Volume 14, Number 1

January 2004 ISSN 0268-0130

THE HERPETOLOGICAL JOURNAL



Published by the BRITISH HERPETOLOGICAL SOCIETY Indexed in Current Contents The Herpetological Journal is published quarterly by the British Herpetological Society and is issued free to members. Articles are listed in Current Awareness in Biological Sciences, Current Contents, Science Citation Index and Zoological Record.

Applications to purchase copies and/or for details of membership should be made to the Hon. Secretary, British Herpetological Society, The Zoological Society of London, Regent's Park, London NW1 4RY, UK.

Instructions to authors are printed inside the back cover. All contributions should be addressed to the Scientific Editor (address below).

Scientific Editor: Wolfgang Wüster, School of Biological Sciences, University of Wales, Bangor, Gwynedd, LL57 2UW, UK. E-mail: W.Wuster@bangor.ac.uk

Associate Scientific Editors: J. W. Arntzen (Leiden/Oporto), R. Brown (Liverpool)

Managing Editor: Richard A. Griffiths, The Durrell Institute of Conservation and Ecology, University of Kent, Canterbury, Kent CT2 7NS, UK. E-mail: R.A.Griffiths@kent.ac.uk

Associate Managing Editors: L. Gillett, M. Dos Santos, J. McKay

Editorial Board: Donald Broadley (Zimbabwe) John Cooper (Uganda) John Davenport (Cork) Andrew Gardner (Abu Dhabi) Tim Halliday (Milton Keynes) Michael Klemens (New York) Colin McCarthy (London) Andrew Milner (London) Richard Tinsley (Bristol)



BRITISH HERPETOLOGICAL SOCIETY

Copyright

It is a fundamental condition that submitted manuscripts have not been published and will not be simultaneously submitted or published elsewhere. By submitting a manuscript, the authors agree that the copyright for their article is transferred to the publisher if and when the article is accepted for publication. The copyright covers the exclusive rights to reproduce and distribute the article, including reprints and photographic reproductions. Permission for any such activities must be sought in advance from the Editor.

ADVERTISEMENTS

The Herpetological Journal accepts advertisements subject to approval of contents by the Managing Editor, to whom enquiries should be addressed.

Herpetological Journal website: http://biology.bangor.ac.uk/~bss166/HJ

FRONT COVER: Male Trimeresurus kanburiensis (top) and female T. venustus (bottom). A. Malhotra

BIAS IN ESTIMATION OF NEWT POPULATION SIZE: A FIELD STUDY AT FIVE PONDS USING DRIFT FENCES, PITFALLS AND FUNNEL TRAPS

KLAUS WEDDELING, MONIKA HACHTEL, ULRICH SANDER AND DAVID TARKHNISHVILI

Zoologisches Forschungsinstitut und Museum Alexander Koenig, Sektion Herpetologie, Adenauerallee 160, D-53113 Bonn, Germany

Drift fences are frequently used to sample amphibians for population studies. Thus, some researchers do not mark animals, but use capture rates at the drift fence as an indicator of population size. Other workers use mark-recapture techniques to estimate population sizes. These approaches require different amounts of effort and lead to different results. Our study compares several estimates of population size for alpine newts (*Triturus alpestris*) and smooth newts (*Triturus vulgaris*) in five breeding ponds surrounded by permanent drift fences and pitfall traps. The estimates based on mark-recapture techniques (Petersen method) do not vary substantially between the two modes of recapture applied (funnel traps, and drift fences with pitfall traps). These estimates give even better results than simple counts if a substantial part of the newt populations remain within the drift fences throughout the year. While unrecognized trespass by newts appears to be a rare event, some newts may leave a pond for a short time even during the breeding season. This is an important source of bias for population estimates in studies based on counts at drift fences when animals are not marked.

Key words: capture methods, mark-recapture, Triturus alpestris, Triturus vulgaris

INTRODUCTION

A common approach for estimating population sizes of newts at the breeding pond involves the use of markrecapture methods. This approach has been regarded as the most precise among indirect methods (Caughley, 1977). The Petersen method (Petersen 1896) or its modifications (e.g. Bailey, 1952) are most often used for newts, as these methods require group marking – rather than individual marking - of animals (Blab & Blab, 1981; Glandt, 1978; Arntzen & Teunis, 1993; Donnelly & Guyer, 1994; Wenzel et al., 1995; Diaz-Paniagua, 1998). One obvious problem associated with group marking in conjunction with the Petersen method is that it only allows the population size to be estimated at one point in time (Caughley, 1977). Some newts leave a pond before breeding is over, and the number of animals found in the pond - even during the peak of reproductive activity may represent only a part of the reproductive population (Schoorl & Zuiderwijk, 1981; Tarkhnishvili, 1986). Another difficulty is that the Petersen method requires a high proportion of recaptures to attain acceptable estimate errors (Caughley, 1977). Estimates based on marking with individual codes (Seber, 1973) are sometimes more accurate. However, individual marking of a few hundred animals often needs amputation of several toes and one or two fingers, especially in studies where populations in several neighbouring ponds are analysed and movement between ponds is of interest. Due to the rapid regeneration of toes in newts (Henle et al., 1997), a complete amputation at the base of a toe is necessary in order to recognize marked animals for a few months after marking. Complete amputation of more than two fingers may potentially affect survival or reproductive functions of newts and, in our opinion, should be done only if individual tracking of animals is essential.

Other methods such as direct counts of newts at night (Cooke, 1995), or even observations of the number of animals surfacing for air (Andreas, 1982) can provide estimates of relative abundance, but their use is rather limited by the type and size of the breeding pond and species-specific habitat preferences (Wenzel *et al.*, 1995).

Since the 1960s, drift fences have been regularly used in population studies of pond-breeding amphibians (Shoop, 1965; Gibbons & Bennet, 1974; Gill, 1978; Verrell & Halliday, 1985; Dodd, 1991; Dodd & Scott, 1994. Kogoj, 1997; Kneitz, 1998; Baker, 1999). Because it is often assumed that drift fences with pitfall traps will catch all individuals entering a pond, some researchers make direct counts of individuals rather than estimating population size. One problem concerning this approach is that breeding animals may stay in or at the pond throughout the year (Gibbons & Bennett, 1974; Baker, 1999). Trespass is also a problem when animals cross the fence (Dodd, 1991; Verrell & Halliday, 1985; Jahn & Jahn, 1997). It was shown that the number of newts (Notophthalmus, Triturus) caught in pitfall traps may represent as little as 15% of the population, with mean values varying between 50 and 70% (Dodd, 1991; Baker, 1999), while in other studies up to 95% of the population could be captured (Gill, 1978). These studies show that this type of census represents a sample that has an unknown estimation error. Additionally, most drift fence

Correspondence: D. Tarkhnishvili, Department of Ecology, Tbilisi State University, @128, 1 Chavachavadze Ave., Tbilisi, Georgia. E-mail: t@gol.ge



FIG. 1. Study area with position of study ponds

studies were based on a single pond, and it is likely that the type of pond and surrounding habitat, along with fence construction and distance between fence and water may affect the quality of population size estimates.

The problem that some newts may not leave the water or the shoreline at all, can be compensated for by marking all animals entering the pond. Then, the proportion of unmarked newts caught in the water by dip-netting or funnel trapping represents how many individuals were already at the pond before pitfall trapping commenced (Verreil & Halliday, 1985; Baker, 1999). Unfortunately, some field researchers omit this procedure (e.g. Kogoj, 1997; Kneitz, 1998). Sometimes newts temporarily leave the pond during the breeding season. As a result, the same individual may fall in a pitfall trap more than once. Marking of newts that appear into pitfall traps helps to avoid the risk that the same specimen is counted several times.

Our study was designed to estimate reproductive population numbers of smooth newts (*Triturus vul*garis) and alpine newts (*T. alpestris*), in five ponds near Bonn, Germany, using standard methods, i.e. toe-clipping, drift fences with pitfalls, and funnel trapping. The main objective of this paper is to evaluate the degree of bias associated with these methods. We did this by comparing several different methods frequently applied in field surveys of pond-breeding amphibians.

MATERIAL AND METHODS

STUDY PONDS

The five study ponds are situated in an agricultural landscape about 15 km south of Bonn, Northrhine-Westfalia, Germany, on the western side of the river Rhine. The ponds are located 275-1800 m from each other (Fig. 1). Ponds 3 and 5 are natural, whereas ponds 1, 2 and 4 were created artificially in 1988. Ponds 1 and 2 lie at the margin of a mixed forest. Ponds 3, 4 and 5 are surrounded by cereal fields and grassland; their distance from forest ranges from 150 to 700 m. Pond 3 is considerably larger than the other four ponds (Table 1). Pond 5 is ephemeral and regularly dries up for 1-1.5 months during the summer. Ponds 3 and 4 dried out occasionally in late summer for shorter time periods, but did not do so during the reported study. All ponds are surrounded by willow (Salix spp.), bramble shrubbery (Rubus fruticosus), reedmace (Typha latifolia) and sedges (Carex spp.). The waterbodies are partly covered by duckweed (Lemna, Spirodela) and broad-leaved pondweed (Potamogeton natans).

In addition to alpine and smooth newts, the common toad (*Bufo bufo*), agile frog (*Rana dalmatina*), common frog (*R. temporaria*) and green frogs (*R. kl. esculenta* complex) reproduce in the ponds. In ponds 2 and 3, great crested newts (*Triturus cristatus*) are also present.

TABLE 1. Characteristics of study ponds during the study year. SM, maximum surface area (m^2) ; DM, maximum depth (m); SF, size of terrestrial fringe inside the fence (m); ** dry up occasionally in late summer.

Pond	SM	DM	origin	status	setting	SF
1	50	1.2	artificial	permanent	forest/arable	0.5-1.5
2	45	1.5	artificial	permanent	forest/arable	1.5-2.0
3	400	1.7	natural	permanent**	arable	0-4.5
4	45	1.0	artificial	permanent**	arable	2.0-3.0
5	150	1.6	natural	temporary	meadow/arable	0-3.0

Small-bodied newts start the breeding migration in February and leave the ponds before the end of June.

DRIFT FENCES, FUNNEL TRAPS AND TOE-CLIPPING

From June 2000 to December 2001, all study ponds were surrounded by permanent drift fences made from a dense, green, non-transparent polyethylene or metallic fabric. The fence (height 45 cm) was embedded 5-10 cm deep into the soil. A U-shape profile at the top prevented newts from climbing over the fence. The distance between the pond margin and the fence ranged from 0 to 4.5 m depending on pond topography and changes in water level throughout the year (Table 1, following Schäfer, 1993). Paired pitfall traps on opposite sides of the fence consisted of plastic buckets (depth 46 cm, volume 23 l) set at intervals of 3-8 m. The rims of the buckets with their U-shape profile reduced the number of newts leaving the pitfalls. The bottoms of the buckets were covered with water to prevent desiccation. Pitfall traps both at the outer and the inner side of a fence were checked daily from the end of January until the end of November 2001. Unmarked individuals caught in pitfall traps were marked by toe-clipping (pond-specific marking) and released on the opposite side of the drift fence. Funnel trapping was used for two weeks between 28 April and 13 May (peak of breeding activity). The funnel traps were made of green coarse plastic fabric with a mesh size of approx. 2 mm. The size of the box-shaped traps was about $40 \times 40 \times 80$ cm and the top was kept above the water level so that captured newts could breath. Newts entered the traps through two funnelshaped entrances with a minimum aperture diameter of 5 cm. A maximum of eight funnel traps was used simultaneously in one pond. Every day during a two-week period the traps were checked, and marked and nonmarked newts were counted and released. Non-marked animals were released again unmarked.

DATA ANALYSIS

We used four different approaches to interpret our data and compared the resulting estimates of population size. The first 'naive' approach assumed that any newt would be caught on a single occasion by a pitfall trap on the way to the pond during immigration and on a single occasion - if it survives - when it leaves a breeding site. According to this approach, the population size is equal to the total number of newts that fall into the outer pitfall traps, irrespective of marking. These results equal those one would obtain without marking animals. The second approach considered that the same animal could fall in to an outer pitfall trap several times. In this case the population size equals the cumulative number of unmarked newts in outer pitfall traps. The third approach is based on mark-recapture techniques and took into consideration the proportion of marked newts among those emigrating from the pond and caught in inner pitfall traps. Using also the total number of animals marked

when entering the pond, one can calculate the total number of newts in the pond using the Petersen method. The fourth approach estimates the proportion of marked newts among those caught in funnel traps in water. The third and the fourth approaches assume that trap catches accurately reflect the proportion of both the number of marked and unmarked newts in the pond.

We used the Petersen method (Bailey, 1952; Caughley, 1977) for estimation of population size with mark-recapture techniques: N = M(n + 1)/(m + 1), where N is the population size; M the number of marked animals; n the number of newts caught during the second trapping session (either in pitfall traps when newts left the pond or in funnel traps); and *m* the number of recaptured newts. Standard errors (SE) and confidence intervals were calculated as recommended by Caughley (1977). It is important to note that recruitment or immigration between two capture sessions leads to an overestimation of the population size, but mortality and emigration do not bias the estimate (Caughley, 1977). Therefore, mortality of newts during the breeding season should not be a problem. In order to meet assumptions of the index, we defined the period of "second capture session" after immigration to the pond was completely - or almost completely - over.

Baker (1999) stressed that the use of different capture techniques between the first and second capture sessions may potentially bias the population estimates. Use of pitfall trap recaptures (instead of funnel trapping) may help to avoid this source of error. On the other hand, there is a risk that newts that were inside the fences before the reproductive season started might show a preference to remain there also after completion of the breeding period. In this respect, recapture in funnel traps may provide a better estimate.

Significance of differences in 'temporary' terrestrial activity between sexes was tested with 2 x 2 contingency tables (χ^2 test; Sokal & Rohlf, 1995).

RESULTS

POPULATION ESTIMATE BASED ON PITFALL TRAPPING

Due to terrestrial activity, the total number of animals in pitfall traps on the outer side of the fence ("first approach") was always much higher than the results obtained by counting only marked individuals ("second approach"). In different ponds, 10-529 individuals of T. vulgaris and 43-2249 individuals of T. alpestris were caught on the outer side of drift fences more than once (compare N and N, in Table 2). The bias was highest in small artificial ponds 1, 2 and 4 and higher in T. alpestris than in T. vulgaris. Marked differences between sexes were recorded in 'temporary' terrestrial activity: analysis in 2 x 2 contingency tables showed significantly higher activity of female T vulgaris in small ponds which provide little shelter inside the fence (Table 3). In T. alpestris, only at pond 3 did terrestrial activity significantly differ between sexes.

TABLE 2. Number of newts estimated from captures in pitfall traps. N, total number of captures on the outer side of a drift fence;
N, total number of unmarked newts caught on the outer side of a drift fence; M, number of newts marked prior to the second capture
session (2 June for T. vulgaris and 15 May for T. alpestris); n, number of newts captured on the inner side of the drift fence during
the second session, with the number of recaptures (m) ; N_2 , population estimate (Petersen method) calculated from recapture rates
in inner pitfall traps, with the standard error (SE).

	Pond	1	2	3	4	5
T. alpestris,	N	2041	1021	622	158	62
males	N_1	949	600	575	89	49
	M	942	599	571	88	42
	n (m)	76(72)	20(16)	208(184)	40(37)	26(15)
	$N_2(SE)$	994 (26)	740(76)	645 (16)	95(4)	71 (11)
T. alpestris,	Ν	2095	1018	696	358	110
females	Ν,	938	584	614	185	80
	M	932	579	606	184	75
	n (m)	113 (106)	27 (22)	221 (186)	97(87)	35 (21)
	$N_2(SE)$	993 (24)	705 (61)	719 (21)	205 (7)	123 (16)
T. vulgaris,	Ν	332	558	1704	518	47
males	N_1	254	478	1640	332	43
	М	254	478	1627	326	41
	n (m)	25 (16)	24(19)	477 (381)	62 (51)	13 (7)
	$N_2(SE)$	388 (54)	598 (58)	2031 (46)	395 (23)	72(16)
T. vulgaris,	N	366	697	2555	794	75
females	Ν,	241	530	2442	451	69
	M	241	528	2423	443	63
	n (m)	46 (38)	23 (21)	772(683)	81 (73)	19(14)
	$N_2(SE)$	290(19)	576 (35)	2738 (36)	491 (18)	84 (11)

The number estimated by the Petersen method, taking into consideration the proportion of unmarked newts among those leaving the pond ("third approach"), was always higher than the total number of newts marked at the fence. The standard error of an estimate was usually (except for pond 5) lower than 10% of the population size. The number of unmarked newts among those migrating from a breeding site reached 5-40% (usually 10-20%) in different ponds. In particular, pond 5 showed high proportions of unmarked individuals. There were unmarked newts leaving the pond during the breeding season (before mid-May). Their number varied from just a few individuals to up to 90 newts per species and sex. For *T. alpestris*, this number was especially high in ponds 1 and 2 (153-159 specimens of each sex); for *T. vulgaris* in ponds 2 and 3 there were 98-112 specimens, respectively (not shown in Table 2).

Among newts entering a pond, there were many returning individuals that had been previously marked and released, especially in small ponds 1, 2 and 4 (difference between N and N, in Table 2).

RECAPTURING BY FUNNEL TRAPS

The number estimated by the Petersen method, if funnel trapping was applied during the second capture session, showed figures similar to those obtained via recapturing by pitfall traps, with comparable values of standard error (Table 4). Differences between these two estimates were never significant: 95% confidence limits of both estimates overlapped for each individual pond.

TABLE 3. Intersexual differences in 'temporary' terrestrial activity: results from several 2 x 2 contingency tables testing differences between sexes in proportions of $(N-N_1)$ and N_2 from Table 2. (M=male, F= Female)

Pond	1	2	3	4	5
T. alpestris	$M \sim F$	M ~F	M < F	M ~ F	M~F
	$\chi^2 = 0.9123$	χ''=0.8350	χ"=5.5827	χ"=0.6315	χ"=0.6232
	P > 0.05	P>0.05	P<0.05	P>0.05	<i>P</i> >0.05
T. vulgaris	M < F	M < F	M ~F	M < F	M~F
	χ''=22.1689	χ"=28.1416	χ''=2.8894	χ"=12.1496	χ"=0.1433
	<i>P</i> <0.01	<i>P</i> <0.01	<i>P</i> >0.05	<i>P</i> <0.01	<i>P</i> >0.05

TABLE 4. Number of newts marked at drift fences and proportion of non-marked individuals in funnel traps. M, number of newts marked at the fences prior to funnel trapping (28 April); n, number of newts captured in funnel traps, with the number of recaptures (m); N, population estimate (Petersen method) calculated from recapture rates in funnel traps, with the standard error (SE).

	Pond	1	2	3	4	5	
T. alpestris,	М	914	591	568	88	39	
males	n (m)	88 (84)	191 (176)	59 (53)	74 (73)	88 (39)	
	$N_3(SE)$	957 (22)	641(13)	631 (27)	89(1)	87 (10)	
T. alpestris,	М	906	567	579	168	72	
females	n (m)	110(108)	87(84)	99(88)	130(129)	132 (98)	
	$N_3(SE)$	923 (12)	587(12)	651(23)	169(1)	97(5)	
T. vulgaris,	М	248	469	1615	313	39	
males	n (m)	44 (35)	70(62)	207 (165)	47(47)	60(31)	
	$N_3(SE)$	310(23)	529(22)	2023 (70)	313 (0)	74 (9)	
T. vulgaris,	М	232	495	2383	407	53	
females	n (m)	15(14)	18(15)	151 (135)	54 (53)	40 (22)	
	$N_{3}(SE)$	247 (15)	588 (57)	2663 (74)	415(8)	94 (13)	

The proportion of unmarked newts in funnel traps was thus comparable with the proportion of unmarked animals at drift fences during migration from the pond.

