James & Shine, 1988). Nonetheless, inter-island variation in reproductive traits per clutch was not entirely attributable to maternal body-size differences: life-history traits varied significantly among islands even after the effect of differing maternal body sizes was removed from the analysis. The current study found significant inter-island differences in life histories, but, as a descriptive study, there were not enough data to elucidate the ultimate and proximate causes of these variations in life histories. To further clarify the causes for the significant inter-island variation in reproductive traits of *T. septentrionalis,* we would need to take account of the between-island differences in both genetic origins and environmental factors such as food availability, predator pressure and population density. Ideally, reciprocal transplant experiments in the field could identify the respective effects of these factors on the reproductive traits of *T. septentrionalis* (Niewiarowski & Roosenburg, 1993).

Given that variation in adult body size accounted for much of the inter-population divergence in life-history traits of *T. septentrionalis*, we need to consider the factors that influence adult body sizes so as to completely understand life-history variation within this species. Both genetic and environmental factors could affect growth rates and ages at sexual maturity (Reznick & Bryga, 1987; Sinervo and Adolph, 1989; Smith *et al.*, 1994), and in turn be responsible for such inter-population variation in body size. For ectotherms, environmental influence plays an important role in determining body size. Such environmental factors include temperature (Ashton and Feldman, 2003; Angilletta *et al.*, 2004), prey availability and size (Case, 1978; McLaughlin & Roughgarden, 1989; Wellborn, 1995), the intensity of predation (Case, 1982) and demography (King, 1989). As predicted by Case (1982), *E. okadae* from the Izu islands attained larger body sizes on islands with low predation pressure than on those with high predation pressure (Hasegawa, 1994). Unfortunately, because of the absence of data on environmental and ecological parameters, we are currently not able to test these ideas in the island populations of *T. septentrionalis*.

The determinants of egg size reflect selective processes such as the trade-off between clutch size and offspring size, as well as proximate constraints including functional and energetic limitation (Fox & Czesak, 2000; Sinervo *et al.*, 2000). Our study indicated that egg size has a relatively low level of variation compared with that of fecundity in both populations. This greater constancy in egg size than in clutch size accords well with optimality models (Smith & Fretwell, 1974). The difference in egg size existed between the two populations after the effects of maternal body size were removed from the analysis, and persisted throughout all clutches after the females had been kept in an identical laboratory environment for a long period. This result suggests either that the difference in egg size is coded genetically or that it is influenced by events (food supply, temperature, etc.) early in a female's life and is thereafter resistant to change.

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CALLING SITES AND ACOUSTIC PARTITIONING IN SPECIES OF THE *HYLA NANA* AND *RUBICUNDULA* GROUPS (ANURA, HYLIDAE)

ITAMAR A. MARTINS[1], SILVIO C. ALMEIDA[2] AND JORGE JIM[2]

[1]*Universidade de Taubaté, UNITAU, Departamento de Biologia, Lab. de Zoologia–IBB, Taubaté, SP, Brazil*

[2]*Universidade Estadual Paulista–UNESP–IB, Departamento de Zoologia, Botucatu, SP, Brazil*

We analysed spatial and acoustic partitioning among four species of *Hyla* belonging to two species-groups: *nana* (*H. nana* and *H. sanborni*) and *rubicundula* (*H. elianeae* and *H. jimi*). Field activities were conducted at three permanent ponds, from 1998 through 2001. Four attributes of the calling sites were analysed: perch height, distance of the perch from the edge of the pond, type of perch (vegetation) and the individual's position on the perch. There was extensive overlap in the four calling-site variables analysed. However, we found spatial segregation did occur in calling site height and the distance of perches from pond edges. Bioacoustic analyses revealed behavioural differences among species in calling activity, both time of onset and peak calling in chorus. There was acoustic partitioning among species the fundamental frequency of the advertisement calls, principally as a function of the temporal structure (e.g. note duration, rate of note repetition, duration and rate of repetition of the calling pulses). We propose that differences in physical attributes of calling site and in characteristics of calls allow these species to exist in sympatry.

Key words: acoustic communication, calling site, niche breadth, treefrogs

INTRODUCTION

The study of closely related, sympatric species is of special interest in understanding the factors that influence mate recognition systems and the evolution of reproductive isolation. The observation that some ecologically similar and phylogenetically related species can coexist has typically been explained by reduction of possibilities for interspecific competition (Duellman, 1978; Rossa-Feres & Jim, 2001). For anuran amphibians, calling site occupancy and attributes, location of oviposition site and foraging area have been shown to be fundamentally important for resource partitioning (Crump, 1974; Cardoso *et al.*, 1989; Rossa-Feres & Jim, 2001).

Studies of anuran communities have established that breeding site location and the physical structure of the advertisement call are the most important factors in species segregation within a single locale (Duellman & Pyles, 1983; Cardoso *et al.*, 1989; Cardoso & Vielliard, 1990; Martins & Jim, 2003). Segregation of calling sites by synchronopatric species of anurans has been reported by several workers (Crump, 1974; Hödl, 1977; Duellman & Pyles, 1983; Heyer *et al.*, 1990; Rossa-Feres & Jim, 2001), and may act as a mechanism of reproductive isolation and allow coexistence of several species in the same environment.

Similarly, partitioning of the acoustic space, achieved by differences in the spectral and temporal attributes of male advertisement calls, is of great importance during the breeding season (Hödl, 1977; Duellman & Pyles, 1983; Márquez *et al.*, 1993; Grafe, 1996; Grafe *et al.*, 2000). Differences in species-specific male calls, coupled with female ability to perceive such differences, is the main mechanism of reproductive isolation among sympatric species of anurans (Hödl, 1977). However, closely related species can emit similar calls, and the specificity of the signal must arise from combined spectral and temporal parameters coupled with differences in behaviour (Cardoso & Vielliard, 1990; Martins & Jim, 2003). Thus, in species that call in choruses in the same environment, acoustic interference among species may be reduced by using different frequency bands, as well as through synchronization of the call temporal parameters, thus avoiding overlap between individuals of different species (Littlejohn, 1977).

Our study examined the calling site characteristics and the acoustic characteristics of the advertisement call of four species of *Hyla* belonging to the *nana* group (*H. nana* and *H. sanborni*) and the *rubicundula* group (*H. elianeae* and *H. jimi*). These closely allied species share several phenotypic and ecological characteristics: body size, breeding season, types and patterns of calls and strategy of occupying their environment. Therefore they comprise a good system for studying the factors implicated in the coexistence of sympatric species.

METHODS

STUDY SITES

Field activities and recordings were carried out in three permanent ponds in open areas in Botucatu, São Paulo State, Brazil.

Environment I. A large pond, 120 × 50 m, its edges covered predominantly by herbaceous vegetation (Poaceae and Cyperaceae). Location 22°53' S and 48°29' W, altitude 860 m.

Correspondence: I. A. Martins, Universidade de Taubaté, UNITAU. Departamento de Biologia, Lab. de Zoologia–IBB, 12030-180, Taubaté, SP, Brazil. *E-mail*: istama@uol.com.br

Environment II. A large pond, 100 × 70 m, a headwater marsh in *cerrado* (savanna) vegetation. The shoreline vegetation consists of herbaceous plants (Poaceae and Cyperaceae) and bushes (Asteraceae and Melastomataceae). Location 22°57' S and 48°27' W, altitude 810 m.

Environment III. A small pond, 50 × 20 m, with herbaceous vegetation (Poaceae) along the shore, and dense emergent vegetation (Cyperaceae). Location 22°50' S and 48°25' W, altitude 780 m.

CALLING SITE

Field observations were carried out throughout the breeding season (August through March), during a four-year period from 1998 to 2001. During the breeding season of each year, ponds were visited weekly, beginning at sunset (1700 hrs) and ending when the activity of the species decreased (2400 to 0200 hrs).

To characterize the calling sites, each pond was traversed along its perimeter. For each individual found, the type and height of the perch, the location of the perch in relation to the distance from the pond edge (outer – perches on land outside of the pond margin – and inner – typically calling from emergent or floating mats of vegetation within ponds), and the individual's position (parallel or perpendicular) in relation to the perch were recorded.

The degree of overlap in the variables of the calling sites was calculated using the Morisita–Horn (C_H) similarity index (Krebs, 1989) for the frequency data by category. Multidimensional overlap was determined by considering all variables simultaneously. We considered the species as highly overlapping when the C_H value was above 0.70, as partly overlapping when the C_H was between 0.50 and 0.70, and as non-overlapping when the C_H was lower than 0.50.

The niche breadth for the calling site variables (type and height of the perch, distance from the pond edge and position in relation to the perch) was calculated by Levin's index (Krebs, 1989): $B=Y^2/SN_j^2$, where B=Levin's measure of niche amplitude, Y=total individuals sampled and N_j=number of individuals found using the resource j. We considered as generalists those species for which the values of B were higher than 2.36 for at least two calling site variables (Rossa-Feres & Jim, 2001).

To compare interspecific characteristics of occupation and height of the perch and of the distance of the perch in relation to the pond edge (outer and inner), the non-parametric Kruskal-Wallis test was used, since data deviated significantly from a normal distribution (Kolmogorov-Smirnov test).

BIOACOUSTIC ANALYSIS

The times when calling activity began and when it peaked (chorus) were recorded, noting: (1) individuals during the first 30 minutes after the beginning of calling activity (initial call – between 1800 and 1830 hrs), and (2) individuals calling in chorus, at a mean of two hours after beginning their calls. To study intra- and interspecific interactions, individuals at the beginning of their calling activity and during chorus calling were compared, and possible differences in both behaviour and spectral and temporal structure were examined.

The calls were recorded under field conditions with a digital (DAT) recorder (Sony TCD-D8) and an analogue cassette recorder (Sony TCM-S64V) coupled to external semidirectional (ME 66) or cardioid (ME 64) Sennheiser microphones. All recorded calls were edited with a sampling rate of 44,100 Hz and 16 bits per sample in the mono pattern. The bioacoustic analyses were performed on a microcomputer using the program CoolEdit 96 (Syntryllium Software Corporation), with a 20,000 Hz sampling frequency. The 256 points option (Fast Fourier Transform, FFT) and, when necessary, the 1024 points option were used, mainly in determining fundamental frequencies.

Six traits of the advertisement calls of the species were quantified: frequency band width, fundamental frequency (=dominant frequency), note duration, note repetition rate, pulse duration and pulse repetition rate. For the analyses and bioacoustic interpretations, the terms used follow Martins & Jim (2003).

The spectral and temporal intra- and interspecific traits of the advertisement call of the four species of *Hyla*, initial calling and chorus calling, were compared statistically using analysis of variance (ANOVA) to test for significant differences in means of pairs of species, and afterwards complemented by the Student-Newman-Keuls test (BioEstat 3.0; Ayres *et al.*, 2003). For analysis of the correlation between the air temperature and rate of call repetition, Spearman's correlation coefficient (r_s) was used, with a 5% significance level (Zar, 1999).

RESULTS

CALLING SITE

Two groups of three or four species occurred in sympatry: in environments I and III, *H. nana*, *H. sanborni* and *H. elianeae*; and in environment II, *H. nana*, *H. sanborni*, *H. elianeae* and *H. jimi*.

Males of *H. nana* called from lower heights, mean height 30 cm (70%, n=330; Fig. 1), and occupied perches at the inner edges of the ponds (Fig. 2). This species showed a preference for calling in emergent vegetation (71%, n=291; Fig. 3), on leaves and stems, with the body situated parallel to the perch (56.5%, n=122).

Most *H. sanborni* were calling at perch heights between 20 and 60 cm (73%, n=116). The males perched on leaves of herbaceous and emergent vegetation, with the body perpendicular to the perch (69.47%, n=66) and on the edge (Figs. 1–3).

Mean perch height differed significantly between the species of the *nana* group (Kruskal-Wallis, H=77.0; P<0.05): males of *H. nana* called at a mean height of 27.04±15.89 cm (n=471), and *H. sanborni* at 43.77±18.83 cm (n=130).

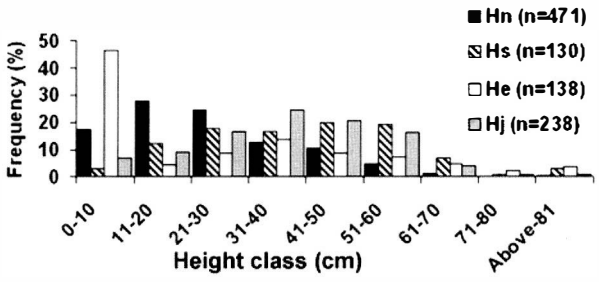

FIG.1. Frequency distribution of the perch heights of *Hyla nana* (Hn), *Hyla sanborni* (Hs), *Hyla elianeae* (He) and *Hyla jimi* (Hj), near Botucatu, São Paulo.

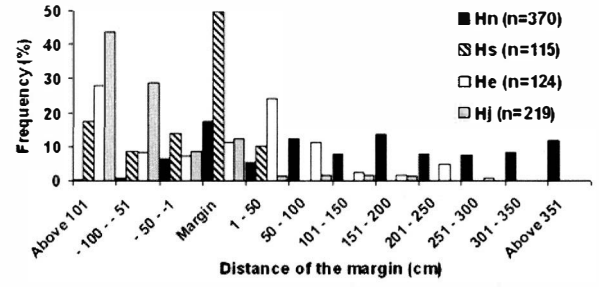

FIG. 2. Distribution of frequencies of occupation of the calling site in relation to distance from the outer and inner edges of the pond, among *Hyla nana* (Hn), *Hyla sanborni* (Hs), *Hyla elianeae* (He) and *Hyla jimi* (Hj), near Botucatu, São Paulo.

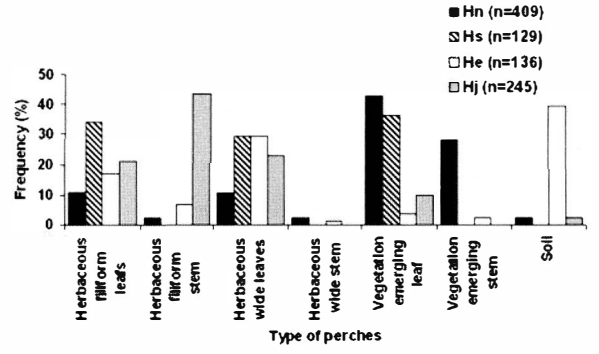

FIG. 3. Relative frequency of the types of perch used as the calling site among *Hyla nana* (Hn), *Hyla sanborni* (Hs), *Hyla elianeae* (He) and *Hyla jimi* (Hj), near Botucatu, São Paulo.

TABLE 1. Niche amplitude, calculated by Levin's index, for the four variables of the calling sites of *Hyla nana* (*Hn*), *Hyla sanborni* (*Hs*), *Hyla elianeae* (*He*) and *Hyla jimi* (*Hj*), near Botucatu, São Paulo.

Variables	*Hn*	*Hs*	*He*	*Hj*
Perch height	5.05	6.30	3.84	5.81
Type of perch	3.96	2.98	3.58	3.35
Distance of the perch from the edge of the pond	9.57	3.18	5.60	3.34
Position on the perch	1.97	1.74	2.00	1.57

Males of *H. elianeae* preferentially called from lower heights, mean height 10 cm (46.4%, n=64; Fig. 1). There was no apparent preference for distance of calling sites from the outer and inner edges of the pond (Fig. 2). The individuals were observed on the ground, among the vegetation (39.7%, n=54; Fig. 3). When they were perching, the males called from leaves of herbaceous vegetation, with the body parallel or perpendicular to the perch (50%, n=32).

Individuals of *H. jimi* mostly called from heights between 20 and 50 cm (77.7%, n=185; Fig. 1). There was a preference for calling sites around the pond (Fig. 2), on the thin stems of grasses or sedges (44%, n=107; Fig. 3), with the body perpendicular to the perch (76%, n=102).

The mean perch heights of *H. elianeae* and *H. jimi* differed significantly (Kruskal-Wallis, H=32.54; P<0.05). The preferred calling height of *H. elianeae* was 27.11±31.22 cm (n=138), and of *H. jimi*, 40.47±17.33 cm (n=238).

The mean height of calling perches did not differ significantly between *H. sanborni* and *H. jimi* (Kruskal-Wallis H=1.53, P>0.05), or between *H. nana* and *H. elianeae* (Kruskal-Wallis H=1.03, P>0.05). The mean distance of the occupied perch from the inner pond edge was significantly different between *H. nana* and the other species, and between *H. sanborni* and *H. elianeae* (Kruskal-Wallis H=160.9, P<0.05). In regard to the mean distance of the perches from the outer pond edge, there was no significant difference (Kruskal-Wallis H=0.48, P>0.05) between *H. elianeae* and *H. jimi*. The other species differed significantly (Kruskal-Wallis H=128.0, P<0.05) when they occupied sites outside the pond.

The four species were generalists in relation to the occupation of the calling site (Table 1). There was extensive overlap in height of calling site and position on the perch (Table 2). Among the species of the *nana* group, the degree of overlap was high only in relation to perch type; for the species of the *rubicundula* group, there was overlap in occupation of the perches in relation to the distance from the pond edge. The multidimensional analysis of the calling sites demonstrated that only two pairs of species showed high overlap (Table 2).

For each of the calling site variables, the grouping analyses resulted in different patterns of similarities among the species (Fig. 4a–d). The greatest similarity in the multidimensional analysis of calling sites was observed for *H. sanborni* and *H. jimi* (Fig. 4e).

BIOACOUSTICS

The advertisement calls of these four species of *Hyla* are composed of simple notes, pulsed and emitted in consecutive series. The males of these species call frequently in large choruses, forming reproductive aggregations around the ponds. The spectral and temporal characteristics of the advertisement calls of *H. nana*, *H. sanborni*, *H. elianeae* and *H. jimi* are presented in Table 3.

TABLE 2. Niche overlap, calculated by the Morisita–Horn similarity index (C_H) for four calling site variables among *Hyla nana* (*Hn*), *Hyla sanborni* (*Hs*), *Hyla elianeae* (*He*) and *Hyla jimi* (*Hj*), near Botucatu, São Paulo.

Species	Perch height	Type of perch	Distance of the perch from the edge of the pond	Position on the perch	Multidimensional overlap
Hn/Hs	0.766	0.721	0.492	0.876	0.711
Hn/He	0.634	0.274	0.433	0.992	0.486
Hn/Hj	0.761	0.343	0.184	0.814	0.505
Hs/He	0.444	0.520	0.599	0.929	0.526
Hs/Hj	0.970	0.553	0.574	0.993	0.737
He/Hj	0.532	0.509	0.725	0.880	0.579

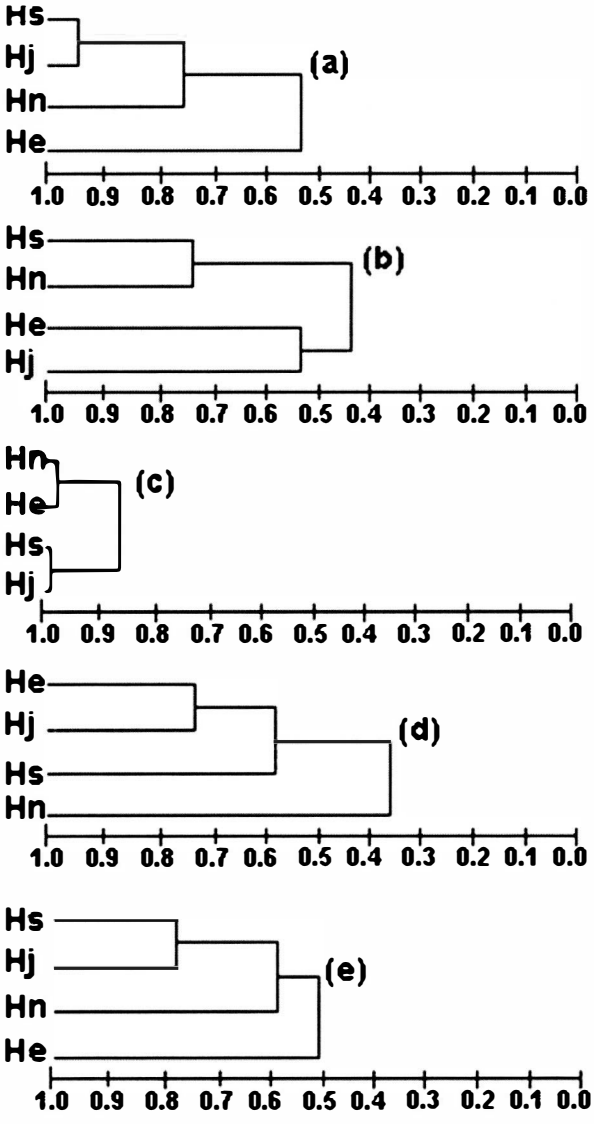

FIG. 4. Similarity of the dimensions of the calling site among *Hyla nana* (*Hn*), *Hyla sanborni* (*Hs*), *Hyla elianeae* (*He*) and *Hyla jimi* (*Hj*), near Botucatu, São Paulo. (a) Height, (b) type of perch, (c) position on the perch, (d) distance of the perch from the pond edge, and (e) similarity of the four variables together.

The four species of *Hyla* have calls with close frequency bands, except for *H. sanborni*, which has calls with higher frequencies. The species of the *nana* group have bands with wider mean frequencies, between 2,950 and 5,950 Hz, whereas the species of the *rubicundula* group have narrower frequency bands, between 2,400 and 4,900 Hz. In both species groups, the notes of the advertisement calls have a fundamental frequency with energy concentration above 3,000 Hz. All the advertisement calls have sound characteristics suited for short or medium distances.

The frequencies band width of the advertisement calls of the four species overlapped. The smallest degree of overlap was recorded for *H. sanborni* and *H. elianeae* (Fig. 5). For the species of the *nana* group and the *rubicundula* group, the fundamental frequency was shown to aid in acoustic partitioning among the species (Fig. 5). The fundamental frequency (=dominant frequency), the band where most of the energy of the notes is concentrated, was shown to be one of the factors that aided in partitioning the acoustic space. In spite of the low degree of overlap observed between *H. nana* and *H. jimi* (Fig. 5), there was a significant difference (ANOVA, F=11.5, P<0.01) between the fundamental frequencies of all the species.

The temporal parameters of the advertisement call were important factors which that the species in partitioning acoustic space. Without phonotaxis experiments we really do not know how these species partition acoustic space, at least insofar as what is important to what conspecific and heterospecific males and females might actually respond to. The intraspecific characteristics of the advertisement call during the beginning of calling activity and chorus activity revealed significant differences (ANOVA, P<0.01) in note duration (Fig. 6a), pulse duration, rate of note repetition (Fig. 6b), and rate of pulse repetition in *H. nana*, *H. sanborni*, *H. jimi* and *H. elianeae*.

In the interspecific analyses of the emissions of the advertisement call at the beginning of calling, there was no significant difference between the rate of repetition of the notes of *H. sanborni* and *H. elianeae* (ANOVA, F=0.82, P>0.01), or of *H. sanborni* and *H. jimi* (ANOVA, F=1.66, P>0.01). Note duration, rate of

TABLE 3. Characteristics of the six advertisement call variables for the four species of *Hyla* studied near Botucatu, São Paulo. The temporal bioacoustic parameters of the advertisement call are presented for songs at the beginning of calling activity and during the chorus. The data represent the mean±one standard deviation and (range).

		Hyla nana (*n*=73)	*Hyla sanborni* (*n*=58)	*Hyla elianeae* (*n*=51)	*Hyla jimi* (*n*=49)
Frequency bands (Hz)		2950–4850±50 (2600–5500)	3860–5950±300 (3600–6450)	2400–4380±140 (2350–4470)	3000–4900±200 (2900–5100)
Fundamental frequency (Hz)		3985±129	5165±136	3396±157	4069±149
Note duration (ms)	Initial call	44.4±9.2 (26–61)	38.2±9.8 (26–65)	16.6±2.6 (10–23)	34.1±6.4 (26–47)
	Chorus	20.5±2.3 (16–26)	30.8±5.5 (20–43)	18.4±2.9 (12–26)	52.1±9.2 (37–72)
Pulse duration (ms)	Initial call	2.54±0.61 (2–3)	4.55±0.86 (3–6)	3.48±0.84 (2–6)	5.2±1.07 (3–6)
	Chorus	2.7±065 (2–4)	4.02±0.94 (3–6)	4.89±0.82 (2–6)	6±1.1 (3–6)
Note repetition rate (notes/sec)	Initial call	1.14±0.31 (0.77–2.01)	1.41±0.44 (0.44–2.08)	1.32±0.3 (0.72–1.88)	1.6±0.3 (0.92–1.98)
	Chorus	4.73±0.87 (2.75–6.51)	3.67±0.7 (2.25–4.76)	3.12±0.45 (2.6–4.02)	2.64±0.51 (2.1–4.93)
Pulse repetition rate (ms)	Initial call	3.98±0.85 (3–5)	6.84±0.69 (6–8)	4.52±0.71 (3–6)	11.37±0.96 (9–12)
	Chorus	4.06±0.66 (3–5)	7.54±0.49 (6–9)	4.89±0.82 (3–6)	15.57±1.23 (12–18)

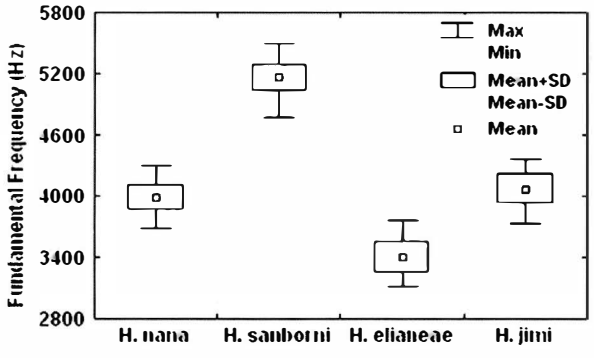

FIG. 5. Fundamental frequencies of the advertisement calls of *Hyla nana*, *H. sanborni*, *H. elianeae* and *H. jimi*, near Botucatu, São Paulo.

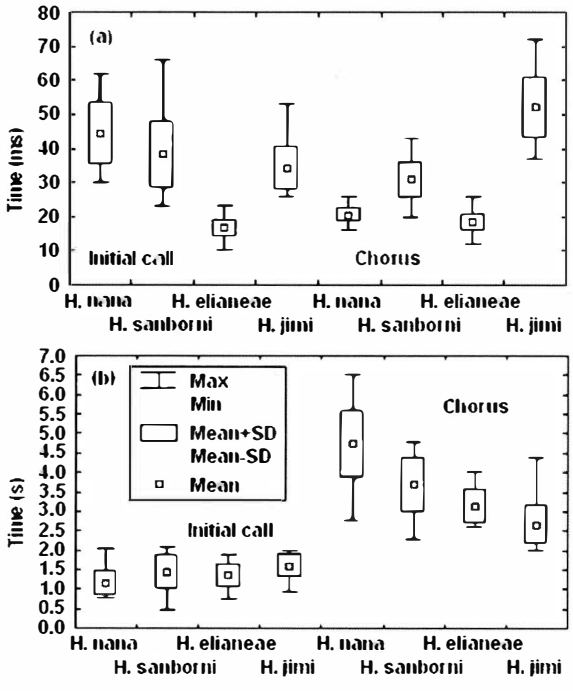

FIG. 6. (a) Note duration (ms) and (b) note repetition rate (s) of the advertisement call of *Hyla nana*, *H. sanborni*, *H. elianeae* and *H. jimi* (Hj), near Botucatu, São Paulo, at the beginning of calling activity and during calling in chorus.

pulse repetition and pulse duration differed significantly among the species during the beginning of calling activity (ANOVA, $P<0.01$).

During chorus calling, the interspecific analyses revealed significant differences among the four species (ANOVA, $P<0.01$) in all the temporal variables of the advertisement call.

There was a positive correlation between air temperature and the rate of note repetition during the chorus for *H. nana* ($r_s=0.62$; $P<0.001$; $n=73$), *H. sanborni* ($r_s=0.78$; $P<0.001$; $n=58$) and *H. jimi* ($r_s=0.80$; $P<0.001$; $n=49$). For *H. elianeae* there was no correlation between the rate of note repetition and air temperature ($r_s=0.29$; $P>0.01$; $n=51$). There was no correlation for any of the four species between rate of note repetition and air temperature during the beginning of calling activity ($P>0.05$).

DISCUSSION

CALLING SITE

Habitat type can be an evolutionarily conservative characteristic among closely related species, so that they tend to share similar life history attributes in the same habitat, but in slightly different locations; i.e. they are spatially separated (Heyer *et al.*, 1990). According

to MacNally (1985), spatial segregation may occur not only through occupying different habitats, but through differences in the behaviour of the species.

The data obtained in the present study for the occupation of calling sites by species of the *nana* group are similar to those presented by other workers in different regions (Barrio, 1962; Bernarde & Anjos, 1999; Bernarde & Kokubum, 1999; Rossa-Feres & Jim, 2001; Bertoluci & Rodrigues, 2002). Ecological information about the species of the *rubicundula* group is scarce (Jim, 2002; Martins & Jim, 2004).

In spite of the overlap recorded for at least one of the calling site variables, there were differences between the species groups in how they occupied their calling sites. The species of the *nana* group differed in height and distance from the pond edge. In the areas where they occurred in sympatry, males of *H. sanborni* tended to call from a higher position than did males of *H. nana*. Distance from the pond edge was the calling-site variable which made possible the greatest spatial segregation between the two species. The males of *H. nana* called predominantly towards the middle of the pond, whereas *H. sanborni* was recorded further out, near the edge.

In the *rubicundula* species group, the segregation occurred in relation to the height and type of the perch. Males of *H. jimi* called from a higher position than did males of *H. elianeae*. The differences in occupation of the perch were closely related to height. Most males of *H. elianeae* were observed on the ground, while males of *H. jimi* predominantly perched on sedges.

Comparing the species of the *nana* and *rubicundula* groups, we observed that there was great similarity between *H. sanborni* and *H. jimi* in the occupation of the calling site, showing the greatest degree of overlap. The mean perch height was similar in the two species. *Hyla sanborni* occupied the region near the pond edge, and *H. jimi* established itself further out from the pond edge.

The species which showed the greatest degree of segregation in the occupation of the calling site was *H. elianeae*. This species has wide behavioural plasticity. In environment II, where *H. elianeae* coexists with *H. sanborni*, *H. nana* and *H. jimi*, the males of *H. elianeae* call on the ground, while the other species call from perches. In environments I and III, where it coexists with *H. nana* and *H. sanborni*, individuals of *H. elianeae* call from perches; although they differ from *H. nana* and from *H. sanborni* in that they use perches located farther from the pond edge.

Ptacek (1992), analysing the use of calling sites in *H. chrysoscelis* and *H. versicolor*, observed that the most important difference between their calling sites was perch height, and that this partitioning of calling site according to perch height may be important in preventing mismatings between these two species. Given (1990) found little evidence for differences in microhabitat use by two species of *Rana*; however, he recorded a differential use in the location of the calling site in relation to distance from the pond edge between the species.

The ecological similarities among the species studied here may be related to adaptive convergence, or may be a consequence of the high degree of relatedness. Zimmerman & Simberloff (1996) argued that phylogenetically close species share morphological and behavioural characteristics, because of the brief time since their speciation event. From this viewpoint, the observed similarity in occupation of the calling site among the species analysed near Botucatu can be interpreted as an interaction between the availability of environmental resources and the limitations imposed by the evolutionary history of the taxonomic groups.

Rossa-Feres & Jim (2001), studying a community of anurans in a temporary environment, observed that in regard to one behavioural variable in calling site use – position on the perch – overlap was greater between closely related species. In the present study, this variable showed the greatest overlap. Nevertheless, this high degree of overlap was not observed for the most closely related species, but rather between a species of the *nana* group and one of the *rubicundula* group (*H. sanborni* and *H. jimi*). An important aspect to be considered is that although the species studied are closely related to each other within the groups, the two groups are also closely related, as opposed to the situation studied by Rossa-Feres & Jim (2001), who did not analyse two closely related species groups.

The most important variables for the coexistence of the species studied were height, type of perch and distance of the calling site from the pond edge. Analysing the distribution of frequencies along the gradients of the resources analysed, we found that the species occupied a wide range of gradients. Within this range, however, each species showed a specific preference for the use of available resources (calling site variables), which differed among the species. These differences were often subtle, resulting in close ecological similarity between two species, such as between *H. sanborni* and *H. jimi*.

BIOACOUSTICS

The wide variety and abundance of some sympatric related species of tropical anurans have attracted attention for studies of interspecies coexistence and their interactions in partitioning acoustic space (Duellman, 1967; Hödl, 1977; Duellman & Pyles, 1983; Cardoso & Vielliard, 1990; Márquez *et al.*, 1993; Schwartz & Wells, 1983, 1984). When many individuals are signalling within an actively partitioned space, the details of the acoustic interactions can be complex. Additional complexities are imposed by changes in the conditions of social interactions and the presence of noises that interfere with the signals. The acoustic space in a community of anurans is characterized precisely by these complexities (Schwartz, 2001).

Calling is the main isolation mechanism among species that occupy the same type of environment. Nevertheless, sympatric species can have similar calls, and the specificity of the signal must be established by combined spectral and temporal parameters (Hödl, 1977).

Littlejohn (1977) suggested mechanisms through which acoustic interference can be reduced: (1) use of different frequency bands by different species; (2) spatial segregation, each species using specific calling sites within the environment; (3) temporal acoustic segregation, synchronization and different temporal structures of the notes, avoiding overlap; and (4) different patterns of specific codes.

For the species of *Hyla* analysed in this study, frequency band width was not a parameter that promoted acoustic isolation, because of the overlap in the frequency range. The fundamental frequency differs significantly among the species, indicating acoustic partitioning and representing different channels of sound communication among them.

Cardoso (1981) observed that the dominant frequency of the song was different in most of the species that he studied, indicating acoustic partitioning among species in the region where the highest energy of the song was concentrated.

According to Littlejohn (1977), the frequent occurrence of superposition of the same frequency band in different anuran species is a result of selection process and ecological pressures, which act on the species and become evident in morphological characteristics such as body size. This relationship between body size and the frequency band of the calls was confirmed by Duellman (1967). Blair (1964) observed that body size and the size of the vocal apparatus affect the frequency used by anurans. Márquez *et al.* (1993) assumed that similar-appearing species have common anatomical and physical factors, and that such factors would determine the close similarity in the use of acoustic and spatial resources. For the populations of the species studied here, body size did not differ very much, all of them being small (15–26 mm), and using similar frequency bands (Martins & Jim, 2003, 2004).

Communication is a dynamic process, in which a signal emitted by one individual can influence the behaviour of another (Schwartz, 2001). Frequently, individuals calling in large chorus groups change the rhythm of their calls in response to the calls of other, nearby males, in order to preserve the integrity of their signals and reduce acoustic interference, in addition to aiding in location of males by females and in maintaining spacing between conspecific individuals (Wells, 1977, 1988; Passmore & Telford, 1981; Schwartz, 1987).

In the present study, the species of the *nana* group showed a wider frequency band width than did the species of the *rubicundula* group. The use of narrow frequency bands, as observed for *H. elianeae* and for *H. jimi*, can increase communication efficiency by minimizing interference from the environment (Straughan, 1973), and by this means can reduce competitiveness among the species. According to Cardoso (1981), sound communication in open areas may favour those species that have calls in wide frequency bands, as recorded in the present study for *H. nana* and *H. sanborni*, because this greater frequency spread allows for more opportunity for adaptation, if there is competition for communication channels.

The temporal structure of the calls of the species studied was shown to be a strong mechanism for effecting partitioning of acoustic space, allowing coexistence in similar habitats during the same activity periods. The variables of pulse duration, rate of pulse repetition, note duration and rate of note repetition differed significantly among the species, mainly during chorus activity, when the competition for acoustic space is greatest in the aggregations of individuals.

The overlap in temporal parameters observed among the species occurred at different moments of calling activity. The rhythm of note emission by individuals in chorus was an important factor in partitioning the acoustic space, and can be considered as yet another parameter of species recognition.

Hödl (1977), studying an anuran community in Amazonia, noted that the songs of four species in which the frequency spectra overlapped were strongly differentiated in temporal structure. Cardoso & Vielliard (1990) observed that in five synchronopatric species of hylids that emitted simultaneous sound signals on the same frequency band, acoustic space was partitioned by means of differentiation of the temporal structures.

The partitioning of spatial and acoustic occupation among the species studied cannot be explained simply through isolated analysis of a single variable, but rather by the interaction among all the variables present. These combinations of variations in the characteristics of the different environments occupied allow for many possibilities for adjustments in the use of spatial and bioacoustic resources by the species (Jim, 2002), as observed in the present study. Nevertheless, even if two species show overlap in one or more dimensions of their niches, there will always be some specific aspect that makes possible coexistence between sympatric, phylogenetically related species.

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EFFECTS OF TEMPERATURE ON HATCHING SUCCESS IN FIELD INCUBATING NESTS OF SPUR-THIGHED TORTOISES, *TESTUDO GRAECA*

C. DÍAZ-PANIAGUA[1], A. C. ANDREU[1] AND C. KELLER[1,2]

[1]*Estación Biológica de Doñana-CSIC, Sevilla, Spain*

[2]*Present address: Instituto Nacional de Pesquisas da Amazônia - INPA, Dep. Ecology, Manaus - AM, Brazil*

Spur-thighed tortoises, *Testudo graeca,* in south-western Spain lay 3-4 clutches in shallow nests from April to June. In the present study the incubation temperature of nests laid in field enclosures in April, May and June was monitored over four years. Mean daily temperature throughout incubation averaged 27.9°C, but displayed a wide daily range, with average maximum values around 41°C (also in nests where hatching success was >0), and an absolute maximum of almost 50°C. Early (April) nests displayed lower mean daily temperatures than intermediate (May) and late (June) nests, although all nests reached similar high temperatures during the hottest month (July). Incubation temperatures were affected by nest vegetation cover. Incubation length varied from 67-129 days. Because the length of incubation was negatively correlated with nest temperature, early nests had longer incubation periods than intermediate and late nests. Hatching success averaged 61% and was mainly affected by variables related to maximum temperatures. Thus unsuccessful nests (i.e. no eggs hatching) were associated with higher temperatures or longer exposure to higher temperatures. Differences in hatching or nest success were not related to the nesting month, but might have been influenced by the location of the nest. Lethal temperatures for embryo development were frequently reached during July, therefore vegetation cover of the nest is likely to play an important role in avoiding deleterious nest environments.

Key words: chelonia, incubation temperature, nesting, reproductive success

INTRODUCTION

Reptile eggs are highly influenced by their nest environment, mainly soil moisture and temperature. While flexible shelled eggs require the absorption of water from the surroundings to complete development, embryos of hard shelled eggs are relatively independent of variation in substrate moisture, relying mostly on the water supplied by the female at oviposition (Packard, 1999; Tracy & Snell, 1985; Congdon & Gibbons, 1990). Temperature, however, significantly affects both types of eggs. Thermal tolerance limits of embryos are known to range between 22 and 35°C in most reptiles for incubation at constant temperatures in the laboratory. However, field incubating embryos may withstand short periods of temperatures below and above these limits (Ewert, 1979; Congdon & Gibbons, 1990). Incubation temperature strongly influences embryo development and growth rates, thus conditioning the length of the incubation period (Gutzke *et al.*, 1987; Packard & Packard, 1988; Deeming & Ferguson, 1991). Fast embryonic development is associated with a less efficient metabolism, producing large residual yolks (Deeming & Ferguson, 1991). Incubation temperature of eggs kept under laboratory conditions also affects sex determination (e.g. Pieau, 1972, 1982; Janzen & Paukstis, 1991; Bull, 1980), as well as other hatchling phenotypic traits, behaviour and survival (e.g. Deeming & Ferguson, 1991; Janzen, 1993; Cagle *et al.*, 1993; Bobyn & Brooks, 1994; Spotila *et al.*, 1994; Shine *et al.*, 1997; Elphick & Shine, 1998; Wilson, 1998; Packard *et al.*, 1999; Rhen & Lang 1999). The variation of temperature in natural nests has been described in some species of reptiles [e.g. *Emys orbicularis* (Pieau, 1982), *Chelydra serpentina* (Packard *et al.*, 1985), *Emydura macquarii* (Thompson, 1988), *Chrysemys picta* (Cagle *et al.*, 1993), *Sphenodon punctatus* (Thompson *et al.*, 1996), *Chelodina expansa* (Booth, 1998), *Chelonia mydas* and *C. caretta* (Kaska *et al.*, 1998), *Amphibolorus muricatus* (Harlow & Taylor, 2000), *Bassiana duperreyi* (Shine, 2004), *Cyclura cychlura* (Iverson, 2004)]. Natural incubation temperatures are known to affect embryo survival (Thompson *et al.*, 1996), as well as sexual differentiation of embryos (Pieau, 1982, Harlow & Taylor, 2000), and may be used to predict sex-ratio at hatching (Hanson *et al.*, 1998, Marcovaldi *et al.*, 1996). Although temperature may frequently reach lethal levels for embryos in wild nests, only a few studies have analysed the effect of incubation temperature on hatching success in field incubating nests (Shine & Elphick, 2001; Shine, 2004, Thompson, 1996, Congdon *et al.*, 1987). Nest temperatures in the field may vary throughout the nesting season, as well as among localities and years. Differences in incubation temperature throughout the nesting season affect the timing of hatching and the developmental trajectories of embryos, eventually affecting hatchling phenotype (Shine, 2004).

In this study we describe the variation of incubation conditions in field nests of *Testudo graeca* in a population of south-western Spain. Each year females in this

Correspondence: C. Díaz-Paniagua, Estación Biológica Doñana, Apdo. 1056, 41080 Sevilla, Spain. *E-mail*: poli@ebd.csic.es

population produce 1-4 clutches of 1-7 hard shelled eggs, laid in shallow nests of about 8 cm depth, usually under some degree of vegetation cover (Díaz-Paniagua *et al.*, 1996). A demographic analysis indicated that egg and/or juvenile survivorship are usually low, while occasional episodes of successful recruitment significantly contribute to revert otherwise declining population growth tendencies (Díaz-Paniagua *et al.*, 2001). Within this framework, the assessment of the factors that influence hatching success is paramount to the understanding of the mechanisms affecting the dynamics of the population and the determination of effective management measures.

Previous studies on egg incubation of this species under laboratory conditions have showed that sex differentiation is temperature dependent, with a pivotal temperature of 30.5±0.5°C (Pieau, 1975). Hatching success, the period from hatching to emergence, and hatchling morphology have been described from field incubating nests of *T. graeca* in a previous study (Díaz-Paniagua *et al.*, 1997). Because rigid egg shells prevent the loss of large amounts of water to dry surroundings (Packard & Packard, 1988), the eggs of *T. graeca* are assumed to be mostly influenced by incubation temperature, and therefore this study is centred on the variation of the thermal environment of field incubating nests. Our objectives were (1) to describe the variation of incubation temperature for clutches laid at different moments of the nesting season and in different years, and (2) to analyze the relation between the profile of incubation temperature and hatching success.

MATERIAL AND METHODS

We monitored hatching success and incubation conditions in 56 *Testudo graeca* nests in four different years: 1996, 1997, 1999 and 2000 (Table 1). Every year we captured female tortoises during the nesting season, from mid-March or early April to June. Female capture was random, thus some of the clutches both among years as well as among nesting periods within the same year were from the same female. The presence of oviductal eggs was determined through X-raying (see Díaz-Paniagua *et al.* (1996) for details on the X-raying procedure). Egg-bearing females were kept in individual field enclosures of approximately 10 m^2 located within the natural nesting area of *Testudo graeca* in Doñana National Park (SW Spain). All enclosures included natural vegetation (mainly shrubs of *Stauracanthus genistoides*, and *Halimium halimifolium*), under which free ranging tortoises usually lay their eggs (Díaz-Paniagua *et al.*, 1996). Vegetation cover was not measured in the enclosures, but every female had the choice between shaded and unshaded spots in its enclosure for egg-laying. All females were weighed daily after the end of their activity period. Egg laying was assumed when a weight loss occurred approximately equivalent to the total mass of the eggs detected on radiography (mean egg mass from Díaz-Paniagua *et al.*, 1996). The enclosure was then thoroughly searched for the nest site through palpation by one or two persons. All females were released at their original capture sites after oviposition.

TABLE 1. Number of nests (*n*), mean hatching rate and nest success for each nesting period of *Testudo graeca* in the four study years.

Year	Nesting period	*n*	Hatching Rate (%)	Nest success (%)
1996	early	1	50.0	100
	intermediate	5	95.0	100
	late	6	75.0	83.3
	all nests	12	81.3	91.7
1997	early	7	72.4	100
	intermediate			
	late	8	74.6	87.5
	all nests	15	73.6	93.3
1999	early	6	55.5	83.0
	intermediate	5	50.0	80.0
	late	1	33.0	100
	all nests	12	49.7	83.0
2000	early	5	48.4	80.0
	intermediate	9	45.9	55.6
	late	3	33.3	33.3
	all nests	17	44.4	58.8
All years	early	19	60.1	89.0
	intermediate	19	59.4	74.0
	late	18	64.4	77.8
	all nests	56	61.3	80.0

Forty-five nests were fitted with a temperature data-logger placed among the eggs (11 in 1996, 7 in 1997, 11 in 1999 and 16 in 2000), usually 2-3 days after egg-laying. Incubation temperature was recorded at 30-minute intervals using Onset Stowaway data-loggers in 1996-1997 and Onset Tidbit data-loggers in 1999-2000. All data loggers were checked for consistency in temperature recording prior to use in the field and after data launching. After introduction of the data-logger nests were covered with a wire grid of about 15cm x 20 cm, to prevent other females from digging at the same point. Vegetation cover above each nest was classified in a gradient from 0-100%, considering the percentage of shade vertically projected over a 50 cm diameter circle centred on the nest. Temperature in nests with vegetation cover >0.5 was compared with that of nests with lower or no cover.

In the first week of July, after all females had oviposited, nests were manipulated after Díaz-Paniagua *et al.* (1997) in order to control egg pipping and hatchling emergence. From 1st August onwards we monitored the nests daily by lifting the sand bag in order to record the date of egg pipping.

We defined the hatching date of each nest as the day when pipping of the first egg was observed. Accordingly, the incubation period was here defined as the number of days elapsed between the day of egg-laying and the day of first egg pipping.

To determine the temperature profile of each nest we used the temperature records from nest detection (usually 1-2 days after oviposition) to the day of first egg pipping. For nests in which no egg hatched, we considered the data recorded up to 15 September. For 12 nests detected 7-16 days after the estimated nesting date, mean, maximum and minimum daily nest temperatures during these first days were predicted through multiple regression, using complete temperature data sets from other simultaneously incubating nests as independent variables. In all cases regressions had R^2>90%.

For each nest we calculated the following parameters: (1) mean ($meanT_d$), maximum ($maxT_d$) and minimum ($minT_d$) daily temperature; (2) the average of $meanT_d$ ($xmeanT_d$), $maxT_d$ ($xmaxT_d$) and $minT_d$ ($xminT_d$) for the whole incubation period; (3) minimum (T_{min}), and maximum (T_{max}) temperatures for the whole incubation period; (4) the number of days in which the mean daily temperature was over 20, 25, 30 and 35°C ($nT_{mean>20}$, $nT_{mean>25}$, $nT_{mean>30}$, $nT_{mean>35}$, respectively) and the corresponding average mean temperatures for these days ($xmeanT_{mean>20}$, $xmeanT_{mean>25}$, etc.); (5) the number of days with maximum temperature over 30, 35, 40 and 45 °C ($nT_{max>30}$, $nT_{max>35}$, $nT_{max>40}$, $nT_{max>45}$, respectively) and the corresponding mean maximum temperatures for these days ($xmax_{Tmax>30}$, $xmaxT_{max>35}$, etc.); (6) the number of days with minimum temperature over 20 and 25°C ($nT_{min>20}$ and $nT_{min>25}$, respectively) and the corresponding mean minimum temperatures for these days ($xminT_{min>20}$, $xminT_{min>25}$).

Nesting dates were grouped in three categories (hereafter referred to as nesting periods): (1) early nests (eggs laid in April); (2) intermediate nests (eggs laid in May); and (3) late nests (eggs laid in June). The hatching rate per nest was calculated as the number of eggs hatched divided by the total number of eggs. Nest success was defined as a discrete variable (0 = no eggs hatched in the nest; 1 = at least one egg hatched in the nest) indicative of viable incubation conditions, apart from other factors that could affect hatching rate (e.g. egg infertility).

The correlation of incubation length with temperature variables was analyzed individually and using multiple regression. We compared temperature variables (groups 2 and 3, see above) among nesting periods and years using ANOVA. Nest success was compared among years and nesting periods using the χ^2 test. In order to assess which temperature variables mainly affected hatching success, we analyzed the individual correlation of temperature variables (groups 2 to 6, see above) with hatching rate, and then compared the significantly correlated variables among successful and unsuccessful nests using ANOVA. The significance level for comparisons was adjusted to P=0.0031 following a Bonferroni correction.

To assess the influence of incubation temperature variables on hatching rates we carried out a logistic regression analysis in which nest success was the response variable, and temperature parameters were predictor variables. In a first approach, the regression was calculated for only one temperature parameter at a time, and in a second step we calculated regressions for two combined parameters among those presenting significant individual relations with the response variable. Only uncorrelated variables were combined in the second analysis.

RESULTS

Variation of Incubation Temperature

Tortoises nested from early April to the end of June. The yearly nesting season, as represented by the sequence of recorded nesting dates of monitored tortoises, differed significantly among years ($F_{3,52}$=3.96, P=0.0129). The sample size for each nesting period (Table 1) reflects the actual monthly availability of females in the field in each year and was related to climatic differences among years. For example, in 1996, exceptionally low early spring temperatures generally delayed the onset of the nesting season to mid-May.

Tortoise nests experienced a wide variation in daily as well as whole-period temperature progression (Fig. 1). Incubation temperature averaged 27.9°C. Very high

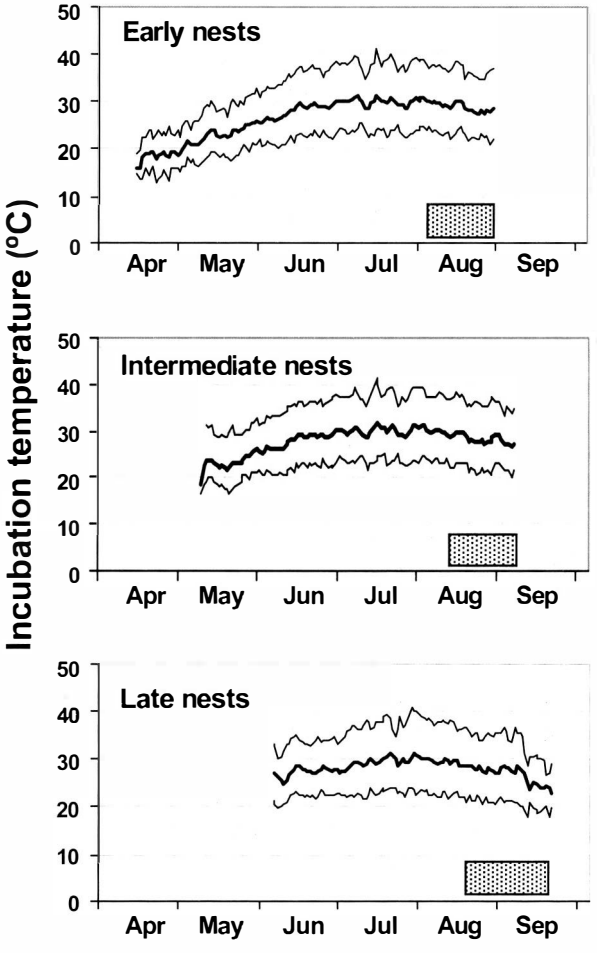

FIG. 1. Evolution of mean, maximum and minimum temperature in field incubating nests of *Testudo graeca* in Doñana National Park (pooled data for nests monitored in 1996, 1997, 1999 and 2000). The grey horizontal bar at the bottom indicates the span of hatching dates.

TABLE 2. Incubation period (in days) and mean incubation temperature (xmeanT_d, in ºC) for nests monitored over different nesting periods (early, intermediate, late and overall) and years. Numbers are the arithmetic mean ± SD, followed by the range (in parenthesis).

Year	Overall	Early	Intermediate	Late
INCUBATION PERIOD				
1996	95.3±10.8 (79-117)	117.0	99.2±1.6 (98-102)	87.0± 8.3 (79-100)
1997	100.0±22.6 (67-129)	118.6±14.5 (89-129)	—	81.4± 9.7 (67-90)
1999	110.0±12.9 (93-128)	121.6±5.4 (114-128)	99.8±1.7 (98-102)	93
2000	107.6±19.3 (76-131)	117.8±16.3 (90-131)	103.8±16.2 (87-126)	76
All years	102.9±18.6 (67-131)	119.1±12.1 (89-131)	101.0±9.3 (87-126)	83.9±9.1 (67-100)
INCUBATION TEMPERATURE				
1996	28.0±1.3 (26.3-30.5)	26.4	28.1±0.9 (27.3-29.8)	28.3±1.5 (26.3-30.5)
1997	27.8±1.4 (25.3-29.1)	26.0±1.0 (25.3-26.7)	—	28.5±0.5 (27.7-29.1)
1999	28.0±2.0 (23.5-30.4)	27.8±1.6 (26.2-30.0)	29.1±1.3 (27.5-30.4)	23.5
2000	27.7±1.6 (24.9-29.2)	26.5±1.5 (24.9-29.2)	28.2±1.3 (25.2-29.2)	29.0±0.3 (28.8-29.2)
All years	27.9±1.6 (23.5-30.5)	27.5±1.6 (24.3-30.3)	28.4±1.2 (25.2-30.4)	28.1±1.7 (26.3-30.5)

temperatures were reached in the nests during the hottest month (July). In 10 nests we recorded T_{max} values over 45ºC. The highest recorded temperature was 49.8ºC, in July in a nest where no egg hatched. However, T_{max} = 47.4ºC was recorded in another nest where hatching success was >0. Nest temperature exhibited a wide daily range, with a mean range of 13.4 ºC. T_{min} never dropped below 10ºC. In general, early, intermediate and late nests were characterized by different incubation temperature regimes, although all of them were exposed to similarly high temperatures during the hottest month (July). Overall early nests displayed lower temperatures than later nests (Table 2). Considering the whole incubation period, nests from different nesting periods differed significantly in xmeanT_d ($F_{2,42}$=6.20, P=0.0044), T_{min} ($F_{2,41}$=10.29, P<0.0002) and xminT_d ($F_{2,41}$=5.20, P<0.01), early nests exhibiting lower values than intermediate and late nests for all three parameters. In contrast, all nests reached similar maximum temperatures. Considering only incubation during July, the only month in which nests from all nesting periods were incubating during the whole month, no significant difference was found in xmeanT_d among nests from different nesting periods. The daily average of xmeanT_d for all nests varied significantly among years ($F_{3,120}$=10.87, P<0.0005), which was mainly due to higher mean daily temperatures in nests in 1999.

Until the first half of May meanT_d stayed under 24ºC. During this period mostly early nests were incubating. Early nests started incubation around 19ºC (Fig. 1). In the second half of May, when incubation of most intermediate nests started, meanT_d values climbed to around 26ºC. Until the end of May minT_d values stayed between 16 and 20ºC, while maxT_d only rarely surpassed 30ºC. In June, when incubation of late nests began, meanT_d climbed from 26-27ºC to 27-29ºC in the first and second halves, respectively. In July and August meanT_d stayed between 29-30ºC, while minT_d and maxT_d values were steadily over 20ºC and 33ºC, respectively. In July maxT_d frequently reached over 40ºC (highest maxT_d=49.8ºC). By the end of August, when eggs of most early and intermediate nests had already hatched, incubation temperatures started to decrease, keeping levels similar to June throughout the remaining incubation time of most late nests.

Tortoise nests were frequently located close to or under the cover of shrubs, which partially shaded them. Vegetation cover of the nests was negatively related to xmeanT_d (r=-0.409, P=0.0079). Nests where vegetation cover was less than 50% shade (46% of the nests) reached significantly higher temperatures than nests with more than 50% shade ($F_{1,39}$=9.26, P=0.0042). Only 10% of the nests had no cover at all (0% shade), while 15 % were completely covered (100% shade) under a dense shrub.

HATCHING DATE AND INCUBATION LENGTH

The hatching date differed significantly among nesting periods ($F_{2,37}$=15.24, P=0.0001), but not among years. The earliest hatchings occurred in the first half of August, and were from early and intermediate nests (which did not differ significantly), while eggs from late nests started to hatch significantly later, towards the end of August (Tukey post-hoc test). Most early and intermediate eggs hatched until the end of August, while late eggs hatched in the second half of September (Fig. 1).

Incubation periods ranged between 67 (a late nest in 1997) and 129 days (an early nest in 1997) and decreased significantly from early to late nests ($F_{2,43}$=45.07, P<0.0001) (Table 2). No significant differences were observed among years.

The length of the incubation period was significantly and negatively correlated with T_{min} (r=0.580, P=0.0003), xminT_d (r=-0.461, P= 0.006), xmeanT_d (r=-0.375 , P=0.02) and positively correlated with $nT_{mean>20}$ (r=0.681, P<0.0001) and $nT_{min>20}$ (r=0.388, P=0.02). A similarly good predictive value was obtained for the two functions: Incubation length = 46.03 -

TABLE 3. Values of temperature variables that differed significantly between 33 successful and 11 unsuccessful *Testudo graeca* nests monitored in 1996, 1997, 1999 and 2000 in Doñana National Park. Numbers are the arithmetic mean ± SD, followed by the range, and ANOVA results of comparisons between successful and unsuccessful nests (df = 1,39). Pearson correlation coefficients and corresponding significance levels are for the relation between temperature variables and hatching rates (significant results following the Bonferroni corrected significance level = 0.0031).

Incubation temperature variables	Successful nests	Unsuccessful nests	*F* (p)	Correlation with hatching rate
Average of maximum daily temperatures ($xmaxT_d$)	34.7±2.4 (29.8-39.1)	37.7 ± 1.7 (34.7-40.5)	13.7 (0.0006)	-0.555 (0.0001)
Absolute maximum temperature (T_{max})	41.5±2.4 (37.1-47.4)	46.5±1.9 (43.4-49.8)	37.7 (<0.0001)	-0.681 (0.0001)
Number of days with mean temperature >20°C ($nT_{mean>20}$)	92.9±16.4 (29-120)	111.9±13.0 (88-132)	12.2 (0.0011)	-0.419 (0.0042)
Number of days with mean temperature >30°C ($nT_{mean>30}$)	21.9±15.4 (0-59)	38.3±19.2 (16-79)	14.5 (0.0004)	-0.536 (0.0002)
Number of days with mean temperature >25°C ($nT_{mean>25}$)	79.6±11.7 (29-95)	101.5±12.6 (75-122)	28.1 (<0.0001)	-0.600 (<0.0001)
Average temperature of days with mean temperature >30°C ($xmeanT_{mean>30}$)	30.9±0.4 (30.2-31.9)	31.5±0.52 (30.7-32.6)	8.3 (0.0062)	-0.524 (0.0002)
Number of days with minimum temperature >20°C ($nT_{min>20}$)	97.0±15.2 (69-115)	81.3±9.5 (66-111)	16.4 (0.0002)	-0.492 (0.0007)
Number of days with maximum temperature >35°C ($nT_{max>35}$)	51.5±24.7 (1-90)	85.3±18.0 (59-114)	16.0 (0.0003)	-0.574 (0.0001)
Number of days with maximum temperature >40°C ($nT_{max>40}$)	14.2±15.3 (0-60)	40.5±21.8 (21-83)	16.4 (0.0003)	-0.614 (0.0001)
Mean maximum temperature in days with maximum temp >30°C ($xmaxT_{max>30}$)	35.8±1.7 (32.3-39.1)	38.7±1.4 (37.4-41.1)	21.7 (<0.0001)	-0.679 (<0.0001)

1.67 $xmeanT_d$ + 1.09 $nT_{mean>20}$ (R^2=0.669) and Incubation length = 58.88 - 2.627$xminT_d$ + 1.08$nT_{mean>20}$ (R^2=(0.669).

Influence of Incubation Temperature on Hatching and Nest Success

Overall hatching rate was 61%, and although a lower percentage was registered in nests from 1999 and 2000, we did not find significant differences among years nor nesting periods, despite the lower values registered for 1999 and 2000 (Table 1). Nest success did not differ significantly among years or nesting seasons. We recorded values over 80% in all seasons of the four study years except in intermediate and late nests in 2000, when no eggs hatched in six of 12 nests.

The comparison of successful and unsuccessful nests mostly revealed significant differences in variables related with maximum temperatures ($xmaxT_d$, T_{max} and $xmaxT_{max>30}$) and with the number of days with high temperatures ($nT_{mean>20}$, $nT_{mean>25}$, $nT_{mean>30}$, $nT_{min>20}$, $nT_{max>35}$, $nT_{max>4}$0), suggesting that egg mortality was associated with the higher temperatures experienced by unsuccessful nests (see Table 3). Similarly those variables related to maximum temperatures were significantly and negatively correlated with hatching rates (Table 3). The highest correlation coefficients were obtained for T_{max}, $x_{max}T_{max>30}$, $nT_{max>40}$ and $nT_{max>35}$, and all had significantly higher values in unsuccessful nests compared to successful ones. The number of days with maximum temperature >40°C was almost three times higher in unsuccessful nests than in successful ones.

Among the logistic regression equations for prediction of successful and unsuccessful nests using individual variables only nT_{mean}>25 and T_{max} obtained correct classification >90% (Table 4). The predictive ability of these two variables increases to up to 95% in combination with $nT_{mean>35}$, T_{min} and $x_{min}T_{min>20}$.

TABLE 4. Results of logistic regression analysis using nest success rate as response variable and incubation temperature variables as explanatory variables for nests of *Testudo graeca* monitored in Doñana National Park (see Material and Methods for definitions of variable names).

	R^2	Correct predictions (%)	Variable coefficients		
			Intercept	V1	V2
One variable					
$nT_{mean>25}$	0.435	91.4	20.08	-0.22	
T_{max}	0.490	90.3	45.70	-1.02	
Two variables					
$nT_{mean>25}$*$nT_{mean>35}$	0.377	95.5	20.99	-0.22	2.19
$nT_{mean>25}$*T_{min}	0.386	95.5	21.97	-0.22	-0.07
T_{max}*$xminT_{min>20}$	0.412	93.0	9.92	-1.01	1.56
T_{max}*$xminT_d$	0.403	90.7	31.48	-1.04	0.70
T_{max}*$nT_{min>25}$	0.406	90.5	45.95	-1.03	0.07

DISCUSSION

Variation of Incubation Temperature

The main period of egg-laying of *Testudo graeca* in Doñana occurs from the beginning of April until the first half of June, with only a few nests recorded at the end of March and during the second half of June (Díaz-Paniagua *et al.*, 2001). This protracted nesting season allows female tortoises to lay up to four clutches in a year (Díaz-Paniagua *et al.*, 1996).

Ambient temperature gradually increases throughout the nesting season of *T. graeca,* so that eggs laid in different months are exposed to different temperature regimes. Notably, early nests were incubated at lower temperatures than intermediate and late nests during approximately the first third of their incubation period, while during the two remaining thirds of incubation nests of different months are exposed to similar temperatures. This adaptation to a wide range of thermal incubation environments enables tortoises in south-western Spain to attain a high clutch frequency before the summer inactivity period (Díaz-Paniagua *et al.*, 1995).

Reptiles excavate their nests in the soil, and depending on the depth, incubation temperature may be more or less influenced by the diurnal cycle of sun radiance and heating/cooling cycles of the ground (Packard & Packard, 1988). Therefore, species with shallow nests have a wider daily variation in temperature than species nesting in deep holes (Ewert, 1979). The location of the nests on bare or vegetated ground also affects the variation of incubation temperature (Ewert, 1979; Congdon *et al.*, 1987; Janzen, 1994; Wilson, 1998; Weisrock & Janzen, 1999). *T. graeca* in Doñana National Park lay eggs in shallow nests with an average depth of 8 cm. Frequently the nests are partially shaded by a shrub, but they are also dug in bare ground (Díaz-Paniagua *et al.*, 1996). Nest temperature experienced a wide variation throughout the day, as well as throughout the whole incubation period. Mean incubation temperature in the field was about 28°C, a value frequently recorded for development of reptile eggs (see e.g. Ewert, 1979; Packard & Packard, 1988), but nest temperatures frequently reached values above the reported tolerance limits for reptile eggs (33-35°C, according to Ewert, 1979; Congdon & Gibbons, 1990; Packard & Packard, 1988). However, thermal tolerance limits have been described for constant incubation conditions in the laboratory, while embryos developing in the field are known to withstand short periods of temperatures above or below thermal limits (Congdon & Gibbons, 1990; Packard & Packard, 1988; Ewert, 1979). Similar high and fluctuating incubation temperatures are also likely to be found for other *Testudo* species arround the Mediterranean, although detailed description of field incubation temperature in other populations has not yet been reported.

Hatching Date and Incubation Length

Embryo development is accelerated by increasing temperature (Packard & Packard, 1988; Congdon & Gibbons, 1990; Deeming & Ferguson, 1991). Embryos from early *T. graeca* nests were exposed to relatively low temperatures during the initial phase of incubation, and are likely to have had very slow or even no development during this period, which is supported by the fact that hatching dates of eggs from early nests did not differ significantly from those of nests laid in May. In contrast, late nests were exposed to relatively higher temperatures during the whole incubation period, which resulted in faster embryo development.

We have found no evidence that incubation conditions of early, intermediate and late nests affect hatching rates in different ways. However, eggs from early nests had slower early development and longer incubation time, which may be expected to influence hatchling phenotype. In a previous study we detected that hatchlings from latest nests had better physical condition than hatchlings from earlier nests (Díaz-Paniagua *et al.*, 1997), probably because their higher mean incubation temperature was related to higher metabolic efficiency during development (Packard & Packard, 1988).

As a consequence of decreasing incubation length from early to late nests the hatching season was much shorter (45 days) and synchronized than the nesting season (80 days). This may be adaptive in south-western Spain, for it prevents hatchling emergence to extend into autumn. On the other hand, emergence from the nest may be delayed for several days (Díaz-Paniagua *et al.*, 1997), which probably enables hatchlings to overcome periods of harsher summer climate.

Influence of Incubation Temperature on Hatching Rate and Nest Sucess

Overall, 39% of eggs did not hatch in this study, while 20% of nests were total failures (no egg hatched). Hatching and nest success rates were unrelated to nesting period and years, and are thus unlikely to be associated with a particular tendency in thermal incubation regime. Unsuccessful nests had significantly higher values for all variables related to maximum temperature, indicating that total nest failure was caused by excessively high temperatures during incubation. This result suggests that lethal temperatures for embryo development may frequently be reached in the soil in Doñana, mainly during July. Some chelonians locate their nests under canopy vegetation cover, close to standing water or in deep holes to avoid extreme temperatures during incubation (Wilson,1998; Morjan, 2003; Weisrock & Janzen, 1999; Kolbe & Janzen, 2002). For *Kinosternon baurii*, a small aquatic turtle with shallow nests, a similar influence of maximum temperature on embryo mortality was described. *K.*

baurii apparently selected nest sites close to vegetation and avoided open sites. Embryo mortality was higher in nests located in open sites, which reached higher maximum temperatures over longer periods than covered nests (Wilson, 1998).

In Doñana National Park protection from lethal temperature peaks in shallow nests can be provided mainly by locating the nests under the shade of vegetation, where nest temperature was significantly lower than in unshaded nests. This is probably why most tortoises in Doñana locate their nests at the base of shrubs (Díaz-Paniagua *et al.*, 1996). Nest success and hatching rate decreased from the first to the last study year. Even though these differences were not statistically significant, they suggest that the study design – females confined each year in the same small enclosures – might have artificially influenced hatching success. The deterioration of vegetation within the enclosures during the four study years due to natural drying of shrubs and cumulative tortoise burying activity probably constrained the availability of adequate nesting sites.

The association of nest failure with higher maximum temperatures and the relation among temperature and vegetation cover suggests that microhabitat structure is important for the successful incubation of *T. graeca* in Doñana. Juvenile survival is generally low in this population (Díaz-Paniagua *et al.*, 2001), but high hatching success has also been observed (Díaz-Paniagua *et al.*, 1997). The stability of the *T. graeca* population in Doñana National Park depends on high adult survival but also on sporadic high juvenile recruitment bouts (Díaz-Paniagua *et al.*, 2001). In this sense, the persistence of high hatching success rates may enhance population stability and this may be achieved by conserving a habitat microstructure that enables that a sufficiently high proportion of nests do not reach lethal incubation temperatures. The same might be applicable to other *T. graeca* populations, which inhabit regions of very hot and dry late spring and summer climate around the Mediterranean. The Doñana National Park population is effectively protected, as is its habitat. However, many other *T. graeca* populations have been suffering severe habitat loss or deterioration (Andreu *et al.*, 2004; Bertolero & Cheylan, 2004; Zwartepoorte, 2004; Bour, 2004*a*,*b*,*c*; Leontyeva, 2004; Shacham, 2004). A consequence of that may be that, even where populations are still able to subsist, ideal conditions for egg incubation may have been compromised.

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COUNTING VENTRAL SCALES IN ASIAN ANILIOID SNAKES

DAVID J. GOWER AND JONATHAN D. ABLETT

Department of Zoology, The Natural History Museum, London, UK

The anteroventral scalation patterns of 48 specimens (24 species) of Asian anilioid snakes (Anomochilidae, Cylindrophiidae, Uropeltidae) were examined. Scales were pinned and X-rayed to allow the position of the neck joint to be determined. Asian anilioids have a pattern of anteroventral scalation that prevents application of the standard Dowling system for identifying the first (anteriormost) ventral scale. No repeated pattern is found between anteroventral scalation and the position of the neck joint. Between four and eight post-mentum midventral scales lie anterior to the neck joint, with intraspecific variation occurring by up to two scales. Variation in the position of the neck joint is probably caused by variation in scalation and preservation, and perhaps ontogeny, with fewer midventral scales anterior to the neck joint in larger specimens. We recommend that counts of Asian anilioid ventral scales for taxonomic purposes include all midventral scales between the mental and anal scales. For precise comparisons of precloacal vertebral numbers among Asian anilioids and other snakes, dissections or X-rays are required.

Key words: methodology, morphology, Serpentes, scalation, Uropeltidae

INTRODUCTION

In a highly influential and widely cited paper, Dowling (1951) proposed a standard system for identifying the anteriormost ventral scale in snakes. As Dowling recognised, the posteriormost ventral is readily identified as the scale adjacent to the anal scale(s), but the anteriormost ventral is not so obvious (see also Peters, 1964: 378). In the majority of alethinophidians, enlarged anterior chin shields are separated from wider scales by a number of small gular scales that are often irregular and not present in a single midventral line. Dowling's system identified the first ventral as the anteriormost wider midline scale that is directly in contact with the first row of dorsal scales. Wide scales lying between this first ventral and smaller gulars were termed "preventrals" by Rasmussen & Howell (1982; see also Largen & Rasmussen, 1993: 317) . As well as being consistent and repeatable across different workers, the first ventral was described by Dowling as corresponding to the first vertebra behind the neck joint of the axial skeleton, at least in ten colubrid genera. Thus, given that the vast majority of alethinophidians have a 1:1 correspondence between vertebrae and ventral scales (Alexander & Gans, 1966), the numbers of ventral scales counted using Dowling's system equals the number of precloacal vertebrae.

In some groups of snakes, the anteroventral pattern of scalation does not correspond to that described by Dowling, and Dowling's system cannot clearly be applied. In this paper, we examine the anterior ventral scales of one of these groups, the anilioids, comprising the South American Aniliidae, and the Asian Anomochliidae, Cylindrophiidae and Uropeltidae (*sensu* McDiarmid *et al.*, 1999). Anilioid monophyly is not well supported, but all but one species (the sole aniliid, *Anilius scytale*) are included in the more probably monophyletic (Gower *et al.*, 2005) "Asian anilioids", which are the focus of this study.

PREVIOUS STUDIES

A precise methodology is generally not presented in even the more significant of previous studies reporting ventral counts in Asian anilioids (e.g. Boulenger, 1890; Wall, 1921; Smith, 1943; Constable, 1949; Rajendran, 1985). Beddome (1886) did not describe a method for counting, but did describe ventral scales as occurring right up to the mental scale or immediately behind the first infralabials (if these meet behind the mental) in several genera of Uropeltidae. Bachman (1985) employed the same method in presenting ventral counts for *Cylindrophis maculatus*. In some cases, new counts made (by DJG) of individual specimens allow us to infer that previously reported ventral counts have included all midventral scales between the mental and anal scales (e.g. at least some of the counts presented by Günther, 1864). However, there are some discrepancies between counts given by other previous workers (without an explicit method) and counts made by one of us (DJG) that include all midventral scales between the mental and anal scales. For example, the type specimen of *Rhinophis fergusonianus* was originally described as having 184 ventrals (Boulenger, 1896) but we count 196, and *Silybura shortii* was originally described as having 134 ventrals (Beddome, 1863) but the four syntypes (McDiarmid *et al.*, 1999) have 137-147. Other possible systems for identifying the anteriormost ventral in Asian anilioids where this has not been clearly described include the first scale that is wider than long (e.g. see Schmidt & Davis, 1941) or the first midventral scale that is the same width as other undoubted (more posterior) ventrals (e.g. see Thompson, 1914). It might be noted that very few taxonomic studies of Asian anilioids have been

Correspondence: D. J. Gower, Department of Zoology, The Natural History Museum, London SW7 5BD, UK. *E-mail*: d.gower@nhm.ac.uk

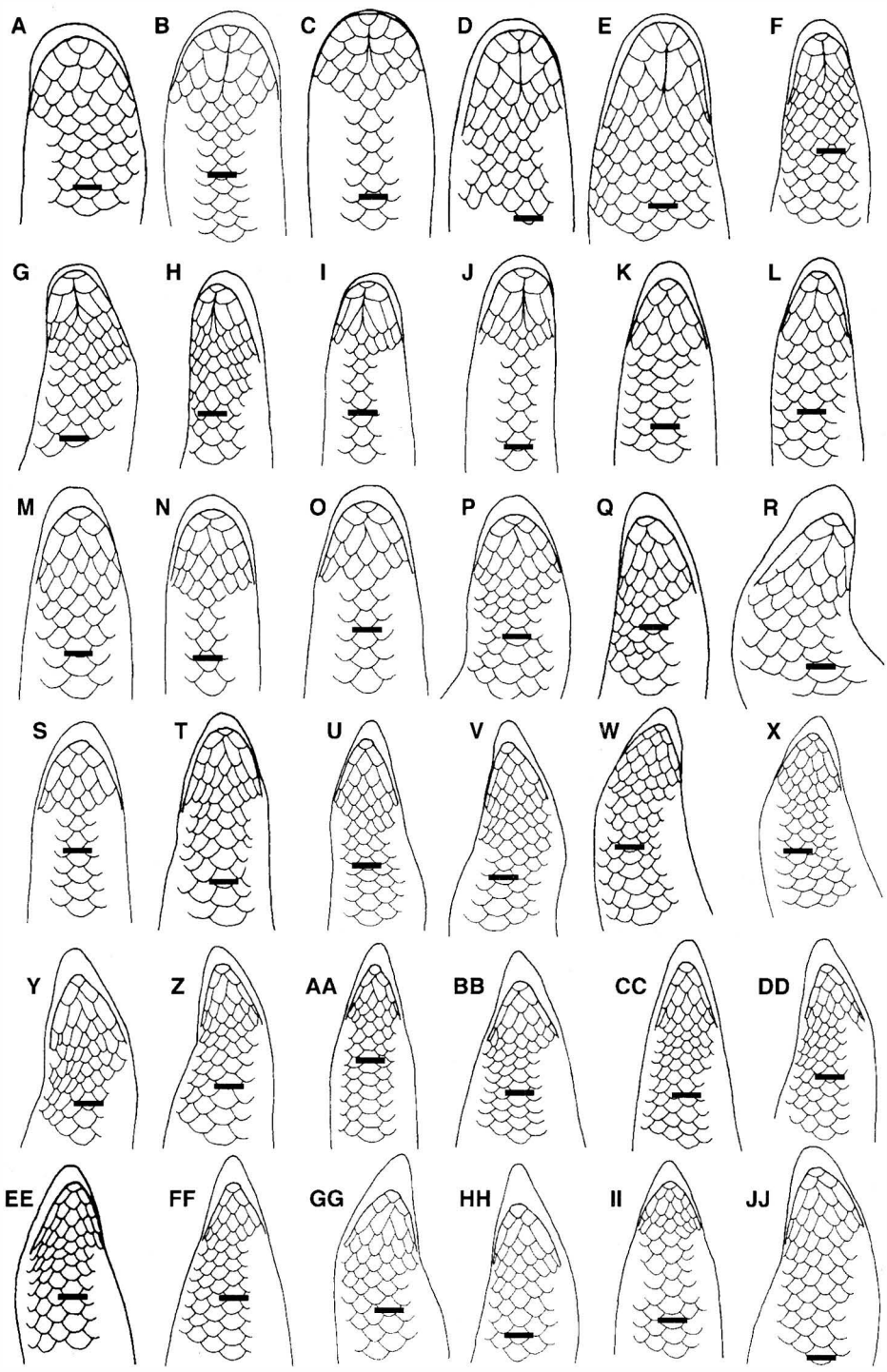

FIG. 1. Outline figures (from camera lucida drawings) of the anteroventral scalation patterns in a range of Asian anilioid snakes. Black bars indicate the position of the occipito-vertebral (neck) joint, as determined by pinning scales and X-raying specimens. The following taxa and specimen numbers (see Appendix 1 for further details) are illustrated, with total length of each specimen reported in parentheses: *A. Anomochilus leonardi* BMNH 1946.1.17.4 (274 mm); B. *Cylindrophis lineolatus* BMNH 1901.5.17.1 (665 mm); C. *C. ruffus* BMNH 87.2.7.1 (415 mm); D & E. *C. maculatus* DNM MW 1762 & 1797 (369 & 407 mm); F. *Melanophidium wynaudense* BMNH field tag MW 2542 (426 mm); G & H. *M. punctatum* BMNH field tags MW 2691 & 2479 (282 & 461 mm); I & J. *M. bilineatum* BMNH 74.4.29.698 & 699 (355 & 175 mm); K & L. *Brachyophidium rhodogaster* BMNH 1923.10.13.33 & 36 (116 & 184 mm); M. *Teretrurus sanguineus* 74.4.29.76 (215 mm); N & O. *Platyplectrurus trilineatus* BMNH 88.1.27.38 & 39 (328 & 395 mm); P. *P. madurensis* BMNH 1923.10.13.29-31 (361 mm); Q & R. *Plectrurus aureus* BMNH 89.7.6.7 & 8 (350 & 215 mm); S. *P. canaricus* BMNH 79.7.4.6-14 (379 mm); T. *Uropeltis macrolepis* BMNH 97.7.19.6 (257 mm); U & V. *U. dindigalensis* BMNH 83.1.12.6 & 7 (358 & 231 mm); W & X. *U. nitida* BMNH 78.1.11.2 & 1 (290 & 295 mm); Y. *U. ocellatus* BMNH 74.4.29.96 (300 mm); Z & AA. *Rhinophis travancoricus* BMNH field tag MW 219 & 221 (183 & 113 mm); BB, CC & DD. *U. phillipsi* DNM MW1759; 1761 & 1757 (294; 184 & 318 mm); EE. *U. melanogaster* BMNH 1905.3.25.66 (176 mm); FF. *R. philippinus* DNM MW 1739 (246 mm); GG & HH. *R. oxyrhynchus* BMNH 95.6.22.1; 233+5 (440 & 419 mm); II & JJ. *Pseudotyphlops philippinus* BMNH1955.1.9.61 & 60 (385 & 203 mm).

published since Dowling's (1951) system was proposed.

METHODS

Forty-eight ethanol preserved specimens representing all genera and a total of 23 species of anomochilids (one species), cylindrophiids (three species) and uropeltids (19 species) were examined (see Appendix 1). Between four and eight fine entomological pins were inserted perpendicular to the long axis of the body, into anterior midventral scales in the estimated region of the neck joint, with adjacent pins generally spaced by one midventral scale. Pinned specimens were subsequently X-rayed, and camera lucida drawings were made of anterior midventral scalation patterns. The radiographs allowed the position of the neck (occipito-vertebral) joint to be related to external scalation. A total of 48 specimens were pinned and X-rayed. Generally, two or three different sized specimens of each included species were examined.

RESULTS

Unlike in most colubroids and other non-anilioid alethinophidians, Asian anilioids do not have intervening scales obviously lying between the anterior scales of the midventral row and adjacent dorsal scale row (Fig. 1). Additionally, Asian anilioids generally have fairly narrow ventral scales, less than twice as wide as adjacent dorsal scale rows, which gradually narrow anteriorly onto the underside of the head. These midventral scales extend far anteriorly, up to the chin where they contact the mental scale or are separated from it by only a single pair of paramedian chin scales and/or the anteriormost infralabials. Thus, Dowling's system cannot be applied.

There seems to be no readily implemented way of recognising the position of the occipito-vertebral joint from external scalation in Asian anilioids. In our sample, the number of midventral scales lying anterior to the occipito-vertebral joint varied from four (some *Cylindrophis*, *Melanophidium*, *Rhinophis*) to eight (only *Pseudotyphlops*). Intraspecific variation was never more than two in our small samples. Variation probably correlates, in part, with variation in the presence (*Cylindrophis*, *Melanophidium*) or absence (other Asian anilioids) of a mental groove, intraspecific variation in whether the anteriormost midventral scale contacts the mental or infralabials (Fig. 1K, II) or is separated from them by at least one paramedian pair of scales (Fig. 1L, HH, JJ), and variation in the length of the stalk of the occipital condyle, which is markedly elongated in many uropeltids (e.g. Rieppel & Zaher, 2002).

For Asian anilioids, attempts to identify the first ventral as the anteriormost midventral scale that is wider than long or the width of typical (further posterior) ventral scales are both problematic because generally the midventral scales narrow anteriorly in a very gradual manner (Fig. 1), and the exposed width of ventral scales varies as specimens are manipulated because of mobile scale imbrication.

In all cases where two or more specimens of substantial different lengths were examined (e.g. Fig. 1D & E, G & H, I & J, N & O, U & V, Z & AA, CC & DD, GG & HH, II & JJ), the larger specimens had fewer midventral scales lying anterior to the occipito-vertebral joint.

DISCUSSION

In the vast majority of extant snakes, there is a 1:1 correspondence between vertebrae and ventral scales, in agreement with knowledge of dermis-vertebral relations during ontogeny (Alexander & Gans, 1966). Thus, establishing standard methods for identifying the anteriormost ventral scale and making repeatable counts of ventral scales is important for two kinds of comparisons that can be made: (1) among conspecifics and closely related species, and (2) among different major lineages of snakes. The former is important in assessments of variation for species- and population-level systematics, while the latter informs broader studies of snake phylogeny and evolution.

We detected some ontogenetic variation in the alignment of the occipito-vertebral joint and anterior midventral scales (see Fig. 1), but it is unclear whether this reflects ontogenetic reduction in relative head length, or is simply a result of preservational differences or is even an artefact of our small sample size.

Asian anilioids have a 1:1 correspondence between vertebrae and ventral scales (Alexander & Gans, 1966 - contra Bellairs & Underwood, 1951), so that a secure method for allowing the position of the occipito-vertebral joint to be determined from external scalation would allow precise comparisons of vertebral numbers with most other lineages of snakes without recourse to X-ray or internal examination. However, based on our results, there seems to be no repeatable system for identifying the anteriormost midventral scale that corresponds with the occipito-vertebral joint in Asian anilioids. In light of this, we make two recommendations: (1) for comparisons at lower levels (especially at or below the genus), ventral counts include all midventral scales between the mental and anal scales; (2) where workers choose not to follow our recommended system, the method should be described. Further, we make three additional observations: (1) individuals of the same species with the same number of precloacal vertebrae might vary in ventral scale counts made using this method because of small amounts of intraspecific variation in precise scalation patterns; (2) for comparisons of numbers of body segments among genera of Asian anilioids, and among anilioids and other snakes, it must be borne in mind that ventral counts for anilioids made in the recommended manner will be (up to eight) higher than the number of precloacal vertebrae; (3) for precise comparisons of vertebral numbers among anilioids and other snakes, specimens must be dissected or X-rayed. Comparisons among major lineages of snakes of numbers of

precloacal vertebrae based on a proxy of ventral scales counted using different methods will be biased in a relatively trivial (though directional, in at least some cases) manner.

Our recommended solution of not excluding any midventral scales between the mental and anal is consistent with some other studies that report ventral scale counts for Asian anilioids, whether described clearly (e.g. Bachman, 1985) or not (e.g. Günther's, 1864 count for the holotype of *Uropeltis bicatenata*). Intraspecific variation in ventral scale counts made using our recommended system for Asian anilioids might not correlate precisely with variation in precloacal vertebrae. This is largely because of intraspecific variation in exact scalation patterns immediately behind the mentum. In addition, variation in the preservation of individuals is likely to cause some differences in how midventral scales align with the occipito-vertebral joint. This might be exacerbated for uropeltids, at least some of which have notably free movement of the anterior end of the vertebral column within the 'integumentary envelope', in association with a particular mode of burrowing (Gans *et al.*, 1978). Thus, small variations in pre-vertebral midventral scale counts for Asian anilioids are probably not of wider relevance.

Some snakes do not have a 1:1 correspondence between vertebrae and ventral scales, for example acrochordids, some marine elapids, typhlopids and anomalepidids (Alexander & Gans, 1966). Some of these and some additional taxa that do have a 1:1 correspondence (e.g. *Anilius*, leptotyphlopids) are superficially similar to Asian anilioids in having anteroventral scalation patterns that do not permit the implementation of Dowling's (1951) system. Novel approaches to counting ventral scales were taken in some studies of these groups, for example Smith (1926: xvi) counted ventral scales in sea snakes "from the first enlarged (bituberculated) scale that can be found upon the neck". In other cases, ventral counts are not regularly recorded, for example middorsal scale counts are often the main or only longitudinal scalation count reported for scolecophidians (e.g. Gower *et al.*, 2004).

Almost all non-anilioid, non-colubroid alethinophidians (pythons, boas and close relatives, possibly not a clade) have a scalation pattern that allows Dowling's (1951) system to be applied, although we are not aware of how consistently this relates to the position of the occipito-vertebral joint. The Xenopeltidae resemble Asian anilioids in not having any obviously intervening gular scales between the anterior midventral scales and the first dorsal scale row (personal observation of *Xenopeltis unicolor*). *Xenophidion*, ungaliophiids and tropidophiids also have scalation patterns amenable to the application of Dowling's system. The latter, along with the monotypic *Anilius* (which has a pattern of anteroventral scalation superficially similar to Asian anilioids), might be the only extant alethinophidians to lie outside of a clade comprising Asian anilioids and all other alethinophidians (e.g. Slowinski & Lawson, 2002; Wilcox *et al.*, 2002; Vidal & Hedges, 2004). Thus the most recent molecular phylogenetic hypotheses for the major lineages of snakes suggest that scalation patterns for which Dowling's system can and cannot be applied are homoplastic.

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APPENDIX 1

MATERIAL OF ASIAN ANILIOIDS PINNED, X-RAYED, AND EXAMINED

BMNH – The Natural History Museum, London; DNM – Department of National Museums, Colombo, Sri Lanka. Taxonomy follows McDiarmid *et al.* (1999). For those BMNH specimens renumbered after 1946, the more recent number is given.

Anomochilus leonardi (BMNH 1952.1.2.63; 1946.1.17.4), *Cylindrophis lineolatus* (BMNH 1901.5.17.1), *C. maculatus* (DNM MW 1762 & 1797), *C. ruffus* (BMNH IV.23.2.b; 87.2.7.1; 1980.909), *Melanophidium bilineatum* (BMNH 74.4.29.698 & 699), *M. punctatum* (BMNH field tag MW 2479 & 2691), *M. wynaudense* (BMNH field tag MW 2542), *Brachyophidium rhodogaster* (BMNH 1923.10.13.33 & 36; 1936.6.11.3), *Teretrurus sanguineus* (two of BMNH 1946.1.16.57-62), *Plectrurus aureus* (BMNH 89.7.6.7 & 8), *P. canaricus* (two of BMNH 79.7.4.6-14), *Platyplectrurus madurensis* (two of BMNH 1923.10.13.29-31), *P. trilineatus* (BMNH 88.1.27.38 & 39), *Pseudotyphlops philippinus* (BMNH1955.1.9.60 & 61), *Rhinophis oxyrhynchus* (BMNH 233+5; 95.6.22.1), *R. philippinus* (DNM MW 1739, 1754 & 1756), *R. travancoricus* (BMNH field tag MW 219 & 221), *Uropeltis dindigalensis* (two of BMNH 1946.1.16.2-4), *U. macrolepis* (BMNH 97.7.19.6; 1958.14.62), *U. melanogaster* (BMNH 61.6.11.1-5; 1905.3.25.66-72), *U. nitida* (BMNH 1946.1.16.30 & 31), *U. ocellatus* (BMNH 74.4.29.95 & 96), *U. phillipsi* (DNM MW1757; 1759 & 1761).

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CONSISTENTLY DIFFERENT LEVELS OF GENETIC VARIATION ACROSS THE EUROPEAN RANGES OF TWO ANURANS, *BUFO BUFO* AND *RANA TEMPORARIA*

EDWARD G. BREDE[1] AND TREVOR J. C. BEEBEE

School of Life Sciences, University of Sussex, Falmer, Brighton, UK

[1]*Current address: Max Planck Institute for Limnology, Tropical Ecology Working Group, Plön, Germany*

We compared the genetic diversities across eight microsatellite loci of two widespread anurans, *Bufo bufo* and *Rana temporaria*, at multiple sites across their western and central European ranges. *Bufo bufo* consistently exhibited less genetic diversity than *R. temporaria*. Our evidence infers that this difference is unlikely to be a feature of the specific marker loci used, nor is it a probable consequence of the different phylogeographic histories of *B. bufo* and *R. temporaria*. No recent bottlenecks were observed in *B. bufo* or *R. temporaria* populations. Both species showed similar levels of differentiation across their European range as estimated by *F*-statistics, but whereas *R. temporaria* exhibited isolation by distance effects, *B. bufo* did not. We suggest that distinct autecological features of the two species are the most likely explanation of the diversity differences, especially more limited historical gene flow among *Bufo* compared with *Rana* populations.

Key words: amphibians, genetic diversity, interspecific variation, microsatellites

INTRODUCTION

Genetic diversity is important to the long-term viability of populations (Amos & Balmford, 2001; Hedrick, 2001). Although many factors can affect genetic diversity (Amos & Harwood, 1998), effective population size and gene flow among populations or sub-populations are among the most important. Differences in either of these features can have substantial effects on comparative genetic diversities between species. We recently found that two broadly similar anurans (*Bufo bufo* and *Rana temporaria*) had very different genetic diversities when multiple populations in Britain were analysed across eight microsatellite loci (Brede & Beebee, 2004). Although *B. bufo* had on average much larger census population sizes than *R. temporaria*, genetic diversity was consistently greatest in *R. temporaria*. This was potentially accounted for by different population structures. *Bufo bufo* had relatively few and isolated populations with little gene flow among them, whereas *R. temporaria* had multiple small populations with extensive interconnecting gene flow.