DISCUSSION

The fact that part of the reproductive population remains at the breeding site throughout the year can bias the estimate of population size when using only drift fences (see introduction). However, it is not clear how to separate this factor from trespass, another potential source of error. Dodd (1991) assumed that newts crawled under the fence using holes produced by plant roots, but he did not provide conclusive evidence of trespass: moreover, his experiments showed that at laboratory newts at least could not climb over or under the fence. Verrell & Halliday (1985) assumed climbing to be a potential source of error and refrained for this reason from estimating the population size of smooth newts. Data presented here demonstrate that the proportion of newts that were not marked at drift fences is high in areas with plenty of terrestrial refugia within the fence (Pond 5), moderate at the large deep site (Pond 3), but quite low at small ponds 1 and 4 with small distances between fence and water edge. Moreover, the proportion of non-marked alpine newts was less than that of smooth newts. As our fence construction was standardized, the effectiveness of a fence depends on the species-specific migration activity and the likelihood of a newt staying in its immediate surroundings. Trespass itself appears to be a relatively unimportant source of bias, estimated in the range of 0.7-3.4%. This is the lowest proportion of unmarked newts in small ponds where there is an absence of refugia between the water line and the fence (e.g. females of *T. alpestris* in small ponds).

It appears that the presence of newts at a pond before migration starts does not strongly bias population estimates by mark-recapture, even if recaptures are done by drift fences. The majority of newts which remain in water throughout winter, however, leave the pond area after the breeding season. This is supported by a good correspondence between estimates based on recapturing in water and in terrestrial habitats.

This correspondence between estimates obtained from funnel trap and pitfall trap recaptures shows that the time between marking and recapture (and, consequently, mortality between two capture sessions) does not significantly bias the estimate. In fact, mode and time of recapture can be planned dependently on the activity period and peculiarities of an individual pond.

Although the majority of authors (Gibbons & Bennett, 1974; Verrell & Halliday, 1985; Dodd, 1991; Baker, 1999) combined drift fence methods with group marking of migrating newts, no results were reported about terrestrial movements during the breeding season. Our data suggest that such movement may be considerable for the populations we studied. During rainy days, some newts (occasionally almost the entire population, as T. alpestris in pond 1) leave a pond for one or several days, travel a short distance, but then return to a pond before the end of the reproductive season. In alpine newts this behaviour is more common than in smooth newts, although intersexual differences occur mainly in smooth newts, where females show significantly higher terrestrial activity. Terrestrial movements are less often recorded at large ponds, but this may be due to more options for moving unnoticed within the fence than at small ponds.

Some publications describe the population size of newts only on the basis of data obtained from drift fences in combination with pitfall traps, without marking animals or taking into consideration the proportion of marked animals at the breeding sites, e.g. Blab & Blab, 1981; Schäfer, 1993; Kogoj, 1997; Kneitz, 1998. We assume that such estimates are strongly biased because part of the population is unaccounted-for. In addition to this, they can give a strong overestimation of population size due to individuals entering the pond more than once during a breeding season (e.g. Kogoj, 1997). Forcing animals to stay inside the fence by keeping them always on the inner side is no solution to the problem because it strongly influences reproductive behaviour of newts and biases observations of movements between ponds and terrestrial habitat.

ACKNOWLEDGEMENTS

We thank Gregor Bosbach, Ruth Rottscheidt, Peter Schmidt and Meike Thomas for helping us during the field work. Stefanie Specht drew the map of the study area. We thank Wolfgang Böhme for leading the project 'Development of amphibian habitats in an agricultural landscape' and Bradley Sinclair for helpful comments on translation problems. Permissions for catching and marking amphibians were granted by the local administration for nature conservation (Untere Landschaftsbehörde, Rhein-Sieg-Kreis, NRW). This project is financially supported as a 'testing and development project' by the Federal Agency for Nature Conservation (BfN) and the Federal Environment Ministry (BMU).

REFERENCES

- Andreas, A. (1982). Eine Methode der quantitativen Bestandsaufnahme von Molch-Populationen in Gewässern. Abh. Ber. Naturk. Museum Mauritanium Altenburg, 1982, 93-97.
- Arntzen, J. W. & Teunis, S. F. M. (1993). A six year study on the population dynamics of the crested newt (*Triturus cristatus*) following the colonization of a newly created pond. *Herpetological Journal* 3, 99-110.
- Bailey, N. T. J. (1952). Improvements in the interpretation of recapture data. *Journal of Animal Ecology* 21, 120-127.
- Baker, J. M. R. (1999). Abundance and survival rates of great crested newt (*Triturus cristatus*) at a pond in central England: monitoring individuals. *Herpetological Journal* 9, 1-8.
- Blab, J. & Blab, L. (1981). Quantitative Analysen zur Phänologie, Erfassbarkeit und Populationsdynamik von Molchbeständen des Kottenforstes bei Bonn. Salamandra 1981, 147-172.
- Caughley, G. (1977). *Analysis of vertebrate populations*. Wiley: Interscience Publication.
- Cooke, A. S. (1995). A comparison of survey methods for crested newts (*Triturus cristatus*) and night counts at a secure site, 1983-1993. *Herpetological Journal* 5, 221-228.
- Diaz-Paniagua, C. (1998). Reproductive dynamics of a population of small marbled newt (*Triturus* marmoratus pygmaeus) in south-western Spain. Herpetological Journal 8, 93-98.
- Dodd, C. K. (1991). Drift fence-associated sampling bias of amphibians at Florida Sandhills temporary pond. *Journal of Herpetology* **25**, 296-301.

- Dodd, C. K. & Scott, D. E. (1994). Drift fences encircling breeding sites. In: Measuring and monitoring biological diversity: standard methods for amphibians.
 W. R. Heyer, M. A. Donnelly, R. W. McDiarmid, L. C. Hayek and M. S. Foster (Eds). Washington and London: Smithsonian Institution Press.
- Donnelly, M. A. & Guyer, C. (1994). Estimating population size: mark-recapture. In: *Measuring and monitoring biological diversity: standard methods for amphibians*. W. R. Heyer, M. A. Donnelly, R. W. McDiarmid, L. C. Hayek and M. S. Foster (Eds). Washington and London: Smithsonian Institution Press.
- Gibbons, J. W. & Bennett, H. D. (1974). Determination of anuran terrestrial activity patterns by a drift fence method. *Copeia* **1974**, 237-243.
- Gill, D. E. (1978). Effective population size and interdemic migration rates in a metapopulation of the red-spotted newt, *Notophthalmus viridescens* (Rafinesque). *Evolution* 32, 839-849.
- Glandt, D. (1978). Notizen zur Populationsökologie einheimischer Molche (Gattung *Triturus*). *Salamandra* **14**, 9-28.
- Henle, K., Kuhn, J., Podloucky, R., Schmidt-Loske, K. & Bender, C. (1997). Individualerkennung und Markierung mitteleuropäischer Amphibien und Reptilien: Übersicht und Bewertung der Methoden; Empfehlungen aus Natur- und Tierschutzsicht. Mertensiella 7, 133-184.
- Jahn, P. & Jahn, K. (1997). Vergleich quantitativer und halbquantitativer Erfassungsmethoden bei verschiedenen Amphibienarten im Laichgewässer, Mertensiella 7, 61-69.
- Kneitz, S. (1998). Untersuchungen zur Populationsdynamik und zum Ausbreitungsverhalten von Amphibien in der Agrarlandschaft. Laurenti-Verlag (Bochum).
- Kogoj, E. (1997). Populationsdynamik von Amphibien an einem Sekundärgewässer der Wiener Donauinsel (Österreich): Ein Vergleich von zwölf Taxa und neun Untersuchungsjahren (1986-1987, 1989-1995). In: 'Hödl, W., Jehle, R. and Gollmann, G. (Eds.), Populationsbiologie von Amphibien. Eine Langzeit-Studie auf der Wiener Donauinsel. Stapfia (Wien).
- Petersen, C. G. J. (1896). The yearly immigration of young plaice into Limfjord from the German sea. *Report of the Danish biological station* **6**, 1-48.
- Schäfer, H. J. (1993). Ausbreitung und Entwicklung von Amphibien-Populationen in der Agrarlandschaft. Ph. D. thesis, Univ. Bonn, 294 p.
- Schoorl, J. & Zuiderwijk, A. (1981). Ecological isolation in *Triturus cristatus* and *Triturus marmoratus* (Amphibia: Salamandridae). *Amphibia-Reptilia* 1981, 235-252.
- Seber, G. A. F. (1973). *The estimation of animal abundance and related parameters*. Griffin: London.
- Shoop, C. R. (1965). Orientation of Ambystoma maculatum: movements to and from breeding ponds. Science 149, 558-559.

- Sokal, R.R. & Rohlf, F. J. (1995). *Biometry (3rd ed.)*. W. H. Freeman and Company, New York.
- Tarkhnishvili, D. N. (1986). Analysis and conservation perspective of the banded newt from surroundings of Tbilisi. - Bulletin of the Academy of Sciences of the Georgian SSR 12, 145-147 (in Russian).
- Verrell, P. & Halliday, T. (1985). Reproductive dynamics of a population of smooth newt, *Triturus vulgaris*, in southern England. *Herpetologica* **41**, 386-395.
- Wenzel, S., Jagla, W. & Henle, K. (1995). Abundanzdynamik und Laichplatzreue von Triturus cristatus und Triturus vulgaris in zwei Kleingewässern einer Auskiesung bei St. Augustin (Nordrhein-Westfalen). Salamandra 31, 209-230.

Accepted: 27.1.03

ANNUAL CYCLE OF NUTRITIONAL ORGAN MASS IN A TEMPERATE-ZONE ANURAN, *RANA CHENSINENSIS*, FROM NORTHERN CHINA

Xin Lu

Department of Zoology, College of Life Sciences, Wuhan University, Wuhan, China

Body reserves of temperate anurans go through an annual cycle in response to highly seasonal environments. Here I describe how changes in relative mass of storage organs of *Rana chensinensis* occurring in northern China contributed to this cycle. Body reserves of both sexes dropped to their lowest levels after hibernation, and experienced a resting period of five months, then the reserves started increasing and attained peaks shortly before hibernating. During hibernation, the frogs' ovaries kept growing and liver and fat bodies declined accordingly. Based on comparable data in other studies, two distinct models of ovarian development in temperate anurans, hibernation-growth and non-hibernation-growth, are suggested. I also show a decreased relative ovarian mass with increased climate harshness.

Key words: body reserves, ecological energetics, environmental physiology, Ranidae

INTRODUCTION

Temporal organization of metabolic activities is a crucial life history trait in temperate-zone organisms. For anurans, liver, fat bodies and gonads (especially of females) are major organs of energy storage, and their relative mass provides a convenient indication of individual nutritional status (Pasanen & Koskela, 1974; Jørgensen *et al.*, 1979; Morton, 1981; Elmberg, 1991; Das, 1996; Tsiora & Kyriakopoulou-Sklavounou, 2001). In response to highly seasonal environments, northern frogs must go through a pronounced annual cycle in the masses of these organs. The pattern of such seasonal changes is a result of species' adaptation to local ecological conditions, and thus shows interspecific and inter-population variation (Pasanen & Koskela, 1974; Jørgensen *et al.*, 1979).

Rana chensinensis was once considered a subspecies of *R. temporaria* (Pope & Boring, 1940), and more recently an independent species endemic to China (Xie *et al.*, 2000). The frogs occur commonly in mountain rivers across northern China (Liu & Hu, 1961). In the present paper, I investigate annual dynamics of storage organ mass of the frogs in a typically seasonal environment, aimed at assessing nutritional strategies of this species in comparison with other temperate-zone anurans.

MATERIALS AND METHODS

Frogs were collected between March 1993 and February 1994, from Jie-xiu (37°04'N, 112°03'E) in Shanxi province, northern China. Climate of the surveyed area is typically continental (Fig. 1a). The frogs lived in two small streams surrounded by loess ravine with shrub and farmland. The spawning period was between mid-February and late March, and the hibernation period extended from early November to mid-February (Fig.

1a). I caught the frogs by hand in streams, ponds or springs (hibernation sites, see Lu, 2001) around the middle of each month. The specimens were brought to the laboratory immediately and were humanely killed. For each frog, after measuring snout-vent length (SVL, to the nearest 0.1 mm) and body mass (to nearest 0.1 g), I opened the body cavity and removed the liver, fat bodies, ovaries and oviducts. These organs were placed on water-absorbing paper for about five minutes and then weighed (to 0.001g). The testis was not estimated because of its small size and less pronounced seasonal changes (e.g. Jørgensen et al., 1979; Geng et al. 1986). I calculated the relative organ mass (percentage of organ mass accounting for body mass) in order to examine the nutritional status of each frog. A frog was classified as a sexually-mature male if it displayed nuptial pads, and as a sexually-mature female if it had developed oocytes (September to pre-spawning) or was over 35 mm SVL (the minimum size of females in amplexus; Lu, 1994, 2001). Most (about 95%) young of the year showed sexual characteristics before their first hibernation and a few (about 5%) did not. Here, I focus solely on the seasonal change of storage organs of sexually mature individuals. In total, 410 (219 males, 191 females) specimens were examined.

I used Pearson's or Spearman's correlation coefficient to evaluate relationships between variables, and Student's *t* test to estimate significance of slopes of regression lines, and analyses of covariance (ANCOVA, with SVL as the covariate to adjust body size of compared specimens to the same level) to compare differences in storage organ mass between sexes or between different stages of the frog's life cycle. Prior to analysis, percentage variables were arcsin transformed and SVL ln-transformed to improve normality.

RESULTS

Correspondence: Xin Lu, Department of Zoology, College of Life Sciences, Wuhan University, Wuhan 430072, China. E-mail: luxinwh@public.wh.hb.cn

After spawning, liver (male: *r*=-0.38, *n*=69, *P*=0.002; female: *r*=-0.49, *n*=68, *P*<0.001) and fat bodies (male, *r*=0.48, *P*<0.001; female, *r*=-0.39, *P*=0.003) declined at

a low rate until August. The regressed period of storage organs was called the resting period. From late August onwards, both organs (liver: male, r=0.61, n=52, P < 0.001: female, r = 0.71, n = 32, P < 0.001; fat body: male, r=-0.48, P=0.001; female, r=0.56, P=0.002) started growing rapidly and attained their maximum size just before hibernation: October in male and September in female (Fig. 1b,c: the accumulating period). After the peaks, they decreased in size rapidly through the winter until emergence (liver: male, r=-0.41, n=126, P < 0.001; female, r=-0.71, n=123, P < 0.001; fat body: male, r=-0.43, P<0.001; female, r=-0.36, P<0.001), compared to the rates during the resting period (comparison of slopes of regression lines, male, March-August vs October-February, liver: t=1.65, df=193, P=0.04; fat body: t=3.32, P<0.001; female, March-August vs September-February, liver: t=5.30, df=189, P<0.001; fat body: t=0.94, P=0.25). In spite of parallel change in the organ mass of both sexes, males had significantly heavier organs than females during November-February (Fig. 1). ANCOVA, liver: all P<0.003; fat body: all P<0.02, except for January P=0.12). However, there was no significant sexual difference in organ size between February and September (ANCOVA, liver: P=0.09-0.78; fat body: P=0.10-0.82).

From spawning (all eggs were released in one spawning) to pre-hibernation, mass of female reproductive organs varied with a similar profile to those of liver and fat bodies (Fig. 1d). However, the ovaries continued to grow through hibernation and reached the maximum size before spawning (r=0.74, n=150, P<0.001), whereas the oviducts peaked by mid-hibernation (r=0.60, n=93, P<0.001) and then remained relatively stable until pre-spawning (r=-0.06, n=30, P=0.76).

DISCUSSION

The present results followed a general trend: energy reserves of temperate-zone anurans reach their lowest point at or shortly after spawning, remain low through a resting period, and then initiate a new growth cycle, with a peak in late autumn from which, before hibernation, the reserves begin to decrease (Jørgensen *et al.*, 1979).

Metabolism should be synchronized with local environmental factors. Northern *R. temporaria* began establishing body reserves immediately after breeding (Pasanen & Koskela, 1974), whereas southern population of this species exhibited a delayed accumulation (Hong *et al.*, 1968). Similarly, for development of female reproductive organs of temperate-zone anurans, the studies on *R. temporaria* by Koskela & Pasanen (1975) showed that there is a reduced ovarian resting period with increased latitude. Jørgensen *et al.* (1979) concluded that a longer ovarian resting period is correlated with earlier occurrence of spawning. In the present study, *R. chensinensis* had a longer post-reproduction resting period (5 months), compared to that (3 months) of a population further north (ovulation timing late



FIG. 1. Relative organ mass of R. chensinensis in relation to the month of year. (a), annual change of monthly average air temperature in the study area. S, R, A and H at the top of the figure represent spawning, resting, accumulating and hibernating periods, respectively; (b), liver; (c), fat body (filled circles represent males, open circles females); (d) female reproductive organs (filled circles represent ovary, open circles oviduct). The vertical bars are 1 SE.

April; Table 1). These observations suggest that higher seasonal constraints due to harsh climatic conditions result in a tight schedule of energy storage for the northern animals. A question arises as to why those frogs experiencing a long resting period do not save any nutrition in their storage organs. Based on an experiment showing that newly spawned *B. bufo* that are well fed may begin a new ovarian cycle immediately, Jørgensen (1973) argued that nutritional constraints upon females are responsible for a delayed onset of vitellogenetic growth. For *R. chensinensis*, however, 1 found that the proportion (89.2%) of individuals with food-filled stomachs during the resting period did not significantly differ from

TABLE 1. Mean relative ovarian mass of pre-hibernation and pre-ovulation in several temperate-zone anuran species and populations.

Species	Locality	Resting period length	Pre-hib- ernation	Pre-ovu- lation	References
R. temporaria	Finland, 64°N, 100 m	0	10.3	10.9	Koskela & Pasanen, 1975
	Sweden, 64°N, 25 m	0	10.0		Elmberg, 1991
	Poland, 52°N		11.4	12.3	Juszcyzk, 1959
	Poland, 50°N, 1000 m	2-3		12.2	Kozlowska, 1971
	Poland, 50°N, 200 m	3-4		14.5	Kozlowska, 1971
	Denmark, 57°N, 100 m		11.7		Jørgensen, 1981
	England, 53°N	3-4	15.0	16.0	Smith, 1950
R. chensinensis	Jilin, China, 43°N, 700 m	3	9.8		Ma, 1982
	Shanxi, China, 38°N, 1650 n	n 3-4	11.2		X. Lu unpubl data
	Shanxi, China, 37°N, 760 m	5	12.5	24.0	This study
R. nigromaculata	Beijing, China, 40°N, 150 m	3-4	10.3	26.7	Wu, 1965
B. bufo	Denmark, 57°N, 100 m	2-3	13.7	18.4	Jørgensen et al., 1979
B. viridis	Denmark, 57°N, 100 m			20.1	Jørgensen, 1981
R. esculenta	Switzerland, 47°N	1-2	15.0	20.0	Cited from Jørgensen et al., 1979
	Poland, 52°N	2	12.0	18.0	Juszcyzyk & Zamachowski, 1973

that (91.8%) during autumn. To assess the mechanism, the underlying metabolism of amphibians during the resting period and to understand its adaptive implication, further quantitative studies on food availability in relation to physiological regulation of metabolic activity should be made.

Using observations on R. temporaria, B. bufo and B. viridis, Jørgensen et al. (1979) and Jørgensen (1981) argued that vitellogenetic growth of oocytes in temperate-zone amphibians has already finished by autumn and thus ovarian mass remains stable throughout the winter. However, significant winter increases in ovarian mass have been detected in R. nigromaculata and several populations of the R. esculenta complex (Table 1). Jørgensen et al. (1979) attributed this increase to accumulation of fluid. In a R. chensinensis population adjacent to the study area, I compared mean ovarian dry mass $(0.53\pm0.05, n=44)$ during pre-hibernation with that (1.46±0.13, n=27) during pre-spawning and found a significant increase (ANCOVA: F_{1.68}=179.67, P<0.001). I therefore hypothesize that there exist two distinct models of ovarian development in temperate-zone anurans: hibernation-growth and non-hibernation-growth.

In amphibians, egg production is energy-consuming and associated with metabolic activities of liver and fat bodies (Krawczyk, 1971; Pasanen & Koskela, 1974; Maruyama, 1979; Elmberg, 1991; Das, 1996). For *R. chensinensis*, females depleted more energy during hibernation than did males and the increase in the size of ovaries (140.6%) was approximately equal to the loss of mass (152.2%) in both liver and fat body (Table 2), suggesting that most expenditure of the stored energy in females was devoted to sustained winter growth of the oocytes.

Metabolic rate depression is a common mechanism for amphibians to cope with environmental stresses in winter, but hibernating animals do not reduce metabolism to zero and thus they must build up body reserves before winter (Pinder et al., 1992). The amount of depleted nutrition during hibernation should depend on the length of winter. Therefore, it might be expected that northern frogs save more energy before hibernation to survive a longer winter (Pasanen & Koskela, 1974). However, relative ovarian mass decreases with increased climate harshness (Jørgensen et al., 1979; Elmberg, 1991; summarized from the data in Table 1 of this study, correlation between length of resting period and relative ovarian mass, R. temporaria: $r_{=}=0.95$, n=5, P=0.01; R. chensinensis: r_s=1.00, n=3, P<0.001), indicating northern female animals have a lower reproduction investment than southern animals. Interestingly, the longer resting period of nutrient organs in

TABLE 2. Change in relative mass of nutritional organs of *R. chensinensis* from pre-hibernation (maximum organ mass, as 100%) to emergence from hibernation.

Nutritional organ	Male (October-February)	Female (September-February)			
Liver	-21.6	-59.1			
Fat body	-78.0	-93.1			
Ovary		+140.6			
Oviduct		-6.4			
Total energy expenditure	-99.6	-18.0			

southern animals due to warmer climates results in a growth period similar in length to that of northern populations. This suggests that despite having similar available foraging time for nutrient acquisition, the animals living in different climate conditions may adopt different strategies of energy allocation.

ACKNOWLEDGMENT

I thank X. P. Guan and B. Li for supplying specimens, and to two anonymous referees for their helpful comments on the manuscript. The study was supported by the Foundation for Natural Sciences of Shanxi Province, China.

REFERENCES

- Das, I. (1996). Reproductive strategy and lipid mobilization in *Rana hexadactyla* at a seasonal locality. *Ethology Ecology and Evolution* 8, 125-133.
- Elmberg, J. (1991). Ovarian cyclicity and fecundity in boreal common frogs *Rana temporaria* L. along a climatic gradient. *Functional Ecology* 5, 340-350.
- Geng, X. L., Zhang, Y. X. & Qiu, S. (1986). Seasonal changes in the testis of *Bufo raddei* in Lanzhou, China. Acta Herpetologica Sinica 5, 189-193.
- Hong, S. K., Park, C. S., Park Y. S. & Kim, J. K. (1968). Seasonal changes of antiuretic hormone action on sodium transport across frog skin. *American Journal of Physiology* 215, 439-443.
- Jørgensen, C. B. (1973). Pattern of recruitment of oocytes to second growth phase in normal toads, and in hypophysectomized toads, *Bufo bufo bufo* (L.), treated with gonadotropin (HCG). *General* and Comparative Endocrinology 21, 152-159.
- Jørgensen, C. B. (1981). Ovarian cycle in a temperate frog, Rana temporaria, with special reference to factors determining number and size of egg. Journal of Zoology, London 195, 449-458.
- Jørgensen, C. B., Larsen L. O. & Lofts, B. (1979). Annual cycles of fat bodies and gonads in the toad Bufo bufo bufo (L.), compared with cycles in other temperate zone anurans. Biologiske Skrifter 22, 1-37.
- Juszcyzk, W. (1959). The development of the reproductive organs f the female common frog (Rana temporaria L.) in the yearly cycle. Annals of University of Maria Curie-Skleodowska, Lublin 14, 169-231.
- Juszcyzk, W. & Zamachowski, W. (1973). Morphological changes in the reproductive organs of the female edible frog (*Rana esculenta* L.) in the annual cycle. *Acta Biologica Cracoviensia Series Zoologia* 16, 167-178.
- Koskela, P. & Pasanen, S. (1975). The reproductive biology of the female common frog, *Rana* temporaria L., in northern Finland. Aquilo Series Zoologica 16, 1-12.

- Kozlowska, M. (1971). Difference in the reproductive biology of mountain and lowland common frog *Rana temporaria* L. Acta Biologica Cracoviensia Series Zoologia 14, 17-32.
- Krawczyk, S. (1971). Changes in the lipid and water content in some organs of the common frog (*Rana temporaria* L.) in the annual cycle. *Acta Biologica Cracoviensia Series Zoologia* 14, 211-237.
- Liu, C. Z. & Hu, S. Q. (1961). *Chinese Amphibia*. Beijing: Science Press.
- Lu, X. (1994). Fecundity and its geographic variation in *Rana* chensinensis. Acta Ecologica Sinica 14, 209-214.
- Lu, X. (2001). Amplexus-like behaviour of hibernating Rana chensinensis in northern China. Herpetological Journal 11, 121-122.
- Ma, C. F. (1982). Studies on reproductive ecology of *Rana* temporaria chensinensis. Acta Herpetologica Sinica 1, 48-62.
- Maruyama, K. (1979). Seasonal cycles in organ weights and lipid levels of the frog, Rana nigromaculata. Annotationes Zoologici Japonenses 52, 18-27.
- Morton, M. L. (1981). Seasonal changes in total body lipid and liver weight in the Yosemite toad. *Copeia* **1981**, 234-238.
- Pasanen, S. & Koskela, P. (1974). Seasonal and age variation in the metabolism of the common frog, *Rana temporaria* L., in northern Finland. *Comparative Biochemistry and Physiology* 47A, 635-654.
- Pinder, A. W., Storey, K. B. and Ultsch, G. R. (1992). Estivation and hibernation. In: *Environmental biology of* the Amphibia, 250-274. Feder, M.E. and Burggren, W.W. (Eds.). Chicago: University of Chicago Press.
- Pope, C. H. & Boring A. M. (1940). A survey of Chinese Amphibia. *Peking Natural History Bulletin* 15, 13-86.
- Smith, G. L. (1950). Seasonal changes in blood sugar, fat body, liver glycogen, and gonads in the common frog, *Rana temporaria. Journal of Experimental Biology* 11, 73-80.
- Tsiora, A. & Kyriakopoulou-Sklavounou, P. (2001). Male reproductive cycle of the water frog *Rana epeirotica* in northwestern Greece. *Amphibia-Reptilia* **22**, 291-302.
- Wu, Y. L. (1965). Change of body and several organ mass in Rana nigromaculata during hibernation. Chinese Journal of Zoology 1965, 116-119.
- Xie, F., Ye, C.Y, Fei, L. Jiang, J. P., Zeng, X. M. & Matsu, M. (2000). Taxonomical studies on Brown frogs (*Rana*) from northeastern China (Amphibia: Ranidae). Acta Zootaxonomica Sinica 24, 224-231.

Accepted 27.1.03

FEEDING ECOLOGY OF *VIPERA LATASTEI* IN NORTHERN PORTUGAL: ONTOGENETIC SHIFTS, PREY SIZE AND SEASONAL VARIATIONS

JOSÉ C. BRITO

Centro de Biologia Ambiental and Dep. de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Lisbon, Portugal

The dict of Vipera latastei was investigated in northern Portugal from 1998 to 2002. Palpation of stomach contents and forced defaecation from 190 snake specimens resulted in the recovery of 83 identifiable previtems. V. latastei prevs on four species of small mammal (76%), two lizard species (14%), three amphibian species (5%) and arthropods s.l. (5%). Estimates of prey availability demonstrated that the most common prey were also the most frequent prey consumed. No differences between the sexes were detected in terms of the proportion of snakes with prey or diet composition. However, there was an ontogenetic shift in diet composition. Juveniles fed mostly on ecthotermic prey(60%), the majority of subadults fed on insectivorous mammals and lizards (60%), and adults fed mainly on rodents (88%). This ontogenetic shift is mostly due to the morphological constraints imposed on the juveniles, which cannot swallow large prey items. There is a positive correlation between snake size and prey size. V. latastei is selective in terms of both the species and size of prey ingested, with larger snakes being more selective than smaller snakes. Larger snakes have a narrower food niche breadth than smaller snakes, but their diet composition overlaps moderately. There is seasonal variation in the diet composition, with snakes taking amphibians mainly in spring and autumn, lizards in spring, and mammals in summer and autumn. Feeding frequencies indicate that both males and females and subadults and adults - consume prey more frequently during summer.

Key words: dietary habits, food selection, prey availability, snakes, Viperidae

INTRODUCTION

Foraging ecology plays a direct or indirect role in virtually all aspects of snake life-history (Mushinsky, 1987). For instance, obtaining sufficient energy is a crucial factor in reproduction and it may influence the frequency of reproduction (Naulleau & Bonnet, 1996), reproductive output (Gregory & Skebo, 1998) or the maternal condition after reproduction (Shine & Madsen, 1997). Feeding also affects demographic traits of populations, especially rates of growth and survival (Madsen & Shine, 2000).

Although the diet of western European vipers (*Vipera* spp.) is relatively well documented (Bea *et al.*, 1992), data on Lataste's viper (*Vipera latastei*) are scarce and mostly anecdotal (Valverde, 1967; Vericad & Escarre 1976; López Jurado & Caballero, 1981), or come from studies carried out in captivity outside their natural distribution area (Saint-Girons, 1980). Although general accounts (Bea & Braña, 1988) state that this species feeds on lizards and small mammals, no study has specifically addressed its foraging ecology.

Vipera latastei is a small-sized viper, with snout-vent length usually not larger than 60.0 cm. It is distributed from north-western Africa (Morocco, Tunisia and Algeria) in the south, to the Iberian peninsula in the north, with the exception of Cantabrian and Pyrenean regions. Despite its former wide distribution in Portugal, it presently only occurs in isolated and small populations, especially in the south (Godinho *et al.*, 1999). This species is threatened mainly by habitat loss, as a consequence of forest fires and intensification of agriculture, and direct human persecution (Brito *et al.*, 2001). Additionally, the Portuguese preliminary conservation status of "Indeterminate" for *V. latastei* (S.N.P.R.C.N., 1991), demonstrates insufficient knowledge concerning this species. Thus, a conservation programme has been established in northern Portugal, which aims to identify priority areas for conservation and to develop management strategies for viper populations and their habitats.

Data on foraging ecology is important because (1) species with very specialized diets usually are unable to use alternative prey types if their habitats are modified or destroyed; and (2) ontogenetic shifts in diet composition may contribute to the endangerment of species, for instance due to declines in a specific prey type needed by a particular age/size class of snakes (Webb & Shine, 1998). Within the framework of the conservation programme for V. latastei, the following questions were asked: (1) is it a size-selective predator and how does prey availability influence the diet composition? (2) Are there ontogenetic shifts in the diet composition? (3) Are there intersexual differences in the diet composition? (4) What is the frequency of feeding and are there seasonal changes in diet composition? These questions should help in understanding the foraging ecology of V. latastei and allow the identification of favourable ecological niches for the species.

Correspondence: J. Brito, Centro de Investigação em Biodiversidade e Recursos Genéticos da Universidade do Porto (CIBIO/UP), Instituto de Ciências Agrárias de Vairão, R. Padre Armando Quintas, 4485-661 Vairão, Portugal. *Email*: jcbrito@mail.icav.up.pt

METHODS

STUDY AREA

The study was conducted in the extreme north-western part of the distribution area of V. latastei (Brito & Crespo, 2002), in a National Woodland and Biosphere Reserve, "Mata de Albergaria", Portugal (41°49'N, 8°07'W; elevation c. 750 m a.s.1.), included in a protected area, Parque Nacional da Peneda-Gerês. The area is a series of granite mountains, with altitudes ranging from 50 to 1500 m a.s.l. The climate is Atlantic Mediterranean (Goday, 1953), characterized by wet winters and hot summers. Mean monthly rainfall ranges from 55 mm in July to 457 mm in January (average annual rainfall above 3200 mm/year), and mean air temperature ranges from 7.9 °C in January to 20.4 °C in July (average annual air temperature 14 °C) (INAG, 2002). The vegetation consists mostly of deciduous oak forests (Quercus robur) or mixed deciduous and coniferous forests (Pinus sp.) with arbutus trees (Arbutus unedo). Major shrubs include heath (*Calluna vulgaris* and *Erica*) sp.), gorse (Ulex sp.) and brooms (Cytisus sp.).

FIELD AND ANALYTICAL METHODS

Data were collected between 1998 and 2002 in an area of around 2.300 ha. 135 snakes were caught by hand, sexed by analysing external tail morphology, measured for snout-vent length (SVL), head length (HL), head width (HD), and head height (HH), and permanently marked for future identification by clipping unique combinations of ventral scales. Snakes were palpated to detect prey in the stomach and intestines, but only forced to defecate intestine contents and never forced to disgorge prey; however, prey presence was recorded in such cases. Additionally, 55 road-killed snakes were dissected to collect stomach and intestine prey items. Mammals were identified using identification keys based on the internal structure of hairs (Teerink, 1991). Reptiles, amphibians and invertebrates were identified through their external morphology. Prey items collected from road-killed snakes were measured (body length), whenever their preservation status allowed.

The availability of potential prey was estimated in a smaller area (2.24 ha), and compared with prey data from the vipers captured in this area (64% of the total examined snakes). Due to logistical reasons, prey availability focuses only on small mammals and lizards. Small mammal abundance was estimated using 100 Shermann traps, placed in line at 10 m intervals, baited and surveyed for six nights, in June 1999, 2000 and 2001. This period should correspond to the annual abundance peak of small manimals in this region (Mathias, 1999). Traps were examined in early morning (at about 07:00 to 08:00 hr) and late afternoon (at about 19:00 to 20:00 hr). Lizard abundance was estimated through visual encounter surveys of 1000 m, in the same area at the same time period. Three lacertid lizard species were found in this smaller study area (Podarcis bocagei, *Lacerta schreiberi* and *L. lepida*), but – due to the large size of adult L. schreiberi and L. lepida – only juveniles were considered as potential prey for V. latastei.

The extent of sexual divergence in viper body morphology was quantified according to the methods outlined by Shine (1991*a*). For SVL, the quotient between female SVL and male SVL was found. For HL, HW and HH, a regression equation linking female measures to the SVL was calculated. An estimate of the female head measure was obtained using the mean male SVL. The ratio of female to male head length, at a mean male SVL, provided an index of the extent of sexual size dimorphism in relative head measures (Shine, 1991*a*).

For analysis purposes, snakes were divided into three size categories according to their sexual maturation status: (1) Juveniles and neonates (SVL <30.0 cm); (2) subadults (SVL 30.0-40.0 cm); (3) adults (SVL >40.0 cm) (Brito & Rebelo, 2003). Differences in diet composition were analysed by χ^2 tests and contingency tables (Siegel & Castellan, 1988). To avoid pseudoreplication of data, no snake was included in the analysis more than once. Niche breadth was measured using the standardized Levin's index (B_A) and niche overlap between categories was measured using Pianka's symmetrical equation (O_{jk}) (for details see Krebs, 1989). The relation between snake size and prey size was determined by calculating Kendall's tau (Siegel & Castellan, 1988),

TABLE 1. Sample sizes, body measures and sexual size dimorphism of *Vipera latastei* containing prey. SVL, snout-vent length, HL, head length; HW, head width; HH, head height (all measures in cm); SD, standard deviation; SSD, sexual size dimorphism. See methods for calculation of SSD.

	11	Mean SVL±SD	Mean HL±SD	Mean HW±SD	Mean HH±SD
Males	62	36.3±9.1	2.02±0.36	1.38±0.26	0.76±0.13
Females	39	37.7±8.3	2.12±0.37	1.40 ± 0.30	0.79 ± 0.17
SSD (f/m)	101	1.04	1.01	0.99	1.00
Juveniles	21	25.0±3.7	1.56 ± 0.19	1.02 ± 0.13	0.57±0.06
Subadults	38	34.3±3.2	1.97±0.16	1.33±0.15	0.75 ± 0.11
Adults	42	45.2±4.7	2.37±0.21	1.59 ± 0.22	0.89±0.09
Total	101	36.9±8.8	2.06±0.37	1.39±0.28	0.78±0.15

testing snake SVL against prey length. In the analysis of feeding frequency and seasonal changes in diet composition, data were pooled into three seasons – spring (March to May), summer (June to August) and autumn (September to November). No data were available between December and February due to the low activity levels of *V. latastei* during the winter (Brito, 2003). Feeding frequencies were inferred from the proportion of snakes containing detectable prey items (Shine *et al.*, 1998). Differences in abundance between snake diet and prey availability were analysed by χ^2 tests contingency table tests. A minimum rejection level of α =0.05 was used in all statistical tests.

RESULTS

A total of 190 individual snakes were analysed in this study, of which 101 contained prey (Table 1). The relative number of snakes with and without prey did not differ significantly between sexes ($\chi^2=0.003$, df=1, P=0.954). *V. latastei* preys on four species of small mammal (76% of the total prey items), two lizard species (14%), three amphibian species (5%) and arthropods s.l.

(5 %) (Table 2), and the differences between the frequencies of prey type consumed were significant (χ^2 =116.8, df=3, *P*<0.001). The rodent *Apodemus sylvaticus* was the most frequent prey, representing 40% of the total prey taken. Analysis by sex shows that females take proportionally more mammals than males, but differences were not significant (all size categories: χ^2 =1.74, df=3, *P*=0.628; adults only: χ^2 =1.94, df=2, *P*=0.164). Since there were no inter-sexual differences in the proportion of snakes with and without prey, or in diet composition, sexes were pooled in the subsequent analysis in the three size categories.

There were significant differences in the dietary composition of the three size categories ($\chi^2=17.5$, df=6, P<0.05) (Table 2). Juveniles fed essentially on ectothermic prey (60%), subadults fed on small mammals (74%), mainly insectivores (43%), but also on ectothermic prey (26%), while adults fed almost exclusively on rodents (88%), and only to a small extent on insectivorous (15%) and ectothermic prey (6%). The importance of small mammals in the diet of *V. latastei* increased with snake size, representing 40% of the diet

TABLE 2. Diet composition and niche breadth (B_A) from different sexes and size categories of *Vipera latastei*. Number of prey items and percentage (in parenthesis). See methods for size categories.

	Males	Females	Juveniles	Subadults	Adults	Total
No. snakes examined	117	73	50	70	70	190
% of snakes with prey	53.0	53.4	42.0	58.6	55.7	53.2
Coleopteran	3 (6.3)	1 (2.9)	2 (13.7)	2 (5.7)	-	4 (4.8)
Total Arthropod	3 (6.3)	1 (2.9)	2 (13.3)	2 (5.7)	-	4 (4.8)
Alytes obstetricans	1 (2.1)	-	1 (6.7)	-	-	1 (1.2)
Chioglossa lusitanica	1 (2.1)	-	1 (6.7)	-	-	1(1.2)
Triturus boscai	-	1 (2.9)	-	-	1 (3.0)	1 (1.2)
Unidentified amphibians	1(2.1)	-	-	1 (2.9)	-	1 (1.2)
Total Amphibia	3 (6.3)	1 (2.9)	2 (13.3)	1 (2.9)	1 (3.0)	4 (4.8)
Podarcis bocagei	4 (8.3)	3 (8.6)	1 (6.7)	6 (17.1)	-	7 (8.4)
Podarcis hispanica	1 (2.1)	-	1 (6.7)	-	-	1 (1.2)
Unidentified Lacertidae	3 (6.3)	1 (2.9)	3 (20.0)	-	1 (3.0)	4 (4.8)
Total Reptilia	8 (16.7)	4 (11.4)	5 (33.3)	6 (17.1)	1 (3.0)	12 (14.5)
Crocidura russula	7 (14.6)	5 (14.3)	2 (13.3)	10 (28.6)	-,	12 (14.5)
Sorex granarius	2 (4.2)	4 (11.4)	1 (6.7)	5 (14.3)	-	6 (7.2)
Microtus lusitanicus	4 (8.3)	3 (8.6)	2 (13.3)	-	5 (15.2)	7 (8.4)
Apodemus sylvaticus	19 (39.6)	14 (40.0)	1 (6.7)	8 (22.9)	24 (72.7)	33 (39.8)
Unidentified mammals	2 (4.2)	3 (8.6)	-	3 (8.6)	2 (14.3)	5 (6.0)
Total Mammals	34 (70.8)	29 (82.9)	6 (40.0)	26(74.3)	31 (93.9)	63 (75.9)
Standardized Levin's B _a	0.287	0.142	0.754	0.236	0.044	0.221



FIG. 1. Seasonal variation in the diet composition of *Vipera latastei* from northern Portugal.



FIG. 2. Seasonal changes in the proportion of *Vipera latastei* from northern Portugal containing prey, by sex (A) and by size category (B). See text for size categories.

of juveniles and 94% of adults, while the importance of ectothermic prey decreases with snake size, representing 60% of the diet of juveniles and 6% of adults.