However, other factors than current population structure can affect levels of genetic diversity. Britain must have been colonized by both of these species in the immediately post-glacial period when there were land connections to mainland Europe (Vincent, 1990). If founder numbers were much smaller for *B. bufo* than for *R. temporaria*, this factor alone could have led to persistent lower genetic diversity in British *B. bufo* populations compared with those of *R. temporaria*. The potential role of founder effects in determining genetic diversity has been understood for a long time, but specific reports mainly relate to recently established populations (e.g. Merilä *et al.*, 1996; Cabe, 1998; Zeisset & Beebee, 2003). Another possible explanation is that there have been extensive recent declines, and subsequent bottlenecks, in *B. bufo* but not *R. temporaria* populations. In this study we tested the hypotheses that differences in genetic diversity between the two study species may be a result of: (1) their different post-glacial colonization histories; or (2) widespread recent population bottlenecks in the less diverse species. Our approach was to make a comparative study of the two species at multiple sites spread across their biogeographical ranges in mainland Europe. If founder effects contributed significantly to the differences in genetic diversity between them in Britain, we expected that such differences would be absent from much or all of mainland Europe, though perhaps also present in Scandinavia. By contrast, if differences were universal across the range there must be more general reasons based on differences in the ecology and population structure of the two species. Bottleneck tests should resolve whether these differences related to widespread recent declines in *B. bufo* but not *R. temporaria*. Our study focused specifically on these two species, and was not intended to test more general hypotheses about postglacial founder effects, which would require a larger number of test organisms.

We chose to use microsatellite markers as indicators of genetic diversity because they are the most polymorphic currently available. However, this carries a potential disadvantage because it was necessary to compare different sets of loci in the two species. This could generate differences based on properties of the markers rather than of the species bearing them. We address this problem by making a quantitative comparison of critical features of the microsatellite loci in the two species that might differentially affect their mutation rates, notably the repeat motifs, mean allele

Correspondence: T. J. C. Beebee, School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK. *E-mail*: T.J.C.Beebee@sussex.ac.uk

lengths, frequency of repeat interruption and relative abundances of rare and common alleles.

MATERIALS AND METHODS

SAMPLING ANURAN POPULATIONS

Mainland European and Irish samples of *B. bufo* and *R. temporaria* were collected at breeding sites by colleagues in various countries (Fig. 1). Samples of the two species were not always from the same ponds, as all that was required was a representative genetic sample of each species from each region. Seven *B. bufo* and six *R. temporaria* populations were sampled altogether. The aim was to obtain a random sample of up to 40 individuals from each location, although this number was not always achieved (Table 1). The samples were either toe or muscle tissues from adults, or entire larvae harvested at stage 26 (Gosner, 1960), and all were stored in 70% ethanol prior to DNA extraction. Larvae were collected by random netting at multiple localities within breeding ponds, to ensure that as far as possible a representative sample of the genetic variation in each population was obtained. This approach has been widely used in earlier studies with amphibians (e.g. Rowe *et al.*, 1998). For comparative purposes, samples from up to seven British populations of both species used in a previous study (Brede & Beebee, 2004) were also included in some analyses (see Appendix 1).

MICROSATELLITE GENOTYPING

DNA was extracted from tissues using a Chelex 100 protocol (Walsh *et al.*, 1991). Microsatellite loci were amplified in the presence of [a^{33}P]-dATP and locus-specific primers previously developed for these species (Brede *et al.*, 2001; Rowe & Beebee, 2001). Eight polymorphic microsatellite loci (*Bbufμ*14, 15, 39, 46, 47, 54, 62, 63) were available for *B. bufo* and a further eight (*Rtempμ*1, 2, 3, 4, 7, 8, 9,10) for *R. temporaria*. Both sets of microsatellites were dinucleotide [CA] repeats although two in *B. bufo* (*Bbufμ*14 and *Bbufμ*39) and three in *R. temporaria* (*Rtempμ*1, *Rtempμ*2 and *Rtempμ*7) had short interruptions within the repeat sequences. PCR products were electrophoresed alongside an M13 marker on standard sequencing gels (6% w/v polyacrylamide) and alleles were scored after visualisation by autoradiography (Rowe *et al.*, 1997).

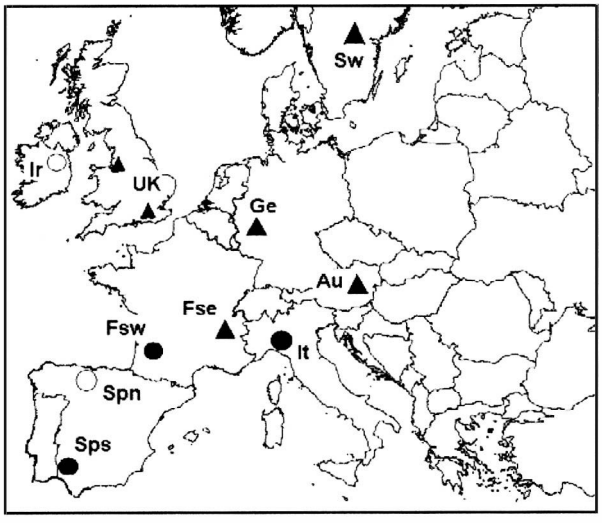

FIG. 1. Sampling site locations. Ir, Ireland; UK, United Kingdom (two sites); Sw, Sweden; Ge, Germany; Au, Austria; It, Italy; Fsw, south-west France; Fse, south-east France; Sps, southern Spain; Spn, northern Spain. Further details are given in Table 1. open circles, *R. temporaria* alone; filled circles, *B. bufo* alone; triangles, *R. temporaria* and *B. bufo*

GENETIC ANALYSIS

Tests for Hardy-Weinberg equilibrium and linkage disequilibrium were performed using BIOSYS-1 (Swofford & Selander, 1981) and GENEPOP 3.1 (Raymond & Rousset, 1995) respectively. Genetic diversity estimates including expected (H_e) and observed (H_o) heterozygosities and allelic richness were carried out using BIOSYS-1 and FSTAT (Goudet, 1995). Allelic richness estimates used samples with the same minimum sizes (n=11) for both species. This was achieved by randomly selecting 11 of the 20 *R. temporaria* samples from Austria, a country from which only 11 *B. bufo* samples were available (Table 1). Population bottleneck events were investigated using BOTTLENECK 1.2.02 (Cornuet & Luikart, 1996). A two-phase mutation model in which the proportion of stepwise mutation (SMM) was set at 70% was employed in this analysis. As with UK populations, we found no significant differences between 70% and 90% SMM assumptions in the results of the bottleneck tests (Brede & Beebee, 2004).

Pairwise estimates of F_{st} together with their statistical significance were obtained using FSTAT, and patterns of isolation by distance using linear geographic distances were compared for the two species using the ISOLDE program (with 10,000 randomisations) in GENEPOP 3.1. We also carried out assignment tests using the program GENECLASS (Cornuet *et al.*, 1999) and the "probability of belonging" facility to estimate numbers of possible immigrants in each population. Runs used the frequency method with 10,000 simulated

TABLE 1. European sampling sites and sample details.

Sampling site	*B. bufo*	*R. temporaria*
Austria (Vienna)	11 adults	20 adults
Italy (Torino)	40 larvae	-
Germany (Koblenz)	40 larvae	40 larvae
South-east France (Chambery)	40 larvae	40 larvae
South-west France (Bordeaux)	40 larvae	-
Northern Spain (Cantabria)	-	40 larvae
South-west Spain (Sevilla)	30 larvae	-
South Sweden (Skane)	24 adults	25 adults
Ireland (Limerick)	-	40 larvae
United Kingdom (Ainsdale)	40 larvae	40 larvae
United Kingdom (Pells)	40 larvae	40 larvae

individuals, a threshold of 0.01 and the "leave one out" procedure, and assumed a constant frequency of 0.01 in cases of null alleles.

Randomisation tests were performed using RT 2.1 (Manly, 1997). In this procedure, the observed mean difference between two samples is compared with the distribution obtained by randomly allocating data values to the two samples. Significant differences (at P=0.05) in a two-tailed test are inferred if the observed mean is in either 2.5% tail of the distribution following a large number (at least 1000) of data randomizations. Standard statistical analyses (Wilcoxon Signed Rank tests and correlations) were carried out using the STATISTIX 7 analytical software package (Tallahassee, USA). Estimates of H_e were arcsin transformed and all data sets were tested for normality before analysis with parametric methods (Pearson moment correlations).

RESULTS

MICROSATELLITE LOCI IN *B. BUFO* AND *R. TEMPORARIA*

The microsatellite loci used for this study were first compared among 400 individuals of each species (250 from seven British populations and 150 from six European populations) chosen at random from the totals of >450 samples available for each species. Because different loci were used in the two species, statistical assumptions based on independent sampling from the same distribution do not strictly apply. Nevertheless, it was useful to make comparisons based on a simplifying assumption that the loci are indeed equivalent as diversity indicators provided that the results are interpreted with caution. We used randomization tests for this purpose. Mean numbers of repeats in the microsatellite loci (unweighted averages across all alleles) were 9.9 for *R. temporaria* and 8.4 for *B. bufo*. These differences between the species were not significant by randomization tests in which >28% of 10,000 permutations yielded larger differences between the species than those actually observed. The proportion of loci with interrupted repeats was higher in *R. temporaria* (0.38) than in *B. bufo* (0.25). Permutation tests indicated no significant differences between species in the mean proportions of alleles present at <1% frequency (0.25 for *B. bufo*, 0.30 for *R. temporaria*) or at >10% frequency (0.19 for *B. bufo*, 0.15 for *R. temporaria*). The distributions of allele frequencies in the two sets of loci were therefore broadly similar.

GENETIC DIVERSITY IN EUROPEAN POPULATIONS OF *B. BUFO* AND *R. TEMPORARIA*

The sample data were first tested for compliance with Hardy-Weinberg equilibrium. The *B. bufo* locus *Bb*μ63 was omitted from three populations (south-east France, south-west France, and Spain) due to difficulties with scoring alleles. One *B. bufo* population (Spain) showed significant discordance from Hardy-Weinberg equilibrium at four of the remaining seven loci after Bonferroni correction. Three of the eight Spanish *R. temporaria* loci were also out of Hardy-Weinberg equilibrium after Bonferroni correction, in this case possibly because the samples were collected from three separate ponds. In all the remaining assessments, only single loci deviated significantly from Hardy-Weinberg equilibrium after Bonferroni correction in three *B. bufo* populations (south-east France, south-west France, Italy) and four *R. temporaria* populations (south-east France, Germany, Ireland, Sweden). In all cases the deviations were an excess of homozygotes. Potential causes of these deviations include sampling bias, in which siblings were over-represented, and the presence of null alleles. We have no rigorous way of distinguishing between these alternatives, but in the Spanish populations of both species, where multiple loci showed deviations, null alleles are arguably the less likely explanation. Random netting around the ponds was designed to minimize over-representation of a few kin groups, but may not have eliminated it altogether. The tests for linkage disequilibrium showed six pairs out of 252 combinations of loci to be significant after Bonferroni correction in the *B. bufo* data set, whilst in the *R. temporaria* data set eight pairs of 224 combinations were significant after Bonferroni correction. Linkage disequilibrium was randomly distributed among populations and pairs of loci, and we therefore concluded it was due to chance effects (such as sampling sibs) rather than being of biological significance.

Within the mainland European populations, the estimated average H_e for *B. bufo* was 0.612 (range 0.431 - 0.748), with an average allelic richness of 3.81 alleles/locus (range 2.21 – 4.83). European *R. temporaria* had an estimated average H_e of 0.687 (range 0.615 – 0.745) with an average allelic richness of 5.47 alleles/locus (range 4.61 – 6.12). Fig. 2 shows in more detail that European *R. temporaria* populations tended to have higher genetic diversities than European *B. bufo* populations. With the same caveats listed above concerning the interpretation of statistics not sampling the same distribution (i.e. with different loci in the two species), randomization tests indicated that 95% of 10,000 permutations yielded smaller differences in mean H_e between European populations of the two species than the mean difference (>0.08) actually observed. Randomisation tests further showed that essentially 100% of 10,000 permutations yielded smaller differences in mean allelic richness than the mean difference (1.66) actually observed. British *B. bufo* populations (n=7) had slightly lower mean expected heterozygosities and allelic richness than European populations (n=7), but in neither case were the differences significant when tested with the group comparison permutation test (1,000 iterations) in FSTAT (for heterozygosity, P=0.530; for allelic richness, P=0.329). Exactly the same situation also held for *R. temporaria* (heterozygosity P=0.878; allelic richness P=0.278) when comparing seven British and six European populations. Genetic diversities of all the European and

British *B. bufo* and *R. temporaria* populations analysed here and in previous studies (Brede & Beebee, 2004) are summarized in Appendix 1.

Within the European populations, there was no significant correlation between mean H_e and allelic richness for *B. bufo* (r=0.497, P=0.172) unless the Spanish sample, with very low allelic richness, was omitted and after which r=0.712, P=0.048. By contrast there was a significant correlation between H_e and allelic richness for the full set of European *R. temporaria* samples (r=0.789, P=0.020).

In bottleneck tests, four of the seven European *B. bufo* populations showed heterozygote excess at >50% of the loci using a 70% stepwise mutation model, but of these only one population (south-east France) was significant (P=0.039). A similar pattern was seen in the *R. temporaria* populations where four of six populations showed heterozygote excess at >50% of loci and once again only one population (Ireland) was significant (P = 0.023). However, after Bonferroni corrections for multiple comparisons no population of either species showed evidence of a significant recent bottleneck effect. Similar results were obtained with higher levels (up to 90%) of SMM in the BOTTLENECK analyses.

Population Structure

The genetic data were analysed to determine whether differences in population structure related to differences in diversity between the two species. Pairwise estimates of F_{st} among populations of both species, from the six localities across Europe where both were sampled in reasonably close proximity, are shown in Table 2. Mean F_{st} estimates across all populations were 0.200 for *R. temporaria* and 0.167 for *B. bufo*. All the F_{st} estimates were significantly greater than zero for both species. There was no significant difference in F_{st} estimates between the species when pairwise estimates were compared (Wilcoxon Signed Rank test, n=15, P=0.118), and there was no correlation of the pairwise estimates between *R. temporaria* and *B. bufo* among the sample localities (Mantel test with 10,000 permutations, P=0.529). However, whereas *R. temporaria* demonstrated significant isolation by distance effects (Mantel test, P=0.04), *B. bufo* did not (Mantel test, P=0.11).

Assignment tests were complicated by the fact that larvae, which were the only samples available from most populations, cannot migrate between ponds. Only hybrid F1 individuals were potentially detectable in these cases. Another problem was that no local potential sources of immigrants were sampled. Overall, the proportion of individuals that could not be ascribed to the population in which they were sampled with P>0.05 was surprisingly high and not significantly different for both species (mean 0.20 for *B. bufo* and 0.24 for *R. temporaria*). Interestingly, the mean proportions for the two populations where adult tissues were available (Austria and Sweden) showed higher proportions of potential migrants (0.45 for *B. bufo*, 0.44 for *R. temporaria*) than for those with larvae (0.16 for *B. bufo*, 0.20 for *R. temporaria*). However, most individuals of both species in most populations were not ascribed unequivocally to a single population either at the default threshold (0.01) or at a threshold of 0.1.

DISCUSSION

Genetic Diversity Differences Between Species

The results of this study show that *R. temporaria* populations across Europe generally maintained greater genetic diversity than *B. bufo* populations (Fig. 2, Appendix 1). This does not support the hypothesis that founder effects generated the differences in diversity between these species in Britain, but points to a more fundamental cause throughout their geographical ranges. No previous studies of genetic diversity across the range have been reported for *B. bufo*, but other work on European *R. temporaria* populations using eight microsatellite loci (including three of those we employed) yielded data broadly similar to ours (Palo *et al.*, 2004). Their results from 29 populations gave an average of 24.8 alleles (range = 9–34) per locus with average H_e estimates between 0.35–0.72. In the present study with 13 populations we found an average of 21 alleles (range = 7 - 30) per locus and average H_e estimates between 0.601–0.745. For *B. bufo* from 14 populations we found an average of 16.7 alleles (range = 11–21) per locus and average H_e estimates between 0.431–0.748. Only one other European anuran has been investigated with respect to genetic diversity at microsatellite loci across its geographical range.

TABLE 2. Pairwise F_{st} estimates. Numbers show mean F_{st} estimates across all loci. Above diagonal, *B. bufo*; below diagonal, *R. temporaria*.

	UK (Ainsdale)	UK (Pells)	Austria	SE France	Germany	Sweden
UK (Ainsdale)		0.113	0.135	0.194	0.037	0.169
UK (Pells)	0.144		0.186	0.271	0.069	0.160
Austria	0.201	0.199		0.240	0.113	0.232
SE France	0.156	0.161	0.171		0.184	0.297
Germany	0.219	0.148	0.179	0.221		0.117
Sweden	0.293	0.260	0.199	0.211	0.241	

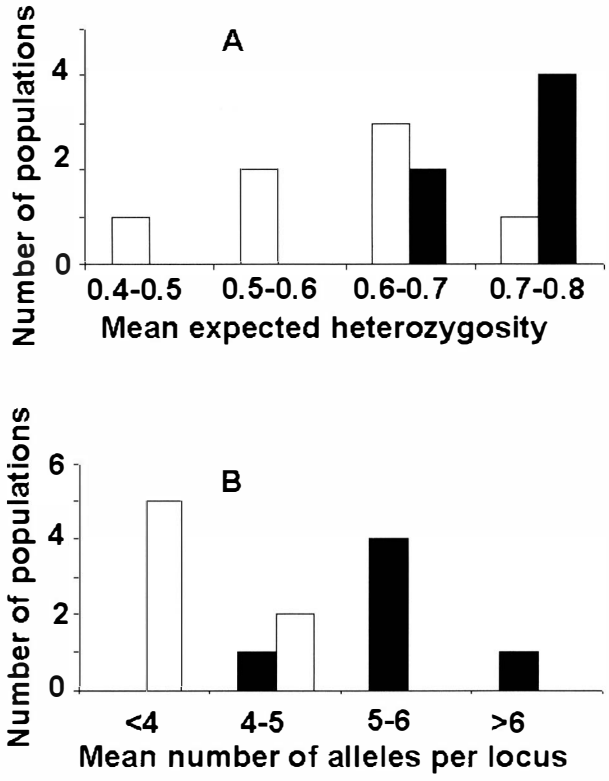

FIG. 2. Genetic diversity of European *B. bufo* and *R. temporaria* populations. (a) Frequency distributions of mean expected heterozygosities; (b) frequency distributions of mean allelic richness. Solid bars, *R. temporaria*; open bars, *B. bufo*.

Beebee & Rowe (2000) showed that across eight loci, 11 *B. calamita* populations had a mean of 13.9 (range = 11–21) alleles per locus and mean expected heterozygosities between 0.431–0.748, broadly similar to the two species studied in the present paper.

Because different loci were compared between the two species, an alternative explanation of our results is that the *B. bufo* microsatellites were inherently less variable than those of *R. temporaria*. We think this unlikely for several reasons. Firstly, the loci were broadly similar with respect to repeat motif, repeat numbers, total numbers of alleles and distributions of allele frequencies. Secondly, a higher proportion of *R. temporaria* loci contained interruptions among the repeats than was the case with the *B. bufo* loci. This factor should predispose the *R. temporaria* loci to lower mutation rates than those of *B. bufo* (Jin *et al.*, 1996), leading to the opposite result of our findings. Thirdly, a similar relationship between the two species is apparent on the basis of earlier allozyme studies. In Britain, Hitchings & Beebee (1998) found a mean observed allozyme heterozygosity of 0.035 and mean allele number per locus of 1.36 across 27 loci in four rural *B. bufo* populations. By comparison, in the same sampling area, Hitchings & Beebee (1997) found a mean allozyme heterozygosity of 0.073 and mean allele number per locus of 1.83 across 19 loci in five rural *R. temporaria* populations. Sixteen loci were common to both species. These differences in heterozygosity and allele numbers were both highly significant, with $P<0.0001$ (Wilcoxon signed rank tests) in both comparisons. Twelve of the allozyme loci were polymorphic in both species when sampled across 12 British populations. Again mean heterozygosity in *R. temporaria* (0.177) was significantly higher than that of *B. bufo* (0.048) by the Wilcoxon rank sum test ($P=0.034$) with these common loci. Difference in mean numbers of alleles per locus (4.17 in *R. temporaria*, 2.17 in *B. bufo*) was close to significance in these common loci ($P=0.052$).

A further possible explanation of interspecific differences in genetic diversity is that one of the two species has experienced widespread recent declines and/or population bottlenecks. However, tests using the BOTTLENECK program gave no indication of such differences. This was similar to the situation in Britain (Brede & Beebee, 2004). It remains possible, of course, that sensitivity to detect such effects was too low or that bottlenecks occurred too far back in time (>4 N_e generations) to be detected by the heterozygosity excess method used in this analysis (Luikart & Cornuet, 1998). The bottleneck test also assumes closed populations, and this may not always be true of *R. temporaria* because local gene flow among ponds might be substantial (Brede & Beebee, 2004).

POPULATION STRUCTURE

Comparison of pairwise F_{st} estimates among the sites where both species were sampled in reasonably close proximity indicated similar levels of differentiation at this geographical scale, where populations were separated by hundreds of kilometers. This contrasts sharply with local F_{st} estimates among ponds in southern England where inter-site migration is possible over short time scales. In these circumstances the F_{st} estimates for *B. bufo* were similar to the larger scale estimates reported here, whereas those for *R. temporaria* were some five-fold lower (Brede & Beebee, 2004). This supports the hypothesis that population structure, with much higher historical rates of gene flow among local demes in *R. temporaria* compared with *B. bufo*, contributes to overall differences in levels of genetic diversity between these species. The similar levels of differentiation observed at the larger geographical scale may reflect the effects of occasional, relatively substantive barriers to movement of both species, such as mountain ranges or major river systems. The lack of isolation by distance in *B. bufo*, but its occurrence in *R. temporaria*, also supports a more fragmented population structure with genetic differentiation dominated more by random drift effects (rather than gene flow) in *B. bufo* than in *R. temporaria*. The assignment tests, however, did not suggest that in current populations there are more first generation migrants in *R. temporaria* than in *B. bufo*. Unfortunately the relatively low power of the tests with our data to ascribe a high proportion of individuals to a unique population strongly limits any interpretation of migrant designations. It may be that more loci are required to carry out this type of analysis with high confidence.

The lack of correlation between the pairwise F_{st} estimates for each species across the European sampling sites may reflect different phylogeographic histories of *R. temporaria* and *B. bufo*. Our sampling was insufficient to generate credible postglacial colonization histories for either species. For *R. temporaria* Palo *et al.* (2004), using mitochondrial DNA sequences and microsatellites, provided clear evidence of two distinct lineages, one in eastern and the other in western Europe, and suggested northerly colonization routes during the postglacial warming. No comparable study has yet been made on *B. bufo*. Partial support for a south-west France/Iberian refugium for this species, and for a Germanic clade derived from Balkan/Italian refugia, has been found with both morphometric data and allozyme analyses (Hemmer & Böhme, 1976; Lüscher *et al.*, 2001).

In conclusion, our studies indicate a very widespread and consistent difference in the genetic diversities of two anurans with broadly similar natural histories and geographical distributions. Autecological differences between *B. bufo* and *R. temporaria* seem more likely to explain this difference than chance events in their population histories. In particular, the preference of *B. bufo* for permanent ponds often results in a lower density of breeding sites in the landscape than is the case for *R. temporaria*. This may result in lower gene flow between ponds in *B. bufo* than in *R. temporaria*. Also, we have found that ratios of effective:census population size are much lower in *B. bufo* than in *R. temporaria*, perhaps because the former but not the latter species has a sex ratio at breeding sites highly skewed in favour of males (Brede & Beebee, 2006). Taken together, these features (low gene flow and low effective population sizes) might be expected to generate lower genetic diversity in *B. bufo* compared with *R. temporaria*.

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APPENDIX 1: Genetic diversity across eight microsatellite loci within *B. bufo* and *R. temporaria* populations.

Population	Average *He* (S.E)	Average *Ho* (S.E)	Average Allelic Richness
BUFO BUFO			
Austria	0.600 (0.040)	0.516 (0.055)	3.62
SE France	0.624 (0.093)	0.595 (0.101)	4.60
SW France	0.431 (0.119)	0.420 (0.120)	3.53
Germany	0.662 (0.041)	0.635 (0.056)	4.05
Italy	0.748 (0.031)	0.625 (0.100)	4.83
Spain	0.599 (0.113)	0.614 (0.124)	2.21
Sweden	0.618 (0.072)	0.509 (0.097)	3.00
Ainsdale (UK)	0.568 (0.052)	0.509 (0.054)	3.27
Crematorium (UK)	0.568 (0.036)	0.503 (0.055)	3.09
Pells (UK)	0.609 (0.041)	0.535 (0.045)	3.62
Saltfleetby (UK)	0.623 (0.041)	0.580 (0.038)	3.50
St Annes (UK)	0.411 (0.100)	0.401 (0.112)	2.24
Whitelands (UK)	0.622 (0.038)	0.586 (0.034)	3.33
Withdean (UK)	0.647 (0.044)	0.611 (0.065)	3.00
RANA TEMPORARIA			
Austria	0.719 (0.055)	0.612 (0.080)	5.44
SE France	0.745 (0.035)	0.699 (0.041)	5.76
Ireland	0.642 (0.055)	0.618 (0.061)	4.61
Germany	0.615 (0.053)	0.576 (0.062)	5.21
Spain	0.702 (0.072)	0.641 (0.080)	6.12
Sweden	0.702 (0.054)	0.605 (0.062)	5.69
Ainsdale (UK)	0.601 (0.090)	0.586 (0.089)	4.56
Crematorium (UK)	0.682 (0.053)	0.645 (0.054)	5.12
Pells (UK)	0.659 (0.093)	0.595 (0.090)	5.46
St Annes (UK)	0.732 (0.060)	0.641 (0.056)	5.99
Halesworth (UK)	0.694 (0.045)	0.594 (0.071)	5.04
Whitelands (UK)	0.668 (0.048)	0.528 (0.064)	4.97
Withdean (UK)	0.687 (0.046)	0.740 (0.064)	5.32

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RESOURCE PARTITIONING OF SYMPATRIC *NOROPS* (BETA *ANOLIS*) IN A SUBTROPICAL MAINLAND COMMUNITY

NEIL C. D'CRUZE[1] AND PETER J. STAFFORD[2]

[1]*Department of Biological Sciences, Imperial College of Science, Technology and Medicine, London, UK*

[2]*The Natural History Museum, Cromwell Road, London, UK*

During an approximately four-week period the ecology and interrelationships of sympatric anoles (*Norops* spp., Beta *Anolis*) was studied at a lowland forest site in Belize. The primary aim was to investigate aspects of niche overlap and resource partitioning among species in a typical mainland forest community by quantifying the dimensions of morphology, structural habitat and microclimate. Through characterization of each ecological niche we aimed to determine how these lizards partition the complex resource base and habitat in which they co-exist. Anole species at the study site clearly appear to partition environmental resources along the three major resource axes of microclimate, habitat structure, and probably also prey size, as originally defined by Pianka (1974). Two of the species also show evidence of sexual size dimorphism, indicating that the 'total' niche of these species is further divided into two 'sub-niches' corresponding to each sex. Further experimental manipulations are required, however to demonstrate conclusively whether interspecific competition alone is responsible for structural patterns within anole communities such as this, and also to define the function of differential susceptibility among species to parasites. In the case of three species, a positive correlation between the number of lamellae on the fourth toe of the hind foot and perch height was observed, supporting the notion that lamella number is highly adaptive for an arboreal lifestyle and related to habitat use.

Key words: ecological niche, lizard, sexual dimorphism, toe-pad morphology

INTRODUCTION

Anolis is a speciose and ecologically diverse clade of Neotropical lizards that has been described as "a model system for addressing biological questions" (Nicholson, 2002). Several aspects of their biology are responsible for this unique distinction. They are often relatively common, and are also of sufficient size to allow direct observation both in the field and in more contrived environmental conditions where they respond relatively well (e.g. Leal *et al.*, 1998). In addition, *Anolis* communities are typically composed of several, often closely related species that successfully coexist in the same ecosystem, presenting numerous opportunities for geographical comparisons, experimental manipulation, and detailed studies of interspecific behaviour. Such is the potential of anoles in helping to improve our understanding of ecological relationships and community structure in lizards that they have been the subject of numerous field studies (e.g. Fitch, 1973, 1975; Talbot, 1976, 1979; Corn, 1981; Guyer, 1986; Pounds, 1988; Vitt *et al.*, 2003; D'Cruze, 2005).

Communities of lizards that are composed of several species can often be observed to partition environmental resources along the three major axes of prey size, microclimate, and habitat structure (Pianka, 1974). These have indeed proven to be important measures that segregate sympatric taxa and have been used in many other studies relating to anoles (e.g. Rand, 1964; Schoener, 1968; Irschick *et al.*, 1997). Understanding the basis of resource partitioning within a complex fauna, however, is complicated by the fact that the 'total' niche of each species can often be further divided into two 'sub niches' corresponding to each sex (Butler *et al.*, 2000). Anoles vary considerably in the extent of sexual dimorphism (Stamps *et al.*, 1997), differences in size between males and females ranging from striking to practically absent. Intraspecific differences in resource use must therefore also be considered in order for resource partitioning at a higher community level to be fully comprehended.

A distinctive feature of anole morphology is the expanded subdigital toe-pad, which exhibits variation among species in both the degree of expansion and number of lamellae (Savage, 2002). Earlier studies have repeatedly demonstrated positive correlations between toe-pad size or the number of toe-pad lamellae with increasing perch height (Collette, 1961; Moermond, 1979; Glossip & Losos, 1997), suggesting that variation in these characters is highly adaptive for an arboreal lifestyle and related to habitat use.

The major aims of this study were to (1) define the extent of inter- and intraspecific resource partitioning among anoles in a subtropical mainland community along the main resource axes outlined by Pianka (1974), and (2) to determine whether the particular anole species found therein demonstrate a positive correlation between the degree of arboreality and the

Correspondence: N. C. D'Cruze, Department of Biological Sciences, Imperial College of Science, Technology and Medicine, London SW7 2AY, UK. *E-mail:* cruzecontrol@gmail.com

number of subdigital lamellae. In this study the name *Norops* is used to distinguish the former beta section of *Anolis* as suggested by Nicholson (2002), which follows the classification advocated by Guyer & Savage (1986; 1992). For other recent assessments of relationships in this complex group see also Glor *et al.* (2001) and Nicholson *et al.* (2005).

MATERIALS AND METHODS

Fieldwork was conducted at the Las Cuevas Research Station, a lowland (ca. 500 m) sub-humid forest site in the provincial district of Cayo, Belize (16°44' N, 88°59' W). Six species of *Norops* are known from the general area of this locality (Stafford & Meyer, 2000), of which the following five were recorded: *N. capito*, *N. lemurinus* (=*N. bourgaei*), *N. rodriguezii*, *N. tropidonotus* and *N. uniformis*. Observations were made between 22 May and 12 June 2004, at the beginning of the summer rainy season. Searches were conducted throughout the day and evening, but lizards were seen only during the day. Most were caught by hand or net in leaf litter near trails, on the surface of broad leaves of creeping vines, stems of vines, buttresses of trees, or on the trunks of trees. Observations were recorded from a distance of 5–10 m, with intervening vegetation used as a screen in order to minimize any possible detrimental effect on natural behaviour caused by human presence. Habitat variables were measured at the point where lizards were sighted, and mean perch height and diameter were gathered following the guidelines outlined by Losos & Irschick (1996). In addition, the following other data were recorded for each individual: habitat type, time of day, whether the perch site was located in a generally wet or dry position, and whether the perch site was located in dense, shaded vegetation or in more brightly lit peripheral situations on the perimeter. Habitats were categorized into three principal groups as described for the area by Penn *et al.* (2004); broadleaf seasonal forest ("Class 2"), broadleaf semi-evergreen forest ("Class 3"), and broadleaf semi-evergreen forest (lowland) ("Class 4a"). Microhabitats included (1) ground, (2) tree buttresses or roots, (3) tree trunks and (4) twigs and branches.

For each species the following morphological variables were recorded: sex (determined by appearance of dewlap, colouration, and extent of swelling at base of the tail), snout-vent length (SVL), body mass, number of lamellae underlying the fourth toe of the hind-foot, and lengths of the forelimb, hind-limb and tail. Length of the forelimb and hind-limb were measured as the distance from the insertion point of the limb to the longest toe of each foot. Tail lengths from lizards with broken or damaged tails were not included. These same morphological traits have been used in other, similar studies of anole ecology and shown to be highly informative (Irschick *et al.*, 1997). Descriptive statistics and statistical analyses were computed using SPSS 11.5 for Windows (SPSS Inc., 2002). Means±SE are given with $\alpha \leq 0.05$ accepted as significant, and to examine whether variables remained statistically correlated once the effect of size was removed, residuals were also used from regressions of each variable against SVL (Macrini *et al.*, 2003).

In order to determine if any pattern was apparent in the ordination of results, and thus illustrate the general extent of niche separation among species, the data collected from captured individuals was assessed using non-metric multidimensional scaling. Sample sizes were restricted to 10 specimens (5 males and 5 females) of each species in order to aid visual interpretation and to ensure that sample numbers remained equal. Assessment focused specifically on the adult population with analysis restricted to the largest 10 individuals of each species. The characters used were SVL, number of lamellae, perch height, perch width, and proportional ratios of tail length (e.g. tail length divided by SVL), forelimb length, and hind-limb length. Values for each character were standardized before analysis to *z*-scores with a mean of 0 and standard deviation of 1, and the ordination of specimens along two NMMDS dimensions was plotted. A two-dimensional NMMDS solution was sought because the alternative hypothesis suspected the existence of three similarity-based groupings.

Correlations and non-parametric tests were carried out using standard statistical procedures rather than phylogenetic comparative methods (Purvis & Rambaut, 1995; Freckleton, 2000). These were chosen for several particular reasons. Firstly, Irschick *et al.* (1997) demonstrated for a very similar set of anole species (several of the same species) that no phylogenetic effect exists for the ecological and morphological variables used in this study. Macrini *et al.* (2003) did not use phylogenetic comparative methods when investigating similar anole species based on these findings. In addition, Losos (1999) stated that phylogenetic comparisons may not always be necessary as closely related species are not necessarily similar ecologically or morphologically. Most importantly, the use of phylogenetic comparative methods is rendered difficult by the fact that our understanding of anole relationships, especially mainland species, is still incomplete (Nicholson, 2002).

RESULTS

Data were collected for a total of 83 lizards representing all five species. However, only *Norops lemurinus*, *N. rodriguezii, N. tropidonotus* and *N. uniformis* occurred in sufficient numbers to allow the collection of meaningful data. Fig. 1 shows that male lizards were more abundant (or alternatively more conspicuous) than females with regard to these four species. Due to the limited sample size, data gathered for *N. captio* was not used in any analysis, and not all data gathered for the other species were included. Despite efforts to increase the sample size of *N. rodriguezii* and *N. capito* these species were least abundant. All species were most active during late morning and early afternoon with few observations before 10.00 hr and after 18.00 hr.

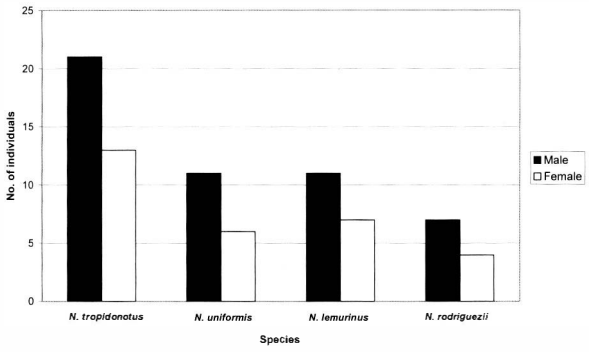

FIG 1. Sex ratios (adult) of captured lizards.

DIFFERENCES IN HABITAT OCCUPATION

One method of examining interspecific spatial differences among species is to list them according to the various habitat categories (as defined by vegetation types) in which they are observed, in the hope that "at least partial two-dimensional allopatry can be demonstrated" (Schoener, 1968). If this concept is applied to the three principal vegetation classes recognized for the area by Penn *et al.* (2004), a clear pattern emerges (Table 1). *Norops lemurinus* was the most widespread and abundant of the four species, and was found in all four vegetation types. It was the only species observed in broadleaf semi-evergreen forest (lowland) vegetation ("Class 4a" of Penn *et al.*, 2004). *Norops tropidonotus* was abundantly present in two of the three vegetation types. *Norops uniformis* and *N. rodriguezii* were also found in two of the three vegetation types, but only rarely sighted in Class 3 vegetation. All four species were classed as abundant in Class 2 type vegetation. Thus the question of how these anole species coexist at Las Cuevas, despite the number of different vegetation types present, can be simplified by addressing the wider issue of coexistence in a broadleaf seasonal forest. If a detailed study is made of microhabitat preferences within this general vegetation type, then the degree of restriction for each species within the range of vegetation available will be possible to determine.

Table 2 presents observations regarding the perch location of individual lizards, and shows that *N. tropidonotus* and *N. uniformis* are predominantly terrestrial forms, the former being most commonly encountered on the ground and the latter on tree buttresses or roots. However, these two species appear to show separation along the microclimate axis as 95% of *N. tropidonotus* were sighted in dry conditions, whereas 94% of *Norops uniformis* were sighted in generally mesic situations. This evidence supports previous claims that *N. uniformis* appears to ecologically replace *N. tropidonotus* in wetter conditions (Stafford & Meyer, 2000). In addition, the two terrestrial species appear to have different preferences with regard to vegetation density, with 82% of *N. tropidonotus* having been found basking in relatively open situations (e.g., at the edges of trails), and 71% of *N. uniforms* in dense vegetation. Table 2 also indicates that *N. lemurinus* and *N. rodriguezii* are predominantly arboreal species, as the former was most commonly encountered on tree trunks and the latter on twigs and branches. Unlike the terrestrial species these arboreal forms do not appear to show the same degree of separation along the microclimate axis, as 67% of *N. lemurinus* and 91% of *N. uniformis* were encountered in dry conditions. However, the two arboreal forms appear to have different preferences regarding vegetation density, as 72% of *N. lemurinus* were first sighted in relatively open situations whereas 91% of *N. rodriguezii* were encountered in dense vegetation.

INTERSPECIFIC AND SEX-BASED VARIATION

All four species differed significantly in snout vent length (two-way ANOVA with species and sex as factors: $F_{7,32}$=222.4, P=<0.005), tail length (two-way ANCOVA with species and sex as factors and SVL as covariate: $F_{8,31}$=342.7, P<0.005), forelimb length ($F_{8,31}$=130.0, P<0.005, hind limb length ($F_{8,31}$=175.8, P<0.005) and mass ($F_{8,31}$=48.5, P<0.005). There was also a significant difference in perch height between the four species ($F_{8,31}$=8.1, P<0.005) and perch width ($F_{8,31}$=5.0, P<0.005).

Inspection of the mean ranks revealed that *N. uniformis* and *N. rodriguezii* obtained the same values for both mass and forelimb length. This suggests that differences between them may not be significant for some of the variables and therefore require further investigation. Statistical analyses revealed that *N. uniformis* and *N. rodriguezii* differed significantly in hind-limb length (two-way ANCOVA: $F_{4,15}$=7.1, P=0.002) and tail length ($F_{4,15}$=8.2, P=0.001), but not in forelimb length ($F_{4,15}$=0.4, P=0.812) or overall snout-vent length (two-way ANOVA: $F_{3,16}$=0.5, P=0.709). Variation among the four species in morphological and habitat parameters are summarized in Table 3.

Differences in certain variables were apparent also between the sexes of two species, *N. tropidonotus* and *N. lemurinus*. Based on the largest five individuals of

TABLE 1. Distribution of species at Las Cuevas according to principal vegetation type (as defined by Penn *et al.*, 2004). A=abundant relative to other areas where the species was seen. R=present but rarely seen. O=not observed.

	N. tropidonotus	*N. uniformis*	*N. lemurinus*	*N. rodriguezii*
Broad leaf: Class 2, Seasonal forest	A	A	A	A
Broad leaf: Class 3, Semi-evergreen forest	A	R	A	R
Broad leaf: Class 4a, Semi-evergreen forest (lowland)	O	O	A	O
Total habitats where seen	2	2	3	2

TABLE 2. Numbers of adult lizards observed at Las Cuevas within specific microhabitat categories.

	N. tropidonotus		*N. uniformis*		*N. lemurinus*		*N. rodriguezii*	
	males	females	males	females	males	female	male	female
Number on ground	13	10	1	1	1	1	0	1
Number on tree buttresses or roots	6	3	8	4	0	2	2	0
Number on tree trunks	2	0	2	1	10	4	1	1
Number on twigs and branches	0	0	0	0	0	0	4	2
Number on perimeter	17	11	3	2	8	5	1	0
Number in dense foliage	4	2	8	4	3	2	6	4
Number in wet conditions	1	0	11	5	4	2	0	1
Number in dry conditions	20	13	0	1	7	5	7	3
Overall sample size	21	13	11	6	11	7	7	4

each sex, male *N. tropidonotus* snout vent lengths were significantly greater than those of females (males 54.8±0.49, range 53-54 mm; females 50.2±0.37, range 49-51 mm; ANOVA: $F_{1,8}$=55.684, P<0.005)., whereas in *N. lemurinus*, female snout-vent lengths were significantly greater than those of males (males 63.2±0.20, range 63-64 mm; females 67.2±1.50, range 65-73 mm; $F_{1,8}$=7.018, P=0.029). However, males and females of these species did not differ significantly in forelimb length, hind limb length, tail length, or mass, and there was also no apparent difference between the sexes in either of the tested perch variables.

TOE-PAD MORPHOLOGY AND DEGREE OF ARBOREALITY

The number of lamellae were significantly correlated with perch height for *N. tropidonotus* (r_S=0.67, P<0.05), *N. uniformis* (r_S=0.66, P<0.05) and *N. lemurinus* (r_S=0.66, P<0.05), but not for *N. rodriguezii* (r_S=0.22, P=0.52). The number of lamellae across all of the species studied in this community were significantly correlated with perch height (r_S=0.77, P<0.05), and when the effect of body size was removed, all correlations remained significant: *Norops tropidonotus* (r_S=0.41, P<0.05), *Norops uniformis* (r_S=0.67, P<0.05), *N. lemurinus* (r_S=0.64, P<0.05), all species (r_S=0.78, P<0.05).

MORPHOMETRIC ANALYSIS

An ordination plot of seven variables (see Material and Methods) based on non-metric multidimensional scaling reveals the existence of four distinct clusters (Fig. 2). These correspond to each of the four species at Las Cuevas for which sufficient data was obtained (i.e. *N. lemurinus*, *N. rodriguezii*, *N. tropidonotus*, and *N. uniformis*). Separation along the first dimensional axis relates mostly to differences between the species in perch height and number of lamellae. Separation along the second dimension primarily separates the species from each other with regards to size, as this axis is related to differences in SVL and the other morphological variables. Three data points corresponding to speci-

TABLE 3. Morphological and ecological data collected for anole species at Las Cuevas, Belize. Data are mean±SE, range (in parentheses), and sample size.

Species	SVL (mm)	Body Mass (g)	Tail Length (mm)	Forelimb (mm)	Hind-limb (mm)	Lamellae number	Perch height (mm)	Perch diameter (mm)
N. tropidonotus	50.2±0.4	2.1±0.04	95.2±1.1	25.1±0.16	47.4±0.4	24.4±0.2	76.2±18.8	75.3±16.9
	(46-56)	(2-3)	(81-104)	(23-27)	(42-50)	(23-28)	(10-440)	(18-410)
	n=34	n=34	n=32	n=34	n=34	n=34	n=34	n=34
N. uniformis	33.5±0.5	1±0	43±0.7	15.3±0.5	28.6±0.5	26±0.5	295.9±50.9	275.6±23.6
	(30-39)	(1-1)	(40-51)	(11-17)	(25-32)	(23-30)	(30-720)	(20-425)
	n=17	n=17	n=15	n=17	n=17	n=17	n=17	n=17
N. lemurinus	63.1±0.9	3.5±0.2	135.9±3.0	28.1±0.36	53.2±0.7	37±0.2	631.9±94.4	232±30.3
	(56-73)	(2-6)	(103-150)	(26-32)	(49-60)	(34-38)	(40-1270)	(30-370)
	n=18	n=18	n=17	n=18	n=8	n=18	n=18	n=18
N. rodriguezii	34±0.9	1±0	38.4±0.9	15.7±0.3	26±0.4	34.1±0.6	885.5±156.9	98.5±18.9
	(30-37)	(1-1)	(31-41)	(15-17)	(25-28)	(31-37)	(170-1380)	(15-230)
	n=11	n=11	n=11	n=11	n=11	n=11	n=11	n=11
N. capito	87	19	160	38	74	40	3100	300
	(n=1)	(n=1)	(n=1)	(n=1)	(n=1)	(n=1)	(n=1)	(n=1)

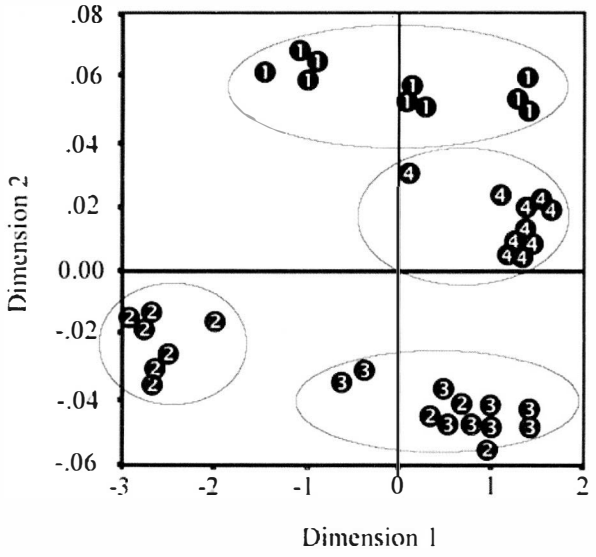

FIG 2. Ordination of specimens of *N. lemurinus* (1), *N. rodriguezii* (2), *N. uniformis* (3), and *N. tropidonotus* (4) based on results of multi-dimensional scaling analysis (Euclidean distance model). For details of characters used see text.

mens of *N. rodriguezii* occur within or very close to the *N. uniformis* cluster; this is because the two species are similar in size and the particular individuals concerned were observed close to the ground. Despite this apparent overlap the ordination pattern nonetheless indicates a significant difference in the extent of niche separation between these two species.

DISCUSSION

The four main species of *Anolis* studied at Las Cuevas clearly appear to partition environmental resources along the major resource axes of habitat structure, microclimate, and probably also prey size. Although a specific analysis of diet was not undertaken (several unsuccessful field trials were attempted), studies of other communities have indicated that prey size in anoles tends to be strongly correlated with body size (e.g. Roughgarden, 1974). These niche dimensions are not independent (Pianka, 1974), but together they separate pairs of ecologically similar sympatric species. Habitat structure appears to be the resource axis along which the greatest degree of partitioning occurs. Each of the five species may be broadly categorized as either predominantly terrestrial or arboreal, but varied significantly in respect of preferred perch height and width.

Both the terrestrial and arboreal microhabitats are occupied by two ecologically similar species that differ greatly in size. It is thus likely that species coexisting in the same microhabitat are able to do so by targeting and consuming prey of different size and possibly type. However, specific dietary analyses are clearly required in order to determine if this is indeed the case. The arboreal species appear to show greater partitioning of resources along this axis than their terrestrial counterparts, conforming to trends observed in Caribbean communities (e.g. Losos, 1994).

The two terrestrial species show clear segregation along the microclimate axis. These species were rarely observed together, suggesting that partitioning along this axis has developed to such an extent that they have become almost allotropic, with overlap at ecotones explaining sightings of the two species together (e.g. Rand, 1964). In contrast to Caribbean communities (Losos, 1994), these closely related forms differ significantly in body size. Resource partitioning in this mainland community conforms to another trend observed in the Caribbean (Losos, 1994) in that the more arboreal species do not show the same distinct level of partitioning along the microclimate axis.

SEX-BASED VARIATION

The anole species studied at Las Cuevas do not show pronounced sex-based separation along either the microclimate or structural habitat resource axes. However, the males and females of two species, *N. lemurinus* and *N. tropidonotus,* may differ significantly along the third prey size axis (ND'C pers. data based on anecdotal observations). Males of the terrestrial *N. tropidonotus* were found to be significantly larger than females; in highly territorial species such as this sexual selection may favour larger males because they typically acquire larger territories containing more females, resulting in greater mating success (Rand, 1964; Trivers, 1976; Butler *et al.*, 2000). The more arboreal *N. lemurinus* also displays evidence of sexual size dimorphism, although in contrast to *N. tropidonotus,* females were found to be significantly larger than males. In non-territorial systems, small male size may be favoured (Zamudio, 1998). However, field observations suggest this scenario is doubtful and other selective pressures relating to prey size are more likely to be responsible.

A possible explanation for the observed sexual size dimorphism in *N. lemurinus* and *N. tropidonotus* is that different habitats can vary greatly in their degree of visibility. As demonstrated by Butler *et al.* (2000) for Greater Antillean species, habitat structures favoured by the two species displaying prominent sexual size dimorphism are both relatively open with a high degree of visibility, *N. lemurinus* being typically found on the trunks of trees and the terrestrial *N. tropidonotus* restricted mostly to open vegetation near paths and clearings (Table 2). Conversely, habitat structures favoured by the two non-dimorphic species are relatively dense, *N. rodriguezii* being typically found amongst interconnecting matrices of branches and twigs, and the terrestrial *N. uniformis* in dense understorey vegetation (Table 2). Degree of visibility may therefore be implicated as a contributing factor towards the development of sexual size dimorphism, and this would benefit from further investigation

TOE-PAD MORPHOLOGY AND HABITAT USE

Variation is evident in both the degree of expansion and number of lamellae among the anoline lizards at Las

Cuevas. A positive correlation between the number of lamellae on the fourth toe of the hind-foot and perch height was observed for three species, and in agreement with Glossip & Losos (1997), statistically robust relationships were observed even when the effect of size was removed. As demonstrated in previous studies this suggests that variation in toe pad morphology is highly adaptive for an arboreal lifestyle and related to habitat use (e.g. Collette, 1961; Moermond, 1979; Glossip & Losos 1997; Macrini *et al.*, 2003). Species with more lamellae are potentially able to utilise higher perch sites because toe-pads with more lamellae have more setae and thus greater adhesive ability (Peterson, 1983). Species that select higher perch sites may also need this greater adhesive ability either because they encounter smooth surfaces more frequently, or because the consequences of falling are much greater (Macrini *et al.*, 2003).

Driving Forces Behind Resource Partitioning

Interspecific interactions, specifically in the form of competition, have been invoked as the causal basis for resource partitioning in communities of organisms since the studies of Gause (1934) and Park (1948). Losos (1994) stated that "ecologically syntopic species compete strongly, creating strong selective pressure for species to diverge in resource use, thereby allowing coexistence". Rapid micro-evolutionary adaptation in response to these shifts in resource utility is typically believed to ensue (Taper & Case, 1992), and these "adaptive shifts" are known to occur along all three of the major axes highlighted in this study (Losos, 1994).

Despite the wealth of evidence supporting interspecific interactions as the major force behind resource partitioning and structuring within *Anolis* communities, are there any other processes that could be responsible for these patterns? Both predation and "intra-guild predation" (Polis *et al.*, 1989) have been largely dismissed as major factors in resource partitioning (Losos 1994), leaving differential susceptibility to parasites as a possible influential determinant of community structure (e.g. Grosholz, 1992; Schall, 1992). The larvae of trombiculid mites in particular are commonly found on a wide range of reptile species in Belize (Stafford, pers. obs.). Among the species of *Norops* studied, however, these small parasites (typically ranging from 5 to 20 in number) were observed only in the deep axillary pockets of *N. tropidonotus* and *N. uniformis*. The presence of mites on only two of the species may thus be altering competitive relationships and facilitating their existence in sympatry. Another potentially influential and unknown factor that may affect ecological relationships among the anoles studied at Las Cuevas is the occurrence of other species that were not directly observed. At least one additional anole, *N. biporcatus*, is known from the general vicinity of this locality (Stafford, pers. obs.). *Norops biporcatus* is a large (90 mm SVL), bright green, arboreal species that is more of a canopy inhabitant, and as well as feeding on invertebrates is known to include other anoles in its diet (Taylor, 1956).

Results of this study are consistent with natural history observations reported elsewhere for the particular species concerned (Lee, 1996; Campbell, 1998; Lee, 2000; Stafford & Meyer, 2000), and mainland communities of these lizards in general (Fitch, 1973, 1975; Talbot, 1976, 1979; Corn, 1981; Guyer, 1986; Pounds, 1988). In order to draw inferences about the processes responsible for community structure and ecological divergence among anoles in this area, however, further studies are clearly required in combination with investigations of other mainland communities. Our principal results can be summarized as follows: (1) five species of anole were observed at the study site and found to be broadly sympatric; (2) male lizards were more abundant (or otherwise more conspicuous) than females with regard to the four species for which sufficient data were obtained – *Norops lemurinus*, *N. rodriguezi*i, *N. tropidonotus*, and *N. uniformis*; (3) these species clearly appear to partition environmental resources along the three major axes outlined by Pianka (1974); (4) the species differed significantly in perch height and perch width; (5) two were predominantly terrestrial (*N. tropidonotus* and *N. uniformis*) and two were predominantly arboreal (*N. lemurinus* and *N. rodriguezii*); (6) the two most terrestrial species differ in microclimate preferences and density of vegetation surrounding the perch site; (7) the two arboreal lizards show the greatest partitioning along the body size axis; (8) *N. uniformis* and *N. rodriguezii* are similar in body size but differed significantly in terms of resource use; (9) male *N. tropidonotus* were significantly larger than females in SVL; (10) female *N. lemurinus* were significantly larger than males in SVL; (11) both species were commonly found in habitats that possessed a high degree of visibility, which may be a driving factor behind the evolution of sexual size dimorphism; (12) the number of lamellae on the toe-pad of the fourth toe (hind foot) was significantly correlated with perch height for *N. tropidonotus*, *N. uniformis* and *N. lemurinus*; (13) none of the correlations listed above were affected when the effects of body size were removed.

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FOOD HABITS, ONTOGENETIC DIETARY PARTITIONING AND OBSERVATIONS OF FORAGING BEHAVIOUR OF MORELET'S CROCODILE (*CROCODYLUS MORELETII*) IN NORTHERN BELIZE

STEVEN G. PLATT[1,3], THOMAS R. RAINWATER[2,4], ADAM G. FINGER[2], JOHN B. THORBJARNARSON[1], TODD A. ANDERSON[2] AND SCOTT T. MCMURRY[2]

[1]*Wildlife Conservation Society, Bronx, New York, USA*

[2]*The Institute of Environmental and Human Health, Department of Environmental Toxicology, Texas Tech University, Lubbock, Texas, 79409-1163, USA*

[3]*Current address: Department of Biology, Sul Ross State University, PO Box C-64, Alpine, Texas, 79832, USA*

[4]*Current address: The Institute of Environmental and Human Health, Department of Environmental Toxicology, PO Box 764, Jefferson, Texas, 75657, USA*

We studied the food habits and size-related dietary patterns of Morelet's crocodile (*Crocodylus moreletii*) in freshwater wetlands of northern Belize (1992–2000). Crocodiles (n=420) were classified as hatchlings, small juveniles, large juveniles, subadults or adults based on total length. Stomach contents were obtained primarily by stomach flushing. Prey items included aquatic and terrestrial insects, arachnids, aquatic gastropods, crustaceans, fish, amphibians, reptiles, birds, and mammals. Based on the percent occurrence of recovered prey items, we concluded that the smallest size classes feed largely on insects and arachnids. Large juveniles broadened their diet to include aquatic gastropods, crustaceans, fish and non-fish vertebrates. Insect and arachnid consumption declined sharply among subadults, and increasing amounts of aquatic gastropods and fish were recovered from this size class. The adult diet consisted mainly of aquatic gastropods, fish and crustaceans. Dietary diversity was greatest among large juveniles and subadults. Conversely, hatchlings and small juveniles had the most specialized (least diverse) diet owing to a reliance on insects and arachnids. Dietary overlap was greatest between adjacent size classes, and lowest between the smallest and largest size classes. We also provide field observations of prey-specific foraging behaviours.

Key words: crocodile, foraging ecology, ontogenetic dietary change, stomach flushing

INTRODUCTION

Morelet's crocodile (*Crocodylus moreletii*) is a large crocodilian (total length [TL] to 410 cm; Perez-Higareda *et al.*, 1991) that inhabits freshwater wetlands throughout much of the Atlantic lowlands of Mexico, Guatemala and Belize (Groombridge, 1987), and many aspects of its life history, including diet and foraging ecology, remain largely unknown (Platt, 1996). Platt *et al.* (2002) investigated the foraging ecology of hatchlings (<2 months old). Schmidt (1924), Shreve (1957), Alvarez del Toro (1974) and Stafford *et al.* (2003) collectively examined the stomach contents of 17 juveniles ranging from 29 to 75 cm TL, and found turtle scutes, snail opercula, fish scales, anuran bones, crustaceans and insects. Alvarez del Toro (1974) recovered the remains of fish, turtles, and an opossum (*Philander opossum*) from the stomach of an adult, Sigler & Marina (2004) documented predation by an adult on a young brocket deer (*Mazama americana*), and Perez-Higareda *et al.* (1989) compiled a checklist of vertebrate taxa consumed by a group of subadults and adults confined in a semi-natural lagoon. However, detailed field investigations have yet to be conducted and more comprehensive dietary data for *C. moreletii* are lacking. Moreover, despite the recognition that increasing body size exerts a strong influence on diet and foraging ecology in many crocodilians (e.g. Cott, 1961; Webb *et al.*, 1982; Platt *et al.*, 1990; Thorbjarnarson, 1993*b*), only Tucker *et al.* (1996) have quantified intraspecific dietary niche overlap among different size classes.

Studies of diet are fundamental to understanding the ecology of an organism (Rosenberg & Cooper, 1990), and among crocodilians, diet has been demonstrated to affect body condition, growth, behaviour and reproduction (Lang, 1987; Delany *et al.*, 1999). Furthermore, behavioural patterns associated with hunting specific prey are poorly documented for most crocodilians (Lang, 1987; Gans, 1989), including *C. moreletii* (Platt, 1996). Field observation of foraging behaviour is difficult because much foraging activity is nocturnal, crocodiles are often wary, and turbidity may obscure underwater behaviour (Magnusson *et al.*, 1987; Thorbjarnarson, 1993*a*; Platt *et al.*, 1990). We present here the results of a dietary study of Morelet's crocodile in freshwater wetlands of northern Belize. In this study we characterize the diet of *C. moreletii*, address ontogenetic dietary differences, quantify dietary niche overlap among size classes and provide field observations of prey-specific foraging behaviours.

Correspondence: T. R. Rainwater, The Institute of Environmental and Human Health, Department of Environmental Toxicology, PO Box 764, Jefferson, Texas, 75657, USA. *E-mail*: thomas.rainwater@tiehh.ttu.edu

STUDY AREA AND METHODS

Fieldwork was conducted from 1992 to 2000 at freshwater wetlands throughout northern Belize (Belize, Cayo, Corozal and Orange Walk Districts), a region characterized by alluvial floodplains and interfluvial swampy depressions and sinkholes (Alcala-Herrera *et al.*, 1994). Natural wetlands are estimated to occupy up to 40% of the lowlands in northern Belize (Alcala-Herrera *et al.*, 1994), and generally contain water throughout the year, although levels fluctuate (Darch, 1983). Freshwater wetlands are often heavily vegetated with *Cladium jamaicense*, *Typha domingensis*, *Eleocharis* spp. and *Nymphaea* spp. (Darch, 1983; Rejmankova *et al.*, 1995). The climate of northern Belize is considered tropical with a mean temperature every month of >18°C. Annual rainfall ranges from 1,300 to 2,000 mm with a pronounced wet season occurring from mid- to late June through late November. Average monthly precipitation is variable and ranges from a maximum of 231 mm in June to a minimum of 31 mm in March (Johnson, 1983). Our study sites are described in greater detail elsewhere (Platt, 1996; Rainwater *et al.*, 1998; Platt & Thorbjarnarson, 2000).

Crocodiles were captured at night with the aid of a headlight. Smaller animals (TL≤100 cm) were taken by hand or dip net, and a noose-pole was used to capture larger (TL≥100 cm) individuals.TL and snout–vent length (SVL; tip of snout to anterior margin of cloaca) were measured, and each crocodile was permanently marked for future identification by notching the dorsal edge of a unique series of caudal scutes (Jennings *et al.*, 1991). Crocodiles were released at the capture site within 12 to 24 hours. Crocodiles were classified as hatchlings (TL<30.0 cm), small juveniles (TL=30.0–50.0 cm), large juveniles (TL=50.1 to 100.0 cm), subadults (TL=100.1–150.0 cm) or adults (TL>150.0 cm); these categories reflect size–age relationships (Platt, 1996).

Stomach contents were obtained using a modification of the stomach flushing technique of Taylor *et al.* (1978). A flexible PVC tube (exterior tube diameter=1.4, 1.9 and 2.1 cm for crocodiles <45, 45–120 and >120 cm TL, respectively) was eased down the oesophagus and into the stomach, and water was slowly poured into the tube until the abdomen became visibly distended. Gently palpating the abdomen caused a mixture of water and stomach contents to surge into the tube. The crocodile was then inverted, the contents expelled, and this mixture deposited onto a fine mesh screen. This process was repeated (usually three to four times) until only water free of stomach contents was obtained. Flushing is a safe, highly effective technique that has been demonstrated to recover >95% of prey and most non-food items from crocodilian stomachs (Fitzgerald, 1989).

We also obtained stomach contents by dissecting a small number (<10) of crocodiles that were killed by poachers, accidentally drowned in fishing nets, or found dead from unknown causes. Stomach contents were sorted and prey items identified to the lowest possible taxonomic category. Each prey item was assigned to one of nine major taxonomic categories (insects, arachnids, gastropods, crustaceans, fish, anurans, reptiles, birds and mammals). The length of every snail operculum recovered from many (but not all) crocodiles was measured to the nearest 0.1 mm and used as an index of snail size (Thorbjarnarson, 1993*b*). Non-food items such as stones, seeds and vegetable matter were also recorded. Additionally, a few (<5) crocodiles were captured with prey held in their jaws prior to swallowing. We assumed these prey would have been consumed had crocodiles not been captured and included them in our analysis.

We calculated the percent occurrence for each prey category by size class. Although often considered synonymous with frequency of occurrence, we follow Rosenberg & Cooper (1990) and define percent occurrence as the number of samples in which a particular prey item occurs divided by the sample size of a particular size-class of crocodile. Percent occurrence is appropriate when individual prey items cannot be quantified (Rosenberg & Cooper, 1990). Because bone, flesh and mollusc shell are rapidly digested, while chitinous remains, hair and feathers are more persistent, differential digestion of prey types is a common source of bias in studies of crocodilian diet (Jackson *et al.*, 1974; Fisher, 1981; Garnett, 1985; Magnusson *et al.*, 1987). To reduce bias from this source, we analysed ontogenetic trends within prey categories under the assumption that the remains of different prey within any one prey category persisted in the stomach for similar periods (Magnusson *et al.*, 1987; Thorbjarnarson, 1993*b*; Tucker *et al.*, 1996). We transformed percent occurrence data using a square root arcsine transformation (Zar, 1996) before searching for correlations in dietary composition across crocodile size classes. The association between crocodile body size and the size of snails consumed as prey was investigated by correlating the mean, minimum and maximum length of snail opercula recovered from each crocodile with crocodile SVL (Thorbjarnarson, 1993*b*). Results were considered significant at $P \leq 0.05$.

We used the Shannon–Wiener diversity index (H') to estimate dietary niche breadth and determine the degree of dietary specialization in each size class (Schoener, 1968). The Shannon–Wiener index is calculated as:

$$H' = - \Sigma p_j \log p_j$$

where p_j is the proportion of individuals using resource j (prey category). Because H' may range from 0 to infinity we standardized the index on a scale of 0 to 1 using the evenness measure J' calculated as:

$$J' = H' (\log n)^{-1}$$

where n is the number of prey categories (Krebs, 1989). The lower the value of J', the more specialized the feeding habits of a particular size class; i.e. the lowest J'

value indicates the least diversity of prey consumed, and hence the greatest degree of specialization (Schoener, 1968; Krebs, 1989).

Dietary niche overlap among size classes was determined using percent overlap (P), which measures the area of overlap of the resource utilization curves of crocodile size class j and k (Krebs, 1989). P is estimated by Σ (minimum p_{ij}, p_{ik}) × 100, where p_{ij} and p_{ik} are the proportion of prey item (i) used by size class j and k, respectively, and ranges from 0 (no overlap) to 1 (complete overlap) (Krebs, 1989).

Observations of foraging behaviour were made opportunistically while capturing crocodiles for this study and others (Platt, 1996; Rainwater, 2003; Finger, 2004), and conducting population surveys (Platt & Thorbjarnarson, 2000). We also provided apple snails (*Pomacea flagellata*) to a group of six captive *C. moreletii* (TL *c.* 75 to 150 cm) in a pond at the Belize Zoo to observe prey handling behaviour.

RESULTS

We obtained stomach contents from 420 crocodiles ranging in size from 23 to 255 cm TL by stomach flushing (412) and dissection (8). Although we captured crocodiles during every month of the year, most were taken in the late dry season (March to mid-June; n=133) and early wet season (late June through mid-August; n=157). Throughout much of the wet season, crocodiles were dispersed in flooded wetlands and proved difficult to capture (Rainwater *et al.*, 1998; unpubl. data). Hatchlings (n=71) were collected from late August to early October, shortly after emerging from the nest (Platt *et al.*, 2002). To our knowledge no mortality resulted from capture or stomach flushing, and numerous

TABLE 1. Prey items identified in the stomach contents of 420 *Crocodylus moreletii* collected in freshwater wetlands of northern Belize (1992–2000). Includes data from Platt *et al.* (2002).

Category	Taxon
Aquatic insects	Belostomatidae (giant water bugs)
	Corixidae (water boatmen)
	Dytiscidae (predaceous diving beetles)
	Gyrinidae (whirligig beetles)
	Hydrophiloidea (water beetles)
	Nepidae (water scorpions)
	Notonectidae (backswimmers)
	Odonata (dragonfly larvae)
	Tabanidae (horsefly larvae)
Terrestrial insects	Caelifera (grasshoppers)
	Carabidae (ground beetles)
	Embioptera (webspinners)
	Ephemeneroptera (mayflies)
	Formicidae (ants)
	Lepidoptera (butterflies and moths)
	Mantidae (mantids)
	Odonata (adult dragonflies)
	Scarabaeidae (scarab beetles)
Arachnids	Unidentified spiders
Gastropods	*Pomacea flagellata* (apple snail)
Crustaceans	*Cardisoma* spp. (freshwater crab)
	Procambarus spp. (crayfish)
	Decopoda (freshwater shrimp)
Fish	*Astyanix fasciatus* (Mexican tetra)
	Belonesox belizanus (alligator fish)
	Cichlasoma spp. (cichlids)
	Gambusia spp. (mosquito fish)
	Ophisternon aenigmaticum (obscure swamp eel)
	Petenia splendida (bay snook)
	Poecillia mexicana (Mexican molly)
	Rhamida spp. (freshwater catfish)
	Synbranchus marmoratus (mud eel)
Amphibians	*Bufo marinus* (marine toad)
	Eleutherodactylus spp. (rainfrog)
	Rana berlandieri (Rio Grande leopard frog)
Reptiles	*Trachemys scripta* (common slider turtle)
	Anolis spp. (anole)
	Basiliscus vittatus (basilisk lizard)
	Ctenosaura similis (spiny-tailed iguana)
	Iguana iguana (green iguana)
	Sceloporus chrysostictus (spiny lizard)
	Coniophanes schmidti (Schmidt's striped snake)
Birds	*Agelaius phoeniceus* (red-winged black bird)
	Butorides virescens (green-backed heron)
	Bubulcus ibis (cattle egret)
	Egretta spp. (egret)
	Phalacrocorax spp. (cormorant)
Mammals	*Coendou mexicanus* (Mexican hairy porcupine)
	Didelphis spp. (opossum)
	Oryzomys spp. (rice rat)
	Philander opossum (gray four-eyed opossum)
	Rattus spp. (Old World rat)
	Sigmodon hispidus (cotton rat)

TABLE 2. Prey items, gastroliths, empty stomachs, dietary diversity and evenness among size classes of *Crocodylus moreletii* (n=420) from freshwater wetlands of northern Belize. Number of crocodiles containing a specified prey followed by percent occurrence (%) within each size class in parentheses. r = correlation of percent occurrence of each prey category with size class. Size classes include hatchlings (TL<30.0 cm), small juveniles (TL=30.0–50.0 cm), large juveniles (TL=50.1–100.0 cm), subadults (TL=100.1–150.0 cm) and adults (TL>150.0 cm). Hatchling data from Platt *et al.* (2002). * P=0.05, ^{NS} Not significant (P>0.05).

Prey category	Size class (n)					r
	Hatchlings (71)	Small juveniles (117)	Large juveniles (121)	Subadults (63)	Adults (48)	
Insects	60 (84.5)	107 (91.4)	83 (68.5)	22 (34.9)	6 (12.5)	
Arachnids	21 (29.5)	31 (26.5)	8 (6.6)	1 (1.5)	0	
Insects/arachnids (total)	69 (97.1)	112 (95.7)	84 (69.4)	23 (36.5)	6 (12.5)	-0.97*
Gastropods	2 (2.8)	6 (5.1)	25 (20.6)	26 (41.2)	34 (70.8)	0.94*
Crustaceans	0	9 (7.6)	22 (18.1)	9 (14.2)	10 (20.8)	0.90*
Fish	12 (16.9)	7 (5.9)	31 (25.6)	20 (31.7)	15 (31.2)	0.79^{NS}
Anurans	0	1 (0.008)	7 (0.05)	2 (0.03)	0	
Reptiles	0	0	8 (0.06)	2 (0.03)	1 (0.02)	
Birds	0	0	2 (0.01)	3 (0.04)	5 (0.10)	
Mammals	0	0	12 (0.09)	2 (0.03)	1 (0.02)	
Non-fish vertebrates (total)	0	1 (0.008)	29 (23.9)	9 (14.2)	7 (14.5)	0.65^{NS}
Non-food items	14 (19.7)	10 (8.5)	34 (28.0)	14 (22.2)	13 (27.0)	
Gastroliths	8 (11.2)	7 (5.9)	22 (18.1)	11 (17.6)	7 (14.5)	
Empty stomachs	0	2 (0.01)	5 (0.04)	10 (0.15)	0	
Diversity (H')	0.96	1.04	1.86	1.73	1.51	0.70^{NS}
Evenness (J')	0.40	0.43	0.77	0.72	0.63	0.70^{NS}

recaptures have since been made (Platt, 1996; Platt *et al.*, 2002; unpubl. data). Although we did not verify the effectiveness of the technique, abdominal palpation indicated that flushing resulted in complete or near-complete gastric emptying. Gastroliths that probably exceeded the tube diameter occasionally remained in stomachs despite repeated flushing.

Prey recovered from crocodile stomachs included aquatic and terrestrial insects, arachnids, aquatic gastropods, crustaceans, fish, amphibians, reptiles, birds and mammals (Table 1). Insects were the most frequently recovered prey and occurred in the stomach contents of 278 (66.1%) crocodiles of all size classes (Table 2). Although whole insects and large fragments were frequently recovered, remains generally consisted of highly macerated pieces of chitin and fleshy material that could not be identified to a particular taxonomic group. Representatives of nine insect orders (Coleoptera, Diptera, Embioptera, Ephemeroptera, Hemiptera, Hymenoptera, Lepidoptera, Odonata, Orthoptera) were found among identifiable remains (Table 1). Arachnids were recovered from 61 (14.5%) stomachs. Because insects and arachnids are functionally similar as prey, these groups were combined for analyses; insects, arachnids, or both were recovered from the stomachs of 294 (70.0%) crocodiles of all size classes. There was a significant negative correlation between size class and the percent occurrence of insects/arachnids (Table 2). Hatchlings and small juveniles feed almost exclusively on insects/arachnids. With the exception of three large ants recovered from a stomach that also contained fresh anuran remains, we found nothing to suggest that insects or arachnids were secondarily ingested.

Gastropods were found in the stomach contents of 93 (22.1%) crocodiles from all size classes (Table 2). *Pomacea flagellata*, a large (ca. 60–70 g) ampullarid snail abundant in freshwater wetlands of northern Belize (Covich, 1983) was the only gastropod recovered. There was a significant positive correlation between size class and the percent occurrence of gastropods (Table 2). In a sample of 72 crocodiles (containing 1–618 opercula) there were significant positive correlations between crocodile SVL and mean (r=0.84), minimum (r=0.69) and maximum (r=0.87) operculum length.

Crustaceans were a relatively minor component of the diet and occurred in only 50 stomachs (11.9%) from all size classes except hatchlings (Table 2). There was a significant positive correlation between the percent occurrence of crustaceans and size class (Table 2).

Fish were the most frequently recovered vertebrate prey, and occurred in the stomachs of 85 (20.2%) crocodiles of all size classes (Table 2). With the exception of an anuran recovered from a small juvenile, fish were the only vertebrates consumed by hatchlings and small juveniles. Although the percent occurrence of fish remains was positively correlated with size class, this relationship was not significant (Table 2). However, the recovery of scales from an adult *Petenia splendida*, undoubtedly consumed as carrion by six hatchlings in a single pod (Platt *et al.*, 2002), inflated the percent occurrence of fish among this size class. If these six

TABLE 3. Percentage of dietary overlap (%) among size classes of *Crocodylus moreletii* from freshwater wetlands in northern Belize. Size classes include hatchlings (TL<30.0 cm), small juveniles (TL=30.0–50.0 cm), large juveniles (TL=50.1–100.0 cm), subadults (TL=100.1–150.0 cm) and adults (TL>150.0 cm).

Size class	Hatchlings	Small juveniles	Large juveniles	Subadults	Adults
Hatchlings	100.0				
Small juveniles	89.0	100.0			
Large juveniles	59.8	58.1	100.0		
Subadults	41.6	38.7	68.4	100.0	
Adults	23.1	18.3	45.1	71.0	100.0

hatchlings are removed from the analysis, the correlation between the percent occurrence of fish and size class becomes significant (r=0.90).

Vertebrates other than fish were poorly represented in the diet and occurred in only 46 (10.9%) crocodiles (Table 2). Amphibians, reptiles, birds and mammals were recovered from 10 (2.3%), 11 (2.6%), 10 (2.3%) and 15 (3.5%) crocodiles, respectively. The percent occurrence of non-fish vertebrates was positively correlated with size class, although this relationship was not significant (Table 2). Non-fish vertebrates were most frequently recovered from large juveniles; these consisted primarily of rice rats (*Oryzomys* spp.). Few subadults or adults contained non-fish vertebrates and with one exception, non-fish vertebrates were lacking from the stomach contents of hatchlings and small juveniles.

Non-food items were present in the stomach contents of 85 (20.2%) crocodiles of all size classes and included fragments of vegetation, hard seeds, pieces of wood, stones and parasites. Gastroliths (stones and hard seeds) were recovered from 55 (13.0%) crocodiles of all size classes. Empty stomachs were rarely encountered among any size class (Table 2).

Dietary diversity (H') and evenness (J') values were not significantly correlated with size class (Table 2). Dietary diversity was greatest among large juveniles and subadults, intermediate in adults, and lowest among hatchlings and small juveniles. Conversely, dietary specialization (evenness) was greatest among hatchlings and small juveniles owing to a reliance upon a limited selection of prey, primarily insects and arachnids. Large juveniles and subadults consumed insects and arachnids in addition to increasing amounts of crustaceans, gastropods and vertebrate prey, and consequently had the most generalized diet of any size class. Dietary specialization was intermediate in adults, due to the high occurrence of snails.

To summarize the general ontogenetic trend based on the percent occurrence of prey items recovered from *C. moreletii*, the diet of hatchlings and small juveniles comprises largely insects and arachnids. Large juveniles likewise rely heavily on insects and arachnids, but broaden the diet to include gastropods, crustaceans, fish and non-fish vertebrates. Consumption of insects and arachnids appears to decline greatly among subadults, and increasing amounts of gastropods and fish were found among the stomach contents; crustaceans and non-fish vertebrates were recovered less often from this size class. Gastropods were the prey most frequently recovered from adults, and although fish and crustaceans were found less often, these are nonetheless important prey for this size class. Insects and non-fish vertebrates appear to be a minor component of the adult diet.

Dietary overlap was greatest among adjacent size classes (Table 3). Near complete overlap occurred between hatchlings and small juveniles. High overlap (>60%) occurred between large juveniles and subadults, and subadults and adults, while moderate overlap (50-60%) was found between hatchlings and large juveniles, and small and large juveniles. Overlap was low (30-50%) between adults and large juveniles, as well as between subadults and hatchlings and small juveniles. The lowest (<30%) overlap occurred between adults, and hatchlings and small juveniles.

DISCUSSION

Our study is the first to examine stomach contents from a large sample of *C. moreletii* ranging in size from hatchlings to mature adults. It should be noted that several factors may confound dietary analyses based on stomach contents in crocodilians. Firstly, differing gut retention times of various prey species may bias results (Garnett, 1985; Janes & Gutkze, 2002), but because we analysed ontogenetic trends within prey categories, bias from this source is probably minimal (Magnusson *et al.*, 1987; Thorbjarnarson, 1993*b*); i.e., any digestibility bias was consistent within prey types regardless of variation among prey types (Tucker *et al.*, 1996).

Secondly, some authors have suggested that insect remains found in crocodilian stomachs were acquired secondarily from anurans consumed as prey (Neill, 1971; Jackson *et al.*, 1974; Wolfe *et al.*, 1987). However, we found nothing to suggest that secondary ingestion is a significant source of insects for *C. moreletii*. Anurans were poorly represented in the stomach contents of all size classes, particularly so among smaller crocodiles in which the occurrence of insects was greatest. Dietary studies of other crocodilians have likewise concluded that consumption of anurans is rare (Webb *et al.*, 1982; Delany & Abercrombie, 1986; Delany, 1990; Platt *et al.*, 1990; Webb *et al.*, 1991;Thorbjarnarson, 1993*b*; Tucker *et al.*, 1996). Moreover, in the single case where we recovered insect and anuran remains from the same crocodile, both were similar in size, suggesting consumption of the insects by

the co-occurring anuran was unlikely. Finally, because prey movement is important in eliciting a feeding response in crocodilians (Fleishman & Rand, 1989), ambush predators such as most anurans (Duellman & Trueb, 1986), which remain motionless for long periods, are likely to escape detection by foraging crocodiles. However, our conclusions and those of others regarding the consumption of anurans should be interpreted with caution owing to the rapid digestion of amphibians in the crocodilian stomach. Delany & Abercrombie (1986) note that sirens (*Siren lacertina*) fed to captive *A. mississippiensis* were completely digested within 24 hours and suggested that this may result in amphibians being under-represented in studies of crocodilian stomach contents.

The results of our study and others (Schmidt, 1924; Alvarez del Toro, 1974; Stafford *et al.*, 2003) indicate that insects and arachnids are especially important prey for the smaller size classes of *C. moreletii*. These results are not unexpected as studies of most crocodilians suggest that insects are the primary food for smaller size classes (e.g. Cott, 1961; Staton & Dixon, 1975; Webb *et al.*, 1982; Delany, 1990; Platt *et al.*, 1990; Thorbjarnarson, 1993*b*). Both aquatic and terrestrial insects are consumed by small *C. moreletii* suggesting that a variety of foraging modes are employed. Terrestrial insects are probably captured when crocodiles forage at the land/water ecotone and among emergent vegetation, or when insects fall into the water (Palis, 1989; Platt *et al.*, 1990).

Ampullarid snails are abundant in freshwater wetlands of northern Belize (Covich, 1983), and have previously been reported as prey for juvenile and subadult *C. moreletii* (Schmidt, 1924; Alvarez del Toro, 1974; Stafford *et al.*, 2003). Alvarez del Toro (1974) considered aquatic snails especially important food for "small" crocodiles. However, snail consumption was not reported in the only previous study of adult *C. moreletii* diet (Perez-Higareda *et al.*, 1989). In contrast to other studies of *C. moreletii* diet, our results indicate that while snails are consumed by all size classes, consumption increases with increasing crocodile body size, and is greatest among the two largest size classes. Schmidt (1924) speculated that the blunt posterior teeth of *C. moreletii* are well adapted for crushing molluscs.

The positive correlations of mean, minimum and maximum snail operculum lengths with crocodile SVL suggests that as *C. moreletii* grow larger they consume increasingly larger snails while excluding smaller snails from their diet. Optimal foraging theory predicts such an ontogenetic shift in the lower size limit of prey when the energy content of individual prey is small in relation to the energetic cost of capture and ingestion (Stephens & Krebs, 1986; Arnold, 1993). Because crocodiles are gape-limited predators (Schmidt & Holbrook, 1984), mechanical constraints undoubtedly define the upper size limit of snails that can be consumed.

It is unclear how *C. moreletii* detect and locate snails underwater, but tactile and chemical cues are probably important. We observed wild *C. moreletii* capturing snails underwater while crawling along the bottom and making frequent lateral head sweeps; contact with a snail elicited snapping behaviour. Specialized sensory organs on the jaws (Soares, 2002) probably facilitate underwater prey capture by functioning as mechanoreceptors that locate prey by touch (Thorbjarnarson, 1993*a*). Because *Pomacea* are known to release alarm pheromones in the presence of crocodilians (Snyder & Snyder, 1971), it is also possible that waterborne chemical cues play a role in locating snails. Waterborne chemicals are detected by taste buds on the tongue and posterior palate of the American alligator (*Alligator mississippiensis*) and stimulate head sweeping behaviour (Weldon *et al.*, 1990) similar to that observed among *C. moreletii*. The importance of visual cues in underwater prey capture is probably minimal as the crocodilian eye is severely hyperopic (farsighted) and usually covered by opaque membranes when submerged (Fleishman *et al.*, 1988; Platt & Brantley, 1991).

Diefenbach (1979) reported that *Caiman latirostris* swallowed intact snails either underwater or after raising the head above the surface, but we observed *C. moreletii* at the Belize Zoo crushing snails prior to swallowing. Snails were seized, held between the jaws, and then crushed in a series of rapid mandibular contractions with the head held at or slightly above the water surface. This was followed by several slow, lateral head sweeps with the jaws slightly agape and just below the surface that appeared to flush shell fragments from the mouth. The head was then tilted upwards and the crushed snail swallowed.

In contrast to our results, previous studies found few fish among the stomach contents of *C. moreletii,* probably owing to the small number of crocodiles examined and the preponderance of juveniles in this sample (Schmidt, 1924; Shreve, 1957; Alvarez del Toro, 1974; Stafford *et al.*, 2003). Perez-Higareda *et al.* (1989) included *Cichlasoma* sp. and *Anguilla* sp. on a checklist of prey consumed by *C. moreletii*, but did not quantify percent occurrence in the diet. Tactile, visual and auditory cues appear important in fish capture by *C. moreletii*. We frequently observed *C. moreletii* snapping at surface disturbance when among dense schools of *Astyanix fasciatus*, *Poecillia mexicana* and *Gambusia* spp., surface-swimming fish that create considerable disturbance when feeding. Crocodiles floated slowly in a "cross-posture position" (Olmos & Sazima, 1990) among schools of fish, making occasional forward lunges or lateral head swipes directed at surface disturbances. Crocodilians are sensitive to vibrations on the water's surface, and anecdotal accounts exist of crocodilians snapping at splashing or dripping water (Hartley & Hartley, 1977; Lazell & Spitzer, 1977). Others have commented on the importance of surface disturbance in fish capture (Whitfield & Blaber, 1979; Schaller & Crawshaw, 1982; Olmos & Sazima, 1990; Soares, 2002), and Platt *et al.* (1990) found that while

surface-swimming fish were a significant component in the diet of juvenile *A. mississippiensis*, fish inhabiting the mid-littoral zone were rarely consumed. Success rates of surface fishing are typically low (Olmos & Sazima, 1990; Thorbjarnarson, 1993*a*) and therefore this behaviour is probably energetically worthwhile only when fish are present in high densities. Although we never observed *C. moreletii* capturing bottom-dwelling fish (e.g. catfish and eels), these occurred in the diet and are probably taken in a manner similar to that described for snails that relies heavily on tactile cues.

Previous studies found non-fish vertebrates among the stomach contents of a limited number of juvenile, subadult and adult *C. moreletii* (Schmidt, 1924; Shreve, 1957; Alvarez del Toro, 1974; Stafford *et al.*, 2003). According to Perez-Higareda *et al.* (1989), wading birds were the principal food of 48 semi-captive subadult and adult *C. moreletii*, but wild and domestic mammals, amphibians and reptiles were also consumed; however, because frequencies of individual taxa in the diet were not reported, comparisons with our results must remain qualitative. Despite their low percent occurrence in the stomachs we examined, some non-fish vertebrates, particularly mammals and birds, may be important prey for *C. moreletii*. As noted by Rosenberg & Cooper (1990), measures of percent occurrence tend to minimize the importance of infrequently consumed larger prey that may nonetheless make significant energetic contributions to the diet.

While avian remains were rarely found among the stomach contents of *C. moreletii*, we observed two instances of crocodile predation on birds during this study. The first occurred when an adult (TL c. 180 cm) crocodile made two near-vertical lunges to snap at grey-breasted martins (*Progne chalybea*) skimming above the surface of a pond. These lunges began with the head resting on the surface, and propelled the crocodile far enough out of the water to expose the forefeet to view; one lunge resulted in prey capture. *Crocodylus niloticus* have likewise been reported to capture small, low-flying birds (Atwell, 1954). We also observed a subadult crocodile (TL c. 120 cm) swimming from a rookery with a freshly killed adult green-backed heron (*Butorides virescens*) in its mouth. Additionally, although predation was not observed, concentrations of crocodiles were frequently encountered at cormorant (*Phalacrocorax* spp.) roosts during spotlight surveys. Predation of adult and juvenile wading birds at rookeries and nocturnal roosts by *A. mississippiensis* is well documented in the literature (McIlhenny, 1935; Hopkins, 1968; Ruckdeschel & Shoop, 1987).

Plant material, small stones and hard seeds are frequently reported among stomach contents in studies of crocodilian diet (Cott, 1961; Webb *et al.*, 1982; Platt *et al.*, 1990; Webb *et al.*, 1991; Thorbjarnarson, 1993*b*; Tucker *et al.*, 1996). Although deliberate frugivory by captive crocodilians has been observed (Brueggen, 2002; Brito *et al.*, 2002), it is generally assumed that plant material is ingested incidental to prey capture and has no nutritional value (Coulson & Hernandez, 1983). Small stones and other hard objects are purposefully consumed and serve as gastroliths (Davenport *et al.*, 1990; Fitch-Snyder & Lance, 1993). While not essential for digestion, gastroliths are thought to facilitate the breakdown of ingested prey in a manner similar to grit in the avian gizzard, and may be especially important for smaller size classes that consume chitin-rich diets (Sokol, 1971; Platt *et al.*, 1990; Fitch-Snyder & Lance, 1993). Davenport *et al.* (1990) found that gastroliths enhance digestion by squeezing fluids from punctured arthropods, but Taylor (1993, 1994) discounted this role and speculated that gastroliths serve primarily as ballast for buoyancy control. More recently, Henderson (2003) used a mathematical and computational model to convincingly demonstrate that the relatively small mass of gastroliths occurring in crocodilian stomachs is inconsequential for maintaining stability and buoyancy in the water column.

Ontogenetic dietary changes have not been previously reported for *C. moreletii*, but are well documented in many species of crocodilians (Lang, 1987), and presumably reflect energetic advantages and the ability of larger individuals to capture larger prey (Webb *et al.*, 1991). In general, smaller size classes subsist primarily on insects and crustaceans, with a pronounced increase in the consumption of vertebrates as individuals mature (Lang, 1987). *Crocodylus moreletii* appears to follow this general pattern except that as crocodiles mature, the diet includes increasing amounts of aquatic snails rather than vertebrates. This trend is undoubtedly exaggerated by the tendency of snail opercula to accumulate in the stomach (Barr, 1997), over-emphasizing the percent occurrence of this item in the diet. However, other vertebrate remains such as fish scales, turtle scutes, bird feathers and mammal hair that are likewise resistant to digestion (Delany & Abercrombie, 1986; Janes & Gutzke, 2002) would also accumulate and be over-represented in the diet if crocodiles were consuming significant numbers of these taxa.

We found high dietary overlap between adjacent size classes of *C. moreletii,* with decreasing overlap as size differences increased; the lowest overlap occurred between the largest and smallest size classes. This is not unexpected in a species such as *C. moreletii* that undergoes an almost 500-fold increase in body size from hatching to adulthood. Similar findings were reported by Tucker *et al.* (1996) for *C. johnstoni* in the only previous study to quantify intraspecific dietary overlap in crocodilians. Despite high dietary overlap between similar-sized *C. moreletii*, niche overlap alone does not necessarily indicate that competition is occurring (Pianka, 1988). Although habitat use by *C. moreletii* has yet to be investigated, intraspecific size-related ecological separation appears commonplace among crocodilians (Lang, 1987) and may function to reduce competition for food (Tucker *et al.*, 1996).

The magnitude of dietary overlap between the largest (adult) and smallest (hatchling and small juvenile) size classes of *C. moreletii* is within the range of differences

typically found between species (MacArthur, 1972; Polis, 1984). If different size classes use sufficiently different resources, they may function as different ecological entities (see review by Polis, 1984). These entities were described by Enders (1976) and Maiorana (1978) as 'ecological species', and defined as intraspecific units whose differences in resource use approximate those of taxonomic species. Because intraspecific competition between 'ecological species' is minimal, interspecific rather than intraspecific interactions may be more important in defining patterns of resource use for these size classes (Polis, 1984). However, these potentially complex community interactions have not been investigated in any crocodilian.

Finally, although we did not investigate seasonal patterns of prey consumption by *C. moreletii* in northern Belize, seasonal changes in diet have been reported in other crocodilians (Valentine *et al.*, 1972; Gorzula, 1978; Hutton, 1987; Thorbjarnarson, 1993*b*; Tucker *et al.*, 1996). Prey availability is often influenced by seasonal fluctuations in water levels that function to concentrate or disperse prey (Valentine *et al.*, 1972; Platt *et al.*, 1990; Thorbjarnarson, 1993*b*). For example, during dry periods crustaceans are often unavailable when aestivating, while fish become concentrated in shallow pools and are readily captured by foraging crocodiles (Thorbjarnarson, 1993*b*). On the other hand, fish are less available when dispersed by rising water levels, while terrestrial insects become accessible in partially flooded vegetation (Platt *et al.*, 1990). Given the pronounced wet-dry seasonality of northern Belize, seasonal differences in the diet of *C. moreletii* are likely and warrant future investigation.

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PHYLOGENETIC RELATIONSHIPS OF *LYGODACTYLUS* GECKOS FROM THE GULF OF GUINEA ISLANDS: RAPID RATES OF MITOCHONDRIAL DNA SEQUENCE EVOLUTION?