The food niche (B_A) varied between 0.044 for adult snakes to 0.754 for juveniles (Table 2), indicating that adults were more specialized than subadults and juveniles. Food niche differences between all size categories of males and females indicate a greater specialization in females, which consumed proportionally more mammals than the males (Table 2). Niche overlap (O_{jk}) was high between subadults and adults (O_{jk}=0.978), and moderate between juveniles and sub-adults (O_{jk}=0.863), and between juveniles and adults (O_{jk}=0.749). Overlap between all size categories of males and females was very high (O_{jk}=0.993).

(O_{jk} =0.993). There was seasonal variation in diet composition, although this was not significant (χ^2 =8.15, df=6, *P*=0.228) (Fig. 1). Snakes fed on an increasing proportion of mammals, representing 50% of the diet in spring, but 72% in autumn. Reptiles were mostly preyed upon in the spring (25%), while in autumn they were only marginally utilized as prey items (9%). A mphibians were mostly preyed upon in the spring (25%) and autumn (14%).

Analysis of feeding frequency demonstrated significant differences in the proportion of males and females with and without prey from spring to autumn (males: χ^2 =6.49, df=2, *P*=0.039; females: χ^2 =6.66, df=2, *P*=0.036) (Fig. 2A), with both sexes consuming prey more frequently in the summer. There were also differences between the size categories (Fig. 2B), but these were only significant for subadults (χ^2 =6.71, df=2, *P*=0.035) and adults (χ^2 =7.97, df=2, *P*=0.019). These size categories of snakes consumed prey more frequently in the summer.

There was a positive correlation between snake SVL and prey size (Fig. 3) (Kendall's tau = 0.562, P < 0.001). Small snakes ate smaller prey, mostly lizards and insectivorous mammals, but mean prey size shifted at an intermediate body size (SVLs around 40.0 cm), with larger snakes feeding almost exclusively on larger prey.

TABLE 3. Percent frequency of prey types consumed by *Vipera latastei* compared to prey availability.* Indicates significant differences between availability and proportion of prey in the diet (P=0.01).** Indicates prey types which varied significantly in abundance over the study period (P<0.001).

		Consumed			Available	
	1999	2000	2001	1999	2000	2001
Podarcis bocagei	100.0	100.0	100.0	93.8	94.3	97.5
Lacerta schreiberi	-	-	-	6.3	3.8	2.5
Lacerta lepida	-	-	-	0.0	1.9	0.0
Total reptiles	2	5	1	16	53	40
Crocidura russula	50.0	15.4	30.0	20.0	2.0	11.1
Sorex granarius	-	15.4	-	-	-	-
Microtus lusitanicus	-	15.4	-	-	2.0	-
Apodemus sylvaticus	50.0	53.8*	70.0	80.0	96.1**	88.9
Total mammals	4	13	10	15	51	9



FIG. 3. Relationship between Vipera latastei snout-vent length and prey length ($r^2=0.424$, n=19, P=0.003), in northern Portugal.

Estimates of availability of the two main prey types (lizards and small mammals; Table 3), and diet composition analysis indicate that the most common prey (*Podarcis bocagei*, *Crocidura russula* and *Apodemus sylvaticus*) were also the species most frequently preyed upon. Combining data for the three years of study, these three prey species accounted for 88.6% of the diet of the snakes, and their availability represents 97.2% of the total sample. Although available, juveniles of *Lacerta schreiberi* and *L. lepida* were apparently not preyed on, and *Sorex granarius* only marginally so.

DISCUSSION

The diet of V. latastei in the study area follows the general diet pattern of the European Vipera (Bea et al., 1992), with the main prey taken being small mammals. Nevertheless, it is significantly different ($\chi^2=13.41$, df=4, P=0.009) from the diet of this species reported in a study of museum specimens from Spain (Bea & Braña, 1988). For instance, amphibians have never previously been reported as prey for V. latastei. However, in the study area they represent 13% of diet of the juveniles and 3% of the diet of subadults and adults, respectively. Bea & Braña (1988) suggested that V. latastei had a low frequency of small mammals in the diet (58%) compared to other European vipers, while in northern Portugal they play an important role, representing up to 76% of the diet of this viper. These differences in diet composition between studies are probably due to annual and/or seasonal differences in prey availability between regions. For instance, mammal abundance in the smaller study area fluctuated significantly during the three years of study.

Seasonal shifts in dietary composition of snakes are expected due to spatial and temporal changes in prey availability throughout the year, and examples include the viper Vipera ursinii (Agrimi & Luiselli, 1992), the python Liasis fuscus (Madsen & Shine, 1996), and the colubrid Coluber constrictor (Shewchuk & Austin, 2001). V. latastei exhibited seasonal changes in diet composition, with snakes taking an increasing proportion of mammals from spring to autumn. Conversely, there is a decrease in the proportion of lizards taken. Amphibians are mainly preyed upon during spring and autumn. These seasonal shifts are most likely related to fluctuations in the availability of prey. Lizards are especially abundant during their mating season, in spring, and after hatching, in summer, and are thus susceptible to predators (unpublished data). Amphibians should be more available during the spring and autumn, when the climatic conditions enhance their movements (personal observation). The two species of mammal most frequently taken (*Crocidura russula* and *Apodemus sylvaticus*) have their offspring in spring and autumn (Mathias, 1999), thus being more available during these seasons.

Intersexual differences in diet composition have been reported for several snake species, such as the acrochordid Acrochordus arafurae (Houston & Shine, 1993), the pythonid Morelia spilota (Pearson et al., 2002), and the colubrid Natrix maura (Santos & Llorente, 1998). These differences are mainly caused by sexual size dimorphism, females being larger than males, and thus able to feed on larger prey. Such sexual differences in diet composition could also correspond to a higher energetic demand for females, which must devote more resources to their reproductive output (Shine, 1989). Females of V. latastei are larger than males in SVL and relative head length, but the extent of sexual size dimorphism is small compared to other snake species (Shine, 1991a). As expected, there are no significant intersexual differences in the diet composition. The high value of diet overlap between all size categories of males and females further demonstrates the absence of intersexual differences in diet composition.

Overall, V. latastei exhibits a main foraging period in the summer, which should be related to environmental temperature. Winter in this montane study area is rainy and long, thus snakes forage mainly in summer (Brito, 2003), when temperatures should be optimal to enhance digestion (Naulleau, 1982). Juvenile V. latastei had a similar feeding frequency compared to the other size categories, while juveniles of other European viperids have been reported to feed less frequently than sub-adults and adults (Prestt, 1971; Luiselli, 1996). It has been suggested that this can be a direct consequence of the generalized feeding activity of juveniles and/or to gape limitation: juvenile snakes are too small to ingest adult rodents, and thus have a smaller range of prey types which can be ingested (Shine & Madsen, 1997). However, since juveniles are not involved in mating activities, they should maximize the rate of prey intake in order to enhance their growth rates and survival during the hibernation period (e.g. Bonnet, 1997).

Ontogenetic shifts in diet composition are a common pattern in snakes, mainly because they are gape-limited predators (Mushinsky, 1987), and the European Vipera usually exhibit a shift from lizards to small mammals (Saint-Girons, 1980). Juvenile V. latastei feed essentially on ectothermic prey, while adults prey on rodents, and there is a significant correlation between snake size and prey size. This ontogenetic shift in the diet compo-

sition could be explained in two ways. First, differences in energetic content of prey types. Optimal foraging theory predicts that a predator will ignore small prey when densities of large prey are high enough for predator survival (Schoener, 1971). Additionally, the ingestion of numerous small prey would increase capture and handling time compared to the ingestion of fewer large prey. Thus, adult snakes should prefer mammals to lizards, due to the larger size and higher energetic content of manimals. Second, morphology may impose constraints on ingestion capacity in juvenile snakes. Snakes are gape-size limited predators, therefore juveniles are forced to eat small prey. With an increase in snake body size the number and size of ingestible prey types increase, allowing larger snakes to utilize a greater range of prey sizes and taxa (Shine, 1991b).

Nevertheless, lizards are not entirely excluded from the diet of adult *V. latastei*, representing about 6% of prey taken, which is a common pattern in other European viperids (Braña *et al.*, 1988; Luiselli & Agrimi, 1991). Estimates of prey availability demonstrate that both small prey (lizards and insectivorous mammals) and large prey (rodent mammals) are readily available. Faced with this availability of prey, adult snakes should not be entirely forced to shift from lizards to rodent mammals.

In conclusion, *V. latastei* is selective in terms of the species and the size of prey ingested, with larger snakes being more selective than smaller snakes. In addition, larger snakes have a narrower food niche breadth than do smaller snakes. The ontogenetic shift in the dietary habits is mostly due to the morphological constraints imposed on juveniles, which cannot swallow large prey items.

ACKNOWLEDGMENTS

This work was financially supported by a PhD grant from Fundação para a Ciência e Tecnologia (PRAXIS XX1/BD/16093/98) and by Parque Nacional da Peneda-Gerês. Instituto da Conservação da Natureza (ICN) granted the permissions for capture of snakes. We acknowledge the Banco de Tecidos de Vertebrados Selvagens (ICN) for providing some of studied specimens and all the people that collected road-killed vipers, especially F. Álvares and A. Rebelo. Thanks to E. Crespo, T. Madsen, R. Rebelo, M. Carretero, S. Thorsteinsdóttir and A. Loureiro for comments on the manuscript.

REFERENCES

- Agrimi, U. & Luiselli, L. M. (1992). Feeding strategies of the viper Vipera ursinii ursinii (Reptilia: Viperidae) in the Apennines. Herpetological Journal 2, 37-42.
- Bea, A. & Braña, F. (1988). Nota sobre la alimentación de Vipera latastei, Boscá, 1878 (Reptilia, Viperidae). Munibe 40, 121-124.

- Bea, A., Braña, F., Baron, J. -P. and Saint-Girons, H. (1992). Régimes et cycles alimentaires des vipères européennes (Reptilia, Viperidae). Année Biologique 31, 25-44.
- Bonnet, X. (1997). Influence of size on survival in newborn asp vipers (*Vipera aspis*): preliminary results. *Amphibia-Reptilia* 18, 107-112.
- Braña, F., Bea, A. & Saint-Girons, H. (1988). Composición de la dieta y ciclos de alimentación en Vipera seoanei Lataste, 1879. Variaciones en relación con la edad y el ciclo reproductor. Munibe 40, 19-27.
- Brito, J. C. (2003). Seasonal and daily activity patterns of *Vipera latastei* in northern Portugal. *Amphibia*-*Reptilia* 24, 497-508.
- Brito, J. C. & Crespo, E. G. (2002). Distributional analysis of two vipers (*Vipera latastei* and *V. seoanei*) in a potential area of sympatry in the Northwestern Iberian Peninsula. In *Biology of the Vipers*, 129-138. Schuett, G. W., Höggren, M., Douglas, M. E. and Greene, H. W. (Eds.). Eagle Mountain Publishing.
- Brito, J. C. & Rebelo, R. (2003). Differential growth and mortality affect sexual size dimorphism in *Vipera latastei*. *Copeia* 2003, 865-871.
- Brito, J. C., Rebelo, A. & Crespo, E. G. (2001). Viper killings for superstitious reasons in Portugal. Boletin de la Asociación Herpetológica Española 12, 101-104.
- Goday, S. R. (1953). Apreciación sintética de los grados de vegetación de la sierra de Gerês. In A Flora e a Vegetação da Serra do Gerês, 449-480. Lisboa: Instituto de Alta Cultura.
- Godinho, M. R., Teixeira, J., Rebelo, R., Segurado, P., Loureiro, A., Álvares, F., Gomes, N., Cardoso, P., Camilo-Alves, C. & Brito, J. C. (1999). Atlas of the continental Portuguese herpetofauna: an assemblage of published and new data. *Revista Española de Herpetologia* 13, 61-82.
- Gregory, P. T. & Skebo, K. M. (1998). Trade-offs between reproductive traits and the influence of food intake during pregnancy in the garter snake, *Thamnophis* elegans. American Naturalist 151, 477-486.
- Houston, D. & Shine, R. (1993). Sexual dimorphism and niche divergence: feeding habits of the Arafura filesnake. *Journal of Animal Ecology* 62, 737-748.
- I. N. A. G. (2002). Dados de Base Sistema Nacional Informação de Recursos Hídricos. Instituto Nacional da Água. Available on-line: http://snirh.inag.pt/snirh/ dados_base/main_nav_fr.html
- Krebs, C. J. (1989). *Ecological Methodology*. New York: Harper Collins.
- López Jurado, L. F. & Caballero, M. R. (1981). Predación de Vipera latastei sobre Mustela nivalis. Doñana Acta Vertebrata 8, 298-299.
- Luiselli, L. (1996). Food habits of an alpine population of the sand viper (*Vipera ammodytes*). Journal of Herpetology **30**, 92-94.
- Luiselli, L. M. & Agrimi, U. (1991). Composition and variation of the diet of *Vipera aspis francisciredi* in

relation to age and reproductive stage. *Amphibia-Reptilia* **12**, 137-144.

- Madsen, T. & Shine, R. (1996). Seasonal migration of predators and prey - a study of pythons and rats in tropical Australia. *Ecology* 77, 149-156.
- Madsen, T. & Shine, R. (2000). Silver spoons and snake body sizes: prey availability early in life influences long-term growth rates of free-ranging pythons. *Journal of Animal Ecology* 69, 952-958.
- Mathias, M. L. (Coord.). (1999). Guia dos Mamiferos Terrestres de Portugal Continental, Açores e Madeira. Lisboa: Instituto da Conservação da Natureza and Centro de Biologia Ambiental da Faculdade de Ciências da Universidade de Lisboa.
- Mushinsky, H. R. (1987). Foraging ecology. In Snakes -Ecology and Evolutionary Biology, 302-334. Seigel, R. A., Collins, J. T. and Novak, S. S. (Eds). New York: McGraw Hill.
- Naulleau, G. (1982). Action de la temperature sur la digestion chez les vipères espagnoles du genre Vipera. Publicaciones del Centro Pirenaico de Biologia Experimental 13, 89-94.
- Naulleau, G. & Bonnet, X. (1996). Body condition threshold for breeding in a viviparous snake. *Oecologia* 107, 301-306.
- Pearson, D., Shine, R. & How, R. (2002). Sex-specific niche partitioning and sexual size dimorphism in Australian pythons (Morelia spilota imbricata). Biological Journal of the Linnean Society 77, 113-125.
- Prestt, I. (1971). An ecological study of the viper Vipera berus in southern Britain. Journal of Zoology 164, 373-418.
- Saint-Girons, H. (1980). Modifications sélectives du régime des vipères (Reptilia: Viperidae) lors de la croissance. *Amphibia-Reptilia* 1, 127-136.
- Santos, X. & Llorente, G. A. (1998). Sexual and sizerelated differences in the diet of the snake Natrix maura from the Ebro delta, Spain. Herpetological Journal 8, 161-165.
- Schoener, T. W. (1971). Theory of feeding strategies. Annual Review of Ecology and Systematics 11, 369-404.

- Shewchuck, C. H. & Austin, J. D. (2001). Food habits of the racer (*Coluber constrictor mormon*) in the northern part of its range. *Herpetological Journal* 11, 151-155.
- Shine, R. (1989). Ecological causes for the evolution of sexual dimorphism: a review of the evidence. The Quarterly Review of Biology 64, 419-461.
- Shine, R. (1991*a*). Intersexual dietary divergence and the evolution of sexual dimorphism in snakes. *American* Naturalist **138**, 103-122.
- Shine, R. (1991b). Why do larger snakes eat larger prey items? *Functional Ecology* 5, 493-502.
- Shine, R. & Madsen, T. (1997). Prey abundance and predator reproduction: rats and pythons on a tropical Australian floodplain. *Ecology* 78, 1078-1086.
- Shine, R., Harlow, P. S., Keogh, J. S. & Boeadi. (1998). The influence of sex and body size on food habits of a giant tropical snake, *Python reticulatus*. *Functional Ecology* 12, 248-258.
- Siegel, S. & Castellan Jr., N. J. (1988). Nonparametric Statistics for the Behavioral Sciences. New York: McGraw Hill.
- S. N. P. R. C. N. (eds.). (1991). Livro Vermelho dos Vertebrados de Portugal. Vol. 1 - Mamíferos, Aves, Anfibios e Répteis. Lisboa: Serviço Nacional de Parques, Reservas e Conservação da Natureza.
- Teerink, B. J. (1991). *Hair of West-European Mammals*. Cambridge: Cambridge University Press.
- Valverde, J. A. (1967). Estructura de una comunidad mediterránea de vertebrados terrestres. *Monografias de Ciencia Moderna*, 1. CSIC.
- Vericad, J. R. & Escarre, A. (1976). Datos de alimentación de ofidios en el Levante sur ibérico. *Mediterranea* 1, 5-32.
- Webb, J. K. & Shine, R. (1998). Ecological characteristics of a threatened snake species, *Hoplocephalus* bungaroides (Serpentes, Elapidae). Animal Conservation 1, 185-193.

Accepted: 20.2.03

REASSESSMENT OF THE VALIDITY AND DIAGNOSIS OF THE PITVIPER TRIMERESURUS VENUSTUS VOGEL, 1991

ANITA MALHOTRA AND ROGER S. THORPE

School of Biological Sciences, University of Wales Bangor, Bangor, UK

Trimeresurus venustus Vogel, 1991 was described from southern Thailand in 1991, and distinguished from the similar T. kanburiensis primarily by the following characters: 21 scale rows at midbody rather than 19 and less irregular and indented supraoculars. However, very few specimens of T. kanburiensis were known at the time of this description, and the name T. venustus has not been universally accepted. Recently, live specimens from the type locality of T. kanburiensis in western Thailand have become available, allowing a reassessment of the status of the southern Thai population. Phylogenetic analysis of two mitochondrial gene regions indicated that specimens from south Thailand are genetically quite distinct from the specimen from the type locality, and the former are more closely related to T. macrops than to T. kanburiensis from the type locality that are now known and twenty specimens from southern Thailand. Despite the small sample size, it is clear that some of the diagnostic characteristics used to define T. venustus are invalid. We conclude that the current evidence indicates that T. venustus is a valid species, and present new diagnostic characters to separate it from T. kanburiensis.

Key words: Crotalinae, systematics, Thailand, Trimeresurus kanburiensis, Viperidae

INTRODUCTION

The genus Trimeresurus (Serpentes: Viperidae; Crotalinae) contains many taxonomically vexing issues that are still awaiting resolution (Malhotra & Thorpe, 1997, 2000). Recently, molecular data have promised to resolve some of these issues. One example is the status of the taxa T. kanburiensis Smith 1943 and T. venustus Vogel 1991. The holotype of *T. kanburiensis* (a female) was collected in 1938 from Kanchanaburi (then known as Kanburi) province in western Thailand (Fig. 1). It was at first identified as T. puniceus and only described as a new species in 1943 (Smith, 1943). It remained the only specimen available for the species until the late 1980s. In the interim, confusion developed over the identity of this species following specimens of another species *T. purpureomaculatus*, that occurs in the same region, being mistakenly labelled T. kanburiensis in books and by dealers in the captive trade (see Warrell et al., 1992, for details). In the late 1980s, two additional specimens, also females, were found in Kanchanaburi Province (Warrell et al., 1992). Specimens apparently referable to the species were also found in southern Thailand, in Nakhon Si Thammarat and Krabi provinces. Ironically, some of these specimens found their into the captive trade labelled "*Τ*. way purpureomaculatus" (Warrell et al., 1992). Vogel (1991) described this southern population as $T_{\rm c}$ venustus, citing the following diagnostic characters to separate it from T. kanburiensis: 21 scale rows at midbody rather than 19; narrower, less indented and divided, supraoculars; slighter body and a distinctive brownish-red banded colour pattern.

However, the name *T. venustus* has not been widely accepted. Among recent checklists of venomous snakes, it has been listed by Golay *et al.* (1993) and David & lneich (1999) but not by McDiarmid *et al.* (1999) or the EMBL taxonomy database (http://www.emblheidelberg.de/~uetz/families/Viperidae.html).

Specimens of *T. kanburiensis* that were available to Vogel for comparison were in poor condition. The holotype of *T. kanburiensis* is in two pieces and clearly has a section of body and the tail tip missing (also noted by Warrell *et al.*, 1992). The head is very distorted and squashed, with torn skin on one side, and any pattern has entirely faded. One of the two additional specimens available is also almost in three pieces and the colour pattern has faded considerably in both (Warrell *et al.*, 1992). Warrell *et al.* (1992) doubted that *T. venustus* was a different species to *T. kanburiensis*, after finding a fourth specimen of *T. kanburiensis* from K anchanaburi province in 1991 (the only male), in which the colour pattern was well preserved and appeared to be identical to that of the southern population.

Further doubt was cast on the validity of *T. venustus* when, during fieldwork in Thailand in the 1990s, we found several specimens in the vicinity of the Khao Luang massif, near Nakhon Si Thammarat (Fig. 1), that also had 19 scale rows at mid-body. One of these, a roadkill, has since been presented to the Natural History Musuem, London (BMNH 2002.52). A specimen from Surat Thani with the same character state was also seen by the senior author at the Queen Savoabha Memorial Institute. Therefore, the status of *T. venustus* required further verification (Malhotra quoted in Gumprecht, 2002*a*). Recently, a number of live specimens of *T. kanburiensis* have become available from the type locality. One of these (a female) was sent to the authors by the

Correspondence: A. Malhotra, School of Biological Sciences, University of Wales, Bangor, Gwynedd, LL 57 2UW UK. E-mail: A.Malhotra@bangor.ac.uk



FIG. 1. Map of Thailand, showing the location of specimens. Circles represent specimens referable to *T. vemustus* (with 21 scale rows at mid-body); triangles indicate the specimens from the south with 19 scale rows; squares indicate specimens referred to *T. kanburiensis*. The type locality was not precise, referring only to limestone hills in the vicinity of Kanchanaburi near the Tennaserim border. Note that the position of the Saiyok military camp where two specimens of *T. kanburiensis* were located, is different from that in Fig. 1 of Warrell *et al.* (1992), which showed the position of Saiyok village rather than the military camp (which is, confusingly, not near Saiyok village).

owner and is the first specimen available for DNA analysis. It has since been presented to the Natural History Museum, London (BMNH 2002.51). In this paper, we present a phylogenetic analysis of mitochondrial sequences to evaluate the relationship of populations referable to *T. venustus* to that from the type locality of *T. kanburiensis.* We also evaluate their morphological similarity by conducting a multivariate morphometric analysis of scalation, head shape and colour pattern, along with an analysis of internal characters (tooth numbers and position of the major internal organs) to evaluate the diagnostic characters proposed by Vogel (1991).

MATERIALS AND METHODS

DNA ISOLATION, AMPLIFICATION AND SEQUENCING

Whole genomic DNA was extracted from blood preserved by the addition of 5% EDTA and 2-4 ml SDS-Tris buffer (100 mM Tris, 3% SDS), or from liver or muscle tissue preserved in 80% ethanol, using standard protocols (Sambrook *et al.*, 1989). Cytochrome *b* (cyt *b*) sequences were obtained as described in Malhotra & Thorpe (2000), NADH dehydrogenase (ND4) as described in Parkinson *et al.* (2000) and 12S small subunit ribosomal RNA (12S) as described in Knight & Mindell (1993). Unincorporated nucleotides and primers were removed using a variety of commercially available kits (e.g. Prep-a-gene [Biorad], Wizard minicolumns [Promega], or QIAquick columns [QIAGen]). The double-stranded product was sequenced using dye-labelled terminators (ABI PRISMTM BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit), and subsequently run on an ABI Prism 377 automated DNA sequencer.

PHYLOGENETIC ANALYSIS

Malhotra & Thorpe (2000) presented a phylogeny of 21 species of Trimeresurus (sensu lato) based on cyt b sequences, and evaluated the taxonomic value of certain morphological characteristics against this tree. On this basis, four species groups were defined within Trimeresurus sensu stricto, which are diagnosed by a combination of the condition of the first upper labial and nasal scale (fused or separate) and the hemipenial structure. By these criteria, the T. kanburiensis/T. venustus complex is a part of the albolabris species group, although it is quite genetically divergent and apparently diverged early in the history of the species group. We therefore analyse a number of species from the albolabris species group, as well as representatives of the other three species groups, together with one specimen of T. kanburiensis, and four specimens of T. venustus, including all three specimens from the Khao Luang area with 19 scale rows at mid-body. A full list of sequences included and their origins is listed in Table 1.

The coding sequences were first translated into amino acid sequences using MEGA version 2.1 (Kumar *et al.*, 2001) to check for the unexpected occurrence of stop codons which might indicate amplification of psuedogenes. The possibility of non-neutral evolution was tested using a variety of tests implemented in the program DnaSP 3.51 (Rozas & Rozas, 1999), including McDonald and Kreitman's (1991) test, Fu and Li's D* and F*, and their modifications for use with an outgroup sequence (Fu & Li 1993), and Tajima's D (Tajima, 1989).

We used both unweighted parsimony and Bayesian Markov Chain Monte Carlo (MCMC) approaches to reconstruct phylogenies, using PAUP* 4.0b8 (Swofford, 2001) and MrBayes (Huelsenbeck & Ronquist, 2001) respectively. We first checked the dataset for homogeneity of base composition among taxa, to detect problems with the assumption of a similar underlying substitutional model. Parsimony searches were heuristic, with starting trees obtained by random addition with 100 replications, and tree-bisection-reconnection (TBR) branch swapping. Confidence in the inferred branches of the optimal trees was obtained by bootstrapping (1000 replication) with the search strat-

Species	Cat.	Location	GenBank accession
			nos. (cyt <i>b</i> , ND4, 125)
Trimeresurus venustus	A74	S Thailand, Nakhon Si Thammarat Pr. (Khao Luang)	AY289224, AY289230, AY289218
T. venustus	A75	S Thailand, Nakhon Si Thammarat Pr. (Khao Luang)	AY289223, AY289229, AY289217
T. venustus	A249	S Thailand, Nakhon Si Thammarat Pr. (Khao Luang)	AY289234, AY289233, AY289235
T. venustus	A237	S Thailand, Nakhon Si Thammarat Pr. (Thung Song)	AY289222, AY289228, AY289216
T. venustus	A241	S Thailand, Nakhon Si Thammarat Pr. (Thung Song)	AF171914, to come
T. kanburiensis	B522	Kanchanaburi Province	AFI 7194, AY 289231, AY 289219
T. macrops	B27	Bangkok '	AF517184, AF517219, AF517163
T. macrops	B161	Champassak Prov., S Laos	AY289221, AY289227, AY289215
T. stejnegeri	A160	Taiwan, Taipei county	AFI 71896, AY059593, AY059539
T. gumprechti	A164	NE Thailand, Loci Province	AF171898, AF157224, AF517168
T. vogeli	B97	NE Thailand, Nakhon Ratchasima Pr.	AY059574, AY059596, AY059546
T. flavomaculatus	B3	Philippines	AF171916, AY059584, AY059535
T. hageni	B33	Thailand, Songkhla Province	AY059567, AY059585, AY059536
T. albolabris	B6	Indonesia, W Java	AF517186, AF517213, AF517158
T. albolabris	B22	Nonthaburi, C Thailand	AF517189, AF517221, AF517165
T. albolabris	B47	Phetburi Province, W Thailand	AF517187, AF517216, AF517160
T. albolabris	A229	N Thailand, Pha Yao Province	AY059566, AY059583, AY059544
T. purpureomaculatus	A83	Satun province, S Thailand	AF517188, AF517218, AF517162
T. septentrionalis	A100	Nepal, Mahattari District	AF171909, AY059592, AY059543
T. insularis	B7	Indonesia, West Timor	AY059568, AY059586, AY059534
T. popeiorum	A203	S Thailand, Nakhon si Thammarat Pr.	AF171904, AY059588, AY059537
T. malabaricus	A218	S India, Tamil Nadu State	AY059569, AY059587, AY059548
T. trigonocephalus	A58	SW Sri Lanka	AF171890, AY059597, AY059549
Tropidolaemaus wagleri	B132	W Malaysia. Perak	AF517191, AF517223, AF517167
Protobothrops mucrosquamatus	B165	N Vietnam, Nghe An province	AY289226, AY289232, AY289220

TABLE 1. List of specimens used in the phylogenetic analysis, their geographic origin and GenBank accession numbers. Cat represents the author's specimen catalogue number.

egy modified to use only 10 replication of the start tree. In the Bayesian analysis, start trees were random, and the time reversible model with gamma distributed rates was used. Four markov chains (three heated and a single cold chain) were run for 1 378 300 generations, sampling every 100 trees. The likelihood scores of all 13 783 sampled trees were plotted against generation number and the first 215 trees, representing the burnin period before the likelihood scores approached stationarity, were discarded. A majority-rule consensus tree of the remaining 13 568 trees was produced in PAUP* 4.0, and the percent of the time that the clade occurs among the sampled trees then represents the posterior probability of that clade existing.

MORPHOMETRIC ANALYSIS

Some specimens were obtained in the field, and morphometric measurements and macro-photographs were taken while the specimens were anaesthetised. As well as the five specimens of T. kanburiensis mentioned above, another female specimen (UF 61846) collected in 1985 from Prathat caves in Erawan National Park near Kanchanaburi (Fig. 1) but mistakenly identified as T albolabris, was correctly identified by the senior author during studies of T. albolabris. This brings the final number of T. kanburiensis specimens from the type locality to six. All specimens used are listed in Appendix 1. Two of the three specimens collected in the Khao Luang area were roadkills; one was too badly damaged to be included in the morphological analysis, while another allowed most scalation characters but not internal or head dimension characters to be recorded. Two specimens without locality data but with 21 scale rows at mid-body, were also included in the analysis.

A list of characters used and their abbreviations can be found in Appendix 2. All morphometric analyses were carried out in BMDP Dynamic v. 7.0 (BMDP Statistical Software Inc., Cork, Ireland). Missing values were substituted with the group mean (for non-allometric characters; the south Thailand group excluded the specimens with 19 scale rows at mid-body). For allometric characters, inserted values were estimated by regression using correlated linear measurements. Missing values were only filled in for characters in which no more than 50% of specimens in that group had missing values. Characters were then checked for significant between-group variation and sexual dimorphism with a two-way analysis of variance (ANOVA) and co-variance (ANCOVA). In the case of characters measuring banding pattern, specimens with no discernible banding (i.e. BAND = 0) were excluded, since it seems likely that the absence of pattern was due to preservation effects. If no significant sexual dimorphism was present, all specimens could be analysed together; a considerable advantage given the small sample size. The assumption of homogeneity of variance was checked using Levene's test, and the Brown-Forsythe variant of the ANOVA, which relaxes this assumption, was used where it was violated (Brown & Forsythe, 1974).

A principal component analysis (PCA) was then carried out on the characters showing significant between-group differences, excluding those that showed sexual dimorphism in the case of joint analyses of both sexes. Although having less discriminatory power than methods that define a priori groups such as canonical variate analysis (CVA), PCA is to be preferred in this case since we do not wish to prejudge the distinctness of the groups. However, PCA does not take between-character correlations into account, so all size-related characters were first adjusted to a common size using the pooled within-group slope, with either snout-vent length (SVL) or head length (LHEAD) as the covariate. Included characters were screened for high correlations with each other (r > 0.7), which would indicate that they do not provide independent information. In CVA, these correlations are taken into account, but in PCA they may result in over-emphasis of the correlated variables (Thorpe, 1983). If this was found, only one of the characters from the correlated character sets was used in PCA.

Adding internal characters can substantially improve the resolution of taxa (Thorpe, 1979, 1989). However, internal characters are not particularly useful for identification in many situations and also substantially reduce the sample size, as internal data were not available for many specimens. Given the generally poor condition of many of the specimens from Kanchanaburi, many headshape characters are also missing in this group. Therefore, two sets of analyses were carried out, the first included scalation and colour pattern characters only, and the second included all types of characters. If the PCA indicated that the groups were distinct, a discriminant function analysis was carried out, using the same subsets of data as in the PCA, in order to find the characters most useful at distinguishing them.

RESULTS

PHYLOGENETIC ANALYSIS

The final data set consisted of 26 taxa and 1612 base pairs of sequence data (587 bp from cyt b, 600 bp from ND4, and 425 bp from 12S). The coding sequences translated into amino acid sequence without the occurrence of stop codons, and were easily aligned by eye. The chi-square test for the homogeneity of base frequencies showed that base composition was not significantly different in all taxa included (P=0.99). None of the neutrality tests showed a significant departure from neutrality. The mean likelihood score was -9419.2581.

The mean values for the parameters of the model, estimated by the program, were: base frequencies (A: 0.33704, C: 0.32727, G: 0.11341, T: 0.22228), alpha = 0.214011.

Most branches in the group of interest were highly supported. These show that while all specimens from south Thailand are closely related, regardless of whether they have 19 or 21 scale rows at mid-body, they are very distinct from the specimen from K anchanaburi Province and indeed are more closely related to *T. macrops* (Fig. 2). Trimeresurus venustus is supported as a distinct species by this analysis. The relationships of these three species with the other species groups is unresolved in this analysis, however, and is addressed in a larger analysis of *Trimeresurus s.s.* that includes more species (Malhotra & Thorpe, 2004).

MULTIVARIATE MORPHOMETRICS

ANOVA and ANCOVA showed that only a few characters that were significantly different between localities were also significantly sexually dimorphic. These included SCS, TAIL, CLOPOST, VS19 to 17, HTANT, and HTPOST. Therefore, to maximise sample size, both sexes were analysed together excluding these characters. To check the effect that these characters had on the discrimination, a parallel analysis on just females, with these characters included, was conducted. In no case did they noticeably affect the results, and these analyses are not discussed further. The final scalation and colour pattern character set for PCA included VS21 to 19, DV17 to 15, BTWSUP2, LAB3, ROST, GEN-IAL, VENTEDGE, and SCR1. Unfortunately, one of the Khao Luang specimens (BMNH 2002.52) had some damage to the rostral scale and could not be included with this dataset. Since the position of these specimens is important, the analysis was repeated with ROST re-



FIG. 2. Bayesian evolutionary hypothesis of the relationships between *T. kanburiensis*, *T. venustus* and *T. macrops*. Despite the close morphological similarity between the former two species, *T. venustus* is actually most closely related to *T. macrops*. Branch lengths are proportional to amount of evolution, and are averaged over all trees. Figures at nodes indicate posterior probabilities (first) of the clades from the Bayesian analysis, followed by parsimony bootstrap support values.

moved, so that both specimens could be included. This had the effect of slightly reducing the separation between the groups, but otherwise the difference was negligible. While the resulting discrimination clearly separated the western and southern population with 21 scale rows, the intermediate position of the specimens from the south with 19 scale rows cast doubt on their distinctness (Fig. 3a). Since the number of scales at midbody was influential in the ordination, we investigated whether the Khao Luang specimens most resembled T. kanburiensis or T' venustus in other characteristics. This was done by repeating the analysis with this character (VS21 to 19, which actually measures the position along the body where the reduction from 21 to 19 scale rows occurs) removed. The only marked effect on the discrimination was to shift the two Khao Luang specimens towards the other southern specimens (Fig. 3b), thus clearly indicating that in other respects they were clearly identifiable as T. venustus. Removal of VS19 to 17 also has a marked effect on the CVA, with the Khao Luang specimens grouping with the specimens from western Thailand when it was included, but with the other southern specimens when it was excluded. Finally, inclusion of internal characters (DENT, RKPOST, LKPOST) substantially improved the discrimination of the two species in the PCA (Fig. 3c) and CVA (not illustrated). In all analyses, the two specimens from an unknown locality clearly grouped with other T. venustus specimens as might be expected from their mid-body scale counts. These were therefore included within the southern Thailand population for the CVA.

The CVA allowed us to assess which characters contribute to the discrimination of *T. venustus* and *T. kanburiensis*, as well as their mean value, and range. Since the use of internal characters substantially reduces sample size, the results of the analysis of external characters are used in preference. However, internal characters that are important in the discrimination when all characters are used are also listed. It can be seen that the difference in any single character is subtle, with largely overlapping ranges (Table 2).

T. venustus is distinguished from T. kanburiensis in both sexes by a scale reduction from 17 to 15 scale rows (DV17to15) that involves scale rows higher on the body in T. kanburiensis than in T. venustus (Table 2), a smaller light blotch on the first dorsal scale row in T. kanburiensis than in T. venustus (SCR1), a rostral scale with a higher ratio (a squarer shape) in T. venustus than in T. kanburiensis (ROST). There also tends to be a higher number of gulars (GULAR), fewer scales between the rear edges of the supraoculars (BTWSUP2), and at least one scale separating the third supralabial and the subocular (LAB3) is more likely to be present in $T_{\rm c}$ kanburiensis than in T. venustus. Of the internal and head dimension characters, only the length of the head (LHEAD) serves to separate the two species, with T. kanburiensis having a relatively longer head than T. venustus. Of the sexually dimorphic characters that are also significantly different between groups, only the



FIG. 3. Plot of first two principal components for both sexes. a) external characters only, including VS21to19; b) external characters excluding VS21to19; c) external and internal characters. Circles: *T. venustus* (with 21 scale rows at mid body); triangles: southern specimens with 19 scale rows; squares: *T. kanburiensis*. Empty symbols indicate females and filled symbols indicate males. The two specimens of unknown locality are indicated by arrows in 3a and are clearly assignable to *T. venustus*. They are therefore not highlighted in the remaining figures.

number of subcaudal scales is important, and tends to be higher in *T. venustus*.

Of the characters that help to distinguish between the sexes of both species (Table 3), the most important is relative tail length (TAIL), which is longer in males than females. The number of teeth on the pterygoid bone (PTER Y) is also sexually dimorphic with higher numbers in males than females. The scale reduction from 8 to 6 rows on the tail (SC8to6) occurs further down the tail in males than females, which is associated with the length of the tail, the ventral surface tends to be more heavily blotched and speckled in males than females, and the position of the front and rear edges of the liver (LVANT, LVPOST) occur more posteriorly in males than females, as does the anterior edge of the left kidney (LKANT).

DISCUSSION

EVALUATION OF DIAGNOSTIC CHARACTERS.

Of the characters stated in the description (Vogel, 1991) to distinguish T. venustus from T. kanburiensis, we have shown that the number of scale rows at midbody is not diagnostic. It is also stated to be slighter in body shape than T. kanburiensis. The analysis shows that this is only discernible in the slightly longer head of 7. kanburiensis, which in practise may not be a very useful diagnostic character. Vogel (1991) also states that T. venustus lacks the indented, divided and very broad supraoculars found in T. kanburiensis. However, in none of the specimens analysed were the supraoculars actually divided. The width (and length) of the supraoculars was also not significantly different between the two species. The irregularity of the inner edge of the supraoculars was not included in the analysis because it is difficult to measure in an objective manner. While it does appear to be more irregular in the specimens of T. kanburiensis examined than in T. venustus, this is not always true. For example, the T. kanburiensis specimen BMHN 1987.943 has broad supraoculars with a smooth margin, while T. kanburiensis specimens BMNH 1988.385 and BMNH 2002.51 have only slightly indented supraoculars on one side. Many of the T. venustus specimens examined had at least slightly indented and irregular inner edges to the supraoculars, and so this character may only be useful when expressed in its extreme form in each species. Finally, the species were said to differ in colour pattern, in the presence of bands and in the ventral surface being more blotched in T. venustus, whereas T. kanburiensis was unbanded and had a plain or speckled ventral surface. However, these aspects of colour pattern can now be shown to be an artefact of small sample size. All the specimens of T. kanburiensis from which Vogel obtained this information have a very faded colour pattern and are females. The new specimens indicate that banding is the norm in T. kanburiensis as well, and although pictures of the live specimens suggest that the colour of the bands may be slightly different in the two species, this cannot be confirmed by analysis at present. The difference in ventral

	-			
Character	T. venustus	п	T. kanburiensis	п
DV17to15	3.38 (3.0 - 5.0)	20	4.0 (3.5 – 4.5)	6
SCR1	0.39(0.1 - 0.8)	20	0.17(0.0-0.2)	6
ROST	0.45 (0.33 - 0.58)	17	0.33(0.22 - 0.47)	6
GULAR	6.9 (5.5 - 9.5)	20	8.6 (7 – 9.5)	6
BTWSUP2	12.85 (11.0 - 16.0)	20	10.33 (9.0 - 12.0)	6
LAB3	0.15(0-1)	20	0.58(0-1)	6
LHEAD	19.74 (17.9 – 23.6)	19	21.62 (19.0 - 22.6)	5
SCS (fernales only)	56.08 (50 - 66)	13	51.25 (50 - 52)	4

TABLE 2. Mean values and range of morphological characters important in multivariate discrimination between *T. venustus* and *T. kanburiensis*. Size-related characters are adjusted to a common size of SVL (47.0 cm). Characters are listed in order of magnitude of their contribution to the discriminant function, and their abbreviations are explained in Appendix 2.

coloration appears to be due to sexual dimorphism rather than being a diagnostic difference. Thus, it seems that the diagnosis and characteristics of the two species requires redefinition.