JOSÉ JESUS[1], ANTONIO BREHM[1] AND D. JAMES HARRIS[2]

[1] *University of Madeira, Penteada, Portugal*

[2] *Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO\UP) and Departamento de Zoologia–Antropologia, Faculdade de Ciências, Universidade do Porto, ICETA, Campus Agrario de Vairão, Portugal*

Mitochondrial DNA (12S rRNA, 16S rRNA and cytochrome *b*) sequences and nuclear sequences (C-*mos*) were analysed within *Lygodactylus thomensis* from three volcanic islands in the Gulf of Guinea that have never been connected to the continent. Our aim was to assess interrelationships between the three subspecies to test a recent hypothesis suggesting high rates of mitochondrial DNA (mtDNA) sequence evolution in geckos. Our results indicate, based on mtDNA sequence data, that the three subspecies are genetically differentiated at a level more typically observed between species. However, the forms cannot be differentiated using the nuclear marker C-*mos*. These results further substantiate the hypothesis of rapid rates of mtDNA sequence evolution in geckos, although the alternative that C-*mos* is evolving more slowly cannot be discounted. They also suggest that present calibrations for molecular clocks are at the upper limit of divergence over time.

Key words: dwarf gecko, genetic analysis, phylogeny, São Tomé, Príncipe, Annobon

INTRODUCTION

The islands of the Gulf of Guinea are part of a volcanic chain formed during the middle to late Tertiary. Bioko (formerly Fernando Pó) is the largest and closest to Africa, only about 32 km from Cameroon. Smaller and more geographically isolated are São Tomé and Príncipe (1001 km^2 combined), which include a number of small islets, and 160 km southwest of São Tomé, Annobon (17 km^2; Fig. 1). Estimated ages for the origins of Príncipe, São Tomé and Annobon are 31 my, 14 my and 4.8 my respectively (Lee *et al.*, 1994). These three islands have never been interconnected, or linked to the continent. This isolation has promoted species divergence and evolution, and they presently harbour several endemic species, including the dwarf gecko, *Lygodactylus thomensis*.

Lygodactylus contains about 60 species, with a centre of distribution in sub-Saharan Africa. Unusually for geckos, dwarf geckos are diurnal. *Lygodactylus thomensis* is the only dwarf gecko known from Príncipe, São Tomé and Annobon. Three subspecies have been recognized, *L. t. thomensis* from São Tomé, *L. t. delicatus* from Príncipe and *L. t. wermuthi* from Annobon.

Although many phylogenetic studies have been performed on the gecko fauna of the more northern Atlantic volcanic islands, such as the Cape Verde archipelago (Carranza *et al.*, 2000; Jesus *et al.*, 2001, 2002) and the Canary Islands (Gübitz *et al.*, 2005), very little is known about the fauna of the islands of the Gulf of Guinea. A recent phylogenetic study of the geckos *Hemidactylus* indicated that the commonest species, *H. mabouia*, was probably introduced, and also indicated the existence of a genetically distinct lineage (Jesus *et al.*, 2005*a*) that may in fact be *H. longicephalus* (Carranza & Arnold, 2006). Like other recent studies on geckos (e.g. Austin *et al.*, 2004; Kasapidis *et al.*, 2005; Kronauer *et al.*, 2005; Lamb & Bauer, 2000; Harris *et al.*, 2004*a,b*) this work highlighted extraordinarily high levels of mtDNA sequence divergence within morphologically conservative geckos. However, in the studies where a comparison with nuclear DNA sequence data has been available (Austin *et al.*, 2004; Harris *et al.*, 2004*b*; Jesus *et al.*, 2005*a*) variation within the nuclear markers has been low or non-existent. This led to the speculation that geckos may have a relative fast rate of mtDNA evolution (Jesus *et al.*, 2002; Harris *et al.*, 2004*a*). Using both mitochondrial and nuclear DNA sequences we aim to (1) examine the levels of variation between forms on the three islands, and compare this with the age of the islands; (2) determine the possible colonization sequence of the islands, and compare colonization rates and patterns to those of other species; and (3) further test the hypothesis of high rates of mtDNA sequence evolution in an additional gecko species.

MATERIALS AND METHODS

The number and geographic locations of the specimens used in this study are given in Table 1 and Fig. 1. Voucher specimens are housed in the collections of the University of Madeira. Total genomic DNA was extracted from small pieces of tail using standard

Correspondence: D. J. Harris, Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO\UP) and Departamento de Zoologia–Antropologia, Faculdade de Ciências Universidade do Porto, ICETA, Campus Agrario de Vairão, 4485-661 Vila do Conde, Portugal.
E-mail:james@mail.icav.up.pt

TABLE 1. Specimens used in this study. Localities refer to Fig. 1. Codes refer to voucher specimens and to Fig. 2.

Species	Locality	Code
L. t. wermuthi	Annobon	638, 639, 640,641, 642, 643, 645, 646, 649, 650
L. t. thomensis	S. Nicolau, São Tomé	725, 727
L. t. delicatus	Montalegre, Príncipe	699, 700
	Terra Velha, Príncipe	720
L. capensis	Tanzania	TZ32
L. luteopicturatus	Tanzania	TZ1

methods (Sambrook *et al.*, 1989). Primers used in both amplification and sequencing of mitochondrial DNA were 16SL and 16SH, 12Sa and 12S*b*, and Cytochrome *b*1 and 3 from Kocher *et al.* (1989). Amplification conditions were the same as those described by Harris *et al.* (1998). Primers used to amplify a fragment of the nuclear gene C-*mos* were G73 and G74, and were used following the conditions given by Saint *et al.* (1998). C-*mos* sequences have been widely used to infer relationships at many levels within geckos (e.g. Austin *et al.*, 2004; Carranza *et al.*, 2002; Han *et al.*, 2004; Harris *et al.*, 2004*b*). Two outgroup species were also sequenced for all four gene regions, *Lygodactylus luteopicturatus* and *Lygodactylus capensis*. Additionally for the analyses based only on C-*mos*, sequences of *Lygodactylus* sp. and *Lygodactylus bradfieldi* were also included (Austin *et al.*, 2004; Han *et al.*, 2004). Amplified fragments were sequenced on a 310 Applied Biosystem DNA Sequencing Apparatus. Sequences were aligned using Clustal W (Thompson *et al.*, 1994). Length variation in loop regions of the rRNAs was relatively limited, and all positions were included in the analysis. Mitochondrial DNA sequences were imported into PAUP* 4.0b10 (Swofford, 2003) for phylogenetic analysis. For the phylogenetic analysis of the combined data, we used maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference. We used the approach outlined by Huelsenbeck & Crandall (1997) to test 56 alternative models of evolution, employing PAUP* 4.0b10 and Modeltest (Posada & Crandall, 1998). Once a model of evolution was chosen, it was used to estimate a tree employing ML (Felsenstein, 1981) with random sequence addition (10 replicate heuristic search). The MP analysis was also performed with random sequence addition (100 replicate heuristic searches). In both MP and ML support for nodes was estimated using the nonparametric bootstrap technique (Felsenstein, 1985) with 1000 replicates. The Bayesian analysis was implemented using MrBayes (Huelsenbeck & Ronquist, 2001). Two independent replicates were conducted and inspected for consistency to check for local optima. Both analyses were conducted with random starting trees, run for 1 x 10^6 generations, and sampled every 1000 generations using a general-time-reversible model of evolution with a gamma model of among-site rate variation. In both searches, stationarity of the Markov Chain was determined as the point when sampled ln-likelihood values plotted against generation time reached a stable mean equilibrium value; "burn-in" data sampled from generations preceding this point were discarded. All data collected at stationarity were used to estimate posterior nodal probabilities and a summary phylogeny. New sequences from C-*mos* for nine individuals were aligned against the published *Lygodactylus* sequences. There were no indels. Because no characters were homoplastic (consistency index=1) only an MP analysis was performed.

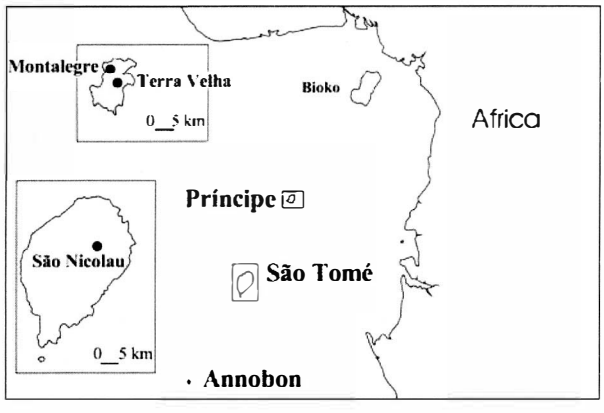

FIG. 1. Map showing the sampling localities of *Lygodactylus* from the Gulf of Guinea. The outgroup samples are both from Tanzania.

RESULTS

For the combined mtDNA gene fragments, 17 individuals were included for a total of 1177 base pairs; ML, MP and Bayesian analyses gave identical estimates of relationships (Fig. 2). The most appropriate model for the combined data was the GTR model with an estimate of invariable sites (0.21) and a discrete approximation of the gamma distribution (0.41). The ML heuristic search using this model found two trees of -Ln 3590. Bayesian analysis produced an identical estimate of relationships to one of these. For MP 176 characters were informative, and the MP search found one tree of 458 steps (Fig. 2). All analyses varied only in relationships between individuals from Annobon. In all analyses all three islands formed monophyletic clades with 100% support. *Lygodactylus thomensis* is a monophyletic group, similarly with 100% support, relative to the included outgroups. Also supported is a sister-taxa relationship between *L. t. delicatus* from Príncipe and *L. t. wermuthi* from Annobon. Average levels of sequence divergence between congeneric rep-

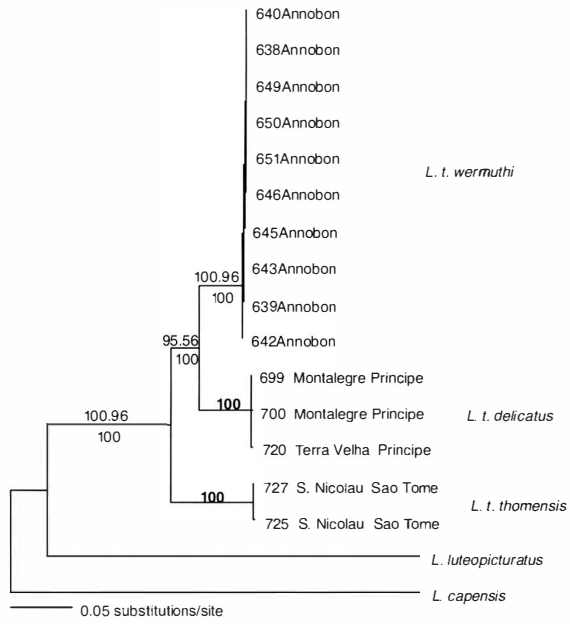

FIG. 2. One of two trees derived from an ML analysis of combined 12S and 16S rRNA fragments using the model described in the text. MP and Bayesian analyses gave identical estimates of relationships. Bootstrap values (>50%) for MP and ML are given above the nodes, and Bayesian probabilities are given below the nodes. When all values were the same, one value is given. The tree was rooted using *Lygodactylus capensis* and *L. luteopicturatus*.

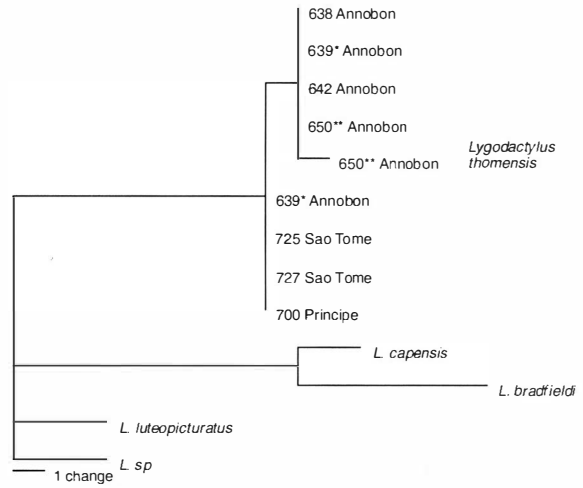

FIG. 3. Single MP tree showing relationships derived from partial sequences of C-*mos*. The * and ** indicates the heterozygous alleles from the same individuals.

tile species is known to average approximately 12% for cytochrome *b* (Harris, 2002). Sequence divergence for cytochrome *b* between populations from Annobon and Príncipe is approximately 10%, and between Príncipe and São Tomé approximately 15%.

For the C-*mos* nuclear DNA sequences 11 characters were parsimony-informative. A heuristic search found a single tree of 28 steps (Fig. 3). Our analyses of variation of C-*mos* indicate minimal variation within *Lygodactylus thomensis*. Only three haplotypes were found, with two individuals being heterozygous. One haplotype was found in individuals from all three islands. *Lygodactylus thomensis* was clearly differentiated from the other species included in the analysis.

DISCUSSION

Analysis of the mtDNA sequences produced a robust estimate of relationships for populations from the three islands. Presently the species appears to be monophyletic although including additional *Lygodactylus* species in the analysis would be necessary to confirm this. The populations from Annobon and Príncipe are sister taxa. Given the geographical remoteness and younger geological age of Annobon, and that the majority of individuals from Annobon had a derived haplotype for the C-*mos*, it seems very likely that Annobon was colonized from Príncipe. Thus *Lygodactylus* on these islands do not fit a classic "stepping-stones" model of island colonization. Nor do they show the same pattern as *Hemidactylus*, where *H. newtonii* that is endemic to Annobon is sister taxon to a form from São Tomé (Jesus *et al.*, 2005*a*) that is either a new species or may correspond to *H. longicephalus*. They also differ from *Mabuya* skinks, which independently colonized each island (Jesus *et al.*, 2005*b*). These differences in colonization patterns highlight the difficulties in drawing general conclusions regarding how islands are colonized from only a few species – clearly stochastic processes play an important role.

Despite the much greater ages of São Tomé and Príncipe relative to Annobon, the time delay between colonization events is relatively similar. Carranza *et al.* (2000), using 12S rRNA and cytochrome *b* sequences calibrated for *Tarentola* geckos in the Canary Islands, estimated 1.96% sequence divergence per million years. Since *Hemidactylus* have similar size and preferred temperatures this estimate is likely to be appropriate in this group also (Gillooly *et al.*, 2005). Based on our estimate of relationships we cannot determine if São Tomé or Príncipe was the first island colonized. However, the 10% divergence between them for 12S and cytochrome *b* combined sequences suggests that there was an approximately five million year delay between colonization of the first and second islands. The 8% divergence between Príncipe and Annobon would indicate that Annobon was colonized approximately four million years ago, less than one million years after its formation. This is a relatively short delay given the small size and isolation of Annobon – the delay before Madeira was colonized by the lacertid lizard *Lacerta dugesii*, for example, was closer to 10 million years (Brehm *et al.*, 2003). This supports the hypothesis that geckos are relatively rapid reptile colonizers, probably due to the ability of their calcareous-shelled eggs to resist salt water and to be able to be rafted from place to place (Brown & Alcala, 1957). It further suggests that calibration of molecular clocks at a slower rate than that used here would be inappropriate, at least for *Hemidactylus*, as they would predict that Annobon was colonized prior to its formation.

Given the high levels of mtDNA sequence divergence between populations from the different islands we would have expected to see some variation within the C-*mos* sequences. Variation within *Lacerta schreiberi*, for example, is less than half the level seen for mtDNA sequences, but four haplotypes at C-*mos* have been reported (Paulo *et al.*, 2002; Godinho *et al.*, 2001). Similar situations occur in *Mabuya* from the Cape Verde Archipelago (Brehm *et al.*, 2001) and in *Lacerta dugesii* (Brehm *et al.*, 2003; Jesus *et al.*, 2005*c*). At the same time almost every study of intraspecific variation within geckos has uncovered extremely high levels of mtDNA sequence variation (e.g. Austin *et al.*, 2004; Harris *et al.*, 2004*a,b*; Kasapidis *et al.*, 2005; Kronauer *et al.*, 2005; Rocha *et al.*, 2005). Such levels are much higher than typically seen in other vertebrates – up to 26.9% for cytochrome *b* in *Thecadactylus rapicauda*, for example (Kronauer *et al.*, 2005). Although there are other possible explanations, such as an artefact of taxonomy due to morphological conservativeness of geckos, combined with low levels of variation with C-*mos* the data are consistent with the theory of an elevated rate of mtDNA sequence evolution in geckos. More nuclear markers from diverse groups of geckos will be needed to test this further.

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DEFENSIVE BEHAVIOUR IN PIT VIPERS OF THE GENUS *BOTHROPS* (SERPENTES, VIPERIDAE)

MÁRCIO S. ARAÚJO[1] AND MARCIO MARTINS[2]

[1]*Programa de Pós-Graduação em Ecologia, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP, Brazil*

[2]*Departamento de Ecologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil*

The genus *Bothrops* encompasses at least six evolutionary lineages that show a great diversification in macro and microhabitat use. We studied the defensive behaviour of one species of each of five lineages within the genus *Bothrops*: *B. alternatus*, *B. jararaca*, *B. jararacussu*, *B. moojeni* and *B. pauloensis*. Specifically, we investigated if this diversification in habitat use was accompanied by a similar divergence in the characters related to defensive behaviour in the genus. Eight behavioural categories were recorded, five of which may be classified as "threatening" (strike, tail vibration, head and neck elevation, dorsoventral body compression and body thrashing); two as "escape" (locomotor escape and cocking); and one as "cryptic" (head hiding). We observed significant differences in four behavioural categories. We also detected a significant difference in the way species elevated their head and neck. Tail vibration and strikes were the most common behaviours presented, and snakes that displayed their tails struck more frequently than those that did not display. A reconstruction of characters related to defensive behaviour on a phylogeny of *Bothrops* indicated an increase in the use of dorsoventral body compression in the groups *alternatus* and *neuwiedi*, which may be associated with the invasion of open areas by these lineages.

Key words: comparative method, Crotalinae, defensive tactics, evolution of behaviour

INTRODUCTION

Snakes are exposed to different kinds of predators in the various habitats they occupy (Greene, 1988), and, as a result, may differ in defensive behaviour. For example, snakes in open habitats may suffer a more intense predation pressure from highly mobile predators than in forested habitats (Greene, 1988). Microhabitat use in snakes (e.g. terrestrial, arboreal) may also be associated with defensive behaviours (Greene, 1979). For instance, an association between gaping behaviour and arboreality has been demonstrated in snakes (Greene, 1997). In the case of the genus *Bothrops*, the ways by which the habitat is used by snakes are diverse (Martins *et al.*, 2001). Within the genus, there are lineages of both open and forested areas and with varying degrees of arboreality (Martins *et al.*, 2002). These differences in use of microhabitat (terrestrial and arboreal) and macrohabitat (open and forested areas) may be associated with differences in defensive behaviour of the different lineages of *Bothrops*. In fact, with the exception of the studies on *B. jararaca* by Sazima (1988, 1992), there are no detailed studies of defensive behaviour in the genus *Bothrops*. Sazima (1992) suggested that comparative studies among some *Bothrops* species typical of forested areas and species of open areas could reveal similarities and differences related to their ecology and their phylogenetic relationships.

Correspondence: M. S. Araújo, Departamento de Parasitologia, Instituto de Biologia, Universidade Estadual de Campinas, CP 6109, 13084-971, Campinas, SP, Brazil.
E-mail: maraujo@unicamp.br

The genus *Bothrops* (including *Bothriopsis*; e.g., Wüster *et al.*, 2002) includes about 45 described species (Campbell & Lamar, 2004). The phylogenetic relationships within *Bothrops* have been explored in the last few years (e.g. Salomão *et al.*, 1997; Vidal *et al.* 1997; Wüster *et al.*, 2002). The genus encompasses at least six lineages, the groups *atrox*, *jararacussu*, *jararaca*, *alternatus*, *neuwiedi* and *taeniatus* (Wüster *et al.*, 2002).

Here we describe and compare the defensive behaviour of one species of each of five lineages within the genus *Bothrops*, namely *B. alternatus* (*alternatus* group), a terrestrial species which inhabits open areas; *B. jararaca* (*jararaca* group), a semi-arboreal forest dweller; *B. jararacussu* (*jararacussu* group), a terrestrial forest inhabitant; *B. moojeni* (*atrox* group), a semi-arboreal species found in open formations, but associated almost exclusively with riparian forests within these areas; and finally *B. pauloensis* (*neuwiedi* group), a terrestrial species exclusive to open areas (Table 1). We also explore the evolution of characters related to defensive behaviour in the genus *Bothrops* and speculate on possible associations between changes in defensive behaviour and changes in habitat use.

MATERIALS AND METHODS

Test subjects were species of *Bothrops* from several localities of southeastern (*B. alternatus*, *B. jararaca*, *B. jararacussu*, *B. pauloensis* and *B. moojeni*) and central Brazil (*B. moojeni*) brought to the Instituto Butantan between April 1998 and February 1999. Ten

TABLE 1. Habitat use, sizes (mm), captivity duration (days), and number of individuals of the five species of *Bothrops* studied. T: terrestrial; SA: semi-arboreal; O: open areas; F: forests; SVL: snout–vent length; SD: standard deviation; CD: captivity duration; *n* = number of individuals.

Species	Habitat use	Mean SVL	SD	Mean CD	SD	*n*
B. alternatus	T/O	708.6	160.35	4	4.8	10
B. jararaca	SA/F	803.2	169.86	1	1.3	10
B. jararacussu	T/F	611.1	187.13	8	9.6	10
B. moojeni	SA/F	946.5	137.48	5	3.6	10
B. pauloensis	T/O	608.0	126.23	5	2.8	10

individuals of each species were tested as they arrived at the Instituto Butantan (Table 1). Upon arrival, individuals were housed in a large plastic container (c. 100 × 70 × 60 cm high) with bark mulch as a substrate. Snakes were not manipulated until they were removed from the container, measured and individually put in small wood containers, and taken to a temperature-controlled laboratory (25±2°C) where the tests were conducted. The snakes were taken to the laboratory during daytime, approximately eight hours before the initiation of the tests, and the tests were carried out on the same day, always at night, from 1758 hr to 0002 hr. The snakes were tested 0–16 days after arrival at the Instituto Butantan, except for one individual of *B. jararacussu* that was kept for 33 days at the Instituto Butantan before tests were performed. Each individual snake was tested only once.

The tests were carried out in an arena set on the ground of the laboratory (Fig. 1). The laboratory wall formed one of the sides of the arena; the other three sides were made of wood and glass. One of the sides adjacent to the wall was opaque and the other two sides were transparent. During trials, we stayed behind the opaque side of the arena to minimize possible disturbance. Two Panasonic NVRJ PR VHS cameras were used, one over the arena set on a tripod and facing the ground, and the other on the ground, lateral to the arena and facing the wall. The ground was covered with a black plastic sheet; both the plastic sheet and the wall had gridlines of 1 and 2 cm, respectively, for distance estimates. The light sources were two 60 W bulbs set on the main axis of the arena, one at each side. Although rather artificial, the light sources were necessary for the recording of the trials on tape.

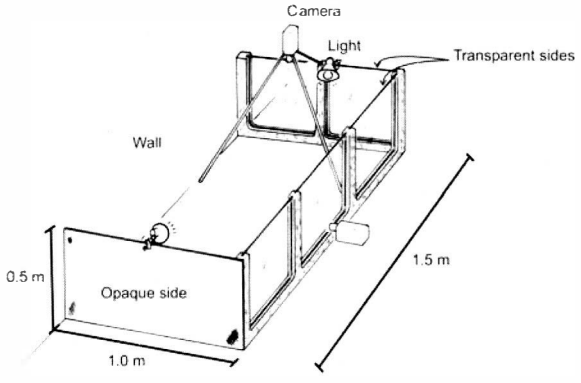

FIG. 1. Arena where the defensive behaviour of five species of *Bothrops* was elicited and filmed.

Defensive behaviour was elicited with using a stimulus object, a plastic bottle (height 15 cm; diameter 10 cm; volume 0.5 l) covered with a 0.5 cm-thick sheet of soft black rubber to which a 1.5 m plastic pipe was attached at a 45° angle. The purpose of the rubber was to minimize injuries to the snakes' fangs during strikes. The bottle was filled with warm water (60°C) shortly before the tests to raise the temperature of the external surface of the rubber to about 37°C (mean±SD = 37.1±0.94°C; n=17; recorded immediately before trials by a Miller & Weber Inc. quick-reading thermometer with an accuracy of 0.1°C). The stimulus object was developed by us and was chosen, among several others, on the grounds that it immediately elicited typical defensive behaviours upon its introduction into the arena. We believe that the stimulus object simulated the head of a mammal approaching the snake horizontally and close to the ground.

Before each test, the internal surfaces of the arena as well as the stimulus object were cleaned with ethanol. The snake was then put in the centre of the arena and a transparent acrylic box (30 cm on each side and 15 cm high), with the open side facing down, was put over the snake using a hook. We used a transparent box to make sure that the snake could see its surrounding environment before the initiation of trials. The acrylic box was also cleaned with ethanol before the tests. The arena lights were on prior to introducing snakes into the arena. Snakes were left undisturbed for 10 minutes before the beginning of the tests. The 10 minute interval was selected arbitrarily, but we believe it was enough for the snakes to settle down before the initiation of trials.

Cameras were turned on by remote control and recorded at 30 frames/s. Trials began when the acrylic cover was removed with a hook and the stimulus object was introduced into the arena and moved towards the snake, parallel to the ground and about 1 cm above it, always by the same person. The stimulus object touched the snake's body and was withdrawn repeatedly, about once every two seconds, 30 times uninterruptedly for each snake. If the snake went out of the camera's field (time out; cf. Martin & Bateson, 1993), the stimulus object was removed from the arena and the snake was brought to its centre with a hook; this will hereafter be termed an intervention. The stimulus object was then reintroduced and the stimulation was resumed.

During trials, we never moved from behind the light bulb, even when the stimulation had to be interrupted

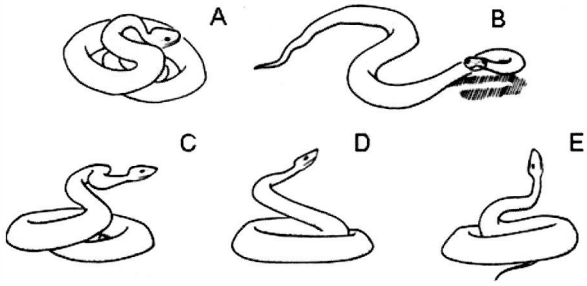

FIG. 2. Defensive behaviour of *Bothrops* spp. Defensive body posture, A: coiled; and B: loose. Head and neck elevation, C: horizontal; D: at angle of 45°; E: vertically.

and the snakes brought to the centre of the arena. Trials were later analysed frame-by-frame with a Panasonic NVSD475 PR VHS player. We measured the duration of each trial with the use of a digital chronometer; time-out periods were not considered in the estimates of time.

We used the continuous sampling method (cf. Martin & Bateson, 1993) and all the behaviours were recorded and quantified. Behavioural responses were categorized according to Greene (1988) and Sazima (1992), and were as follows: (1) strike: a rapid movement of the snake's head towards the stimulus object with its jaws wide open, as the lateral curves of its anterior body straightened, and the posterior part of the body remained stationary; (2) tail vibration: the tail was moved rapidly back and forth against the substrate, with production of sound; (3) head and neck elevation: the head and anterior part of the body were lifted from the substrate; this could be horizontal (Fig. 2C), at an angle of approximately 45° (Fig. 2D) or vertical (Fig. 2E); (4) dorsoventral body compression: the snake flattened its body dorsoventrally; (5) locomotor escape: a flight response in which snakes moved away quickly from the stimulus object; (6) cocking: the snake retreated backwards employing the posterior part of its body, while keeping the anterior portion of its body in an S-coil, and facing the stimulus object; (7) head hiding: the snake hid its head under one or more parts of its body; and (8) body thrashing: the snake made sudden and erratic movements.

Depending on their type, behavioural categories were quantified as the frequency of occurrences during trials (strikes, head and neck elevation, locomotor escape, cocking and body thrashing) or as the proportion of the trial time during which the behaviour was exhibited, varying from 0 to 1 (tail vibration, dorsoventral body compression and head hiding).We carried out a two-factor analysis of covariance (ANCOVA) in order to compare the behaviours among snakes, and test the effects of sex, snake size, captivity duration and the number of interventions during trials on the snakes' behaviours. Factors were species and sex, whereas snake size, captivity duration and the number of interventions were covariates. We used snout–vent length (SVL) in mm as a measure of size. Captivity duration was measured in days from the arrival of a given specimen at the Instituto Butantan until the day of the trial, and the number of interventions as the number of times we had to introduce the hook into the arena and pull the snake back to the camera's field during a trial. The frequency of the types of head and neck elevation were compared with a Pearson chi-square test. The number of strikes made by snakes that tail vibrated during tests and of those that did not tail vibrate was compared with a *t*-test. Because of the small number of individuals that did not tail vibrate, we pooled the data of all species in this latter analysis. Variables were all transformed to fulfil test assumptions. We did a ln transformation on snake size; square-root transformations on captivity duration, number of interventions and the behavioural variables strikes, head and neck elevation, locomotor escape, cocking and body thrashing (frequencies); and finally arcsine transformations on the behavioural variables tail vibration, dorsoventral body compression and head hiding (proportions; Zar, 1999) . Due to the high degree of asymmetry in the distributions of the raw values of variables, we decided to use their medians, instead of the means, on the character optimization onto a phylogeny of the genus *Bothrops* (adapted from Wüster *et al.*, 2002) using Linear Parsimony Analysis with the use of MacClade 4.0 (Maddison & Maddison, 2000). We were not able to optimize the characters head hiding and body thrashing, because the median values for all five species were zero.

RESULTS

During trials the snakes remained with the anterior part of the body in an S-shape position either coiled (Fig. 2A) or in a loose posture (Fig. 2B), and could change from one position to the other. We were not able to record the penetration of the snake's fangs into the rubber of the stimulus object (bite) through the analysis of the videotapes. However, it certainly occurred, since the rubber always presented marks of perforation from which venom drained following the tests.

Strike and tail vibration were the most used defensive behaviours by the five species studied (Table 2). Frequency of head and neck elevation, dorsoventral body compression, locomotor escape and cocking varied among species, whereas head hiding and body thrashing were rarely used by all species (Table 2). The ANCOVA revealed significant differences among species in head and neck elevation, dorsoventral body compression, locomotor escape and cocking (Table 3). The differences were related to the prevalence of head and neck elevation and cocking in *B. moojeni* (Fig. 3), of dorsoventral body compression in *B. alternatus* and *B. pauloensis* (Fig. 3) and of locomotor escape in *B. jararacussu* (Fig. 3). There was no effect of sex on the variables, nor any interactions between the factors sex and species (Table 3). However, we found a significant effect of interventions on both locomotor escape and head and neck elevations, as well as an effect of captivity duration on head hiding (Table 3). We detected a significant difference in the frequency of horizontal and 45-degree angle head and neck elevations among spe-

TABLE 2. Number of individuals of each *Bothrops* sp. that displayed each of the defensive behaviours (n=10 of each species). Total: number of individuals of all species which displayed the behaviour; values in parentheses are percentages of the total number of individuals (n=50).

Defensive behaviour	*B. alternatus*	*B. jararaca*	*B. jararacussu*	*B. moojeni*	*B. pauloensis*	Total
Strike	10	10	9	10	8	47 (94)
Tail vibration	10	7	10	10	8	45 (90)
Head and neck elevation	5	9	5	10	7	36 (72)
Dorsoventral body compression	10	4	6	6	7	33 (66)
Locomotor escape	5	7	9	5	5	31 (62)
Cocking	3	10	4	9	5	31 (62)
Head hiding	4	2	4	1	3	14 (28)
Body thrashing	3	2	3	0	2	10 (20)

cies (χ^2=64.6; df=4; P<0.0001): *Bothrops alternatus* and *B. pauloensis* elevated the head and neck parallel to the ground in most cases (Fig. 2; Table 4), *Bothrops jararacussu* showed horizontal and 45° elevation in similar proportions, whereas *B. jararaca* and *B. moojeni* elevated the head and neck at 45° more frequently (Table 4). Additionally, *B. moojeni* was the only species to elevate the head and neck vertically (Table 4). Snakes that vibrated their tails during tests struck more than those that did not vibrate their tails (t_{48}=2.3; P=0.026).

In the character optimization, *B. alternatus* did not show any change in relation to the ancestor of the genus in the median occurrence of any of the behaviours (Fig. 3). On the other hand, *B. pauloensis*, *B. jararaca* and *B. jararacussu* showed four changes each, and *B. moojeni* presented five changes (Fig. 3).

DISCUSSION

In the present study, the escalation of the defensive sequence reported for *B. jararaca* (Sazima, 1988) and *C. viridis* (Duvall *et al.*, 1985) was obviously not observed, since the snakes were already in a restrained situation at the beginning of observations. This experimental constraint may be responsible for the generally high tendency of snakes to strike during trials, as reported for *B. jararaca* when constrained in the field (Sazima, 1988). Our observations support the suggestions made by Duvall *et al.* (1985) and Sazima (1988) that pit vipers are able to evaluate their chances of escape during an encounter with a potential predator and make a decision on which defensive tactic to adopt, which was also suggested in relation to another viperid, *Agkistrodon piscivorus* (Gibbons & Dorcas, 2002).

TABLE 3. F-values and levels of significance of a two-factor analysis of covariance (ANCOVA) on the defensive behaviour of *Bothrops alternatus*, *B. jaracaca*, *B. jararacussu*, *B. moojeni* and *B. neuwiedi* (n=10 of each species). Factors are species and sex; covariates are size, captivity and interventions. Species x sex is the interaction between the two factors. See text for details of the quantification of variables. *Variables where significant differences among species were found.

Dependent variables	Factors and covariates					
	Species	Sex	Species x sex	Size	Captivity duration	Interventions
Strike	$F_{4,45}$=1.18 P=0.34	$F_{1,45}$=1.44 P=0.24	$F_{4,45}$=0.45 P=0.77	$F_{1,45}$=0.03 P=0.69	$F_{1,45}$=0.16 P=0.87	$F_{1,45}$=2.73 P=0.11
Tail vibration	$F_{4,45}$=2.01 P=0.11	$F_{1,45}$=1.05 P=0.31	$F_{4,45}$=0.38 P=0.82	$F_{1,45}$=0.26 P=0.62	$F_{1,45}$=0.00 P=0.95	$F_{1,45}$=2.3 P=0.14
Head and neck elevation*	$F_{4,45}$=8.08 P<0.0001	$F_{1,45}$=0.29 P=0.59	$F_{4,45}$=0.70 P=0.60	$F_{1,45}$=1.45 P = 0.24	$F_{1,45}$=0.13 P=0.72	$F_{1,45}$=4.57 P=0.039
Dorsoventral body compression*	$F_{4,45}$=6.38 P=0.001	$F_{4,45}$=2.02 P=0.16	$F_{4,45}$=0.78 P=0.55	$F_{1,45}$=0.22 P=0.64	$F_{1,45}$=0.65 P=0.42	$F_{1,45}$=0.92 P=0.34
Locomotor escape*	$F_{4,45}$=2.64 P=0.05	$F_{4,45}$=3.53 P=0.07	$F_{4,45}$=0.23 P=0.92	$F_{1,45}$=1.05 P=0.31	$F_{1,45}$=1.34 P=0.26	$F_{1,45}$=55.91 P<0.0001
Cocking*	$F_{4,45}$=5.16 P=0.002	$F_{4,45}$=0.17 P=0.68	$F_{4,45}$=0.15 P=0.96	$F_{1,45}$=0.43 P=0.52	$F_{1,45}$=0.24 P=0.63	$F_{1,45}$=0.50 P=0.49
Head-hiding	$F_{4,45}$=0.67 P=0.61	$F_{4,45}$=0.85 P=0.36	$F_{4,45}$=0.16 P=0.96	$F_{1,45}$=0.26 P=0.62	$F_{1,45}$=5.90 P=0.02	$F_{1,45}$=2.38 P=0.13
Body thrashing	$F_{4,45}$=0.92 P=0.46	$F_{4,45}$=0.58 P=0.45	$F_{4,45}$=0.82 P=0.52	$F_{1,45}$=0.07 P=0.79	$F_{1,45}$=0.05 P=0.83	$F_{1,45}$=0.52 P=0.47

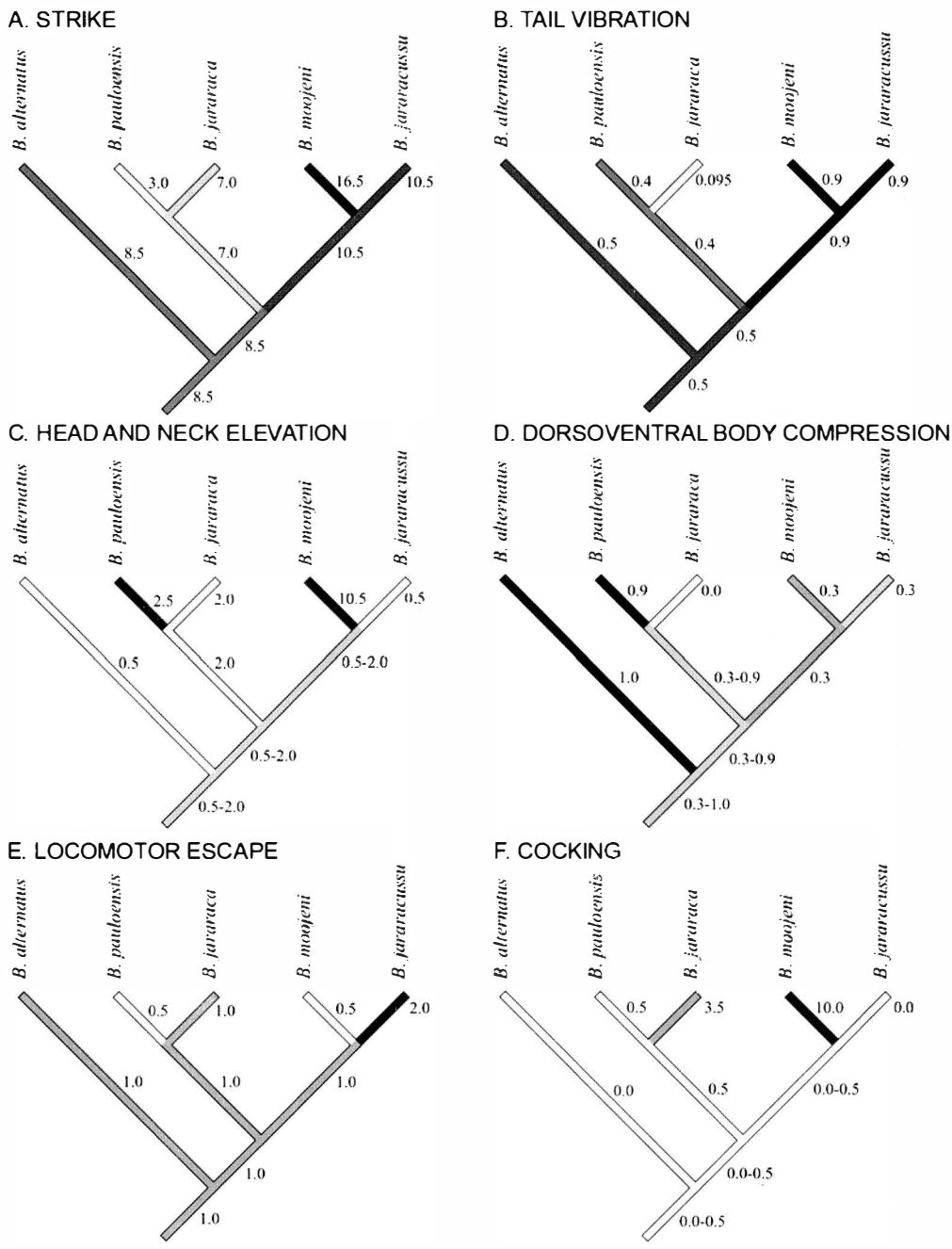

FIG. 3. Optimization using linear parsimony of defensive behaviours on a phylogenetic hypothesis for the species of *Bothrops* treated herein (adapted from Wüster *et al.*, 2002). The values for each species are medians. Tail vibration and dorsoventral body compression were quantified as the proportion of time they were exhibited by snakes during trials (varying from 0 to 1), the remaining characters as the frequency of occurrence during trials. A: strike; B: tail vibration; C: head and neck elevation; D: dorsoventral body compression; E: locomotor escape; F: cocking.

TABLE 4. Types of head and neck elevation in *Bothrops* spp. shown as percentages in relation to the total number of head and neck elevations in each species. Values in parentheses are the number of elevations.

Species	Horizontal	Angle of 45°	Vertical
B. alternatus	88.9 (8)	11.1 (1)	0
B. jararaca	26.9 (7)	73.1 (19)	0
B. jararacussu	44.4 (4)	55.6 (5)	0
B. moojeni	8.0 (9)	91.1 (102)	0.9 (1)
B. pauloensis	67.9 (19)	32.1 (9)	0

Although not a primary goal of this study, our data suggest that the defensive behaviours of the studied species may be altered by captivity duration and manipulation of the snakes, which has already been reported for other viperid snakes (Glaudas, 2004). These undesired effects constitute an important caveat of our study and may be taken as a warning by investigators who are designing and conducting behavioural studies on snakes in captivity.

The behavioural categories we observed were the same as those described by Sazima (1988, 1992) for *B.*

jararaca in field conditions. Moreover, in field conditions, individuals of *B. jararaca* showed an increase in the frequency of strikes (90% of the individuals struck at the observer) when they did not have access to escape routes (Sazima, 1988), a percentage very similar to that observed in our study, considering *B. jararaca* alone (100%, Table 2) or all species pooled (94%, Table 2). It seems reasonable, therefore, that the defensive behaviour observed in our study can be interpreted as that of a cornered individual in the field. In spite of the caveats previously mentioned, we believe that behavioural data obtained in captivity are indeed reliable, at least for some types of behaviour (e.g. defensive), contrary to the suggestion of Shine *et al.* (2002) that responses of captive animals do not provide a viable alternative to behavioural field studies. In fact, there are a high number of behavioural studies with snakes housed in captivity (Ford, 1995). Furthermore, encounters with lanceheads of the genus *Bothrops* in the field are generally rare (see Nogueira *et al.*, 2003) and depend on long-term studies, which are time-consuming and costly. Studies in captivity, therefore, may be useful and necessary in such cases.

Following the functional definitions of Mori & Burghardt (2004), of the eight behavioural categories observed herein five may be classified as "threatening" (strike, tail vibration, head and neck elevation, dorsoventral body compression and body thrashing), two as "escape" (locomotor escape and cocking) and one as "cryptic" (head hiding). When cornered, snakes of the genus *Bothrops* will readily defend themselves with strikes; however, they also rely on warning signals such as tail vibration to warn potential predators of their willingness to defend themselves. As observed in the viperid *Gloydius shedaoensis* (Shine *et al.*, 2002), tail vibration was also associated with striking in the species of *Bothrops* we studied. This may indicate that in the genus *Bothrops*, tail vibration provides a warning of an individual's likelihood to strike. Our results, however, must be interpreted with caution, because of the fact that we pooled all species in this analysis.

The differences in the types of head and neck elevation (Table 4) may be associated with microhabitat use in the studied species. The terrestrial *B. alternatus* and *B. neuwiedi* (Martins *et al.*, 2001) tended to use horizontal head and neck displays, whereas the semi-arboreal *B. jararaca* and *B. moojeni* tended to position the head and neck at a 45-degree angle. *Bothrops jararacussu*, which belongs to a terrestrial lineage that descends from a semi-arboreal ancestor (Martins *et al.*, 2001), used both head elevation patterns at the same frequency.

The defensive behaviour of the five species studied was qualitatively very similar, since all species presented all types of behaviour, the only exception being the absence of body thrashing in *B. moojeni*. However, we observed quantitative differences in four behavioural categories (head and neck elevation, dorsoventral body compression, locomotor escape and cocking), which is in accordance with the idea that behavioural differences in snakes, at the generic or specific levels, are mainly quantitative instead of qualitative (Arnold & Bennett, 1984).

Bothrops alternatus was the most and *B. moojeni* the least conservative lineage in relation to the ancestor of the genus (Fig. 3). There seems not to be a clear pattern relating the evolution of overall defensive behaviour (Fig. 3) and the great divergence of size, shape and habitat use in the genus *Bothrops* (see Martins *et al.*, 2001, 2002). Nevertheless, we believe that there is an association between habitat use and one of the observed behaviours, namely dorsoventral body compression. *Bothrops alternatus* and *B. pauloensis* showed a high prevalence of dorsoventral body compression (Fig. 3D). *Bothrops itapetiningae*, a species related to B. *alternatus* (Wüster *et al.*, 2002), also flattens the body frequently (M. Martins, personal observation), as does *B. mattogrossensis* (I. Sazima, unpublished data), of the *neuwiedi* group, which further indicates the prevalence of this behaviour in the groups *alternatus* and *neuwiedi*. The ancestor of *Bothrops* was most likely a forest species (Martins *et al.*, 2001, 2002) that subsequently invaded open areas, giving rise to the *alternatus* and *neuwiedi* groups. We believe that there was an increase in the use of this behaviour in these groups in relation to their ancestors (Fig. 3D), and that this increase is associated with the invasion of open habitats by these lineages. Perhaps the common occurrence of dorsoventral body compression in *B. alternatus* and *B. pauloensis* is an adaptation to a habitat where the predation pressure by birds of prey is higher than in the forests inhabited by the other species of *Bothrops*. Indeed, four predation attempts by the owl *Athene cunicularia* on *B. alternatus* were recently described (Valdujo & Nogueira, 2000; Martins *et al.*, 2003). An additional possibility is that the efficiency of body flattening may be increased in open habitats relative to forested habitats because snakes are possibly more visible to predators in the former. These two factors combined may account for the higher occurrence of this behaviour in the open habitat species. This hypothesis could be further explored by searching for convergent behaviours in other snake lineages that are also known to have invaded open areas.