REDESCRIPTION OF VARIATION WITHIN T. KANBURIENSIS

Colour in preservative. The banding pattern seems to be easily lost especially after some time in formalin (Warrell et al., 1992), leaving a uniformly brown dorsal and lateral coloration. In these specimens, the ventral surface appears white, although it is still possible to detect a pale blotch on the first dorsal scale row, and some darker pigment extending onto the edge of the ventral scales. The darker colour also extends onto the sublabial scales and some of the scale rows between these and the genial scales, although it may be patchy. In better preserved specimens, darker blotches are visible on the head and form irregular bands on the body.

Colour in life. This description is based on a specimen freshly preserved in alcohol and on pictures of two living males and one female specimen from the same population. The ground colour of the body is a shade of olive or greyish green. The head is heavily blotched with dull brown or orange brown, and bands of the same colour occur on the body. The ventral surface is creamy white. Brown pigment encroaches onto the edges of

TABLE 3. Mean values and range of morphological characters in *T. venustus* and *T. kanburiensis* which do not discriminate among species but distinguish the sexes. Data are for the maximum number of specimens available for that character. Size-related characters are adjusted to a common size of SVL (42.0 cm). Characters are listed in order of magnitude of the F-ratio in the ANOVA/ANCOVA.

Character	Male	п	Female	п
TAIL	9.15 (± 0.23)	7	6.83 (± 0.22)	15
PTERY	14.8 (14-16)	6	13.2 (12–14)	9
SC8to6	16.4 (14.0–18.5)	8	9.9 (5.0–15.5)	18
DARKVE	NT 64.4 (0–100)	8	43.1 (0-100)	18
LVANT	63.25 (62-64)	4	61.6 (58-66)	9
LKANT	138.75 (133–142)	41	35.5 (125–145)	8
LVPOST	96.5 (91-99)	4	95.1 (93–103)	9

some of the ventrals. This is more extensive in males than females, in which the encroachment is just a sprinkling of brown spots. Males and females both have white or bluish spots on the first dorsal scale row, the overall effect being a blurred margin between dorsal and ventral scales. Labial scales are also scattered with white or bluish pigment, and in males are boldly marked with two or more patches of dark pigment. A broad and irregular dark stripe extends from the rear edge of the eye, again this is bolder in males than females. The eye is light orange heavily speckled with brown. The tail is similarly patterned to the body but the colour of the bands is brighter than on the rest of the body. One of the males has a series of white vertebral spots.

Morphometric and meristic characters. Females have relatively shorter tails than males, and this is also reflected in the number of subcaudals (ranging in females between 50-52, compared to 61 in the male. Maximum recorded size for females is 58.2 cm SVL compared to 41.5 cm for the male. The number of ventral scales varies between 172 and 177 in both sexes. Body scales are keeled, although this may vary from weak to strong. Temporal scales and scales on the side of the head between the temporals and supralabials are also usually keeled, as are scales on the rear of the head. The ratio of the upper and lower edges of the rostral scale varies between 0.2 and 0.5 (note that this character is not noticeably allometric as it is not correlated with any linear measurement on the head or body). Supralabials vary between 10 and 11 and sublabials between 11-13. The minimum number of scales between supraoculars varies between 7-9 and there are 9-12 scales between the posterior edges of the supraoculars. The number of scales between the nasals and shield bordering the anterior of the pit varies between 0 - 1 and there may be up to one scale between the internasals. The third supralabial may be separated from the subocular by up to one scale, but there is always one scale between the fourth and fifth supralabials and the subocular. The supraoculars may be heavily indented on the inner margin with the adjacent head scales, making the edge appear very uneven and irregular. However, a few specimens have regular supraoculars, and they may also be very broad. A few specimens have a suture run-



FIG. 4. Comparison of scalation features of the head in a) *Trimeresurus kanburiensis* and b) *Trimeresurus venustus*. There tend to be fewer scales between the rear edges of the supraoculars, and there is more likely to be at least one scale between the third supralabial and the subocular, in *T. kanburiensis* than in *T. venustus*. Although the latter is not apparent in the specimen pictured, there is a distinctly narrower portion of the first labial in contact with the subocular than in *T. venustus*. In addition, note that the inner edges of the supraoculars are not necessarily more indented in *T. kanburiensis* than in *T. venustus*, contrary to the original description of *T. venustus*.

ning partially across the scale, but the supraoculars are never completely divided. There are always 19 scale rows at mid-body, with the reduction from 21 to 19 scale rows occurring between 10 and 28% of the distance between the first ventral scale and the anal scale. Some of these scalation patterns are illustrated in Fig. 4a. There are 2-3 postocular scales, 5-8 scales bordering the subocular scale (not counting the pre- and post-oculars) and 12-14 teeth on the pterygoid and 12-15 on the dentary bone.

REDESCRIPTION OF VARIATION WITHIN T. VENUSTUS

Colour in preservative. Some specimens have a uniformly dark dorsal and lateral coloration. This is almost certainly a preservation effect since no specimen of T. venustus without bands has ever been found (although one aberrant striped individual has been recorded; Gumprecht, 2002b). The ventral surface appears white, with a pale blotch extending onto some scales of the first dorsal scale row, and some darker pigment extending from the dorsal surface onto the edge of the ventral scales. In better preserved specimens, darker blotches are visible on the head and form irregular bands on the body. Supralabials are lighter than the rest of the head, but may have a number of darker blotches (usually no more than one). Sublabial scales are generally white and unmarked, although in some specimens they appear darker.

Colour in life. This description is based on macrophotographs of five specimens (one male, four females) captured alive in south Thailand. The ground colour of the body is dull olive or bluish-green (male) to grass-

green (female). The head is heavily blotched with dull brown, and bands of the same colour occur on the body. The ventral surface is a similar, slightly lighter colour. than the dorsal surface. Brown pigment encroaches onto the edges of some of the ventrals, although the extent of the encroachment is variable and some females have virtually immaculate ventral scales. Males and females both have a white spot on the first dorsal scale row, which is more regular than in T. kanburiensis and therefore forms a clear margin between the dorsal and ventral scales although in the male there are also white flecks on some ventral scales. The labials are the same green colour as the rest of the dorsal surface, and in both sexes are boldly marked with at least one brown patch. A broad and irregular dark stripe may extend from the rear edge of the eye, not appearing to be different in intensity in males and females. The eye is light orange heavily speckled with brown. The tail is patterned and coloured identically to the body.

Morphometric and meristic characters. Females have relatively shorter tails than males, also reflected in the number of subcaudals (ranging in females between 50-66, compared to 68-72 in males). Females reach a slightly larger size than males but this is not a marked difference (maximum recorded 48.6 cm SVL compared to 43.4 cm for males). The number of ventral scales varies between 166 and 183 in both sexes. Body scales are always keeled, with a range from weak keeling to strong keeling. Temporal scales, scales between the temporals and supralabials, and scales on the rear of the head are also weakly to strongly keeled. The ratio of the upper and lower edges of the rostral scale varies between 0.3 and 0.6 (note that this character is not noticeably allom-

etric as it is not correlated with any linear measurement on the head or body). Supralabials vary between 9 and 11 and sublabials between 10-13. The minimum number of scales between supraoculars varies between 6-10 and there are 11-16 scales between the posterior edges of the supraoculars. The number of scales between the nasals and shield bordering the anterior of the pit varies between 0 and 1, and there may be 0-1 internasal scale. There may be up to 1 scale between the third and fourth supralabial and the subocular, and 1-2 scales between the fifth supralabials and the subocular scale. The inner edge of the supraoculars may be smooth or slightly indented by the adjacent head scales (especially towards the rear of the scale). There may be 21 or 19 scale rows at mid-body, with the reduction from 21 to 19 scale rows occurring between 59 and 67% of the distance between the first ventral scale and the anal scale in the former case, or between 4.5 - 23% of this distance in the latter. There are 1-4 postocular scales, 5-8 scales bordering the subocular scale (not counting the pre- and post-ocular's) and 12-16 pterygoid and 13-16 dentary teeth. Some of the head scalation patterns are illustrated in Fig 4b.

DIAGNOSIS

T. kanburiensis can best be distinguished from T. venustus by its coloration, which is always less saturated in the former (Fig. 5). The ventral colour is white or cream rather than a shade of green. Males also have more boldly marked labial scales in T. kanburiensis. The white lateral stripe is less obvious in T. kanburiensis, appearing rather as a blurring of the boundary between dorsal and ventral coloration. There is no single scalation character that can unequivocally distinguish between the two species. However, the rostral tends to be more triangular in shape, there tends to be fewer scales between the rear edges of the



FIG. 5. Top: Male *T. kanburiensis* from Kanchanaburi province, Thailand. Photo: Kamphol Udomritthiruj (AquariCORP, Thailand). Bottom: female *T. venustus*, Nakhon si Thammarat province, Thailand (A. Malhotra).

supraoculars, and more likely to be at least one scale between the third supralabial and the subocular, in *T. kanburiensis* than in *T. venustus*. The scale reduction from 17 to 15 scale rows (the most posterior on the body) is more likely to involve higher scale rows in *T. venustus* than in *T. kanburiensis*.

COMPARISONS

Although this paper is focused primarily on the species T. kanburiensis and T. venustus, Vogel (1991) has provided diagnostic characters to separate T. venustus from some other species with which it co-occurs and/or has been confused in the past. These include T. purpureomaculatus, T. erythrurus, T. macrops and T. albolabaris, T. popeiorum, T. stejnegeri and T. sumatranus. The first four of these are all members of the albolabris group (sensu Malhotra & Thorpe, 2000). Vogel (1991) stated that 7'. venustus could be separated from members of the albolabris group by separation of the first supralabial shield from the nasal shield. However, this is incorrect. In fact it is very rare that these scales are not fused to some extent in both T. venustus and T. kanburiensis. Only one out of the 26 specimens examined had a completely divided nasal and first labial scale, and even this only showed this character state on one side. Thus this character actually serves to diagnose T. venustus/T. kanburiensis from all other Trimeresurus species except members of the albolabris group. Trimeresurus purpureomaculatus is very distinctive and Warrell et al. (1992) have provided a table of characters that serves to distinguish this species from T. venustus/ T. kanburiensis. Trimeresurus erythrurus can be distinguished by a higher number of scales at mid body (23 and above), and is not currently known to overlap in range with T. kanburiensis, although this is possible. However, confusion with T. albolabris and T. macrops is still likely, especially in preserved specimens of T. venustus which have 21 scale rows at mid-body and in which the distinctive banding pattern has faded. Trimeresurus albolabris may be distinguished by its narrow supraoculars and larger head, but T. macrops is similar to T. venustus in head shape and in having broad supraoculars. Trimeresurus macrops can be distinguished by a number of scalation differences (Table 4) such as the scale reduction from 10 to 8 rows on the tail (SC10to8) and from 6 to 4 rows (SC6to4) occurring further towards the tip and a lower ventral scale count (VSC). Although the range of T. macrops does not appear to overlap with that of either T. kanburiensis or T. venusius according to Regenass & Kramer (1981), in fact it has a much wider distribution (Viravan et al., 1992; personal observation).

DISTRIBUTION AND NATURAL HISTORY

Based on verifiable records, *T. venustus* and *T. kanburiensis* are presently known only from Thailand. *Trimeresurus kanburiensis* is known from the western province of Kanchanaburi, which is on the border with Myanmar, and it seems likely that it will also be found in

			C		
Character	T. venustus	n	T. macrops	n	
SC10to8	5.15 (2.9-8.3)	17	9.17 (5.8–13.6)	15	
DV17to15	3.28 (3-4)	17	4.26 (3.5-4.5)	15	
VSC	174.4 (166–183)	17	166.1 (159–173)	15	
BSCK	0.9(0.5-1)	17	0.6 (0.5 – 1)	12	
VENTEDGE	8.2 (7-9)	17	7.4 (7-8.5)	14	
SC6to4	62.7 (50.9-71.8)	17	73.0 (60.1-85.9)	15	
SOCBORD	6.4 (5.5-8)	17	7.2 (5.5–9)	15	
Dv21TO19	3.9 (3.5-5)	17	4.3 (3-5)	15	

TABLE 4. Mean values and range of scalation characters distinguishing between T. venustus (with 21 scale rows at mid-body) and T. macrops (from central and northeastern Thailand), but are not sexually dimorphic. Data are for the maximum number of specimens available for that character. Characters are listed in order of magnitude of the F-ratio in the ANOVA/ANCOVA.

Myanmar eventually. Although precise localities are not available for some specimens, all specimens with known localities come from a relatively short distance around the provincial capital Kanchanaburi. In the course of searching for the species, we found very few villagers who recognised pictures of them; those who did were engaged in bamboo cutting in the limestone hills. This is in accord with the details in Warrell *et al.* (1992) which describes the case history of a woman who was bitten by this species while cutting bamboo. The scarcity of the species until recently is more likely to be due to the fact that they are confined to higher parts of these hills, and may also have a limited activity period since these hills get extremely arid during the dry season.

T. venustus is currently only known from the southern provinces of Nakhon Si Thammarat, Surat Thani and Krabi. Considering the popularity of T. venustus in captivity, it is surprising how little has been written about its natural history, possibly because almost all captive specimens have been bought from dealers. In December 1997, the authors were taken to a limestone outcrop near Thung Song, where local people often find the snakes while collecting forest produce. After a short but difficult vertical climb, four specimens were found in a very small, slightly more level, area. All were coiled in an alert posture on rocks, and did not seem alarmed by our arrival. A final specimen was found under a rock near the base of the outcrop on our descent. This suggests that the species are primarily ground-living ambush hunters, and that they are not high altitude species as suggested by Vogel (1991). Although some specimens have been found on the outskirts of the Khao Luang massif, these were not at any great elevation. One was found at the base of a tall limestone cliff, the others (roadkills) were found on narrow roads passing through rubber plantations. Bulian (2001) describe his observations of the species, which largely agree with our observations above. He also adds that they can be found up to 700 m above sea level, although much more common at lower altitudes, and can be found in high densities in particular localities, often narrow shaded and humid valleys with rocky substrate. He mentions that they are mostly diurnal, although they may also be active during the night, and are particularly active after rain.

It has been speculated that the presence of limestone hills almost throughout the peninsula between the locations of the known distributions of the two species suggests that they may also be present here. In this case, it would be interesting to see where the distributions of the two species meet. However, increasing numbers of herpetological surveys in the region (Grossman & Tilllack, 2002; Pauwels *et al.*, 2000) have so far failed to find any evidence of these species in other southern provinces.

Note added in proof. Since this paper was accepted, changes to the taxonomy of the species discussed within have been made. The new generic names can be found in Malhotra & Thorpe (2004).

ACKNOWLEDGEMENTS

We are grateful to the large numbers of people who have assisted us in the field, or supplied us with tissue samples for analysis. These include Lawan Chanhome (Queen Savoabha Memorial Institute, Thailand), Jenny Daltry (Flora and Fauna International), Wolfgang Wüster (University of Wales Bangor), Tanya Chan-ard, Jarujin Nabhitabhata (National Science Museum of Thailand), Kumthorn Thirakhupt and Peter Paul van Dijk (Chulalongkorn University, Thailand), Kamphol Udomritthiruj (AquariCORP, Thailand), Merel J. Cox, and Jonathan Murray. We also thank Kamphol Udomritthiruj for providing details of coloration and photographs of live T. kanburiensis, Gernot Vogel for making some of his personal collection of *T. venustus* available to us for examination, and Andreas Gumprecht for providing a translation of papers in German. We gratefully acknowledge the National Science Council of Thailand for permission to carry out fieldwork. This study was funded by the Leverhulme Trust (F174/I and F174/0), the Wellcome Trust (057257/Z/99/Z and060384/Z/00/Z), and the Darwin Initiative (162/6/65), with additional support for fieldwork from the Royal Society, the Percy Sladen Trust, the Bonhote Trust, and the Carnegie Trust.

REFERENCES

Brown, M. B. & Forsythe, A. B. (1974). Robust tests for the equality of variances. *Journal of the American Statistical Association* 69, 364-367.

- Bulian, J. (2001). Hinweise zur Lebenweise und Haltung der Bambusotter *Trimeresurus kanburiensis*. *Reptilia* 6, 62-66.
- David, P. & Ineich, I. (1999). Les serpents venimeux du monde: systématique et répartition. *Dumerilia* **3**, 3-499.
- Dowling, H. G. (1951). A proposed system for counting ventrals in snakes. *British Journal of Herpetology* 1, 97-99.
- Fu, Y.-X. & Li, W.-H. (1993). Statistical tests of neutrality of mutations. *Genetics* 133, 693-709.
- Golay, P., Smith, H. M., Broadley, D. G., Dixon, J. R., McCarthy, C., Rage, J. C., Schätti, B., & Toriba, M. (1993). Endoglyphs and other venomous snakes of the world: a checklist. Geneva: Azemiops S.A., Herpetological Data Centre.
- Grossman, W. & Tillack, F. (2000). Bemerkungen zur Herpetofauna des Khao Lak, Phang Nga, thailändische Halbinsel Teil I: Einführung; Amphibia; Reptilia: Sauria. Sauria 22, 23-38.
- Gumprecht, A. (2002a). Trimeresurus kanburiensis. Reptilia 7, 4-5.
- Gumprecht, A. (2002b). Eine ungewöhnliche Zeichnung bei *Trimeresurus kanburiensis* Smith, 1943. Sauria 24, 45-46.
- Huelsenbeck, J. P. & Ronquist, F. R. (2001). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17, 754-755.
- Knight, A. & Mindell, D. P. (1993). Substitution bias, weighting of DNA sequence evolution, and the phylogenetic position of Fea's viper. Systematic Biology 42, 18-31.
- Kumar, S., Tamura, K., Jakobsen, I. B., & Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. Tempe, Arizona: Arizona State University.
- Malhotra, A. & Thorpe, R. S. (1997). New perspectives on the evolution of South-East Asian pit-vipers genus Trimeresurus from molecular studies. In Venomous Snakes: Ecology, Evolution and Snakebite, 115-118. Thorpe, R. S., Wüster, W. & Malhotra, A. (Eds.). Symposia of the Zoological Society of London 70, Oxford: Clarendon Press.
- Malhotra, A. & Thorpe, R. S. (2000). A phylogeny of the *Trimeresurus* group of pit-vipers: new evidence from a mitochondrial gene tree. *Molecular Phylogenetics and Evolution* 16, 199-211.
- Malhotra, A. & Thorpe, R. S. (2004). A phylogeny of four mitochondrial gene regions suggests a revised taxonomy for Asian pit vipers (*Trimeresurus* and *Ovophis*). *Molecular Phylogenetics and Evolution* 32, 83-100.
- McDiarmid, R. W., Campbell, J. A., & Toure, T. A. (1999). Snake species of the world. A taxonomic and geographic reference. Volume 1. Washington D.C.: Herpetologist's League.
- McDonald, J. H. & Kreitman, M. (1991). Adaptive protein evolution at the *Adh* locus in *Drosophila*. *Nature* **351**, 652-654.
- Parkinson, C. L., Zamudio, K. R. & Greene, H. W. (2000). Phylogeography of the pitviper clade *Agkistrodon*: historical ecology, species status, and conservation of cantils. *Molecular Ecology* 9, 411-420.