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COMPARISON OF SKULL MORPHOLOGY IN NINE ASIAN PIT VIPERS (SERPENTES: CROTALINAE)

PENG GUO[1,2] AND ER-MI ZHAO[1]

[1]*College of Life Sciences, Sichuan University, Chengdu 610064, People's Republic of China*

[2]*Department of Biotechnology, Yibin University, Sichuan 644000, People's Republic of China*

The relationships of nine Asian pit vipers are discussed using a comparison of skull morphology. *Protobothrops xiangchengensis* shares more characters with other *Protobothrops* species than with the other genera. It is morphologically distinct from *P. mucrosquamatus*. *Zhaoermia mangshanensis* shows many similarities with the members of the genus *Protobothrops*, supporting its close relationship with *Protobothrops*. *Ovophis monticola* is unique in several skull characters among the species examined. The relationships indicated by skull morphology between *Viridovipera stejnegeri*, *V. yunnanensis* and *Cryptelytrops albolabris* are consistent with their previous reclassification based on molecular results and hemipenial comparison.

Key words: morphometrics, snake, taxonomy, *Trimeresurus*

INTRODUCTION

Trimeresurus (*sensu lato*), which consists of over 40 species (David & Ineich, 1999; McDiarmid *et al.*, 1999; Gumprecht *et al.*, 2004), represents a major evolutionary radiation (Malhotra & Thorpe, 2000), and ranges widely over southern, eastern and south-eastern Asia (Gumprecht *et al.*, 2004). The species of this group occupy a wide range of habits and display a variety of lifestyles (terrestrial, semi-arboreal and arboreal) and reproductive modes (oviparous and ovoviviparous). Originally, all were considered to be congeneric in *Trimeresurus* (*sensu lato*). Subsequently, several new genera have been proposed (*Tropidolaemus*, Wagler, 1830; *Ovophis*, Burger in Hoge & Romano-Hoge, 1981; *Protobothrops*, Hoge & Romano-Hoge, 1983; *Triceratolepidophis*, Ziegler *et al.*, 2000; *Zhaoermia*, Gumprecht & Tillack, 2004) based on morphological studies (Burger, 1971; Gumprecht & Tillack, 2004; Hoge & Romano-Hoge, 1981, 1983; Zhang, 1993, 1998; Ziegler *et al.*, 2000). More recently, a revised taxonomy for *Trimeresurus* (*sensu stricto*) and *Ovophis* (*sensu lato*) has been published on the basis of hemipenial features and molecular phylogeny (Malhotra & Thorpe, 2004). This taxonomy is followed here.

The skull is one of the most important structures available for the taxonomy and phylogenetic analysis of pit vipers (Brattstrom, 1964; Burger, 1971; Guo *et al.*, 1999; Hoge & Romano-Hoge, 1981, 1983; Zhang, 1993; Zhang & Zhao, 1990). Although several skull morphological studies of *Trimeresurus* (*sensu lato*) have appeared in past decades, a limited number of specimens or species were included (Burger, 1971; Hoge & Romano-Hoge, 1981, 1983; Zhang & Zhao, 1990; Zhang, 1993, 1998).

In the present paper, we report on a comparative study of the skulls of nine Asian pit vipers. Our aim is to re-evaluate the relationships within *Trimeresurus* (*sensu lato*) by skull morphological comparison methods, and propose diagnostic characters for some valid genera. Although the number of specimens and species is not enough to clarify all of the relationships, it is an important step toward resolving the taxonomy and phylogeny of this group.

Correspondence: Er-Mi Zhao, College of Life Sciences, Sichuan University, Chengdu 610064, PRC. *E-mail:* zem006@163.com

MATERIALS AND METHODS

Thirty-one individuals representing nine species and five genera of Asian pit vipers were examined, including four specimens of *Ovophis monticola*, one specimen of *Zhaoermia mangshanensis*, one specimen of *Protobothrops flavoviridis*, six specimens of *P. jerdonii*, five specimens of *P. mucrosquamatus*, three specimens of *P. xiangchengensis*, four specimens of *Viridovipera stejnegeri*, four specimens of *V. yunnanensis* and three specimens of *Cryptelytrops albolabris*. Detailed information on the studied specimens is listed in Appendix 1. All specimens are adults without anomalies and injuries to the head. Vernier callipers were used to measure different bones using the methods suggested by Brattstrom (1964) (Fig. 1). The descriptive methods for skull characters follow Guo *et al.* (1999). Accurate tooth counts can be obtained by counting the sockets and the teeth present, rather than by just counting the teeth present. The line drawings of skulls are based on the photographs and skull samples.

All specimens and skull samples are deposited in the Sichuan University Museum.

RESULTS

The skull structure and feature of nine Asian pit vipers is consistent with the other pit vipers; for example, they share movable maxilla, hollow fang, pit cavity. The skull of each species is not described here in detail, but rather illustrated in both ventral and dorsal view (Figs. 7–15); some single bones are also illustrated. In Table 2, some characters are compared for

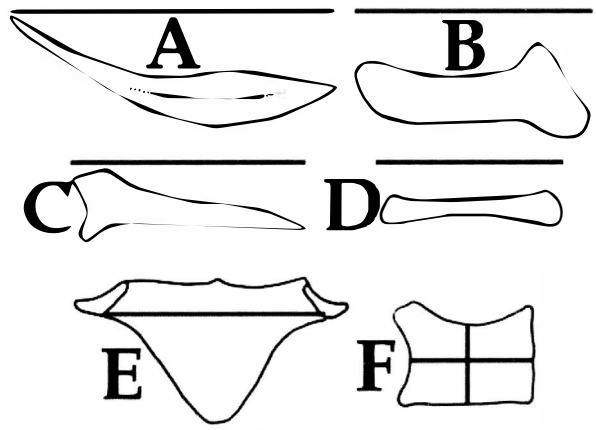

FIG. 1. Measurement of skulls (horizontal and vertical lines indicate length and width respectively). A: Pterygoid; B: Squamosal; C: Ectopterygoid; D: Quadrate; E: Lower jaw; F: Parietal.

each species. A detailed description of some bones, with variation noted, is given below. In Fig.3 to Fig. 15, the horizontal line bars indicate 0.5 cm

TEETH

In Crotalidae, teeth are found on the maxilla, pterygoid, palatine and dentary bones. The fang, which is on the maxilla, is not very different between species. Brattstrom (1964) and Zhang (1993) proposed that the fangs of various species were different in the length and curvature. However, these characters are very difficult to described and compared in practice. The number of palatine teeth varies from 0 to 5 in the specimens examined. Palatine teeth are absent in *Zhaoermia mangshanensis*, *Ovophis monticola*, *Protobothrops xiangchengensis*, *P. mucrosquamatus*, *P. flavoviridis*; 3–5 are present in *Viridovipera stejnegeri*, *V. yunnanensis* and *Cryptelytrops albolabris*. However, in *Protobothrops jerdonii*, some have one or two teeth (SCUM035028-29, SCUM035041), while in others, the palatine teeth are absent (SCUM035075, SCUM035078, SCUM035081).

Generally, the number of dentary teeth is 8–14 (10 on average), but *Ovophis monticola* has more (17–18). The pterygoid teeth vary greatly in numbers among species. *Ovophis monticola* has the greatest number of pterygoid teeth (14 on average), but *Protobothrops mucrosquamatus* has only five, *Protobothrops flavoviridis*, *V. stejnegeri*, *V. yunnanensis* and *C. albolabris* have more than 10, while the others have about eight. The positions of the first and last pterygoid teeth are very stable traits. For example, the pterygoid teeth of *Ovophis monticola* begins immediately at the articulation of the pterygoid with the palatine, and extends beyond the posterior end of the articulation of the ectopterygoid with the pterygoid; however, those of *Protobothrops mucrosquamatus* begin at a distance from the articulation of the pterygoid with the palatine, and do not extend to the anterior articulation of the ectopterygoid with the pterygoid.

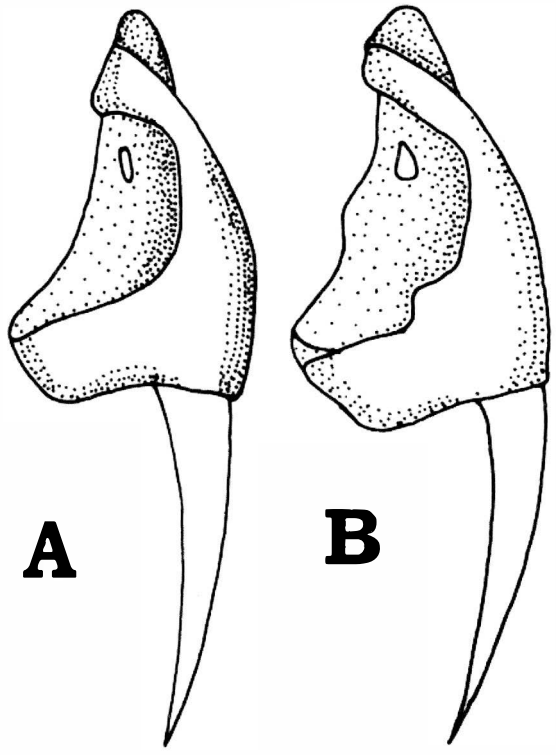

FIG. 2. Two types of maxilla. A: no projection; B: with projection.

MAXILLA

The maxilla is located in front of the prefrontal. This bone has a large lateral opening that contains the heat-sensitive facial pit. The shape of the anterior edge of the pit cavity is of some taxonomic importance (Brattstrom, 1964). Two states of this character were detected among the specimens examined (Fig. 2). Some species are smooth on the edge of the pit cavity (Fig. 2A), e.g. *Ovophis monticola*, *Protobothrops mucrosquamatus*; the others have a projection or process on the border of the pit cavity (Fig. 2B), e.g. *Zhaoermia mangshanensis*, *Protobothrops xiangchengensis*.

FRONTAL

The frontal bone, which is flat and quadrate, articulates posteriorly with the parietal (sometimes with the postfrontal), anteroventrally with the nasal and anterolaterally with the prefrontals. The shape of the frontal is constant within each species (Table 1). The frontals of four species of *Protobothrops* (*flavoviridis*, *jerdonii*, *mucrosquamatus*, *xiangchengensis*) are elongate (longer than wide); those of *Viridovipera stejnegeri*, *V. yunnanensis* and *Cryptelytrops albolabris* are square; those of *Zhaoermia mangshanensis* and *Ovophis monticola* are generally wider than long.

POSTFRONTAL

The postfrontal is present in all crotalids (Brattstrom, 1964). This bone either touches the frontal or not, and

TABLE 1. Skull comparison of nine Asian pit vipers. Abbreviations: *Zm*: *Z. mangshanensis*; *Om*: *O. monticola*; *Pf*: *P. flavoviridis*; *Pm*: *P. mucrosquamatus*; *Px*: *P. xiangchengensis;* *Pj*: *P. jerdonii*; *Vs*: *V. stejnegeri*; *Vy*: *V. yunnanensis*; *Ca*: *C. albolabris*. BMC: border of maxillary cavity; PR: parietal ridge; PS1: parietal shape; PTF: postfrontal touches frontal; SEBB: squamosal extends beyond the posterior end of the braincase; PS2: palatine shape; PT1: palatine teeth; DT: dentary teeth; PT2: pterygoid teeth; BP1: basisphenoid process; BP2: basioccipital process; EALP: ectopterygoid anterior lateral process; RLW: the ratio of skull length to width; RF: the ratio of frontal length to width; RQ: the ratio of quadrate length to skull length; RM: the ratio of mandible length to skull length; RE: the ratio of ectopterygoid length to skull length; RS: the ratio of squamosal length to skull length; RP: the ratio of pterygoid length to skull length.

Species	*Zm*	*Om*	*Pf*	*Pm*	*Px*	*Pj*	*Vs*	*Vy*	*Ca*
BMC	Projection	No	No	No	Projection	Projection	Projection	Projection	Projection
PR	Strong, Flanged	Moderate	Moderate	Moderate	Weak	Weak	Moderate	Moderate	Moderate
PS1	Triangle	T-shape	Triangle	Triangle	Triangle	Triangle	T-shape	T-shape	T-shape
PTF	Yes	Yes	No	Yes or no	Yes or no	Yes or no	No	Yes or no	Yes or no
SEBB	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
PS2	Triangle	Triangle	Triangle	Triangle	Triangle	Triangle	Crescent	Crescent	Triangle
	Not forked	Forked	Not forked	Not forked	Not forked	Not forked	Forked	Forked	Forked
EALP	Not broad	Broad	Not broad	Not broad	Not broad	Not broad	Broad	Broad	Broad
BP1	Strong	Strong	Strong	Strong	Moderate	Moderate	Weak	Weak	Moderate
BP2	Strong	Strong	Strong	Strong	Moderate	Moderate	Weak	Weak	Moderate
PT1	0	4 (3–4)	0	0	0	0, 1, 2	5 (4–5)	5	4 (3–5)
DT	11	17 (17–18)	13–14	9 (8–11)	11 (10–12)	11 (10–11)	12 (10–14)	13 (11–14)	12 (11–13)
PT2	8/9	14 (13–15)	11	5 (4–7)	8 (7–9)	8 (6–9)	12 (9–14)	13 (12–14)	10 (9–11)
RLW	1.81	1.95	2.03	2.45 (2.36–2.51)	2.07 (2.05–2.10)	2.08 (1.94–2.20)	1.60 (1.52–1.65)	1.72 (1.63–1.87)	2.11 (2.11–2.14)
RF	0.93	0.93	1.34	1.50 (1.40–1.58)	1.48 (1.47–1.50)	1.25 (1.1–1.33)	1.06 (1.0–1.15)	0.97 (0.93–1.0)	1.17 (1.11–1.23)
RQ	0.59	0.44	0.45	0.47 (0.44–0.48)	0.45	0.43 (0.40–0.45)	0.44 (0.43–0.45)	0.49 (0.47–0.52)	0.52 (0.51–0.54)
RM	1.58	1.36	1.40	1.44 (1.42–1.45)	1.39 (1.37–1.41)	1.30 (1.29–1.36)	1.40 (1.35–1.43)	1.42 (1.36–1.51)	1.45 (1.43–1.47)
RE	0.72	0.57	0.66	0.69 (0.63–0.74)	0.62 (0.60–0.64)	0.63 (0.60–0.64)	0.68 (0.65–0.71)	0.68 (0.66–0.70)	0.73 (0.72–0.73)
RS	0.32	0.38	0.30	0.31 (0.29–0.33)	0.29 (0.28–0.30)	0.32 (0.28–0.34)	0.34 (0.32–0.39)	0.31 (0.30–0.32)	0.30 (0.30–0.31)
RP	1.08	1.0	0.92	0.96 (0.93–0.99)	0.96 (0.95–0.98)	0.89 (0.84–0.91)	1.02 (0.97–1.08)	0.98 (0.94–1.0)	1.0 (0.98–1.05)

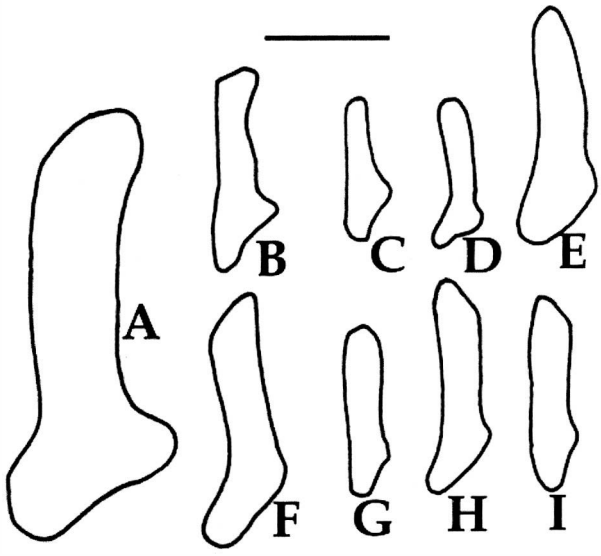

FIG. 3. Squamosals of nine Asian pit vipers (right, dorsal view). A: *Zhaoermia mangshanensis* (SCUM035024); B: *Ovophis monticola* (SCUM035030); C: *Protobothrops jerdonii* (SCUM035041); D: *P. xiangchengensis* (SCUM035043); E: *P. mucrosquamatus* (SCUM035026); F: *P. flavoviridis* (SCUM035056); G: *Viridovipera stejnegeri* (SCUM035053); H: *V. yunnanensis* (SCUM035045); I: *Cryptelytrops albolabris* (SCUM035008).

the distinction is usually considered to be taxonomically important (Brattstrom, 1964; Zhang & Zhao, 1990). The postfrontal touches the frontal in *Zhaoermia mangshanensis* and *Ovophis monticola*, and does not in *V. stejnegeri*. In the other species, both conditions (touches or not) are present within a species, even within an individual (e.g. SCUM035043).

PARIETAL

The parietal is the largest and heaviest bone of the crotalid skull. The parietal ridge is strongest in *Zhaoermia mangshanensis*, and is wing-shaped on both sides. The shape of the dorsal surface of the parietal is characteristic for each species (Figs. 7–15). It is T-

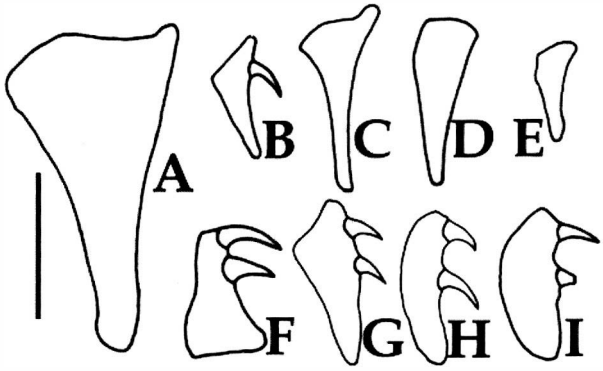

FIG. 4. Palatines of nine Asian pit vipers (side view). A: *Zhaoermia mangshanensis* (SCUM035024); B: *Protobothrops jerdonii* (SCUM035041); C: *P. flavoviridis* (SCUM035056); D: *P. mucrosquamatus* (SCUM035026); E: *P. xiangchengensis* (SCUM035043); F: *Ovophis monticola* (SCUM035030); G: *Cryptelytrops albolabris* (SCUM035008); H: *Viridovipera yunnanensis* (SCUM035045); I: *V. stejnegeri* (SCUM035053).

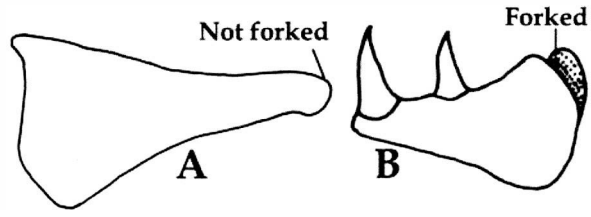

FIG. 5 . Two types of palatine (A: not forked; B: forked).

shaped in *Viridovipera stejnegeri*, *V. yunnanensis*, *Cryptelytrops albolabris* and *Ovophis monticola*, but triangular in the others.

SQUAMOSAL

The squamosal is a thin, flat bone, lying on the posterolateral corner of the parietal. The shape and relative length of this bone vary for each species. In *Ovophis monticola* and *Cryptelytrops albolabris*, the squamosal has an externally lateral process at its end. In *Protobothrops flavoviridis* and *Protobothrops mucrosquamatus*, it has a hook at its end. Some species (for example, *Zhaoermia mangshanensis*, *Protobotrops xiangchengensis*) have both the above conditions (Fig. 3).

The relative length of the squamosal to the skull is about 0.30 in most species, but that of *Ovophis monticola* is 0.38. With the exception of *Protobothrops xiangchengensis*, the squamosals of all species examined extend beyond the braincase.

QUADRATE

The shape of this bone shows little variation among the species examined. The ratio of the quadrate length to the skull is about 0.45 for most species except *Cryptelytrops albolabris* and *Zhaoermia mangshanensis* (Table 1).

PALATINE

This lies between, but does not articulate with, the medial wall of the maxilla and the lateral edge of the vomer. The shape of the palatine and whether it is posteriorly forked or not are characters of some mportance. In the four *Protobothrops* species, *Zhaoermia mangshanenis*, *Cryptelytrops albolabris* and *Ovophis monticola*, the palatines are triangular, but only the latter two are forked; those of *Viridovipera stejnegeri* and *V. yunnanensis* are crescent-shaped and forked (Figs 4 and 5).

PTERYGOID

The pterygoid is a toothed bone. It is narrow, and articulates with the palatine anteriorly and joins with the articular bone. The teeth are present anteriorly. Previous studies indicated that the shape of the posterior portion of the pterygoid, the curvature of the medial and lateral edges, the position of the ectopterygoid junction and the size and shape of the ridge on the ven-

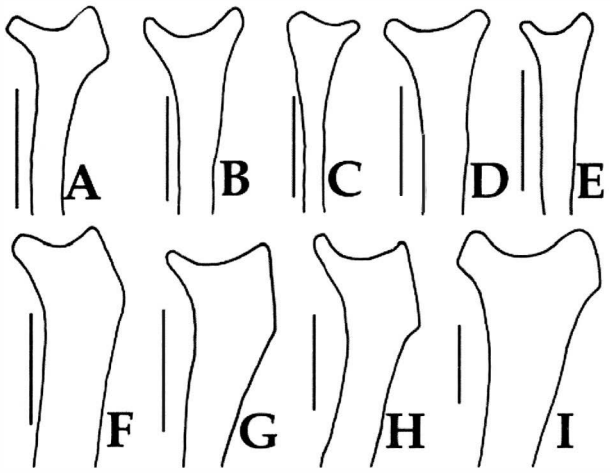

FIG. 6. Ectopterygoids of nine Asian pit vipers (right dorsal view except C). A: *Ovophis monticola* (SCUM035030); B: *Protobothrops flavoviridis* (SCUM035056); C: *P. jerdonii* (SCUM035041, left); D: *P. mucrosquamatus* (SCUM035026); E: *P. xiangchengensis* (SCUM035043); F: *Cryptelytrops albolabris* (SCUM035008); G: *Viridovipera stejnegeri* (SCUM035053); H: *V. yunnanensis* (SCUM035045); I: *Zhaoermia mangshanensis* (SCUM035024).

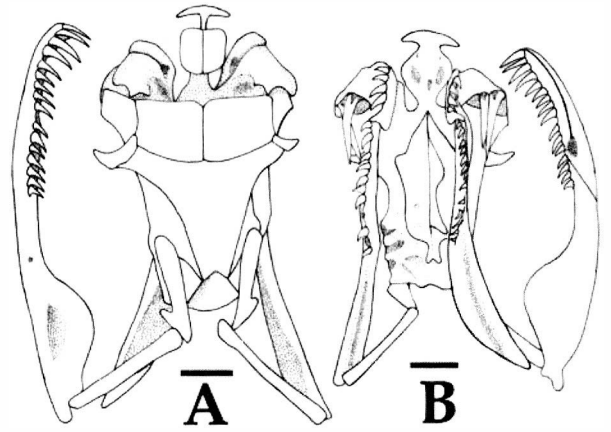

FIG. 7. The skull of *Ovophis monticola* (SCUM035083). A: dorsal view; B: ventral view.

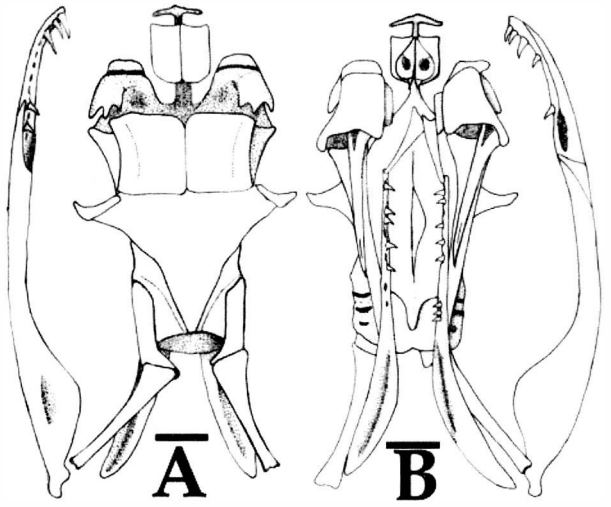

FIG. 8. The skull of *Protobothrops flavoviridis* (SCUM035056). A: dorsal view; B: ventral view.

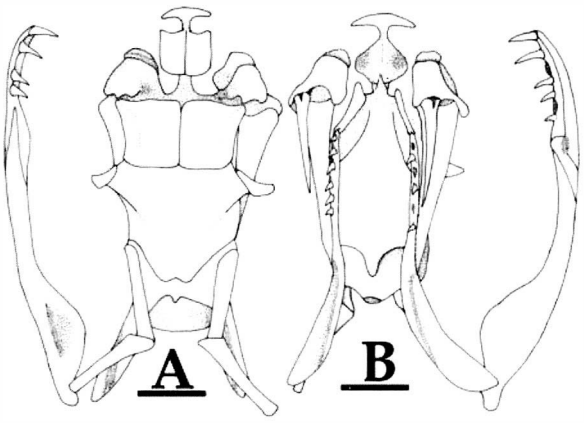

FIG. 9. The skull of *P. jerdonii* (SCUM035075). A: dorsal view; B: ventral view.

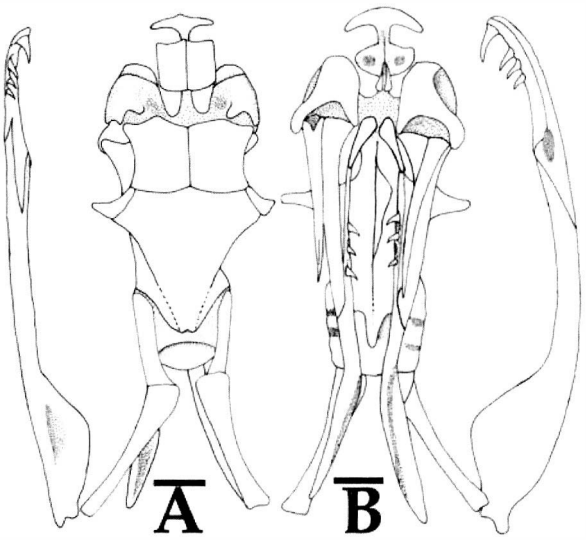

FIG. 10. The skull of *P. mucrosquamatus* (SCUM035050). A: dorsal view; B: ventral view.

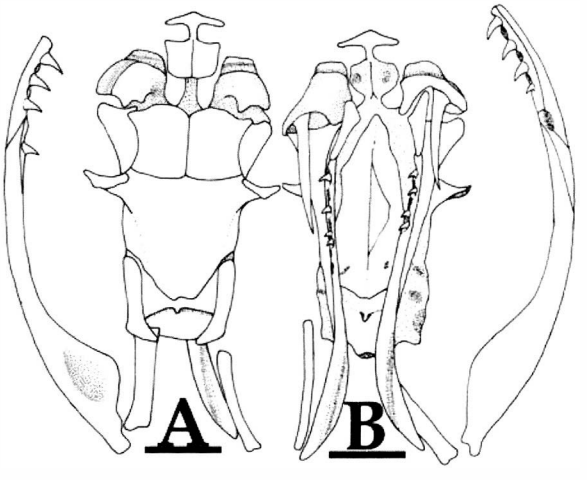

FIG. 11. The skull of *P. xiangchengensis* (SCUM035042). A: dorsal view; B: ventral view.

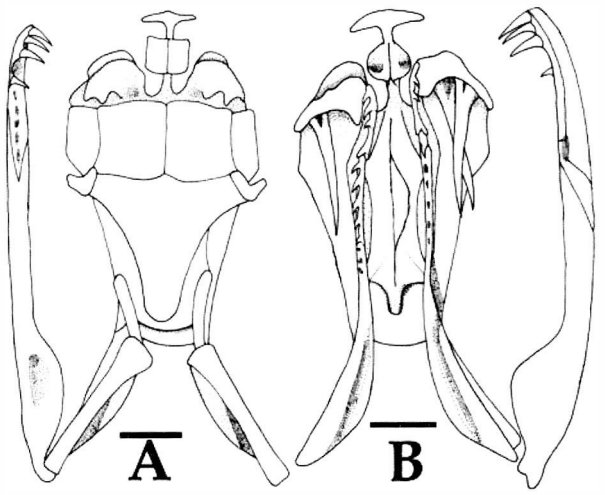

FIG. 12. The skull of *Cryptelytrops albolabris* (SCUM035009). A: dorsal view; B: ventral view.

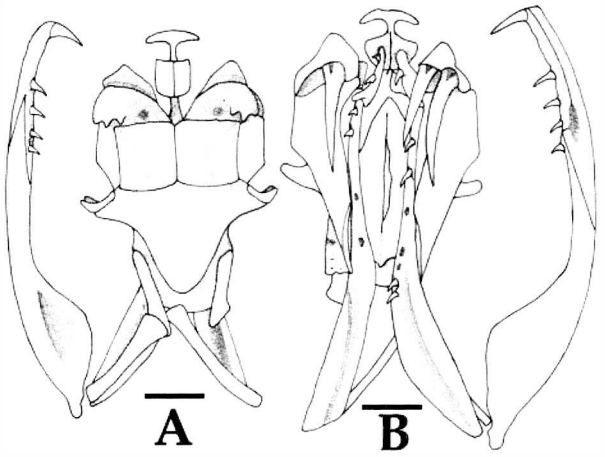

FIG. 13. The skull of *Viridovipera stejnegeri* (SCUM035079). A: dorsal view; B: ventral view.

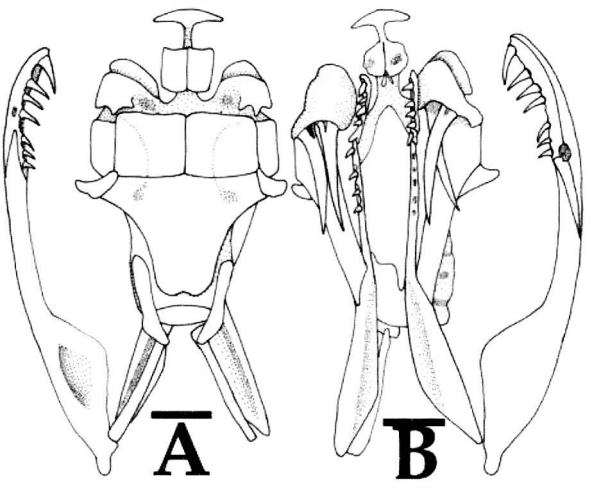

FIG. 14. The skull of *V. yunnanensis* (SCUM035045). A:

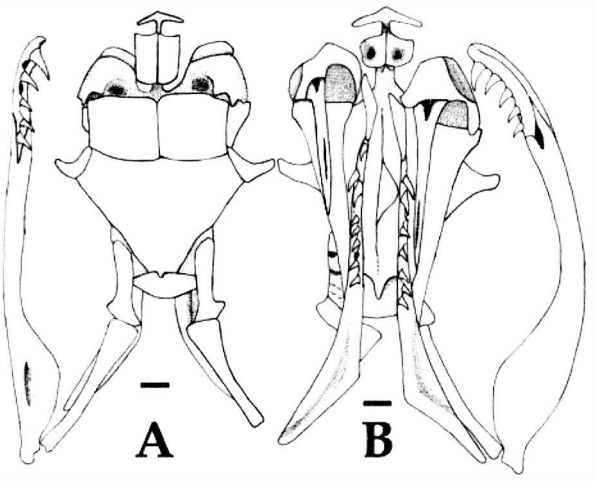

FIG. 15. The skull of *Zhaoermia mangshanensis* (SCUM035024). A: dorsal view; B: ventral view.

tral surface of the pterygoid were all quite characteristic of each species (Brattstrom, 1964). However, these characters are difficult to clarify and describe, and thus their value in determining relationships is limited.

The relative length of the pterygoid to the skull varies slightly, and in most species is about 1.0.

ECTOPTERYGOID

The ectopterygoid is usually forked anteriorly. In all species examined in this study the shape of this fork shows no variation, but the lateral process of the fork is much different among species (Fig. 6). This process is narrow in the four species of *Protobothrops* and *Zhaoermia mangshanensis,* whereas it is broad in *Ovophis monticola*, *Viridovipera stejnegeri*, *V. yunnanensis* and *Cryptelytrops albolabris.*

LOWER JAW

The lower jaw is compound, containing four bones: the angular, splenial, dentary and articular. Both the angular and splenial are located on the medial side of the lower jaw. The angular and splenial are distinctly separate in all the species examined except *Ovophis monticola.*

The ratio of the lower jaw length to the skull is between 1.30 and 1.40, but in *Zhaoermia mangshanensis* is 1.58.

BASISPHENOID AND BASIOCCIPITAL

Both bones are located in the ventral braincase. A thin ventral process is present in both. The height of the ventral process shows interspecific variation: it is lowest in *Viridovipera stejnegeri* and *V. yunnanensis*, moderate in *Protobothrops xiangchengensis*, *P. jerdonii* and *Cryptelytrops albolabris*, and highest in the other four species.

DISCUSSION

The description of some bones and the comparison of 19 characters (Table 1) indicate that (1) skull morphology differs among species, and each species can be identified by its skull features, although the degree of interspecific differentiation varies to some extent; (2) no intraspecific differences in most skull characters can

be detected among the species examined with theexception of whether the postfrontal touches the frontal or not; (3) the shape of the palatine, the size of the ectopterygoid anterior lateral process and the shape of the frontal are stable characters within species, and even within genera: they are therefore important for specific identification and classification.

Comparison With Previous Studies of Skull Morphology

The present results are consistent with most of the previous conclusions (Brattstrom, 1964; Burger, 1971; Hoge & Romano-Hoge, 1983; Zhang, 1993; Zhang & Zhao, 1990) with the exception of those described below. Zhang (1993) proposed that "the squamosal of *O. monticola* is short and narrow, its posterior end becomes thin and does not extend beyond the braincase; its length relative to skull length 0.24; the dentary teeth 11–12". However, on the basis of the four specimens of *O. monticola* studied here, the posterior end of the squamosal extends beyond the braincase, the relative length of the squamosal to the skull is 0.38, and the dentary teeth number is 17–18. 2. Based on a skull morphological comparison of six species of *Trimeresurus sensu lato*, Zhang & Zhao (1990) suggested that the postfrontals of these species touched the frontal except in *Protobothrops xiangchengensis*. However, the results presented here show that only *Zhaoermia mangshanensis*, *O. monticola*, *P. flavoviridis* and *Viridovipera stejnegeri* share the stable condition that the postfrontal touches frontal. Among other species, intraspecific variation was detected. Even in *P. mucrosquamatus* and *P. xiangchengensis*, both conditions of this character (postfrontal touches frontal or not) were found in an individual.

Protobothrops (Hoge & Romano-Hoge, 1983)

The systematic position of *Trimeresurus xiangchengensis* (Zhao *et al.*, 1978), is controversial (see Guo & Zhao, 2004). Recently, based on three mitochondrial gene fragments (12S rRNA, 16S rRNA, cytochrome *b*), phylogenetic analysis indicated that *xiangchengensis* should be a member of *Protobothrops*, and that it is more closely related to *P. jerdonii* than to *P. mucrosquamatus* (Guo *et al.*, unpubl. data). In skull morphology, *Protobothrops xiangchengensis* is greatly distinct from *Viridovipera stejnegeri*, *V. yunnanensis* and *Cryptelytrops albolabris* in the shape of the palatine and the ectopterygoid anterior lateral process. However, this species shares many characters with *P. flavoviridis*, *P. jerdonii* and *P. mucrosquamatus*. These include: (1) palatine triangular, not forked, and generally edentulous; (2) ectopterygoid anterior lateral process not broad; (3) frontal rectangular (longer than wide); (4) head length twice the width. Additionally, they show some similarities in external features. Hence, the placement of *xiangchengensis* into *Protobothrops* is supported morphologically.

Among *Protobothrops* species, *P. xiangchengensis* is very different from *P. mucrosquamatus* in the following skull characters: (1) the projection on the border of the cavity is absent in *P. xiangchengsis*, but present in *P. mucrosquamatus*; (2) the squamosal of *P. xiangchengensis* does not extend beyond the braincase, but that of *P. mucrosquamatus* does; (3) *P. xiangchengensis* has many more pterygoid teeth than *P. mucrosquamatus* (eight versus five on average) and its pterygoid teeth extend beyond the anterior margin of articulation with the ectopterygoid; (4) the two species are distinct in the height of the ventral process of the basioccipital and basisphenoid, and the ratio of head length to head width. Obviously, *P. mucrosquamatus* and *P. xiangchengensis* should be considered two morphologically distinct species, which is consistent with molecular analysis (Guo *et al.*, unpubl. data).

In the description of *Protobothrops*, Hoge & Romano-Hoge (1983) proposed that a projection was absent on the border of the pit cavity. However, another state, in which a projection is present on the border of the pit cavity, was detected in *P. jerdonii* and *P. xiangchengensis* (Table 1) in this paper. Hence, the presence of a projection in the pit cavity cannot be regarded as one of the generic characters of *Protobothrops*.

Additionally, the species of *Protobothrops* share several characters that can distinguish them from the other genera; for example, the relative ratio of the frontal length to width is 1.25–1.50, and the head is clearly elongate, with its length being twice its width.

Zhaoermia (Gumprecht and Tillack, 2004)

Zhang (1993) proposed the genus *Ermia*, which was later replaced by *Zhaoermia* (Gumprecht & Tillack, 2004), based on external and skull features of *Trimeresurus mangshanensis*. *Zhaoermia mangshanensis* is distinct from the other species examined in its unique parietal shape (see above). The shape of the frontal and the relative length of the quadrate to the skull are also different from those of the other species examined.

Among the nine species studied, *Zhaoermia mangshanensis* shares more characters with *Protobothrops* species than with the others. First, the palatine is triangular, not forked, and generally without teeth. Second, the ectopterygoid anterior lateral process is not broad. Third, the shape of the parietal is triangular. The above similarities further strengthen the suggestion that *Zhaoermia* is closely related to *Protobothrops* (Malhotra & Thorpe, 2004; Guo *et al.*, unpubl. data).

Ovophis (Burger, in Hoge and Romano-Hoge, 1981)

In his unpublished dissertation, Burger (1971) put forward *Ovophis*. Subsequently, Hoge & Romano-Hoge (1981) formally published the diagnosis of this genus. Combining Burger's (1971) work with the

present study, *Ovophis monticola* shows the following characters that are distinct from other genera: (1) the maxillary does not possess a projection on the border of the cavity; (2) it has a triangular, tall, forked palatine (Fig. 4F); (3) the palatine teeth number 3--4; 4) it has many more dentary teeth (17–18) and pterygoid teeth (13–15); (5) the ectopterygoid anterior lateral process is very broad; (6) it has a T-shaped parietal. The shape of the palatine and the number of dentary and ectopterygoid teeth are unique for this species among the species examined.

TRIMERESURUS (LACEPEDE, 1804)

Viridovipera stejnegeri, *V. yunnanensis*, and *Cryptelytrops albolabris* were assigned to *Trimeresurus* (*sensu stricto*) previously (David & Ineich, 1999; McDiarmid *et al.*, 1999). Recently, taking hemipenial characters and molecular analysis results into account, Malhotra & Thorpe (2004) proposed a new genus *Viridovipera*, and revalidated *Cryptelytrops*. According to their description of the two genera, *stejnegeri* and *yunnanensis* should be placed into the former, and *albolabris* into the latter. The hemipenis of *V. stejnegeri* and *V. yunnanensis* is forked shallowly, calyculate distally and spinous proximally; but that of *C. albolabris* is long and slender, the distal third is calyculate and the remainder is papillose (Guo & Zhang, 2001; Guo, 2000; Guo *et al.*, 2006). In this study, *V. stejnegeri* shares several characters with *V. yunnanensis* relative to *C. albolabris*: (1) the palatine is crescent shaped (Fig. 4D, I); (2) a wider head (the ratio of skull length to width is 1.60 in *V. stejnegeri* and 1.72 in *V. yunnanensis*, versus 2.11 in *C. albolabris*) (Table 1); (3) a lower process on the basiocciptical and basisphenoid. The distinct morphological differences between the two species of *Viridovipera* and *C. albolabris* indicate that they should be placed in different groups, and thus support the reclassification of these three species proposed by Malhotra & Thorpe (2004).

CONCLUSION

The skulls of nine Asian pit vipers were examined, described and figured in order to re-evaluate the taxonomy of pit vipers. Most bones, such as the palatine, squamosal and parietal, are relatively constant and distinctive for each species and hence useful in identifying species, taxonomy and determining relationships. The relationships indicated by skull morphology among these nine pit vipers are mostly consistent with those proposed by molecular analyses. Although the specimens and species examined in this study are not enough to clarify all of the relationships, it is an important step toward resolving the taxonomy and phylogeny of this group.

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APPENDIX 1: SPECIMENS EXAMINED

SCUM: Sichuan University Museum.

Ovophis monticola
Huili, Sichuan: SCUM035082-83, SCUM035040. Anxian, Sichuan: SCUM035030.

Protobothrops flavoviridis
Japan: SCUM035056.

P. jerdonii
Anxian, Sichuan: SCUM035028-29. Huili, Sichuan: SCUM035041, SCUM035075. Ruoergai, Sichuan: SCUM035081. Qingling, Shaanxi: SCUM035078.

P. mucrosquamatus
Yibin, Sichuan: SCUM035026. Hongya, Sichuan: SCUM035031-32, SCUM035076. Chengdu, Sichuan: SCUM035050.

P. xiangchengensis
Jiulong, Sichuan: SCUM035042-43, SCUM035046.

Cryptelytrops albolabris
Danzhou, Hainan: SCUM035007-9.

Viridovipera stejnegeri
Qunzhong, Hainan: SCUM035013-14. Hejiang, Sichuan: SCUM035053. Guangdong: SCUM035079.

V. yunnanensis
Huili, Sichuan: SCUM035037, SCUM035045, SCUM035114. Kunming, Yunnan: SCUM035077.

Zhaoermia mangshanensis
Yizhang, Hunan: SCUM035024.

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THE PYGMY CHAMELEONS OF THE EASTERN ARC RANGE (TANZANIA): EVOLUTIONARY RELATIONSHIPS AND THE DESCRIPTION OF THREE NEW SPECIES OF *RHAMPHOLEON* (SAURIA: CHAMAELEONIDAE)

JEAN MARIAUX[1] AND COLIN R. TILBURY[2]

[1] *Département des Invertébrés, Muséum d'Histoire Naturelle de Geneve, Switzerland*

[2] *Evolutionary Genomics Group, University of Stellenbosch, South Africa*

The pygmy chameleons of the Eastern Arc Range forests in Tanzania are reviewed on the basis of known and newly collected material. Two new species belonging to *Rhampholeon* (*Rhinodigitum*) and one to *Rh.* (*Rhampholeon*) from the Pare, Nguru and Mahenge Mountains are described. The status and distribution of the other species known in the area are reviewed, and an identification key is provided. The phylogenetic relationships between these taxa are discussed on the basis of small and large mt-rDNA subunits sequences and the relative importance of some morphological characters is evaluated. Hypotheses about the evolution of the group in the area are presented.

Key words: biodiversity hotspot, biogeography, molecular systematics, *Rhampholeon*, *Rieppeleon*

INTRODUCTION

The Eastern Arc Range of Tanzania (EAR) is composed of more than a dozen isolated massifs arrayed in an arc across the north-eastern and central regions of Tanzania (Fig. 1), geologically separated from the Southern Rift Mountains (like the Poroto and Livingstone ranges). The relict montane and sub-montane forests of the EAR (Pócs, 1976) are well known for their extraordinary biodiversity and high level of endemism (Mittermeier *et al.*, 1999; Myers *et al.*, 2000; Newmark, 2002). The East and West Usambara Mountains, which are the closest inland from the coast, are perhaps the best known and most intensively studied areas of the Eastern Arc. Their herpetological fauna has been explored in detail since the late 1800s by a number of distinguished German and American scientists (e.g. Barbour & Loveridge, 1928; Mertens, 1955; Loveridge, 1957 and references therein). However other massifs of the range, some rather far inland, are comparatively little explored and their fauna is still largely unknown.

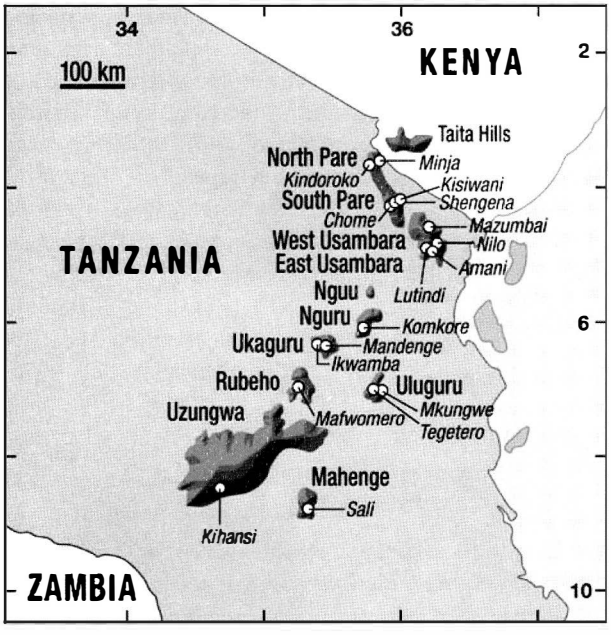

FIG.1. Geographical position of the Eastern Arc Range with massif names and sampling localities (map modified from the Bugwood Network, 2002).

Correspondence: J. Mariaux, Département des Invertébrés, Muséum d'histoire Naturelle de Geneve, CP 6434, CH-1211 Geneva 6, Switzerland. *E-mail*: jean.mariaux@ville-ge.ch

Good examples of highly diversified groups of organisms in the area are the pygmy or leaf chameleons of the genera *Rhampholeon* (Günther, 1874) and *Rieppeleon* (Matthee *et al.*, 2004). There are presently 14 species described in these two genera (Tilbury & Mariaux, 2004; Uetz, 2005) and their phylogenetic relationships have been derived from molecular analyses based on one nuclear and and two mitochondrial genes by Matthee *et al.* (2004), who also split *Rhampholeon* into three subgenera (*Rhampholeon*, *Rhinodigitum* and *Bicuspis*). *Rieppeleon* is mostly a lowland taxon, while *Rhampholeon* is primarily a montane genus.

Seven species have been reported in the EAR, among which five are endemic (Broadley & Howell, 1991; Tilbury & Emmrich, 1996; Flemming & Bates, 1999; Menegon *et al.*, 2002; Spawls *et al.*, 2002; Matthee *et al.*, 2004; Tilbury & Mariaux, 2004). The external morphological differences between some of these species are subtle and the assessment of characters is often open to subjective interpretation.

Although the genus is well known from the East Usambara forests, specimens of *Rhampholeon* from many sub-montane forests to the west of the range were not known until more recently, when these forests were targeted for biodiversity and other research surveys. Interestingly the EAR endemic species known so far seem to have a restricted distribution, and are found in a few massifs only. Given the high level of morphological similarity and the relative inconspicuousness of these lizards, we thought it possible that undescribed taxa might be found in the few remaining forests of the

more remote EAR massifs, and that the diversity of the groups could in fact be more important than recorded until now. An accurate systematic coverage of the group would also allow a better understanding of the evolutionary relationships of its members, as well as their precise geographical distribution.

During the course of several expeditions between 1999 and 2002 we collected numerous reptiles and amphibians in the EAR, and studied their evolutionary relationships as well as their parasitofauna. In the collection were 65 pygmy chameleons belonging to nine species, only six of which have been previously described. A few other specimens collected earlier by other workers in the Usambaras and Pares, tissue samples provided by M. Menegon (Trento, Italy) and comparative material from outside the EAR are also included in this study.

In this contribution we describe three new species, discuss the taxonomic status of several taxa and, more generally, discuss the distribution of the pygmy chameleons in the EAR, as well as the evolutionary relationships of these taxa as derived from mitochondrial DNA sequences.

MATERIALS AND METHODS

SPECIMENS AND LOCALITIES

Collection data for all unpublished material are given in Appendix 1 (specimens) and Appendix 2 (localities). All specimens mentioned in the text are adults unless specified otherwise. Abbreviations: FR: Forest Reserve; *Rh.*: *Rhampholeon*; *Ri.*: *Rieppeleon.*

List of acronyms used for Collections – BMNH: British Museum, Natural History, London; KMH: Collection Kim Howell, Dar es Salaam; MHNG: Muséum d'Histoire Naturelle de Genève, Geneva; MNHN: Museum National d'Histoire Naturelle, Paris; MTSN: Museo Tridentino di Scienze Naturali, Trento; NMB: National Museum, Bloemfontein; NMZB: National Museum of Zimbabwe, Bulawayo; PEM: Port Elizabeth Museum; UDSM: University of Dar es Salaam.

METHODS

Pygmy chameleons were caught at night by hand during torchlight searches. Most specimens were dissected in the field for parasitological investigations within 24 hours of capture. During this process all abdominal soft organs, including gonads, were removed and examined for parasites (except for *Rh. viridis* specimens with field tags other than TZ). A small piece of tissue (usually liver) of each animal was removed and conserved in 80% ethanol for further molecular studies. The specimens were then labelled and fixed in 2-4% buffered formaldehyde for one week to one month, then transferred to 70% ethanol for long-term conservation. They are kept at the MHNG, UDSM, NMZB and PEM. All measurements for morphological studies were made on alcohol-preserved material.

For molecular analyses, DNA extractions were made out of liver samples with the DNeasy Tissue kit® (Qiagen) according to the manufacturer's instructions. DNA fragments were amplified in a Techgene thermocycler (Techne). A 0.56 kbp fragment of the 16S rDNA was amplified using the universal primers L2510 and H3059 designed by Palumbi *et al.* (1991). PCR conditions were as follows: 94° for 3 min, then (93°, 45 seconds; 55°, 45 seconds; 72°, 1 min) × 37 cycles, and final extension 72° for 5 min.

For 12S, we used the primers 12S1 (CTAGGATTAGATACCCTACTATGC) and 12S2 (GATGAGGGTGACGGGCGGTGTG) that are modified versions of the universal primers designed by Kocher *et al.* (1989). PCR conditions were as for 16S except that annealing temperature was 60°. PCR products were checked on a 1% agarose gel, then purified with the QUIAquick® purification kit (Qiagen) and resuspended in a final volume of 30 µl.

Cycle sequencing reactions on both strands were performed using the BigDye® cycle sequencing kit (Applied Biosystems), and sequences were obtained with an ABI 377 automated sequencer. All sequences are deposited with EMBL under accession numbers AJ609595, AJ609597 to AJ609600, and AM55644 to AM55698.

Sequences were treated and aligned with Sequencher™ v. 4.1.2 (Gene Codes Corp.), and minor corrections were done by hand. The final matrix was analysed with PAUP* v4.0b10 (Swofford, 2002), including tests for base composition heterogeneity and for checking the compatibility of partitions using the PHT test (Farris *et al.*, 1994). Evolutionary relationships were inferred under the parsimony criterion. Heuristic parsimony analyses (100 repeats, random addition order) were performed on the whole matrix, with the following settings: uninformative characters excluded, characters unordered and unweighted, gaps treated as missing (or fifth base), multistate treated as uncertainty. Nodal support was estimated with 1000 bootstrap pseudoreplicates (each with five repeats). Comparative maximum likelihood (ML) heuristic analyses (with 50 repeats) were performed on the same matrix. The best-fit model was determined by Modeltest v. 3.6, using the Likelihood Ratio Test (LRT) (Posada & Crandall, 1998). ML bootstrapping was limited to 100 pseudoreplicates (each with three repeats).

RESULTS

DIVERSITY AND DISTRIBUTION OF PYGMY CHAMELEONS IN THE EAR

Up until recently, only seven species (*Rh. boulengeri*, *Ri. brachyurus, Ri. brevicaudatus*, *Ri. kerstenii*, *Rh. moyeri*, *Rh. temporalis* and *Rh. uluguruensis*) were reported from the EAR (Broadley & Howell, 1991; Spawls *et al.*, 2002; Uetz, 2005). To this list we must add *Rh. spinosus,* which was transferred to *Rhampholeon* by Tilbury & Mariaux (2004). These taxa were reported from six mountain chains only (East and West Usambara, Nguru, Uluguru, Udzungwa and Ukaguru) until Loader *et al.* (2004*b*) reported *Ri.*

TABLE 1. Known distribution of pygmy chameleons in the EAR, by main massif (+ indicates presence). a, ssociated with non-montane elevations around the bases of these mountain ranges; b ,specimens found by M. Menegon (Italy), see Menegon *et al.* (2003); c, *boulengeri*-like specimens from the Nguru appear to belong to the *uluguruensis* complex (see text); d, described as *Rh. moyeri*.

	Ri. brachyurus	*Ri. brevicaudatus*	*Ri. kerstenii*	*Rh. spinosus*	*Rh. temporalis*	*Rh. uluguruensis* complex	*Rh. acuminatus*	*Rh. viridis*	*Rh. beraducci*
N Pare			+ a					+	
S Pare			+ a					+	
W Usambara			+ a	+				+	
E Usambara		+	+ a	+	+				
Nguu	+ a		+ a			+ b			
Nguru	+ a		+ a			+ c	+		
Ukaguru	+ a					+			
Rubeho						+ d			
Uluguru		+				+			
Udzungwa		+				+ d			
Mahenge		+							+

brevicaudatus and another undetermined species from the isolated Mahenge mountains. This situation is summarized in Table 1. In the course of the present work we found almost all these taxa again, and also noted new localities and range extension for several species. Although recently discovered in north-western Tanzania, we have discounted the presence of *Rh. boulengeri* in the EAR, its occurrence being based on misidentified specimens. We also report three completely new taxa, bringing the total number of described pygmy chameleons in the EAR to 10; however, the exact status of these species is not always certain, as discussed below. A key allowing the identification of all EAR pygmy chameleons is provided in Appendix 3. One other species (*Rhampholeon nchisiensis*) occurs in Tanzania, inhabiting the forests of the Poroto and Livingstone mountains and the Tukuyu volcanic complex. Since these mountain ranges are not included in the EAR, further discussion of this species is not provided.

Rieppeleon brachyurus (Günther, 1893)

Although known from a diverse geographical range within Tanzania, none of the reported localities could be considered to be montane. It has been collected from the Miombo woodlands at the base of several massifs of the EAR including the Nguru, Ukaguru and near the Nguu.

Rieppeleon brevicaudatus (Matschie 1892)

This species is the most widely encountered in the EAR mountains. It is known from many lowland forests extending from south-eastern Kenya into Tanzania, penetrating into sub-montane forest in the East Usambara, Uluguru, Nguru and Udzungwa up to 1300 m (Spawls *et al.*, 2002). Loader *et al.* (2004*b*) recently reported its presence in the Mahenge and found it in all the above-mentioned massifs up to a maximal altitude of about 1200 m in the Uluguru, which is compatible with the recent observations of Emmett (2004). Although *Ri. brevicaudatus* is common in the East Usambara we were unable to confirm its presence in the West Usambara, and, until further collecting proves otherwise we regard *Ri. brevicaudatus* as being absent from this massif.

Rieppeleon kerstenii (Peters, 1868)

Although not a montane species, this pygmy chameleon is widely distributed in north-eastern Tanzania and may be found in acacia scrub and grasslands on the lower slopes of several of the EAR massifs including Uluguru, Nguru, Nguu, Ukaguru, Usambaras and Pares ranges. It is likely that its range extends into the foothills of the Rubeho as well.

Rhampholeon (Rhinodigitum) boulengeri (Steindachner, 1911)

This species is currently considered to be widely distributed in eastern central Africa, including Burundi, Rwanda, Uganda, the eastern Democratic Republic of Congo and in remnant forest patches in western Kenya up to 2000 m. At least one report from Tanzania (Kange Estate, Nguru mountains) is known (Witte, 1965), and we have recently become aware of a new Tanzanian record from Minziro Forest in Bukoba (J. Beraducci, *in litt.*, 2005). The homogeneity of this widely distributed species has not to date been tested and it is considered possible that *boulengeri* could represent a species complex. A population of pygmy chameleons from the upper reaches of the forests on the Nguru mountains bears a striking morphological resemblance to *boulengeri* and has an almost identical hemipenal structure (Tilbury, unpublished observations). A further population of pygmy chameleons from the Nguu mountains is likewise very similar to *boulengeri* (Menegon *et al.*, 2003).

We collected *boulengeri*-like specimens from the Nguru and included them in our molecular analysis together with specimens from the core populations of the species (DRC, Rwanda) in order to check their conspecificity. The results of the mtDNA analysis clearly show that the Nguru specimens, as well as those from the Nguu, are unequivocally members of the

uluguruensis complex (see below). We therefore consider that *Rh. boulengeri* is absent from the EAR.

RHAMPHOLEON (RHINODIGITUM) MOYERI (MENEGON *ET AL.*, 2002)

This recently described species (Menegon *et al.*, 2002) is currently only known from two localities in the Udzungwa but DNA analyses suggest that the population present in the Rubeho mountains is also of this form (Matthee *et al.*, 2004). It may be relevant to note that the only morphological characters allowing the differentiation between *Rh. moyeri* and *Rh. uluguruensis* are found at the level of the hemipenis (10-12 vs. 9 papillae) and the interorbitals (15-19 vs. 11-13 tubercles) (Menegon *et al.*, 2002), and should be considered with caution given the variation known to exist at least for the second criterion. We included two specimens from *Rh. moyeri* provided by M. Menegon in our mtDNA analysis and obtained equivocal results, as the two samples did not form a clade in our tree (Fig. 12). The specimen from Kihanga is shown to be related to *uluguruensis*, but the specimen from Kitolomero is basal to the *uluguruensis* complex, thus, in theory, possibly justifying its specific status. In any case the differentiation of this species with other members of the *uluguruensis* complex remains at best difficult (see also discussion).

RHAMPHOLEON (RHAMPHOLEON) SPINOSUS (MATSCHIE, 1892)

This species was recently transferred to *Rhampholeon* (from *Bradypodion*) by Tilbury & Mariaux (2004). It is an endemic of the West and East Usambara, and although somewhat more common in the former mountains, it is rare and vulnerable to environmental changes in both places. We found it between about 1000 and 1500 m. Its position in our tree, as well as its morphological characteristics, makes it a member of the *Rh.* (*Rhampholeon*) subgenus (Matthee *et al.*, 2004, appendix C).

RHAMPHOLEON (RHAMPHOLEON) TEMPORALIS (MATSCHIE, 1892)

A poorly known endemic of the East Usambara (and a few neighbouring relict forests) found at up to 1400 m in the East Usambara (Emmett, 2004). Although this species is surrounded by habitats rich in other species of pygmy chameleon, a recent comparative DNA study of the pygmy chameleons (Matthee *et al.*, 2004) showed that its closest relative was the West African species *Rhampholeon spectrum*. Our augmented database shows however that both *Rh. spinosus* and *Rh. viridis* n. sp. (described below) are closer relatives of *Rh. temporalis*, thus demonstrating the radiation of the *Rh.* (*Rhampholeon*) lineage in the easternmost extremity of the EAR.

The morphological homogeneity of this lineage is reinforced by the hemipenis anatomy of its members. The hemipenes of *Rh. temporalis* and *Rh. spectrum* are figured here for comparative purposes to emphasize the unique morphology within the subgenus *Rhampholeon* (*Rhampholeon*) (Figs 2-3). The hemipenes of the subgenus would appear to combine features commonly associated with the typical chameleons, viz: elongate and calyculate truncus with a capitate apex, and the paired apical structures more typical of *Rhampholeon*. *Rh spectrum* has a rather more complex hemipenal apex indicative of its divergent history. In this species a dual arrangement of short blunt apical horns each flanked laterally by a denticulate crest and medially by a smaller blunt horn (Fig. 3) is much more evocative of the apical rotulae seen in typical chameleons.

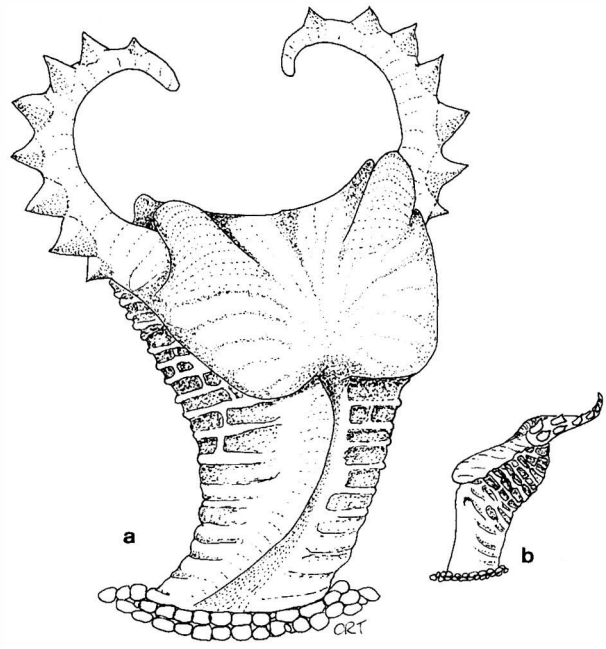

FIG. 2. *Rhampholeon temporalis* (BM 1988.641). Left hemipenis: a, sulcal view; b, lateral view.

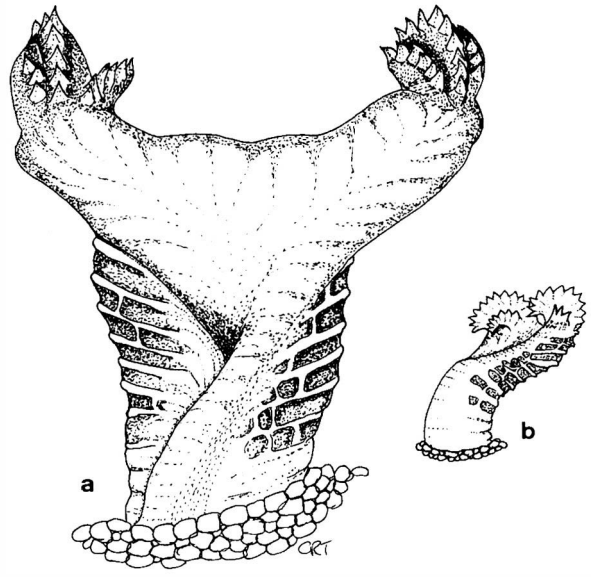

FIG. 3. *Rhampholeon spectrum* (PEM R 15701). Right hemipenis: a, sulcal view; b, lateral view.

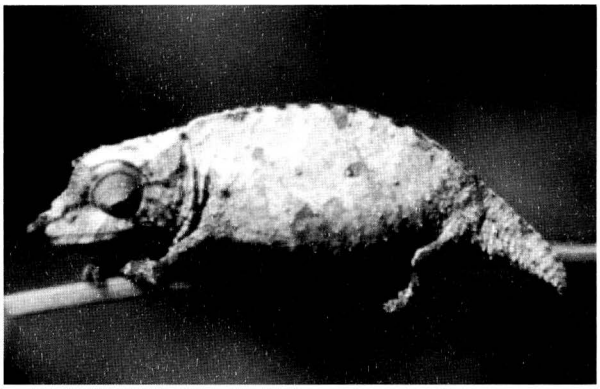

FIG. 4. *Rhampholeon* (*Rhinodigitum*) *beraduccii* n. sp. Male; Sali, Mahenge mountains.

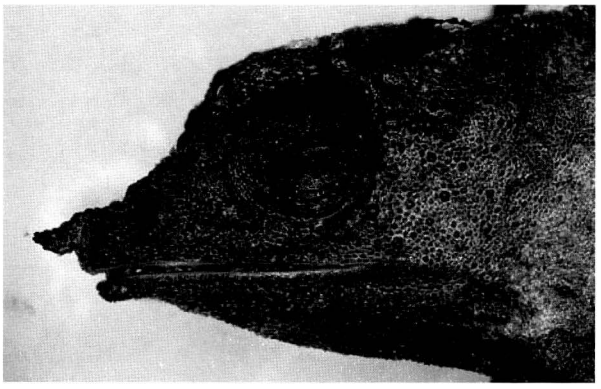

FIG. 5. *Rhampholeon* (*Rhinodigitum*) *beraduccii* n. sp. (paratype, MHNG 2655.021). Head detail. Adult male; Sali, Mahenge mountains.

RHAMPHOLEON (RHINODIGITUM) ULUGURUENSIS TILBURY & EMMRICH 1995

Tilbury and Emmrich (1996) described this species from the Uluguru. We have since recorded this taxon from other neighbouring massifs with slight morphological variations, and our sampling from the Uluguru (in the isolated Mkungwe massif), Rubeho, Nguru and Ukaguru mountains, as well as additional samples from very similar specimens from the Nguu (provided by M. Menegon) allowed for a test of the validity and limits of this taxon (see below). With the latter specimens, all EAR massifs are now known to harbour a pygmy chameleon fauna.

RHAMPHOLEON (RHINODIGITUM) BERADUCCII SP. NOV. (FIGS 4-5)

Holotype. MHNG 2655.019 (field tag TZ 343), female. Tanzania, Morogoro region, Mahenge Mountains, Sali FR [8°57'57.4" S, 36°41'17.9" E], about 1000 m, 9 October 2001. Collected by J. Mariaux & S. Loader.

Paratypes. Two males, MHNG 2655.020–021 (TZ 344, TZ 345) same locality and date.

Etymology.The new species is named in honour of Joe Beraducci, Arusha, Tanzania, as an appreciation for his generous assistance and help provided to us and to numerous other scientists working in the EAR.

Diagnosis. Chamaeleonidae, *Rhampholeon* (*Rhinodigitum*). With the characters of the subgenus. A tiny brown chameleon with snout–vent length (SVL) 20.5–28 mm, maximum total length (TL) 36 mm, and a very short tail, 19–22% of TL. The smallest known *Rhampholeon*. Head with a well-developed nasal process and short supra-optical peaks. Head flat with very slightly marked crests, temporal crest very weak. Dorsal keel weakly undulated. Body with sub-homogeneous granules, but conspicuous shoulder spine present. Deep axillary and inguinal pits present. Claws bicuspid with small accessory spines.

Description of the holotype. Head (Fig. 4): Casque discrete, flat, with smooth edges. Weak temporal crest, first a horizontal line then forming an upward angle, without marked ornamentation except for three larger tubercles just behind the eyes. No parietal crest. Supra-orbital ridge well marked, peaking anteriorly in flattened, short and thick horn-like clusters of tubercles. Two larger tubercles on inferior orbital rim. Supra-orbital peaks connected by an interorbital ridge composed of 14 small granular tubercles marking a prominent frontal line. Rostral ridge well marked, forming a small bump over the nostrils and joining anteriorly in a 1.5 mm long triangular, pointed rostral appendage, about 12 small granules long and seven wide at its base. Nares opening posteriorly. No gular or mental appendages.

Body: TL 36 mm, (SVL 28 mm, tail length 8 mm. Tail 22% of TL. Dorsal crest weakly undulating – almost smooth, without clusters of spines, smooth on the lumbar area, and again weakly undulated on the tail. Deep axillary and inguinal pits present.

Flank scalation homogeneous, composed of small stellate granules with occasional slightly larger ones. One conspicuous enlarged dark tubercle over shoulder and another one on upper mid-flank. Scalation somewhat more irregular on the limbs. One or two larger tubercles on forearms. Claws strongly bicuspid with small accessory plantar spines.

Variation in paratypes. Males, TL 28-29, and tail 19-20% of TL. Very similar to holotype. Interorbital ridge up to 16 granules. Lateral crest more developed. Up to four tubercles behind and below the eye. Rostral appendage with slightly curved lower border. Tubercles on limbs a little more developed although still discrete. The hemipenal morphology is unknown at present.

Colour in life (Fig. 5). Generally yellowish to pale brown with various darker spots, especially on the back. May present two thin blackish diagonal lines on the flanks (antero-dorsal to postero-ventral).

Differential diagnosis. *Rh. beraduccii* can be differentiated from other members of the genus by its smaller size, small optical peaks and the shape of its rostral appendage. Furthermore, members of the similar *uluguruensis* group, including *Rh. moyeri*, do not show inguinal pits, which are clearly marked in *Rh. beraduccii*.

Distribution and ecology. *Rh. beraduccii* is to date only known from the vicinity of Sali in the Mahenge

FIG. 6. *Rhampholeon* (*Rhinodigitum*) *acuminatus* n. sp. Adult male; Nguru South FR, Nguru mountains.

mountains, an isolated massif separated from the Udzungwa range by the Kilombero valley. All animals were found alone, on low shrubs or herbs, within a few centimetres of the ground, in open land, in the immediate vicinity of the village.

Remarks. No faunistic surveys of the Mahenge were available until 2004 when Loader *et al*. (2004*b*) reported the presence of *Ri. brevicaudatus* as well as of *Rh. cf. moyeri* around Sali FR. Although morphologically close to *moyeri*, specimens from the latter group are clearly distinct from the other members of the *uluguruensis* complex. Meanwhile our mtDNA analysis confirmed that the Mahenge specimens were unequivocally distinct from *moyeri* or *uluguruensis*. The very small size of our specimens might also indicate that only juveniles or immature animals were collected. Although this cannot be completely excluded, we note that our specimens were collected in several distinct locations around Sali and, although more specimens were spotted, no significantly larger individuals were seen. Even if *Rh. beraduccii* is remarkably small, other tiny chameleons are known; *Ri. brachyurus*, for example, does not reach 6 cm, and *Brookesia minima* is of a size similar to the new species. Thus, on the basis of both our morphological and molecular evidence, and despite a very limited sampling, we propose to designate this material as a new species.

FIG. 7. *Rhampholeon* (*Rhampholeon*) *viridis* n. sp. Adult male; Chome FR, South Pare mountains.

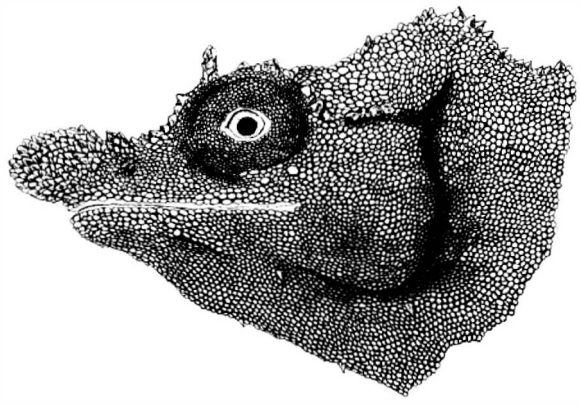

FIG. 8. *Rhampholeon* (*Rhinodigitum*) *acuminatus* n. sp. (Holotype, MHNG 2645.001). Head detail.

RHAMPHOLEON (RHINODIGITUM) ACUMINATUS SP. NOV. (Figs 6, 8, 9)

Holotype. MHNG 2645.001 (field tag TZ 414), male. Tanzania, Morogoro region, Nguru mountains, Nguru South Catchment FR, Komkore Forest above Ubili village [6°2'29" S; 37°30'40.5" E], 1500–1600 m, 21 October 2000. Collected by J. Mariaux & S. Loader.

Paratypes. Three males, MHNG 2645.002–004 (TZ 412, 413, 417), two females MHNG 2645.005–006 (TZ 415, 416), and one male PEM-R 16271. All same locality and date.

Other material. Three specimens collected by David Moyer (Iringa, Tanzania), 25-26 August 1997, 6 km SW of Ubili, 1500 m. These specimens were the first ever recorded for the species but can no longer be localized and are presumed to be lost.

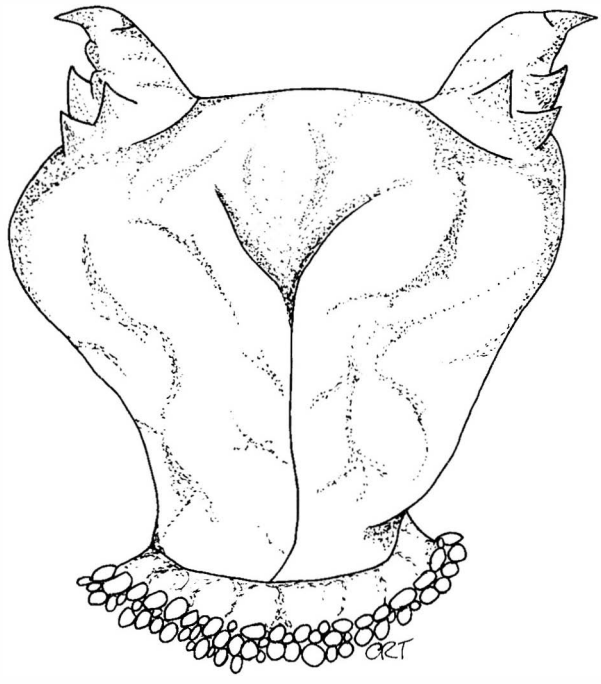

FIG. 9. *Rhampholeon* (*Rhinodigitum*) *acuminatus* n. sp. (PEM-R 16271). Hemipenis: sulcal view.

Etymology. From Latin *acuminare* (to sharpen), in reference to the numerous sharp spines found on the head and body.

Diagnosis. Chamaeleonidae, *Rhampholeon* (*Rhinodigitum*). With the characters of the subgenus. A small chameleon with SVL 47–57 mm (maximum TL 82 mm) and a tail 25–30% of TL. Adults are unmistakable due to their large discoid and vertically flattened rostral process (up to 5 × 3 mm) projecting forward off the rostrum (Figs 6-7), spinous supra-orbital and other cranial projections, prominent casque, exaggerated dorsal crest and numerous spines on the body, limbs and tail. No axillary or inguinal pits. Claws bicuspid. Parietal peritoneum unpigmented.

Description of the holotype. Head (Fig. 8): Elongated with a particularly prominent pyramidal casque formed by upward extensions of the posterior orbital/lateral crests. The parietal region of the head is slightly concave. Sharply acuminate vertical spines are distributed along the lateral edges of the casque (three on each side) and one at the peak. Weak postero-orbital transversal crest. No parietal crest. Supra-orbital ridge strong, marked by large rounded tubercles in its posterior half, peaking anteriorly in prominent, thin, horn-like tufts of tubercles 4-5 rows of tubercles high, just posterior to a markedly enlarged tubercle. Supra-orbital peaks connected by a row of 12 flattened tubercular plates. Orbits almost touching each other anteriorly, only separated by 1-2 granules. Temporal crest prominent, composed of a fin-like triangular ridge formed of 6–8 tubercles in a horizontal line behind the postorbital rim, bending upwards posteriorly. 2-3 enlarged tubercles below the eyeballs. Pre-orbital ridge well marked peaking above the nostril in a short conical cluster, 2-3 tubercles high. Rostral appendage oval (12 granules along its maximal length × 10 granules at its maximum height, 4 × 3.5 mm), with its longer axis horizontal, 6-7 tubercles wide at its base, becoming 2-3 tubercles thick anteriorly. Nares opening posteriorly. No submental appendage. No gular crest but a few randomly distributed spinous tubercles along the mandible and the upper throat.

Body: TL 82 mm, SVL 57 mm, tail length 25 mm. Tail 30% of TL. Dorsal crest preceded anteriorly by three paired simple tubercles commencing at the nape, followed by nine prominent pyramidal clusters of tubercles each positioned over a vertebral body, the most prominent in the middle of the back, separated from each other by 4-6 granules, becoming smooth on the lumbar area, then followed by 14 smaller clusters distributed along the length of the tail. Largest cluster formed of about 10 tubercles. No axillary or inguinal pits. Flank scalation homogeneous, composed of small interlocking stellate granules with about 10-12 enlarged spiny tubercles, half a dozen of them forming an indistinct row on the upper flank. Tail with a row of prominent isolated spinous tubercles along the infero-lateral side of the tail on each side. One inconspicuous spine above the shoulder. Forearms with 3-4, and forelegs with 1-2, large isolated spinous tubercles. Upper arms and legs with 1-2 spines. Claws bicuspid. Palms and soles smooth but 1-2 small accessory plantar spines present at the base of each claw. The holotype hemipenis is not everted.

Variation in paratypes. Males: TL 63-71 mm, and tail 25-30% of TL. Casque sometimes with only two lateral spines on each edge, the most basal one more prominent than in holotype. Some specimens have a noticeable interorbital ridge of up to 8-12 tubercles across the ridge to the bases of the supra-orbital horns. Parietal crest always absent but some very small irregular lines may be present on top of head. Temporal crests may be reduced to only 3-4 tubercles on a straight line, the most posterior one being the most prominent. Up to six large spines on forearms and forelegs, up to three on upper arms, and four on upper legs. Up to about 20 small body spines per flank.

Females. TL 67-69 mm, and tail 25-28% of TL. Variation as for males.

Hemipenis. PEM-R 16271 (Fig. 9). Short, bag-like. No truncal calyces. Apex adorned with two short outwardly curved horns. Each horn has a cluster of three prominent thorn-like papillae at the base and 1-2 other papillae along the outer curvature of the horn.

Eggs. Both female specimens with four eggs (up to 11 × 6 mm).

Colour in life (Fig. 6). Background colour varies from rather bright shades of green, especially on head, to light brown. Generally paler on lower parts with feet pale yellow and belly almost white. Blue patches may be present on casque and shoulders, and occasionally yellow to orange spots at the level of eyes. Very small regular dark spots sometimes present on the body, seen especially in chameleons with a greenish background. Two prominent wide dark antero-dorsal to postero-ventral parallel lines are almost always visible.

Differential diagnosis. Among the pygmy chameleons, only *Rh. spinosus* presents a similar rostral process. However, *Rh. spinosus* has a more rounded rostral process, numerous spiny tubercles on the gular region, a slender overall appearance, and a significantly longer tail (up to more than 40% of TL); furthermore it is not sympatric with *Rh. acuminatus*. Although several other species, like *Rh. uluguruensis* and related taxa, also have rather conspicuous naso-rostral processes, these are more cylindrical and much smaller. Furthermore these species do not show the characteristic body spines seen in *Rh. acuminatus*, thus making confusion unlikely.

Distribution and ecology. So far *Rh. acuminatus* is known from a single population in an Afro-montane rainforest between 1500 and 1600 m above the village of Ubili in the Nguru mountains. The species seems to be locally abundant. Six specimens have been collected for the present description; another six have been transferred to a reptile park in Arusha to attempt captive breeding. Most animals have been found between 50 cm and 2 m high on large ferns and shrubs, although several

have been spotted up to an estimated 3-4 m high. This spatial distribution is rather unusual for pygmy chameleons, which generally stay closer to the ground. Interestingly it is comparable in its arboreal inclination to the morphologically similar *Rh. spinosus* from the Usambara mountains. The living specimens laid 2-4 eggs, hatching in January (J. Beraducci, Arusha, *in litt.*).

RHAMPHOLEON (RHAMPHOLEON) VIRIDIS SP. NOV. (FIGS 7, 10, 11)

Holotype. NMZB 16905 (field tag CT 119), male; allotype NMZB 16906 (CT 120), female. Tanga region, South Pare mountains, from a patch of forest next to the Hingili stream, just north of the Shengena Mountain FR [4°14' 50" S, 37°59'28" E], 1450 m, 4 July 2001. Collected by Colin and Douglas Tilbury.

Paratypes. One male UDSM 1641, and one female UDSM 1642, same data as holotype.

Other material. One male BMNH 1982.1426 (KMH 1514), West Usambara, Mazumbai FR, 02 June 1980, collected by Kim Howell; one female NMZB 16700 (KMH 19586), South Pare, Chome FR, 1800 m, and one male NMZB 14059 (KMH 7935), North Pare, Ngofi Peak, Minja FR, 31 July 1993, collected by N. Cordeiro; two males NMB 7913 & 7914, South Pare, forest above Kisiwani, 18 April 1996, collected by Alexander Flemming; four males MHNG 2617.090, 093 and 2619.031-2 (TZ 139, 140, 142, 147) and two females MHNG 2617.091-092 (TZ 145–146). South Pare, Chome FR, 1840–2070 m, 29–30 September 2000; four males MHNG 2624.059, 2624.074, 2624.076-07 (TZ 495, 510, 512–3) and one female MHNG 2624.075 (TZ 511) North Pare, Kindoroko FR, 1600–1700 m, 10 May 2002.

Rh. temporalis examined for comparative purposes (all from East Usambara): NMZB 14820, female, and NMZB 14821 (KMH 12178), male, Bamba FR; NMZB 16362 (KMH 17875), female, Kwamkoro/Kwamsambia FR; NMZB 14068 (KMH 11224), juvenile male, and NMZB 14069, male, Magrotto Hill, Muheza; KMH 21313, male; BMNH 1935.4.1.35, male, and BMNH 1974.526, juvenile female, Amani; BMNH 1988.641-643 male, Monga estate; MHNG 2617.034, female, Lutindi Peak.

Note. The 16S rRNA sequences (AY524868-9) of the specimens listed as *Rh. sp. nova* by Matthee *et al.* (2004) are 99-100% identical to our sequences of *Rh. viridis*. Therefore their material from the South Pare can safely be identified as *Rh. viridis*.

Etymology. The specific name derives from Latin *viridis* (green) and refers to the rich green colour of the males.

Diagnosis (Fig. 7). Chamaeleonidae, *Rhampholeon* (*Rhampholeon*). With the characters of the subgenus. A small chameleon (maximum TL 89 mm) with a tail 34-46% of TL in males and 33-34% in females. Low casque. Small rostral process represented by a bulge barely projecting over the front of the snout, barely visible in males, somewhat larger in females. Temporal

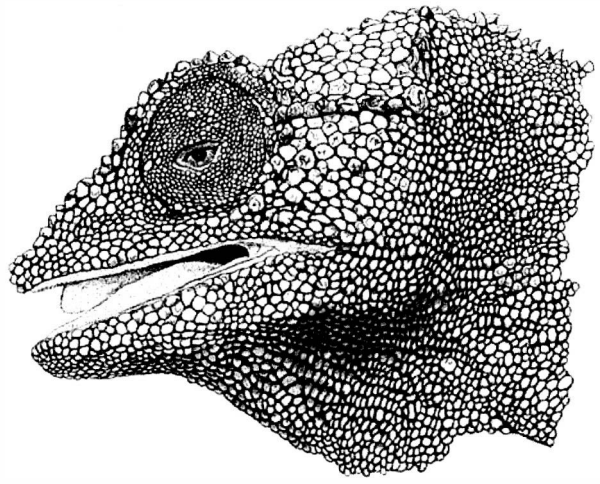

FIG. 10. *Rhampholeon* (*Rhampholeon*) *viridis* n. sp. (MHNG 2624.059). Head detail.

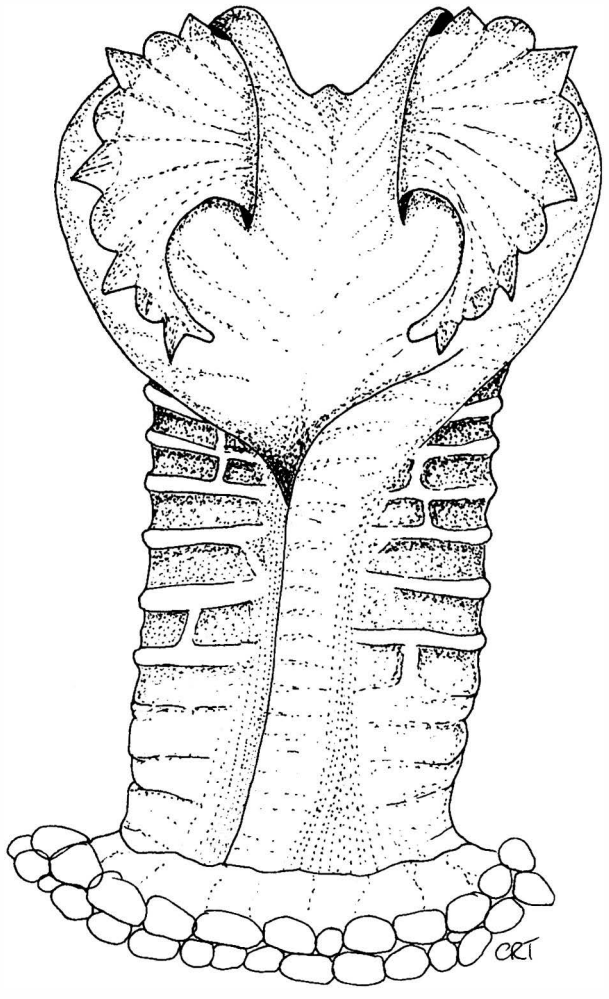

FIG. 11. *Rhampholeon* (*Rhampholeon*) *viridis* n. sp. (MHNG 2624.059). Hemipenis, sulcal view.

crest is distinct. Dorsal keel variable in outline from almost smooth to strongly crenulated. Hemipenis with prominent calyces on the truncus and broad paired apical horns arising from mucosal folds bearing up to nine papillae typically alternating rounded and sharp papillae on the outer edge of the horn. Axillary pits and inguinal pits present, the latter less distinct. Claws simple. There may be one or two slightly enlarged accessory plantar tubercles present at the base of the claws. Soles of feet smooth/cobblestoned as opposed to spinous. The male hemipenis is distinct from other species of *Rhampholeon*. The specimens from North Pare bear typical reddish patches.

Description of the holotype. Head (Fig. 10): Casque flat, not elevated above the nape. No parietal crest present. The supra-orbital crest is composed of subconical to conical tubercles. The supra-orbital ridge is relatively smooth with no supra-optic peak. The orbital ridges are connected across the top of the head by a row of 14 tubercles. The canthal ridge is sharply delineated, terminating anteriorly at the base of a rostral bulge. This bulge, which is covered with sub-conical tubercles, projects forward barely clearing the tip of the snout. The nares open infero-posteriorly from within a low nasal bulge. The gular region is smooth and unadorned with spines or tufts of scales. A prominent temporal crest is present formed by a row of seven conical tubercles, the most posterior of which is by far the largest. The temporal crest continues upwards as a well-marked posterior temporal or squamosal crest of enlarged conical to subconical tubercles to the apex of the casque. The skin of the eyeball is clad with small relatively homogeneous rounded tubercles.

Body: TL 68.5 mm (SVL 44.5 mm + tail 24 mm), the tail comprising 35% of the TL. The dorsal keel is only weakly crenulated. A low cluster of slightly enlarged tubercles is present over each vertebral spinous process commencing from above the shoulder area, fading over the sacrum and re-appearing along the tail. The cluster of tubercles may be centred on either a single low cone or a pair of smaller cones. The flanks are clad in tightly packed sub-homogeneous granules with scattered enlarged conical tubercles. The granules are largely rounded but there are scattered clusters of stellate granules. There is no enlarged tubercle above the shoulder. A vague row of four slightly enlarged tubercles is present along the infero-lateral aspect of the proximal half of the tail. The claws of the feet are simple with no evidence of cusp formation. The tubercles on the soles of the feet are rounded to give the appearance of a cobblestoned surface. There are no prominent accessory plantar spines present at the bases of the toes, rather low plantar tubercles. Deep wide-mouthed dermal pits/invaginations are present in both axillae and the inguinal regions. The hemipenes are not fully everted.

Variation in paratypes and other material. Head: Narrow occipital concave surface may be present. Temporal crest typically with 4-5 large conical postocular tubercles, then typically three more on a upward line, with the lowest one being the most prominent. Vestigial parietal crest, sometimes formed by three ridges. One isolated tubercle above jaw articulation. Interorbital ridge a shallow V, formed of 8-14 granules. A small but distinct rounded rostral appendage in females (1 × 1 mm), less marked or absent in males.

Body: TL 63-89 mm, SVL 38-47 mm, tail 25-32 mm, tail 34-46% of TL for males. TL 65-72 mm, SVL 43-48 mm, tail 22-24 mm, tail 33-34% of TL for females. Thus males slightly larger, but females with a shorter tail. Dorsal keel weakly to strongly crenulated in males (9-12 clusters) may be almost smooth in females. Axillary pits present, but only less conspicuous inguinal depressions. Flanks of one specimen with a few enlarged pyramidal clusters of tubercles on each side. Claws simple. Two gravid female with four eggs each (10-11 × 4.5-6 mm).

Hemipenis. (Fig. 11) (MHNG 2624.059). Hemipenal truncus with prominent calyces on the asulcal (posterior) aspect becoming smooth in the para-sulcal zone. The sulcal lips are smooth. Apex capitate. A pair of apical horns arise from between prominent mucosal folds sited towards the asulcal side of the apex and which curve inwardly over the apical plateau. The outer margins of the horns are adorned with a series of alternating thorn like and button like papillae – nine on one horn and six on the other.

Colour in life (Fig. 7) . When first seen in undisturbed conditions the males of this species have a background colour of emerald green. Two thin dark stripes are angled postero-inferiorly over the flanks from the dorsal keel. North Pare specimens harbour several characteristic reddish/rusty coloured patches on the head, belly, tail and around the main lateral cones, and occasionally one thin transversal reddish stripe from above the shoulder to inguinal region, or some whitish areas on shoulder and occiput.

Differential diagnosis. The simple claws of this species immediately place this form within the group of pygmy chameleons that only includes *Rh. spinosus* and *Rh. temporalis*. The former species differs from *Rh. viridis* by the prominent ovoid rostro-nasal projection found in both sexes. Apart from the striking hemipenal differences between males of *viridis* and *temporalis* (breadth of the apical horns and shape of the papillae on the horns, see Figs 2 and 11), they appear very similar in external morphology. Differences between the two are subtle but may be seen in the more pronounced dorsal crest and the conspicuous temporal crest of *viridis*. Perhaps the best distinguishing feature between them is that the accessory plantar spines in *temporalis* are usually well developed and prominent but are inconspicuous to rudimentary in *viridis*.