- Pauwels, O. S. G., Laohawat, O.-A., David, P., Bour, R., Dangsee, P., Puangjit, C. & Chimsunchart, C. (2000). Herpetological investigations in Phang-Nga Province, southern Peninsular Thailand, with a list of reptile species and notes on their biology. *Dumerilia* 4, 123-154.
- Regenass, U. & Kramer, E. (1981). Zur Systematik der grünen Grubenottern der Gattung *Trimeresurus* (Serpentes, Crotalidae). *Revue Suisse de Zoologie* 88, 163-205.
- Rozas, J. & Rozas, R. (1999). DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* 15,174-175.
- Sambrook, J., Frisch, E. F. & Maniatis, T. E. (1989). Molecular cloning: a laboratory manual. Second ed. New York: Cold Spring Harbour.
- Smith, M. A. (1943). The Fauna of British India, Ceylon and Burma, including the whole of the Indochinese sub-region. Reptilia and Amphibia. Vol. 3, Serpentes. London: Taylor & Francis Ltd.
- Swofford, D. L. (2001). PAUP*: Phylogenetic Analysis using Parsimony (*and Other Methods). Version 4.0b8. Sunderland, Massachussetts: Sinauer.
- Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123, 585-595.
- Thorpe, R. S. (1979). Multivariate analysis of the population systematics of the ringed snake Natrix natrix. Proceedings of the Royal Society of Edinburgh 78B, 1-62.
- Thorpe, R. S. (1983). A review of the numerical methods for recognising and analyzing racial differentiation. In: Numerical Taxonomy: Proceedings of a NATO Advanced Studies Institute, 404-423. Felsenstein J. (ed.) NATO ASI Series, Vol. Gl. Berlin: Springer-Verlag.
- Thorpe, R. S. (1989). Multivariate analysis of geographic variation: pattern congruence of six character systems in relation to character number. *Copeia* **1989**, 63-70.
- Viravan, C., Looareesuwan, S., Kosakarn, W., Wuthiekanun, V., McCarthy, C. J., Stimson, A. F., Bunang, D., Harinasuta, T. & Warrell, D. A. (1992). A national hospital-based survey of snakes responsible for bites in Thailand. *Transactions of the Royal Society* of Tropical Medicine and Hygiene 86, 100-106.
- Vogel, G. (1991). Eine neue Trimeresurus- Art aus Thailand, Trimeresurus venustus sp. nov. (Reptilia: Serpentes: Crotalidae). Sauria 13, 23-28.
- Warrell, D. A., Looareesuwan, S., Stimson, A. F. & Hutton, R. A. (1992). Rediscovery and redefinition of Malcolm Smith's *Trimeresurus kanburiensis* in Thailand, with a report of envenoming. *Transactions* of the Royal Society of Tropical Medicine and Hygiene 86, 95-99.

APPENDIX 1

SPECIMENS USED IN THE MORPHOLOGICAL ANALYSIS

Museum abbreviations are as follows: UF: State University of Florida at Gainesville; BMNH: the Natural History Museum, London; PSGV: Gernot Vogel's private collection; AFS: author's field collection number. Figures in parentheses are the number of males and females respectively. The holotype of *T. kanburiensis* is indicated in italics.

KANCHANABURI PROVINCE

No precise locality: *BMNH 194.6.1.8.91* (F); BMNH 2002.51; Erawan National Park (Prathat Caves): UF61846 (1F); SaiYok Military Camp: BMNH 1987.943 (F), BMNH 1992.535; Tanousri, 25 km NW of Kanchanaburi: BMNH 1988.383 (F).

NAKHON SI THAMMARAT PROVINCE

Thung Song: PSGV 220-222 (2M, 1F), BMNH 1988.384-386 (1M, 2F); BMNH 1987.944 (1F); AFS97B.8-12 (2M, 3F); Khao Luang area: BMNH 2002.52 (M), AFS4 (F).

KRABI PROVINCE

BMNH 1992.536-539 (1M, 3F).

NO LOCALITY

UF (uncatalogued) (2F).

APPENDIX 2

MORPHOLOGICAL CHARACTERS USED, AND THEIR ABBREVIATIONS.

(A) SCALATION

Scale counts recordable on both sides of the body were averages of right and left counts.

- VSC: the number of ventral scales (VS), not including anal scale, recorded by the Dowling (1951) method (i.e. the first VS is the one which contacts the first dorsal scale row on both sides).
- SCS: the number of pairs of subcaudal scales. Any unpaired scales are treated as a pair.
- SUPLAB : the number of supralabials on the left and right hand side.
- SUBLAB: the number of sublabials on the left and right hand side.
- POSTOC: number of postocular scales.
- PREOC: number of preocular scales.
- BORSUPOC: the number of scales bordering the supraocular scales, not counting pre- or post-oculars.
- BTWSUPOC1: the minimum number of scales between the supraoculars.

- BTWSUPOC2: the number of scales between the posterior edge of the supraoculars.
- **JNTNAS:** the number of scales separating the internasal scales.
- NASPIT: the number of scales between the nasal and the scale bordering the anterior edge of the pit (formed by the fused second supralabial and loreal scale).
- LABNAS: the degree of fusion of the first supralabial and nasal scale (1: fully fused, 0: not fused).
- LAB3: minimum number of scales separating 3rd supralabial and subocular.
- LAB4: minimum number of scales separating 4th supralabial and subocular.
- LAB5: minimum number of scales separating 5th supralabial and subocular.
- ROST: the ratio of the anterior margin of the rostral scale to the posterior margin.
- SOCBORD: the number of scales bordering the subocular scale (not including pre- or post-oculars).
- BSCK: the keeling of scales at mid-body.
- KTEMP: the keeling of the temporal scales.
- KHEADSC: the keeling of the scales on the back of the head.
- VENTEDGE: the number of scales between the edge of the mouth and the ventral scales, starting at and including the last sublabial.
- GULAR: the number of scales between the first ventral (see above) and second pair of sublabials (which meet ventrally).

(B) SCALE REDUCTION FORMULA

Recorded as a series of characters, each refering to a specific reduction. Each position will have two characters, the dorso-ventral (DV) position of the reduction (the lowest of the two merging scale rows), and the ventral scale (VS) position (counted from the head), which is the ventral scale to which the scale reduction traces diagonally. Before analysis, the VS position was transformed into the percentage of the total number of ventral scales (%VS), to control for variation.

- VS23to21: ventral scale position of the reduction from 23 to 21 scale rows.
- DV23to21: dorsoventral position of reduction from 23 to 21 scale rows.
- VS21to19: ventral scale position of the reduction from 21 to 19 scale rows.
- DV21to19: dorsoventral position of reduction from 21 to 19 scale rows.
- VS19to17: ventral scale position of the reduction from 19 to 17 scale rows.
- DV19to17: dorsoventral position of reduction from 19 to 17 scale rows.
- VS17to15: ventral position of the reduction from 17 to 15 scale rows.
- DV17to15: dorsoventral position of reduction from 17 to 15 scale rows.

- SC10to8: subcaudal scale position of the reduction from 10 to 8 scale rows.
- DV10to8: dorsoventral position of reduction from 10 to 8 scale rows.
- SC8to6: subcaudal scale position of the reduction from 8 to 6 scale rows.
- SC6to4: subcaudal scale position of the reduction from 6 to 4 scale rows.

(C) BODY DIMENSIONS

All measurements are made on the right side of the head only unless this was damaged, in which case they were done on the left.

- SVL: distance between the tip of the snout and the cloaca.
- TAIL: distance between the anterior edge of the first subcaudal scale and the tip of the tail.
- WHEAD: width of the head measured between the outer edges of the supraoculars.
- LHEAD: length of the head measured between the tip of the snout to the posterior edge of the lower jawbone.
- DEYE: diameter of the eye measured between the edges of the scales surrounding it.
- EYE2NOS: distance between the eye and the nostril, measured between the suture between the second and third preocular (from the bottom) and the inner edge of the nostril.
- WSUPOC: the width of the supraoculars measured in mm, at the widest part.
- LSUPOC: the length of the supraoculars measured in mm.
- WINTNAS: the width of the internasals (in mm).

(D) INTERNAL CHARACTERS

VS positions are transformed to % VS before analaysis (see scale reductions).

- PTERY: the number of pterygoid teeth.
- DENT: the number of dentary teeth.
- HTANT: VS position of the thyroid gland.
- HTPOST: VS position of the rear edge of the ventricle.
- LVANT: VS position of the anterior tip of the liver.
- LVPOST: VS position of the tip of the superficial lobe of the liver.
- RKANT: VS position of the anterior tip of the right kidney.
- CLOPOST: SC position of the posterior tip of the cloacal gland in the tail base (females only).
- (E) COLOUR PATTERN
- STRIPE: presence of a lateral stripe (0, asbent; 1, indistinct; 2, distinct).
- SCRSTR: number of scale rows involved in stripe.
- OCSTRIPE: presence of postocular stripe (0, absent; 1, indistinct; 2, distinct).
- SCROC: number of scale rows involved in postocular stripe.
- BAND: the number of bands (counted on the right side) between the head and vent.
- WBAND: the average width of three bands at mid-body, counted in numbers of scales covered.
- WGAP: the average width of the gap between three bands at mid-body, counted in numbers of scales covered.
- SCR1: the proportion of the first scale row covered by the light area.
- DARKVENT: the percentage of ventral scales with dark pigment.

THE INTER- AND INTRASPECIFIC STATUS OF AEGEAN MAUREMYS RIVULATA (CHELONIA, BATAGURIDAE) AS INFERRED BY MITOCHONDRIAL DNA SEQUENCES

GEORGIA MANTZIOU^{1,2}, NIKOS POULAKAKIS¹, PETROS LYMBERAKIS¹, EFSTRATIOS VALAKOS³ AND MOYSIS MYLONAS^{1,2}

Natural History Museum of Crete, University of Crete

²Department of Biology, University of Crete

³Section of Animal and Human Physiology, Department of Biology, University of Athens

The genus *Mauremys* (Chelonia, Bataguridae) is widely distributed throughout Asia, Europe and NW Africa. Three species (*Mauremys caspica, Mauremys rivulata* and *Mauremys leprosa*) are discontinuously distributed around the Mediterranean region. Present distributions are much smaller than those documented within the fossil record of *Mauremys* in the Mediterranean region. All three extant species are identified on the basis of morphology. In the present study we compare partial mitochondrial DNA sequences of cyt-*b* from 16 populations of *Mauremys rivulata* from Greece, one from Jordan (*M. rivulata*), two from Syria (*M. caspica*) and one from Morocco (*M. leprosa*). Comparison of cyt-*b* partial sequences supports the monophyly of the three species considered, as well as their proposed taxonomic status (i.e. separation at the species level). *Mauremys leprosa* is the most differentiated of the three, *M. caspica* and *M. rivulata* being more closely related. Climatic changes during the Pleistocene influenced the distribution of *M. rivulata* and resulted in a latitudinal oscillation of the populations in a north – south direction in Greece, and consequently in a mixing of their genetic material. This hypothesis is confirmed by the absence of correlation between genetic distances and geographical origin of the specimens studied.

Key words: Acgean region, cytochrome b, Mediterranean, phylogeography

INTRODUCTION

Mauremys caspica (Gmelin, 1774), Mauremys rivulata (Valenciennes, 1833) and Mauremys leprosa (Schweigger, 1812) are the sole European representatives of the diverse family Bataguridae. The family consists of 23 genera, distributed in the Palearctic region, with the exception of the genus Rhinoclemmys, which is distributed in Central and South America (Pough et al., 1998; Fritz, 2001). The systematic position of the family is very problematic (McDowell, 1964; Gaffney & Meylan, 1988; Pough et al., 1998; Fritz, 2001). It has been traditionally considered to be a subfamily of Emydidae (Hirayama, 1984; Iverson, 1992), or a separate paraphyletic family (Gaffney & Meylan, 1988; Pough et al., 1998), but nowadays the opinion of other authors (Shaffer et al., 1997; Fritz, 2001) - who consider Bataguridae as a separate monophyletic family - prevails, although they all agree that to resolve this issue, additional data from more complete sampling are required. The family Bataguridae is known at least from the Eocene in the Old World and the Nearctic. It is possible that the Paleocene "Emydidae", described from China, actually belong to the Bataguridae family (Fritz, 2001). The genus Mauremys is known in the Western Palearctic from the Oligocene (Fritz, 2001), but

Melentis (1966) places its origin earlier in the Eocene. Prior to the Pleistocene, it was widely distributed in Europe, North Africa and the Arabian Peninsula.

Until very recently *M. rivulata* and *M. leprosa* were treated as subspecies of *M. caspica. Mauremys leprosa* was the first to be raised to species level. According to Fritz (2001), this elevation had been considered by Boulenger (1889) and later by Siebenrock (1909), but was not widely acknowledged until after 1980 (Busack & Ernst, 1980). It was based on morphometric studies as well as on the biochemical studies of Merkle (1975). *Mauremys leprosa* is geographically separated from the other two species by a gap in the present distribution of the genus (Fig. 1). According to Busack & Ernst (1980), the geographic isolation, which led to cessation of gene flow, started in the Pliocene.

Mauremys rivulata was raised to the species level more recently (Fritz & Wischuf, 1997). The authors stated that morphometric features could not separate the two species, but suggested species status on the basis of the colour pattern of the carapace and plastron. Although the two species are separated by geographic barriers (Fritz & Wischuf, 1997) and occupy ecologically different habitats (Busack & Ernst, 1980), a narrow contact zone exists, from which Fritz & Wischuf (1997) report two hybrids.

Apart from the morphological studies (Busack & Ernst, 1980; Fritz & Wischuf, 1997; Tok, 1999), Merkle (1975) examined these species by protein electrophoresis. He examined 17 proteins and found that *M. caspica*

Correspondence: G. Mantziou, Natural History Museum of Crete, University of Crete, Knossou Av., P. O. Box 2208, GR71409 Irakleio, Crete, Greece. *E-mail*: mantziou@nhmc.uoc.gr; mantziou@edu.biology.uoc.gr

and *M. rivulata* were identical; *M. leprosa* shared only 13 of these proteins with the other two taxa.

Fritz and Wischuf (1997) noted that the coloration of specimens preserved in ethanol degrades and is not as clear in old animals as it is in young animals. Moreover, turtles of the genus *Mauremys* exhibit great intraspecific variation in colour patterns (Schleich *et al.*, 1996). There are no molecular studies on the Mediterranean *Mauremys* species. We therefore considered that the use of a molecular approach to independently test the present taxonomy would be of great interest. In the present study we investigate the intraspecific relationships of Aegean *M. rivulata* and their relation to the other two Mediterranean *Mauremys* species. We address questions on genetic distances among the species and attempt a reconstruction of their phylogenetic relationships.

We use partial sequences of cytochrome-b (cyt-b) of the mitochondrial DNA (mtDNA), a gene already used in several similar studies (Lamb *et al.*, 1994; Lamb & Lydeard, 1994; Lenk *et al.*, 1998; Lenk *et al.*, 1999). Mitochondrial DNA is a very useful tool in detecting genetic differences and phylogeographic patterns at the intraspecific level, or in closely related species, due to its non-recombining mode of inheritance, rapid pace of evolution and extensive intraspecific polymorphism (Avise et al., 1987). Mitochondrial DNA evolution rate appears significantly slower in Testudines, relative to other groups of vertebrates (Avise et al., 1992; Lamb et al., 1994; Lenk et al., 1999). Nevertheless, mtDNA can be very informative in cases where morphological data seem to be inconclusive (Lenk et al., 1998), which is the case with Mauremys species (Fritz & Wischuf, 1997).

MATERIALS AND METHODS

SAMPLES

Twenty-eight specimens of *Mauremys* were collected from 20 localities (Table 1). Of these localities, 24 contained *M. rivulata*, two contained *M. caspica* and two contained *M. leprosa*. Two published sequences of other Batagurids were used in the analysis database: *Heosemys spinosa* (GenBank U81362, Shaffer *et al.*, 1997) and *Cuora aurocapitata* (GenBank AF043262, Wu *et al.*, unpublished).

Homologous sequences of the emydid turtle *Emys* orbicularis (Linnaeus, 1758) and the tortoise *Testudo* marginata (Schoepff, 1795) were included in the study as outgroups.

TABLE 1. Museum number, exact locality and accession number of each specimen, used in the analysis (code is used in Figs. 1, 3, 4, 5 and Table 2)

Species	Museum number	Locality	Code	Accession number
Mauremys rivulata	NHMC 80.3.15.41	Grecce, Crete, Almyros River	1	AF487640
Mauremys rivulata	NHMC 80.3.15.26	Greece, Crete, Almyros River	2	AF487638
Mauremys rivulata	NHMC 80.3.15.16	Greece, Crete, Almyros River	3	AF487639
Mauremys rivulata	NHMC 80.3.15.125	Greece, Crete, Preveli	4	AF487633
Mauremys rivulata	NHMC 80.3.15.71	Greece, Crete, Gavdos	5	AF487637
Mauremys rivulata	NHMC 80.3.15.66	Greece, Crete, Gavdos	6	AF487641
Mauremys rivulata	NHMC 80.3.15.93	Greece, Crete, Krya Vrysi	7	AF487632
Mauremys rivulata	NHMC 80.3.15.45	Greece, Crete, Zakros	8	AF487627
Mauremys rivulata	NHMC 80.3.15.79	Greece, Crete, Georgioupoli	9	AF487629
Mauremys rivulata	NHMC 80.3.15.77	Greece, Crete, Georgioupoli	10	AF487628
Mauremys rivulata	NHMC 80.3.15.75	Greece, Crete, Akrotiri	11	AF487625
Mauremys rivulata	NHMC 80.3.15.128	Greece, Chios isl.	12	AF487630
Mauremys rivulata	NHMC 80.3.15.127	Greece, Chios isl.	13	AF487631
Mauremys rivulata	NHMC 80.3.15.98	Greece, Lesvos isl.	14	AF487636
Mauremys rivulata	NHMC 80.3.15.53	Greece, Kyklades, Naxos isl.	15	AF487634
Mauremys rivulata	NHMC 80.3.15.83	Greece, Dodekanisa, Rodos isl.	16	AF487635
Mauremys rivulata	NHMC 80.3.15.100	Greece, Dodekanisa, Ikaria isl.	17	AF487619
Mauremys rivulata	NHMC 80.3.15.103	Greece, Samos isl.	18	AF487622
Mauremys rivulata	NHMC 80.3.15.104	Greece, Samos isl.	19	AF487620
Mauremys rivulata	NHMC 80.3.15.132	Greece, Dodekanisa, Kos isl.	20	AF487621
Mauremysrivulata	NHMC 80.3.15.135	Greece, Thessalia, Larisa	21	AF487623
Mauremys rivulata	NHMC 80.3.15.136	Greece, Thessalia, Larisa	22	AF487626
Mauremys rivulata	NHMC 80.3.15.137	Greece, Peloponnisos, Kakkavas	23	AF487624
Mauremys rivulata	NHMC 80.3.15.56	Jordan, 25 km south of Jarash	24	AF487642
Mauremys caspica	NHMC 80.3.112.1	Syria, 5 km after As Suwar towards Margadah	25	AF487644
Mauremys caspica	NHMC 80.3.112.62	Syria, lake Al Asad	26	AF487643
Mauremys leprosa	NHMC 80.3.113.119	Morocco, Qued Tensift	27	AF487645
Mauremys leprosa	NHMC 80.3.113.120	Morocco, Qued Tensift	28	AF487646
Emys orbicularis	NHMC 80.3.16.6	Greece, Thessalia, Larisa	29	AF487648
Emys orbicularis	NHMC 80.3.16.7	Greece, Thessalia, Larisa	30	AF487649
Testudo marginata	NHMC 80.3.22.6	Greece, Kythira isl.	31	AF487647



FIG. 1. Present distribution of the three Mediterranean *Mauremys* species and geographic origin of the studied specimens (numbers correspond to the code listed in Table 1).

This material was collected from 1999 to 2001 during several field trips. The geographical origin of the studied specimens is shown in Fig. 1.

Blood samples were obtained from every animal, except from animals with numbers NHMC 80.3.112.62, NHMC 80.3.15.98 and NHMC 80.3.22.6, which died . and from which tissue sample was isolated and kept in ethanol (95%). The blood was collected by coccygeal vein puncture as described by Haskell & Porkas (1994), preserved in ethanol and stored at 4°C. After blood sampling the animals were released. Museum numbers were given to each animal that was captured and although they were released the numbers still correspond to the tissue samples taken from each one (Table1).

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Blood samples were first centrifuged at 13 000 rpm for 4 min. Ethanol was removed and samples were left at 37°C for 1 hour. Total genomic DNA was extracted following standard proteinase-k protocol, with standard salt extraction method (Sambrook *et al.*, 1989).

PCR amplification, targeting a segment of the cyt-*b* gene of the mitochondrial genome (mtDNA) was done on all extractions. The universal primers L14724 and H15175 (Palumbi, 1996) were used to amplify a 451 bp fragment of the mitochondrial cyt-*b* gene.

Amplifications were performed in a 10µl total reaction volume, where 1µl of template DNA was mixed with 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 4 pmol of each primer and 0.5 units of Taq Polymerase (GibcoBRL). Thermocycling was then performed in a PTC-100 (MJ- Research) thermocycler. The cycle programme comprised of an initial denaturation step of 2 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, extension for 1 min at 72°C and a final extension at 72°C for 10 min.