Distribution and ecology. This species inhabits the undergrowth and lower story vegetation of the submontane evergreen forests of the South and North Pare mountains. Its occurrence in the West Usambara is based on a single specimen in the British Museum collected in 1980 but its presence in these mountains has

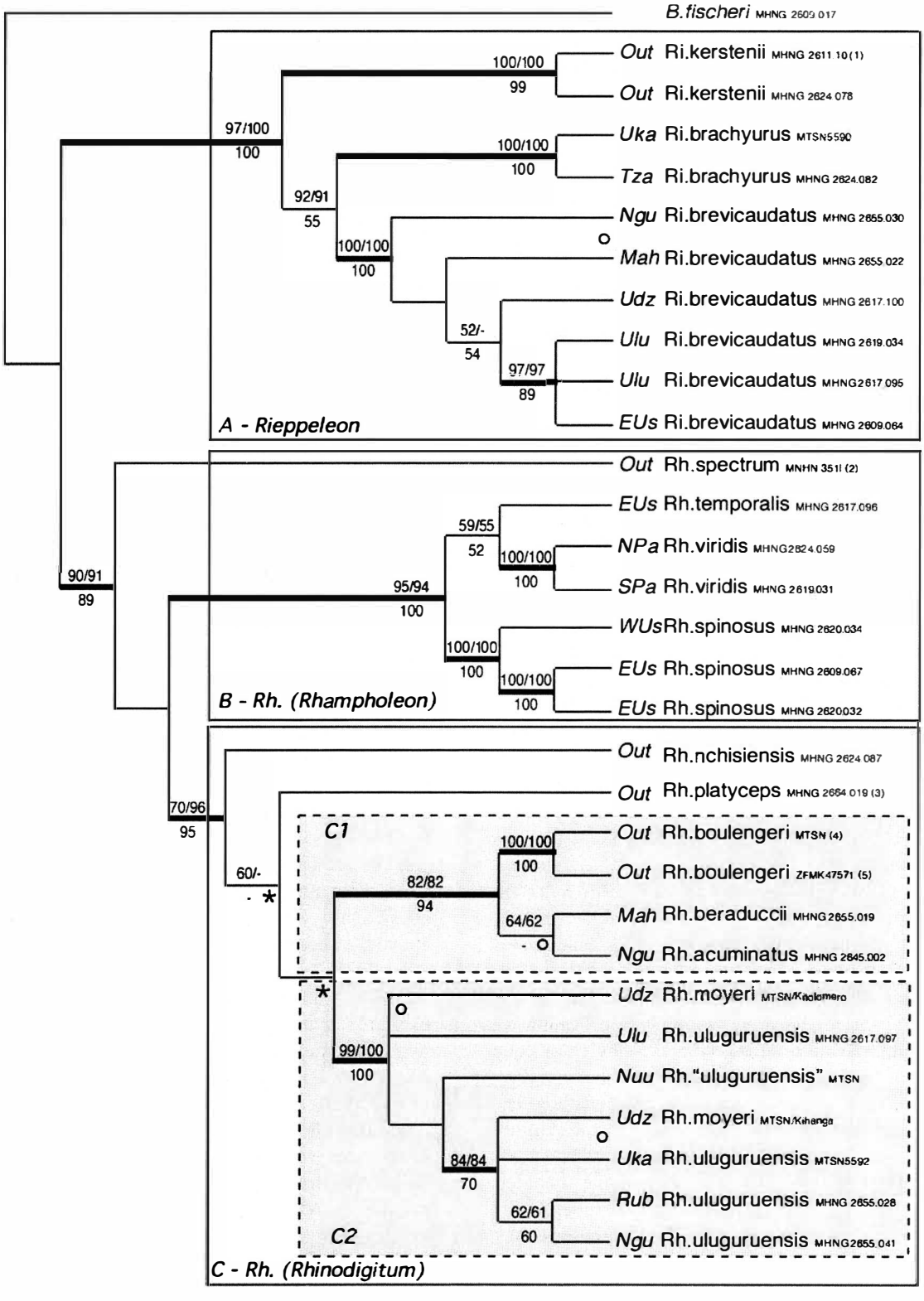

FIG. 12. Molecular tree. Parsimony analysis, gaps treated as missing. Strict consensus of four shortest trees. Numbers above branches are bootstrap values over 50% for heuristic parsimony searches, 1000 repeats (with gaps treated as missing or fifth base) and below branches bootstrap of ML searches (100 repeats). Branches leading to strongly supported nodes by all methods (over 70% bootstrap) are in bold. * Indicates different results found in the analysis where gaps were treated as fifth base (branches collapsed), and ° indicates such variations in the ML best tree (branches collapsed; or sister group relationship between *Ri. brevicaudatus* MHNG 255.022 and 030, between *Rh. moyeri*, Kihanga and *uluguruensis* MTSN5592; or basal position of *Rh. moyeri*, Kitolomero in the clade). Massif of origin of specimens is abbreviated as follows: NPa: North Pare; Spa: South Pare; WUs: West Usambara; EUs: East Usambara; Nuu: Nguu; Ngu: Nguru; Ulu: Uluguru; Uka: Ukaguru; Rub: Rubeho; Udz: Udzungwa; Mah: Mahenge, Tza: unknown origin in Tanzania; Out indicates an origin from outside the EAR. (1) Captivity, (2) Cameroon, (3) Mozambique, (4) Rwanda, (5) Democratic Republic of Congo.

not since been reconfirmed. The holotype was collected at an altitude of 1450 m, but specimens have been found up to 2070 m in the South Pare and 1700 m in the North Pare. These forests are typical examples of the Afromontane forests that are dominated by emergent trees such as *Albizia gummifera, Macaranga kilimandscharica, Xymalos monospora, Ocotea usambarensis, Podocarpus latifolius* and *Chrysophyllum gorongosanum.*

When handled these chameleons produced an easily felt "buzzing" vibration, particularly if touched lightly on the back. At the time of collection in early July, an adult male and female chameleon were found sleeping within a few centimetres of each other, indicating pairing off and thus possible recent past or potentially future mating activity.

Mating. A single pair was observed mating in Kindoroko FR (North Pare) on 10 May 2002 at 7 pm. Exact duration of the copulation was not recorded but was longer than three hours. The pair was on a narrow branch about one metre high; the male was on the back, slightly to the right and parallel to the female.

Parasitology. All specimens from North Pare were parasitized both by intestinal nematodes and acanthocephalans. The acanthocephalans have been found to represent a new species of *Acanthocephalus* (*Pseudacanthocephalus*) recently described by Smales (2005). In the South Pare all specimens but one harboured *Cylindrotaenia* sp. (Nematotaeniidae) tapeworms.

ADDITIONAL SPECIES

Rhampholeon (*Rhinodigitum*) *nchisiensis* (Loveridge, 1953), which is present in Tanzania but not in the EAR, *Rhampholeon (Rhampholeon) spectrum* (Bucholz, 1874), and *Rhampholeon (Rhinodigitum) platyceps* (Günther, 1892), which are not found in Tanzania, have opportunistically been included in our molecular analysis because of their close geographical distribution and possible relatedness to our species of interest.

MOLECULAR SYSTEMATICS

Results. Sequences for 12S (414 bp) and 16S (523 bp) were obtained for 45 *Rhampholeon/Rieppeleon* and one outgroup (*Bradypodion fischeri*). A PHT found both partitions to be compatible, and those sequences were thus concatenated in a single matrix 938 characters long, from which 44 positions were removed for analyses due to uncertain alignment. Distances between in- and outgroups averaged 13.7% (11.9–15.5%). Interspecific distances within the ingroup averaged 11.0% (7.3–15.4%), and intraspecific distances varied between 0 and 3.5%, and up to 5.9% within the *uluguruensis* complex (which was treated as a single species in this case). Distances between members of *Rieppeleon* and *Rhampholeon* (14.1% on average) were as high as between any pygmy chameleon and the outgroup. Among sequences from conspecific specimens distances of 0 to less than 0.35% (0-3 changes) were found between 6 out of 10 *Ri. brevicaudatus*, 4 out of 8 *Rh. uluguruensis*, 3 out 4 *Rh. viridis* and all *Rh. beraduccii* (2), *Rh. acuminatus* (2) and *Rh. "uluguruensis"* from Nguu (4). Removing these identical, or nearly identical, sequences from the analyses reduced the final matrix to 31 taxa and 894 positions.

A heuristic parsimony search of this dataset returned nearly identical results whether gaps were treated as missing (four shortest trees, L 906, CI 0.433, RI 0.704) of "fifth base" (six shortest trees, L 1001, CI 0.447, RI 0.721) (Fig. 12): a first well-supported basal clade comprises three species, a basal *Ri. kerstenii* sister taxon of *Ri. brachyurus* and *Ri. brevicaudatus* (clade A, corresponding to the genus *Rieppeleon*). The second clade is rooted, although very weakly, by *Rh. spectrum*, which is the sister taxon of a large clade comprising *Rh. temporalis*, *Rh. viridis* and *Rh. spinosus* on one side (group B, together with *Rh. spectrum*) and the rest of the ingroup on the other (clade C), corresponding respectively to Matthee *et al.*'s (2004) subgenera *Rhampholeon* and *Rhinodigitum*. Technically, the subgenus *Rh. (Rhampholeon)* is thus paraphyletic. Within the latter group relationships are less clearly resolved, although two subgroups, one with *Rh. boulengeri*, *Rh. acuminatus* and *Rh. beraduccii* (C1) and the other with *Rh. uluguruensis*, including *Rh. moyeri* and *Nguu* specimens (C2) are supported. The positions of *Rh. platyceps* and *Rh. nchisiensis* are uncertain within *Rh. (Rhinodigitum)*, although both the parsimony analysis with gaps treated as missing and the ML analysis place them basal.

The maximum likelihood best-fit model was SYM+I+G with the following parameters: Base=e qual, Nst=6, Rmat =(0.6538 5.2133 0.5304 0.0728 3.7009), Rates=gamma, Shape=0.7680, Pinvar=0.4361. The analysis gave similar results as parsimony except for the clade C1, in which *Rh. acuminatus* is basal, and a few minor details within *Ri. brevicaudatus* and the *Rh. uluguruensis* complex (see caption to Fig. 12).

DISCUSSION

Field studies of the pygmy chameleons are few and our understanding of the group is weak. A single recent paper addressed the overall systematics and evolution of the group (Matthee *et al.*, 2004), and, to our knowledge, no paper dealing specifically with the taxa from the EAR has been published apart from occasional species descriptions and a recent comparative study on the ecology of *Rh. temporalis* and *Ri. brevicaudatus* in the East Usambara (Emmett, 2004).

At the morphological level, the descriptions presented herein clearly demonstrate the frailty of external morphology for differentiating species, and the important role that hemipenal analysis can play in discriminating between most species in this group. For example whilst the hemipenal differences are striking, the external morphological differences between *Rh. viridis* sp. n. and *Rh. temporalis* are subtle and to a cer-

tain extent subjective. The two species are sister taxa and *Rh. temporalis* differs only by showing more derived character states in the degree of development of the rostral process and plantar spines.

However, the problem of external similarity is not always simply solved just by examining the soft tissues. The phenotype and hemipenes of a population of Nguru pygmy chameleons (shown here to belong to the *uluguruensis* complex) cannot reliably differentiate this population from typical *Rh. boulengeri* from Rwanda. Notwithstanding the observation that the nearest recognized population of *boulengeri* is over 600 km away, the question must be asked as to why the Nguru population should not be assigned to *boulengeri* (see also Menegon *et al.*, 2003). Tolley *et al.* (2004) demonstrated that phenotype in chameleons (the Southern African *Bradypodion*) was a relatively plastic expression sensitive to environmental selection pressures. We consider it likely that this observation in *Bradypodion* is mirrored in the pygmy chameleons and particularly in the EAR species. For example, on comparing *Rh. boulengeri* and *Rh. uluguruensis* within their stable forest habitats, it would appear that their external phenotype has been under hardly any environmental pressure to evolve. Similarly, and despite their importance, the relatively simple hemipenis structures observed in these species are not sufficient to characterize them. Their evolutionary differentiation has been rather at the genetic level as indicated by a sequence divergence of about 9%, a level that is clearly within the interspecific range in our data set. Although morphology easily indicates a placement within the subgenus *Rhinodigitum*, it does not allow for specific differentiation.

At the molecular level, Matthee *et al.* (2004) published a complete analysis of the pygmy chameleons and demonstrated their basic organization in two main clades that they proposed to consider as distinct genera, *Rieppeleon* and *Rhampholeon*, in accordance with the earlier observations of Rieppel (1987) and Tilbury (1992). They also distinguished three distinct and well supported lineages (*Rhinodigitum*, *Bicuspis* and *Rhampholeon*) within *Rhampholeon*. Our sampling is different from the one of Matthee *et al.* (2004), in two main ways: first, we do not include members of their "*Bicuspis*" lineage whose members are not found in the EAR (and in Tanzania), and second, a few new species described herein are added to the dataset. Nevertheless our results (Fig. 12) confirm most of Matthee *et al.*'s (2004) observations. We find strong support for the *Rhampholeon* and *Rieppeleon* lineages. In the latter one, though, our data (as well as unpublished preliminary cytochrome b sequences) support a (*Ri. brachyurus* – *Ri. brevicaudatus*) sister-group relationship instead of (*Ri. kerstenii* – *Ri. brachyurus*) as in Matthee *et al.* (2004). This node, however, was relatively weakly supported by their 16S data, and their RAG1 data suggested the same clustering as found here.

Within *Rhampholeon sensu stricto* a possible important difference lies with the position of the West African species *Rh. spectrum*, which is a sister group of the *temporalis/viridis* (Pare Mountain) clade in Matthee *et al.* (2004), and is basal to the whole *Rhampholeon* genus in our work, even if bootstrap support for this position is weak. Matthee *et al.* (2004) explained that the close relationship between *Rh. spectrum* and *Rh. temporalis* was the result of historic climatic changes that resulted in the desiccation of the pan-African forests about 25 million years ago. The position of *Rh. spectrum* in our analysis is speculative given the weak support of this node, which might be due to saturation in this case. We must also note that our sequence is 3-4% different from the Equatorial Guinea sequences from Matthee *et al.* (2004), which may explain the slightly different position of this taxon on our tree. In any case, assuming our placement of *Rh. spectrum* is correct, this would suggest that this species might be the most ancient sister group to all other *Rhampholeon,* an interesting hypothesis given the wide distribution of this taxon. This would also imply that *Rh. (Rhampholeon)* is paraphyletic and might therefore have further taxonomical consequences. Globally, the diversification of the genus in the easternmost extremity of the EAR (i.e. at least with *Rh. temporalis*, *Rh. spinosus* and *Rh. viridis*) is more extensive than previously thought. In Tanzania, *Rh. (Rhampholeon)* is restricted to the eastern/northeastern EAR.

We confirm the existence of two main EAR lineages within *Rh. (Rhinodigitum*), one comprising *Rh. boulengeri* and other species, and the other with *Rh. uluguruensis*, and find the subgenus to be rooted with the non-EAR species *Rh. nchisiensis* and *Rh. platyceps* (although again with weak support). All species included in this subgenus are found in the western/southwestern part of the EAR (and beyond). Interestingly, we show that both newly described *Rh. beraduccii* and *Rh. acuminatus* are more closely related to *Rh. boulengeri* than to the *uluguruensis* complex. Given the overall similarity of *Rh. beraduccii* with the members of the *uluguruensis* complex at the level of the head and appendages, this is again an indication that these morphological characters can be deceptive, and that very similar morphologies may have evolved independently. Convergences can also occur for characters looking very original, like the discoid rostral appendages of *Rh. spinosus* and *Rh. acuminatus*.

We have not found any decisive argument to resolve the status of the taxa included in the *uluguruensis* complex. Relatively high genetic distances between its components obviously plead for a heterogeneous assemblage encompassing more than a single species; however, no satisfactory nomenclatural system can be derived yet. The status of both *Rh. moyeri* and of the new Nguu specimens remains equivocal within this group.

Faunistically, it appears that the genus is more species rich in the EAR than previously expected, and that a combination of ancient colonization and recent, or ongoing, speciation processes can explain this situation. In

the eastern EAR (East and West Usambaras and South and North Pares), at least six species of pygmy chameleon are described. These species belong to both pygmy chameleon genera and are representative of the most ancient lineages of these lizards in our sampling, thus suggesting that the colonization of the EAR started in this geographical area. In parallel with these ancient events it seems that a further diversification of *Rh. (Rhampholeon)* is still ongoing and is facilitated by the complete separation of the East and West Usambara as well as the North and South Pare by lowland valleys. In the former mountain we observe a rather high DNA distance (3.5%) between *Rh. spinosus* specimens sampled on each side of the Lwengera valley (as compared to 0.7% between two specimens from the East Usambara). In the Pare, *Rh. viridis* from the southern massif are clearly distinguishable at the mtDNA level (as well as, to a certain extent, morphologically) from conspecific specimens from the northern massif. In one of the few comparable studies in the area, Johanson & Willassen (1997), working on the Helicopsychidae (caddis flies), also reached the conclusion that the East and West Usambara formed distinct endemic areas. On the other hand, Gravlund (2002), who studied the snake *Crotaphopeltis tornieri* (Colubridae), found no evidence that populations on both sides of the Lwengera valley were genetically distinct. He nevertheless concluded that they were most probably isolated.

A similar scenario might explain the situation in the western EAR with a fauna originating from the south or west of the area and a recent differentiation within the Uluguru–Nguru–Rubeho–Udzungwa massifs. The best example of such a scenario would be the "*uluguruensis*" complex where morphology is minimally useful for the identification of lineages but for which mtDNA shows that some populations (i.e. *Rh. moyeri* from Kitolomero or the Nguu specimens) are relatively well characterized. As many isolated forest reserves in this area have not yet been fully explored, especially in the Rubeho/Ukaguru massifs, we should expect the discovery of more populations in this group.

These hypotheses of relatively recent diversification in the mountains are corroborated by similar observations made for various groups of animals such as birds (Roy, 1997), insects (Johanson & Willassen, 1997) or amphibians (Loader *et al.*, 2004*a*) and support an important role of the "mountain speciation model" (Fjeldså & Lovett, 1997). In any case, the fact that no montane species of pygmy chameleons is shared between the eastern and western parts of the EAR tends to confirm the status of distinct "Evolutionary Significant Units" for the distinct mountains blocks of the range, as suggested by Gravlund (2002). This, added to the fact that the distribution area of most taxa discussed herein is, most probably, very reduced, should imply the strongest possible protection for the remaining forests in the EAR.

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APPENDIX I : NEWLY COLLECTED SPECIMENS

Location	Species	Field no.	No.	Date	Locality	Sex
MHNG	*Rh. acuminatus* n .sp.	TZ412	2645.002	21.10.01	Komkore	M
	Rh. acuminatus n. sp.	TZ413	2645.003	21.10.01	Komkore	M
	Rh. acuminatus n. sp.	TZ414	2645.001	21.10.01	Komkore	F
	Rh. acuminatus n. sp.	TZ415	2645.005	21.10.01	Komkore	F
	Rh. acuminatus n. sp.	TZ416	2645.006	21.10.01	Komkore	
	Rh. acuminatus n. sp.	TZ417	2645.004	21.10.01	Komkore	M
	Rh.beraduccii n. sp.	TZ343	2655.019	09.10.01	Sali	F
	Rh.beraduccii n. sp.	TZ344	2655.020	09.10.01	Sali	juv
	Rh.beraduccii n. sp.	TZ345	2655.021	09.10.01	Sali	M
	Ri. brachyurus	TZ331	2655.018	2001	Ngurus	
	Ri. brachyurus	TZ525	2624.082	14.05.02	Tanzania	
	Ri. brachyurus	TZ526	2624.083	14.05.02	Tanzania	
	Ri. brachyurus	TZ527	2624.084	14.05.02	Tanzania	
	Ri. brevicaudatus	TZ053	2609.062	28.11.99	Amani	juv
	Ri. brevicaudatus	TZ054	2609.063	28.11.99	Amani	F
	Ri. brevicaudatus	TZ060	2609.064	28.11.99	Amani	M
	Ri. brevicaudatus	TZ061	2609.065	28.11.99	Amani	F
	Ri. brevicaudatus	TZ173	2617.087	02.10.00	Nilo	
	Ri. brevicaudatus	TZ174	2617.088	02.10.00	Nilo	
	Ri. brevicaudatus	TZ175	2617.089	02.10.00	Nilo	M
	Ri. brevicaudatus	TZ176	2619.033	02.10.00	Nilo	M
	Ri. brevicaudatus	TZ185	2617.094	03.10.00	Nilo	
	Ri. brevicaudatus	TZ220	2619.034	09.10.00	Tegetero	M
	Ri. brevicaudatus	TZ221	2617.095	09.10.00	Tegetero	F
	Ri. brevicaudatus	TZ284	2619.035	20.10.00	Kihansi	F
	Ri. brevicaudatus	TZ291	2617.100	21.10.00	Kihansi	F
	Ri. brevicaudatus	TZ292	2618.001	21.10.00	Kihansi	F?
	Ri. brevicaudatus	TZ293	2618.002	21.10.00	Kihansi	F
	Ri. brevicaudatus	TZ346	2655.022	09.10.01	Sali	F
	Ri. brevicaudatus	TZ347	2655.023	09.10.01	Sali	M
	Ri. brevicaudatus	TZ348	2655.024	09.10.01	Sali	F
	Ri. brevicaudatus	TZ349	2655.025	09.10.01	Sali	F
	Ri. brevicaudatus	TZ350	2655.026	09.10.01	Sali	F
	Ri. brevicaudatus	TZ374	2655.027	09.10.01	Sali	M
	Ri. brevicaudatus	TZ405	2655.030	20.10.01	Komkore	F
	Ri. brevicaudatus	TZ406	2655.031	20.10.01	Komkore	M
	Ri. brevicaudatus	TZ422	2655.038	21.10.01	Komkore	F
	Rh. brevicaudatus	TZ432	2655.044	22.10.01	Komkore	M
	Ri. kerstenii	TZ517	2624.078	13.05.02	Masai plain	M
	Ri. kerstenii	TZ518	2624.079	13.05.02	Masai plain	M
	Ri. kerstenii	TZ530	2624.085	14.05.02	Tanzania	
	Rh. nchisiensis	TZ531	2624.086	14.05.02	Poroto Mtns	M
	Rh. nchisiensis	TZ532	2624.087	14.05.02	Poroto Mtns	
	Rh. spinosus	TZ24	2609.067	27.11.99	Amani	
	Rh. spinosus	TZ329	2620.032	06.10.01	E. Usambara	
	Rh. spinosus	TZ438	2620.034	26.10.01	Mazumbai	
	Rh. spinosus	TZ440	2620.036	26.10.01	Mazumbai	
	Rh. temporalis	TZ197	2617.096	01.10.00	Lutindi Pk	F
	Rh. temporalis	TZ198	not kept	01.10.00	Lutindi Pk	juv
	Rh. uluguruensis	TZ267	2617.097	12.10.00	Mkungwe	M
	Rh. uluguruensis	TZ268	2617.098	12.10.00	Mkungwe	M
	Rh. uluguruensis	TZ269	2619.036	12.10.00	Mkungwe	M
	Rh. uluguruensis	TZ270	2617.099	12.10.00	Mkungwe	M
	Rh. uluguruensis	TZ394	2655.028	17.10.01	Mafwomero	M
	Rh. uluguruensis	TZ395	2655.029	17.10.01	Mafwomero	M

Location	Species	Field no.	No.	Date	Locality	Sex
MHNG	*Rh. uluguruensis*	TZ427	2655.039	21.10.01	Komkore	F
(cont...)	*Rh. uluguruensis*	TZ428	2655.040	21.10.01	Komkore	M
	Rh. uluguruensis	TZ429	2655.041	21.10.01	Komkore	M
	Rh. uluguruensis	TZ430	2655.042	21.10.01	Komkore	juv
	Rh. uluguruensis	TZ431	2655.043	21.10.01	Komkore	M
	Rh. uluguruensis	TZ481	2624.047	04.05.02	Ikwamba	M
	Rh. uluguruensis	TZ482	2624.048	04.05.02	Ikwamba	F
	Rh. uluguruensis	TZ483	2624.049	04.05.02	Ikwamba	M
	Rh. uluguruensis	TZ484	2624.050	04.05.02	Ikwamba	M
	Rh. uluguruensis	TZ492	2624.056	05.05.02	Mandenge	M
	Rh. uluguruensis	TZ493	2624.057	05.05.02	Mandenge	F
	Rh. viridis n. sp	TZ139	2617.090	29.09.00	Chome	M
	Rh. viridis n. sp	TZ140	2619.032	29.09.00	Chome	M
	Rh. viridis n. sp	TZ142	2619.031	30.09.00	Chome	M
	Rh. viridis n. sp	TZ145	2617.091	30.09.00	Chome	F
	Rh. viridis n. sp	TZ146	2617.092	30.09.00	Chome	F
	Rh. viridis n. sp	TZ147	2617.093	30.09.00	Chome	M
	Rh. viridis n. sp .	TZ495	2624.059	10.05.02	Kindoroko	M
	Rh. viridis n. sp.	TZ510	2624.074	10.05.02	Kindoroko	
	Rh. viridis n. sp.	TZ511	2624.075	10.05.02	Kindoroko	M
	Rh. viridis n. sp.	TZ512	2624.076	10.05.02	Kindoroko	F
	Rh. viridis n. sp.	TZ513	2624.077	10.05.02	Kindoroko	M
NMZB	*Rh. viridis* n. sp.	CT119	16905	04.07.01	Shengena	M
	Rh. viridis n. sp.	CT120	16906	04.07.01	Shengena	F
	Rh. viridis n. sp.	KMH19586	19586	-	Mazumbai	F
	Rh. viridis n. sp.	KMH7935	14059	31.07.93	Ngofi Pk	M
USDM	*Rh. viridis* n. sp.	-	1641	04.07.01	Shengena	M
	Rh. viridis n. sp.	-	1642	04.07.01	Shengena	F
NMB	*Rh. viridis* n. sp.	-	7913	18.04.96	Kisiwani	M
	Rh. viridis n. sp.	-	7914	18.04.96	Kisiwani	M
PEM-R	*Rh. acuminatus* n. sp.	-	16271	21.10.01	Komkore	M

APPENDIX 2 : LOCALITIES

The specimens included in this study come from the following localities [locality, mountain (region), coordinates, altitude]:

Kindoroko FR, North Pare (Kilimanjaro), 3°43'44" S , 37°39'16" E, 1600 m; Ngofi Pk, Minja FR, North Pare (Kilimanjaro), 3°36' S, 37°43' E; Chome FR, South Pare (Kilimanjaro), 4°17'29" S, 37°55'16" E, 1850 m; Shengena FR (Hingili Stream), South Pare (Kilimanjaro), 4°14'50" S, 37°59'28" E; above Kisiwani, South Pare (Kilimanjaro), 4°7' S, 38°5' E; Mazumbai FR, West Usambara (Tanga), 4°48'45" S, 38°30'13" E, 1500 m; Amani, East Usambara (Tanga), 5°5'58" S, 38°37'55" E, 1000 m; Nilo FR, East Usambara (Tanga), 4°54'38" S, 38°39'49" E, 750 m; Lutindi Pk, East Usambara (Tanga), 4°53' S, 38°38' E, 1300 m; Komkore (above Ubili), Nguru (Morogoro), 6°2'51" S, 37°31'43" E, 1000 m; Mafwomero FR (above Mbuga), Rubeho (Dodoma), 6°56'27" S, 36°35'14" E, 1900 m; Tegetero, Uluguru (Morogoro), 6°56'30" S, 37°43'11" E, 1000–1200 m; Mkungwe, Uluguru (Morogoro), 6°52'41" S, 37°55'15" E, 1000 m; Mandenge, Ukaguru (Dodoma), 6°21'14" S, 36°57'54" E, 1600 m; Ikwamba FR, Ukaguru (Dodoma), 6°20'31" S, 36°58'58" E, 1500 m; Kihansi gorges,Udzungwa (Morogoro), 8°35'10" S, 35°51'2" E, 800 m; Sali, Mahenge (Morogoro), 8°57'57" S, 36°41'18" E, 900–1000 m. Additional comparative material comes from: Bamba FR, Magrotto Hill, Kwamkoro/Kwamsambia and Monga Estate, all East Usambara; Kitolomero, Udzungwa; Kihanga, Udzungwa; Mamiwa Kisara FR, Ukaguru; Nguu FR, Nguu; Ukalini Forest, Namuli, Mozambique; Cyangugu/Cyamudongo Forest, Rwanda; Cameroon; and Irangi, Kivu, Democratic Republic of Congo.

APPENDIX 3: KEY TO THE PYGMY CHAMELEONS OF THE EASTERN ARC RANGE

1a. Soles of feet covered with sharply pointed/ spinous (acuminate) tubercles..... 2
1b. Soles of feet covered with sub-conical to rounded tubercles..... 4
2a. A single small beardlike tuft of tubercles present under chin/mentum..... *Rieppeleon brevicaudatus*
2b. Gular region either smooth or with conical tubercles scattered or in divergent rows.....3
3a. Tail very short, averaging less than 20% of the total length of the chameleon..... *Rieppeleon brachyurus*
3b. Tail averaging 25–30% of the total length of the chameleon, supracilliary process in adult males .....*Rieppeleon kerstenii*
4a. Claws of feet are simple non-bicuspid..... 5
4b. Claws of feet are strongly bicuspid..... 7
5a. Rostral process prominent cushion-like (East and West Usambara Mountains)..... *Rhampholeon* (*Rh*) *spinosus*
5b. Rostral process short stubby or indistinct..... 6
6a. Accessory plantar spines well developed and prominent (Eastern Usambara)..... *Rhampholeon* (*Rh*) *temporalis*
6b. Accessory plantar spines weak or indistinct, (Pare Mtns and West Usambara)..... *Rhampholeon* (*Rh*) *viridis*
7a.Deep dermal pits in the groin/inguinal region.....*Rhampholeon* (*Rhin*) *beraduccii*
7b. Groin/inguinal dermal pits absent or indistinct..... 8
8a. Axillary dermal pits prominent..... 9
8b. Axillary dermal pits absent.....*Rhampholeon (Rhin) acuminatus*
9a. 11–13 tubercles between bases of supra-optic peaks (Uluguru, Nguru, Ukaguru, Nguu Mountains) .....*Rhampholeon (Rhin) uluguruensis*
9b. 15–19 tubercles between bases of supra-optic peaks (Udzungwa, Rubeho Mtns) ..... *Rhampholeon (Rhin) moyeri*

SHORT NOTE

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LARVAL TRANSPORT DOES NOT AFFECT LOCOMOTOR PERFORMANCE IN THE STREAM FROG *MANNOPHRYNE TRINITATIS*

J. M. SMITH[1], J. BUCHANAN[1], J. R. DOWNIE[1] AND M. O. RIEHLE[2]

[1]Divisions of Environmental and Evolutionary Biology and [2]Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow UK

The jumping performance of *Mannophryne trinitatis* (Anura: Dendrobatidae), assessed by the parameters of take-off angle, height, length and speed, did not differ significantly between females and males, whether or not males were transporting larvae or had just deposited their larvae. The results are discussed in the context of the possible costs of larval transportation in dendrobatids.

Key words: larval transport, reproductive costs, Trinidad

The stream frog *Mannophryne trinitatis*, Trinidad's only dendrobatid, occurs in and around small mountain streams (Murphy, 1997; Jowers & Downie, 2004). Calling males attract females to rocky crevices where eggs are laid and the males guard them until hatching. Males then transport the whole batch of larvae on their backs (Fig. 1) until a suitable pool or stream is found, where the tadpoles are deposited and then grow until metamorphosis (Wells, 1980). Downie *et al.* (2001) showed that an important characteristic in making a pool or stream suitable for deposition is the absence of tadpole predators, particularly the fish *Rivulus hartii* and the shrimp *Macrobrachium carcinus*. *M. trinitatis* carried their larvae up to 4 days (in laboratory conditions) in the absence of a suitable pool, but then deposited the larvae on damp leaf litter. Downie *et al.* (2005) then tested which factors might limit the duration of larval transport in this species. They found that males were able to forage for food during transportation, that an extended period before feeding began had no harmful effects on the ability of larvae to grow, but that larvae were at risk from dehydration during extended transportation. *A priori*, one of the most obvious limitations seemed to be on male mobility, given that a full load of larvae adds about 30% to the mass of a male frog. Downie *et al.* (2005) were surprised not to be able to detect any effect of tadpole transportation on male frog mobility but measured this only in terms of the jump distance.

FIG 1. *Mannophryne trinitatis* male carrying larvae (eight larvae visible).

Correspondence: J. M. Smith, Division of Environmental & Evolutionary Biology, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK. *E-mail*: J.Smith@bio.gla.ac.uk

A number of studies have considered the effects of increasing mass on jumping ability in frogs; in general, jump distances increase but acceleration and velocity decrease (Wilson *et al.*, 2000; Choi *et al.*, 2000; Emerson, 1978). Field observations on *Mannophryne trinitatis* suggested that transporting males were a little slower and easier to catch than non-transporting frogs (M. Jowers, pers. comm.) and if this were the case might reflect a substantial predation cost of tadpole transport. However, as *M. trinitatis* from Trinidad's north coast sites tend to attempt to escape into crevices and under leaf litter (Jowers & Downie, 2004), distance may not be the most important parameter in jumping ability as it relates to predator avoidance. In this note, we report on follow-up experiments to determine whether locomotor costs of larval transport might be detected by considering additional jump parameters to those in Downie *et al.* (2005); particularly speed, height and take-off angle – these might all be expected to be lower in heavier frogs.

Ten transporting male and ten female *M. trinitatis* were captured at three locations in Trinidad's Northern Range, known to be frequented by the frogs due to presence of suitable tadpole deposition sites. Numbers were limited by the availability of transporting males within the field season. Captured frogs were transported in individual tubs, together with damp leaf litter taken from the site, to our laboratory at the University of the West Indies. Frogs were maintained singly in vivaria, as described in Downie *et al.* (2005). All initial assessments of locomotor performance were carried out within at least 24 hours of the frogs' capture and frogs re-released to original collection sites the day after tadpole deposition occurred.

Locomotor performance was measured on a bench-top runway 90 cm long by 30 cm wide (Fig. 2). At the back, we placed a board showing 5 cm height and 10 cm distance markers. At the end was a shaded area containing a tub of water set in leaf litter and rocks, to act as a positive directional stimulus for an escape jump. At the start was a glass circle of 11.5 cm diameter to act as a fixed starting point. Above the runway, a mirror was set at a 45° angle: this allowed accurate determination of jump length even when frogs did not

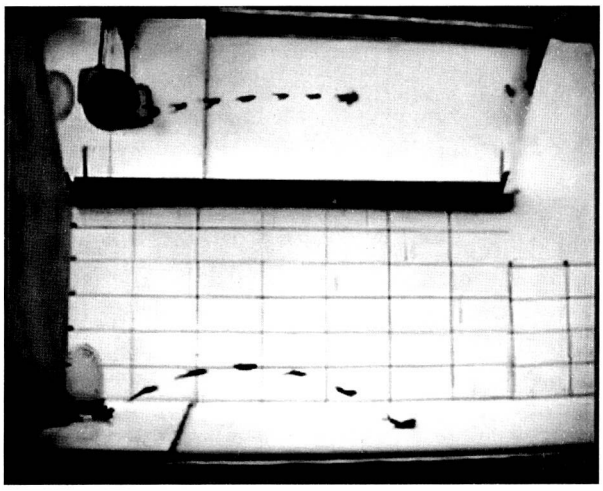

FIG. 2. Z-projection image showing the runway, backing grid and mirror (above) with stacked sequence of images of a jumping frog.

jump straight along the runway. Before locomotor assessment, each frog was placed in a Petri dish with a measured grid base, weighed to 0.01g using an electronic balance, and photographed from below using a digital camera, so that length measurements could be recorded later using the software Image J (Rasband, 2005). Frogs were placed on the glass circle at the start of the runway under a Petri dish base darkened all over except at one end, which was orientated towards the end of the runway, to encourage the frog to face in that direction. Frogs were kept there for 3-5 minutes to allow them time to settle, then the Petri dish base was removed. Frogs were encouraged to jump by moving a hand net above and behind the frog to induce an escape response; this was repeated three times within a short period for each frog. After assessment, transporting males were kept overnight in tanks and provided with a tub of rainwater, to allow them to deposit their tadpoles: their locomotor performance was re-assessed the next day. Each frog was 'jumped' three times to ensure that values were typical,

All jumps were filmed at 60 Hz using a Canon XL2 video camera. Films were edited using Windows Movie Maker and analysed using Image J. To try and ensure that for each assessment we were considering the maximal escape response, we analysed the initial, longest jump made by the frog. A single image was created from each video sequence of a jump by stacking the individual frames together using Image J (Fig. 2). The resultant projection image showed the position of the frog at 0.017 sec intervals throughout each jump, and allowed calculation of all parameters using a single image, again using Image J. Speed was calculated by dividing jump distance by time (calculated from the number of frames it took to complete and frame frequency). Statistical analyses were undertaken for all parameters using the mean values of three jumps for each frog, using SPSS v11.5 software. This sample size was chosen to minimise the stress to the transporting males and to reduce any conditioning effects; jumps were variable but similar within individuals (i.e. for distances, percentage standard errors typically represented 10-15% of the mean).

Males collected from north coast sites were (mean±SD) 21.94±1.53 mm SVL, and weighed an average of 1.27±0.16 g pre-deposition and 1.06±0.08 g post-deposition. Female frogs were, on average, 20.59±1.30 mm SVL and comparable to pre-deposition males in body mass at 1.23±0.23 g. Results for four jump parameters (take-off angle, length, maximum height and speed) for male and female frogs are shown in Table 1. Jump lengths, height and speed were lower in transporting males compared to the two other categories, but differences were not statistically significant in any case (One-way ANOVAs, $P > 0.05$).

A more detailed analysis of males alone, comparing the performance of individuals while transporting larvae and post-deposition, is shown in Table 2. Take-off angle decreased post-deposition in 8 out of 10 frogs, but differences were not statistically significant (Wilcoxon's matched pairs, T=11, NS, n=10). Maximum height increased in 5 out of 10 frogs post-deposition, though again differences were not significant (Wilcoxon's matched pairs: T=21, NS, n=10). Jump distance increased in 8 out of 10 frogs, and jump speed in 6 out of 10 frogs, but differences were not significant (Wilcoxon's matched pairs: T=13 and T=18 respectively, both NS, n=10). From visual inspection of the data, there is a suggestion that the post-deposition frogs might jump lower, faster and further. However, we did not find evidence of this tendency in our formal statistical test, although this may be due to the low power of the non-parametric test that was necessary. It might have been expected that the biggest differences would occur in frogs carrying the largest number of larvae, but Table 2 shows this not to be the case. Frog 10 carried the largest number in our sample (11) but

TABLE 1. Descriptive statistics for size and jump parameters. For each frog, the data point analysed was the mean value from three jumps.

Frog category	n	Jump parameter (mean±SD)			
		Take-off	Length	Maximum	Speed
		angle (°)	(cm)	height(cm)	($msec^{-1}$)
Females	8	32.81±3.18	32.22±10.79	7.78±6.21	2.83±0.60
Transporting males	10	32.80±4.48	31.32±13.68	7.48±3.86	2.72±0.79
Post-deposition males	10	30.73±5.65	36.86±12.52	8.37±4.10	2.95±0.42

TABLE 2. Morphometric and jump characteristics of males (L = males transporting larvae; P-D = post-deposition males).

Number	Mass (g)	SVL (mm)	No. Tadpoles	Tadpoles as % of frog mass	Jump parameters (each data point is the mean of three) Take-off angle (°)		Maximum height (cm)		Distance (cm)		Speed ($msec^{-1}$)	
					L	P-D	L	P-D	L	P-D	L	P-D
1	0.97	22.8	3	9.1	36.0	34.6	9.72	5.99	24.81	29.85	2.12	2.99
2	1.02	22.1	3	9.1	26.5	21.4	4.21	3.53	19.33	21.52	2.14	2.55
3	1.06	24.6	4	13.3	32.3	30.4	7.94	13.34	39.88	55.27	2.99	3.32
4	1.00	21.8	5	14.5	32.3	35.3	6.80	12.22	26.95	49.80	2.17	3.19
5	1.00	20.1	6	15.8	33.3	30.5	3.76	5.26	13.39	21.55	1.59	2.27
6	1.05	21.4	7	17.2	41.2	37.8	15.69	15.24	59.55	44.50	4.01	2.87
7	1.03	22.5	7	17.5	33.6	36.8	11.51	10.04	45.78	50.29	3.73	3.64
8	1.4	21.1	9	19.3	35.9	31.1	4.23	8.03	24.17	38.71	2.23	3.32
9	1.17	19.5	8	20.3	26.0	25.7	4.67	5.34	26.45	30.2	2.90	2.59
10	1.18	23.5	11	24.7	30.9	23.6	6.22	4.66	32.90	26.94	3.29	2.79

jumped lower, slower and for a shorter distance post-deposition.

Overall the results reported here support the earlier conclusion of Downie *et al.* (2005) that larval transportation has no significant effect on jumping performance in *M. trinitatis* males, at least using the parameters tested of jump distance, speed, height and take-off angle in an initial escape jump.

A possible caveat is that we have not tested locomotor endurance. However, we feel that this would be of limited biological relevance to predator avoidance. These frogs live in and alongside mountain streams lined by complex boulders with overhangs and crevices. In order to escape into hidden crevices, they would normally need to jump for less than 1 m, so the runway distances we used were realistic. Comparing the variable measured both in this study and by Downie *et al.* (2005), – jump length – the earlier report found distances in females, post-deposition males and transporting males consistently around 10 cm shorter than those in our study. Downie *et al.* (2005) calculated mean jump length by dividing the length of the runway by the number of jumps taken; the smaller values in this earlier study are likely to be due to the inclusion of sub-maximal jumps subsequent to the initial escape jump. As our study found that initial escape jumps are not different between the three groups, and Downie *et al.* (2005) found no differences in the total number of jumps taken to traverse the runway this may suggest there is little effect of tadpole transport on submaximal jumps, especially over short distances. It may be that costs to endurance would be observed over longer distances. Frogs travel as far as 20 m from streams to deposit tadpoles (Jowers & Downie, 2005). It is likely that frogs in our study were sampled at different times within the transport period, which may explain some inter-individual differences, but since there is no way of knowing how long they had been carrying tadpoles prior to assessment, it is not possible to draw any conclusions about this.

As in the study by Downie *et al.* (2005), the number of tadpoles carried by male frogs was very variable (present study, 3-11; previous study, 3-10) but there was no obvious relationship between jump parameters and tadpole number or frog size (SVL or mass). It may be that restricting observations to frogs with the same number of tadpoles could reduce variability in the data, but obtaining such a sample would be very time-consuming, and inspection of the data in Table 2 does not actually suggest such an interpretation. To some extent this may be due to the combination of a narrow range of values in tadpole number and a high variability in individual jump performance in a small sample, but as normal numbers of tadpoles range from 2-12 (Wells, 1980), a much wider range would be difficult to achieve for this species.

In a study somewhat comparable to ours, where weight changes within normal biological parameters occurred, Buchanan & Taylor (1996) found that emptying the bladder (13.9% of body mass) allowed squirrel tree frogs to jump 18.5% further. Our study found no similar effect in unloaded frogs. There are biomechanical factors such as size, muscle mass, tendon elasticity, leg length and joint morphology that all have effects on jump performance in frogs (Choi *et al.* 2000; Wilson *et al.*, 2000; Marsh & John-Alder, 1993; Emerson, 1978). Small changes in any of these in male frogs transporting tadpoles may well be sufficient to compensate for a load of 30% of the body mass.

Although it seems likely that the costs of larval transport in stream frogs do not include effects on locomotor performance in escape jumps, limitations to the duration of larval transport may still be due to higher predation risk for transporting males. Egg-carrying bugs and spiders are more visible and are consequently significantly more susceptible to predation than non-carrying conspecifics (Li & Jackson, 2003; Kaitala *et al.* 2000). Although tadpole-carrying frogs are generally fairly cryptic, increased visibility to predators might be incurred behaviourally; for example, if they

spend proportionally more time in exposed areas in stream or river beds. Once threatened though, male stream frogs can jump comparable distances and speeds whether loaded or unloaded, which is crucial, since their fitness is highly dependent on their ability to protect their tadpoles.

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THE HERPETOLOGICAL JOURNAL

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(revised January 2004)

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4. For all papers the title page should contain only the following: title of paper; name(s) of the author(s); address of the Institution where the work was done; a running title of five words or less, and the name and address of the corresponding author with (if available) an email address. The text of the paper should begin on page 2 and be produced in the following order: Abstract, Keywords, Text, Acknowledgements, References, Appendices. Full papers and reviews should have the main text divided into sections. The first subhead will be centred in capitals, the second shouldered in lower case, and the third run on in italics. Footnotes are not permitted. *Short Notes* (generally less than six manuscript pages and accompanied by a single data set) should be produced as continuous text, preceded by an abstract of no more than 100 words. A *sans serif* font (e.g. Universe or Helvetica) is preferred.

5. The usual rules of zoological nomenclature apply.

6. Tables are numbered in arabic numerals, e.g. TABLE 1; they should be typed double spaced on separate sheets with a title/short explanatory paragraph above the table. Horizontal and vertical lines should be avoided.

7. Line drawings and photographs are numbered in sequence in arabic numerals, e.g. FIG. 1. Colour photographs can only be included at cost to the author (quotes can be obtained from the Managing Editor). If an illustration has more than one part, each should be identified as (a), (b), etc. The orientation and name of the first author should be indicated on the back. They should be supplied camera-ready for uniform reduction of one-half on A4 size paper. Line drawings should be drawn and fully labelled in Indian ink, dry-print lettering or laser printed. Illustrations produced using other types of computer printer are not usually of suitable quality. A metric scale must be inserted in micrographs etc. Legends for illustrations should be typed on a separate sheet.

8. References in the text should be given as in the following examples: "Smith (1964) stated —"; "—as observed by Smith & Jones (1963)." "—as previously observed (Smith, 1963; Jones, 1964; Smith & Jones, 1965)". For three or more authors, the first author's surname followed by *et al.* should be used (Smith *et al.*, 1972). In the list of references the full title of the journal should be given. Articles 'submitted' or 'in prep' may not be cited in the text or reference list. The following examples will serve to illustrate the style and presentation used by the Journal:

Bellairs, A. d'A. (1957). *Reptiles*. London: Hutchinson.

Boycott, B. B. & Robins, M. W. (1961). The care of young red-eared terrapins (*Pseudemys scripta elegans*) in the laboratory. *British Journal of Herpetology* **2**, 206–210.

Dunson, W. A. (1969*a*). Reptilian salt glands. In *Exocrine glands*, 83–101. Botelho, S. Y., Brooks, F. P. and Shelley, W. B. (Eds). Philadelphia: University of Pennsylvania Press.

Dunson, W. A. (1969*b*). Electrolyte excretion by the salt gland of the Galapagos marine iguana. *American Journal of Physiology* **216**, 995–1002.

9. Final acceptance of a paper will depend upon the production by the author of a typescript, illustrations and computer file(s) ready for the press.

10. Proofs are usually prepared as PDF files and corrections should be returned to the Managing Editor by return of post or email. Alterations should be kept to the correction of errors; more extensive alterations will be charged to the author.

11. A PDF file of the paper will be provided free of charge to the corresponding author on publication.

12. All submissions are liable to assessment by the editorial board for ethical considerations, and publication may be refused on the recommendation of this committee. Contributors may therefore need to justify killing or the use of other animal procedures, if these have been involved in the execution of the work. Likewise, work that has involved the collection of endangered species or disturbance to their habitat(s) will require full justification.

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