Sequencing of double-stranded DNA was performed in both directions in a PE-ABI377 sequencer (using dyeterminator chemistry). The primers in the sequencing reactions were the same as in the amplification procedure.

SEQUENCES ALIGNMENT AND PHYLOGENETIC ANALYSIS

Multiple sequence alignment was performed using a ClustalX program package (version 1.8: Thompson *et al.*, 1997), using the default parameters, alternative gap opening and gap extension penalties, with minor modifications made by eye.

Pairwise sequence comparisons were made for the cyt-b data set using MEGA (v.2, Kumar *et al.*, 2001) in order to determine the number, nature, distance and distribution of base substitutions. Genetic distance was estimated using the Kimura two-parameter model (Kimura, 1980).

Evolutionary relationships, which result from DNA sequence data, are reliable only if sites are not saturated by multiple substitutions (Swofford *et al.*, 1996). To assess potential saturation of substitutions of the cyt-b sequences, the numbers of transitions (Ts) and transversions (Tv) were plotted against the corresponding uncorrected *P*-distances for all pairwise comparisons.

Phylogenetic relationships among specimens were inferred via neighbour-joining (NJ, Saitou & Nei 1987), maximum parsimony (MP, Swofford *et al.*, 1996), and maximum likelihood methods (ML, Felsenstein 1981). NJ trees were implemented by MEGA (v. 2.0, Kumar *et al.*, 2001) using Kimura's (1980) two-parameter distance estimate, even though the distance metric used in NJ had no effect on topology. MP and ML trees were constructed using PAUP (Windows Version 4.0b8a, Swofford, 2002).

Nucleotides were used as discrete, unordered characters. The shortest tree was looked for with the branch and bound search. When more than one minimal length tree was found, the strict consensus tree was presented. Confidence estimates were obtained via bootstrapping with 1000 replicates (Felsenstein, 1985).

For maximum likelihood (ML) analysis (Felsestein, 1981), the best fit model of DNA substitution and the parameter estimates used for tree construction were chosen by performing hierarchical likelihood ratio tests (Huelsenbeck & Crandall, 1997) using Modeltest 3.06 (Posada & Crandall, 1998). Heuristic ML searches were performed with 10 replicates of random sequence addition and tree bisection-reconnection (TBR) branch swapping. ML bootstraps employed 1000 iterations. The model parameters (substitution rate matrix, gamma distribution approximation with four rate classes, and empirical nucleotide frequencies) were estimated initially from the starting trees generated by the approach described above (Huelsenbeck & Crandall, 1997). These estimates were used in a ML analysis to produce a tree from which the parameters were then reestimated. In an iterative fashion, these steps were repeated until the ML score converged to its maximum value (Swofford et al. 1996).

A minimum spanning network was constructed among *M. rivulata* haplotypes, by using Arlequin v.2000 (Schneider *et al.*, 2000).

Tajima's relative rate test (Tajima, 1993; Nei & Kumar, 2000) was carried out using MEGA (v.2.0) in order to assess differences in rates among separate lineages. Statistical estimation of the validity of the molecular clock hypothesis was performed using the χ^2 test proposed by Fitch (1976). In addition, the maximum likelihood model was used to test the null hypothesis that the sequences were evolving at constant rates and therefore fit a molecular clock (Muse & Weir, 1992). This hypothesis may be tested once we have chosen one of the models of evolution, simply calculating the log likelihood score of the chosen model with the molecular clock enforced and comparing it with the log likelihood previously obtained without enforcing the molecular clock. In this case, the molecular clock is the null hypothesis. The number of degrees of freedom is the number of OTUs - 2. It should be mentioned that in this analysis we used not only the unique haplotypes, but all 33 sequences.

Clock assumptions must be treated cautiously since the differences in mtDNA evolution in higher vertebrate groups have not yet been fully identified, and many studies have shown considerable rate heterogeneity (Hillis *et al.*, 1996). Nevertheless, the use of clock assumptions for closely related taxa is generally considered to be more reliable than for distantly related taxa (Caccone *et al.*, 1997), which stems from the premise that rates of evolution of a particular gene are likely to be stable in closely related taxonomic groups, with similar life histories, metabolic rates, and generation times. In this respect, the estimation of "local" rates for closely related taxa might be preferable over a "universal" rate (Hillis *et al.*, 1996). In the present study we use an evolutionary rate suggested for turtle mtDNA (Avise *et al.*, 1992; Lamb *et al.*, 1994; Lenk *et al.*, 1999), instead of the universal rate.

The published sequence used in the relative rate test as outgroup (one specimen), was that of *Staurotypus triporcatus* (GenBank U81349, Shaffer *et al.*, 1997).

The sequence data from this study were deposited in the GenBank Data library under the accession numbers AF487619–AF487649.

RESULTS

BASE COMPOSITION

A total of 11 unique haplotypes from the 28 specimens of *Mauremys* were obtained in this study (Table 2), the lengths of which ranged from 365 to 427 bp. Within the cyt-b gene of the presented sequences, no insertions, no deletions and no premature stop codons were encountered.

A total of 435 base pairs were aligned, of which 42 sites (9.65%) were variable among the *Mauremys* species (26.66% including outgroups) and 25 (5.75%) were parsimony informative (16.78% including outgroups). Nine (21.43%) of the 42 variable positions represent changes in the first codon position, 8 (19.05%) in the second and 25 (59.52%) in the third.

Mean base composition of the fragment of cyt-b of the three codon positions is provided in Table 3. There is a strong bias in base composition (Bias C of Irwin et al., 1991), a feature characteristic of cyt-b and other mitochondrial protein-coding genes in mammals and reptiles. This fact supports the authenticity of the mitochondrial sequences (Irwin et al., 1991; McGuire & Heang, 2001; Lenk et al., 2001; Surget-Groba et al., 2001). As expected, the abundance of G's was low (12.9%), whereas the percentages of A, T and C were quite similar (26.9-31.0%). However, a significant compositional bias exists at the second and especially the third codon position. The frequency of guanine at the first position is 21.1%, while a marked under-presentation of guanine was observed at both second (16.2%) and third position (1.8%).

GENETIC DIVERGENCE AND SATURATION

Summarized Kimura two-parameter distances between all pairs are given in Table 4. Sequence divergence ranged from 0.24% within *M. rivulata* TABLE 2. Grouping of selected *Mauremys* samples into 11 unique haplotypes (numbers in parenthesis correspond to the code listed in Table 1)

	Haplotype	Samples	Frequency	
M. rivula	'a		nan maananing damp oppidae oo safa maan	
	hl	Samos (19)	25%	
		Akrotiri (11)		
		K os (20)		
		Gavdos (5)		
		Gavdos (6)		
		Krya Vrysi (7)		
	h2	Ikaria (17)	29.16%	
		Samos (18)		
		Larissa (21)		
		Larissa (22)		
		Peloponissos (23)		
		Jordan (24)		
		Preveli (4)		
	h3	Chios (13)	8.33%	
		Lesvos (14)		
	h4	Zakros (8)	12.50%	
		Georgioupoli (9)		
		Georgioupoli (10)		
	h5	Naxos (15)	8.33%	
		Chios (12)		
	h6	Rodos (16)	4.16%	
	h7	Almyros (1)	12.50%	
		Almytos (2)		
		Almyros (3)		
M. caspica	1			
	h8	Syria (25)	50%	
	h9	Syria (26)	50%	
M. leprosa				
	h1()	Morocco (27)	50%	
	h11	Morocco (28)	50%	

haplotypes to 16.97% between *E. orbicularis* and *M. leprosa.* If we consider only *Mauremys* species, sequence divergence ranged from 0.24% (within *M. rivulata*) to 7.45% between *M. leprosa* and *M. caspica.*

The results of saturation analysis are presented in Fig 2. Both transitions and transversions show an approxi-

TABLE 3. Percentage base composition at first, second and third codon position for all 33 specimens. Compositional bias index (CBI) is calculated as $C=(2/3)\Sigma|c_i - 0.25|$ where C is the compositional bias index and c_i the frequency of *i*th base (Irwin *et al.*, 1991).

Nucleotide		Mean		
	First	Second	Third	
А	30.0	21.4	40.8	31.0
Т	26.8	37.5	16.6	26.9
С	22.1	24.9	40.8	29.2
G	21.1	16.2	1.8	12.9
CBI	0.091	0.167	0.42	1



FIG. 2. Relationships between genetic distance, transitions and transversions.

mately linear relationship with distances, which indicates that saturation has not occurred.

PHYLOGENETIC RELATIONSHIPS

For the phylogenetic analyses, a data set of 16 cyt-*b* sequences (11 unique haplotypes of *Mauremys* spp.,

TABLE 4. Genetic distances (Kimura two-parameter) between the different taxa. In-group sequence divergence is given in diagonal. The range of genetic distances is given in the parentheses.

	M. rivulata	M. caspica	M. leprosa (°. auroca pitata	H. spinosa	7. marginata	E. orbicularis
M. rivulata	0.7						
	(0.24-1.78)						
M. caspica	3.36	1.83					
	(2.45-3.95)						
M. leprosa	6.16	7.14	0.28				
	(5.9-6.66)	(6.83-7.45)					
C. aurocapitata	9.56	8.94	10.49	n/c			
	(8.41-10.26)	(8.81-9.07)	(10.34-10.63)				
11. spinosa	11.49	11.03	12.53	12.91	n/c		
	(10.23-12.37)	(10.56-11.5)	(12.36-12.71)				
T. marginata	12.88	13.06	14.51	15.42	13.91	n/c	
	(12.41-13.89)	(12.36-13.76)	(14.34-14.68)				
E. orbicularis	14.64	14.54	16.23	16.44	12.87	15.47	0.25
	(12.72-16.31)	(12.70-16.63)	(15.49-16.97)				



FIG. 3. Phylogenetic analysis of the mitochondrial cyt-*b* gene of *Mauremys*. Tree inferred by the neighbour-joining (NJ) method, based on 1000 replicates.

two of *Emys orbicularis* and one of *Testudo marginata* from this study, two of other Batagurid species from the literature) was used. Tree length distribution, determined from random sampling of 10^6 unweighted trees, was significantly skewed to the left (g1=-0.471), suggesting strong phylogenetic signal in the data (*P*<0.01; Hillis & Huelsenbeck, 1992).

In the phylogenetic analysis carried out by the neighbour joining method, the resulting tree (Fig. 3), rooted by *E. orbicularis*, showed *Mauremys* species as a monophyletic group (83% bootstrap support, b.s.). Three lineages are evident in the tree: an early offshoot of *M. leprosa* (100% b.s.), followed by *M. caspica* (89% b.s.) and *M. rivulata* (95% b.s.).

The parsimony analysis of the 16 cyt-*b* sequences, using *E*. *orbicularis* as outgroup, resulted in 176 equally parsimonious trees of 170 steps (consistency index CI=0.788 and homoplasy index HI=0.212), the strict consensus of which is shown (with bootstrap values) in Fig 4. The topology of this tree is similar to the NJ tree, regarding the main clades.

For the maximum likelihood analysis, likelihood ratio tests indicated that the Tamura-Nei model with general time reversible option was the most appropriate for subsequent ML analyses (Table 5). The phylogeny recovered by the ML analysis was similar to that recovered by the MP and NJ analysis and is illustrated in Fig.



FIG. 4. A Maximum Parsimony (MP) tree derived from cyt-*b* sequences of *Mauremys* species. The strict consensus of the 176 equally most parsimonious trees is presented. Probability percentages of bootstrap replicates (1000) supporting each branching pattern are given beside the corresponding nodes (numbers correspond to the code listed in Table 1).

5. One ML tree was identified ($-\ln L=1383.01$; final parameters estimates: base frequencies A=0.30, C=0.30, G=0.14, T=0.26, a=0.3159, P_{inv}=0.000, and A/G=3.59, C/T=11.04).

Geographic distribution of *M. rivulata* haplotypes is shown in Fig. 6. The minimum spanning network among *M. rivulata* haplotypes is presented in Fig.7.

TABLE 5. Test of hypotheses relating to the model of evolution appropriate for phylogeny reconstruction (Huelsenbeck & Crandall, 1997). *P*- values were obtained with Modeltest (Posada & Crandall, 1998).

Null hypothesis	Models compared	-lnL _o	-lnL	df	Р
Equal nucletide frequencies	H _o :JC69, H ₁ :F81	1513.1719	1481.9972	3	0.0000
Equal Ti and Tv rates	H. 81, H. HK Y85	1481.9972	1425.8398	1	0.0000
Equal Ti rates	H.HKY85, H.:TrN	1425.8398	1417.3188	1	0.0000
Equal Tv rates	H _o :TrN, H ₁ :TIM	1417.3188	1416.5848	1	0.2257
Equal rates among sites	H ₀ :TrN, H ₁ :TrN+G	1417.3189	1387.3484	1	0.0000
Proportion of invariable sites	H ₀ :TrN+G, H ₁ :TrN+I+G	1387.3484	1387.2443	1	0.3241

AEGEAN MAUREMYS RIVULATA







FIG. 6. Geographic distribution of *M. rivulata* haplotypes. Note the absence of geographic patterns.



0.1

FIG. 7. Minimum spanning network for the 7 *M. rivulata* haplotypes. Size of each circle is proportional to the frequency of the haplotypes.

TABLE 6. Estimated splitting time between clades. The splitting time is estimated using a divergence rate of 0.3%-0.4% per Myr. A: *M. rivulata* clade, B: *M. caspica* clade, C: *M. leprosa* clade.

Clades	Da	Splitting Time			
		Rate: 0.3% per Myr	Rate: 0.4% per Myr		
(A&B) vs. C A vs. B	- 5.88% 2.1%	19.6 Myr ago 7 Myr ago	14.7 Myr ago 5.3 Myr ago		

Tajima's relative rate test was carried out for many different pair-combinations of the examined clades and resulted that all these clades evolve with the same rate ($\chi^2 < 3.84$, P > 0,05). The likelihood ratio test was employed to investigate the rate of homogeneity for the analysed species (Huelsenbeck & Crandall, 1997). Because the simpler (clocklike) tree cannot be rejected at a significance level of 5% (LRT=17.66, df=31, $\chi_{critical} = 19.28$), we do not reject the application of a molecular clock to the species used in the analysis.

Since our results are compatible with the molecular clock hypothesis, we can use the suggested evolving rate for mtDNA of Emydidae (0.3% -0.4% per Myr) (Lenk *et al.*, 1999). The resulting estimated splitting times between clades are summarized in Table 6.

DISCUSSION

PHYLOGENETIC RELATIONSHIPS

Our results indicate that *M. rivulata*, *M. caspica* and *M. leprosa* are indeed genetically isolated taxa. These three taxa constitute a monophyletic group, which splits



into two genealogical lineages. The first corresponds to the lineage leading to *M. leprosa*, whereas the second corresponds respectively to the lineage from which the *caspica-rivulata* group emerged. The very small withingroup divergence of *M. rivulata* and the multifold between-group divergence (3.36% between *M. caspica* and *M. rivulata*) support the elevation of *M. rivulata* to the species level, as suggested by Fritz & Wischuf (1997), although further sampling in the area of contact is necessary to fully resolve this issue.

The elevation of *M. leprosa*, which was based on electrophoretic and morphometric data (Merkle, 1975; Busack & Ernst, 1980), is further supported from our results.

Specimens of *M. rivulata* (from the Middle East to Greece) cluster with a small intraspecific differentiation ranging from 0.24% to 1.78% (mean=0.7%). This divergence is not related to the geographic origin of the specimens (see Fig. 6, Fig. 7 and Appendix 1). The small intraspecific divergence observed in *M. rivulata*, is comparable to results reported by Lenk *et al.* (1999) for *Emys orbicularis*. The authors compared a great number of populations from Europe and N. Africa, which split to seven groups of haplotypes without being separated geographically.

In general, phylogeographic patterns are considered to be the result of a multifactorial process, which is somewhat arbitrary and variable among different species (Taberlet *et al.*, 1998). The combination of existing paleontological data with the results we present, permits a preliminary interpretation of the phylogeographic pattern of M. *rivulata* in the Eastern Mediterranean and especially in the area of the Aegean Sea.

According to Lenk *et al.* (1999), the climate change in Europe 3.2 Myr ago triggered a sudden radiation of *Emys orbicularis*. During the climatic oscillations of the Pleistocene, the range of *E. orbicularis* probably fragmented recurrently, with isolated populations along a slender belt throughout southern Europe. This belt has been shaped by cold climates to the north and by barriers of inappropriate habitat to the south. Thus, southern Italy and Greece served as refugia for the populations of *E. orbicularis*.

Kotsakis (1980) claimed that the lower Pleistocene glaciations, which provoked the southward shift of distribution of *E. orbicularis* in Italy, drove *Mauremys* populations to extinction in the peninsula.

Thereby, the main factor that influenced the present distribution of *Mauremys* in the Mediterranean region, and particularly the distribution of *M. rivulata* in the Balkans, is climatic change, which prevailed during the glacial periods of the Pleistocene. The cold periods resulted in the latitudinal shift of *Mauremys* populations in the Italian Peninsula towards the south, and ultimately led to their extinction. Something similar occurred in the Balkan Peninsula with *M. rivulata*, resulting in a latitudinal shift of the populations towards the south and east, but it did not result in extinction since they found refuges on the coast of Asia Minor and in the Aegean islands.

Consequently, the shifting and rearrangements of *M. rivulata* populations led to their mixing and the probable establishment of new populations on islands, where they did not exist before and where populations of *E. orbicularis* were not favoured due to ecological factors. According to Lenk *et al.* (1999), *E. orbicularis* is more favoured by continental climate conditions.

On the other hand, the genetic distances observed among *M.*. *rivulata* haplotypes, and their relationships based on minimum spanning network, are not related to respective geographic distances. This fact cannot be explained by the vicariance approach of the distributional pattern of *M. rivulata*, but can be better explained by a dispersal model. To propose a dispersal model we need to have evidence that *M. rivulata* is easily dispersed.

According to Lenk *et al.* (1999), marine straits represent no absolute barriers for *E. orbicularis* and coastal corridors could have promoted genetic exchange. This is probably also the case for *M. rivulata* since this species inhabits also brackish waters (Gasith & Sidis, 1983; Sidis & Gasith, 1985; Engelmann *et al.*, 1993). Consequently, the easily accessible marine straits, which appeared in the Aegean area repeatedly during Pliocene and Pleistocene, and the great dispersal capacity of *M. rivulata* through coastal corridors, are supporting the dispersal model. The extant populations of *M. rivulata* are probably still under the influence of this dispersal procedure.



Phylogenetic Relations Inferred by Palaeontological Data

Bergounioux (1955) suggested that there are three parallel evolutionary lines in the Western Palearctic turtles of the genus Mauremys (Fig. 8): (1) the lineage of Mauremys italica, which starts in the Eocene with the species M. italica and M. vidali and ends in the Oligocene with M. chainei; (2) the lineage of Mauremys sophiae, which consists of M. batalleri from the Oligocene, goes on in the Miocene with M. batalleri and M. sophiae and ends in the Pliocene with *M. sophiae*; (3) the lineage of M. pygolopha, which starts in the Oligocene, with M. subpyrenaica, continues in the Miocene with M. rotundiformis and M. pygolopha and in the Pliocene with M. romani and M. gaudryi. Broin (1977) suggests that M. romani and M. gaudryi are synonymous. According to Melentis (1966), M. steinheimensis from the Miocene belongs to this lineage as well. Among these we can also place M. sarmatica from the Miocene, which is supposed to be the direct ancestor of M. gaudryi from the Pliocene (Kotsakis, 1980). The fossils considered most akin to the extant Mauremys species belong to this third lineage (Melentis. 1966; Kotsakis, 1980).

Combining our results with the available paleontological data allows us to suggest a hypothesis on the phylogenetic relationships of the three species in question. The three species share a common ancestor, which is situated in the lineage of M. pygolopha. The first to separate from this lineage is the branch that led to the extant M. leprosa with the intermediate form of M. gaudri (Broin, 1977; Kotsakis, 1980). When applying the above mentioned evolutionary rate (0.3%-0.4% per Myr) to our data, the common ancestor of the two main genealogical lines dates to the Lower Miocene/Middle Miocene (from 19.8 Myr ago to 14.9 Myr ago; Fig. 8). This contradicts Busack & Ernst's (1980) suggestion that this isolation started during the Pliocene. The information on the second branch, which led to *M. caspica* and *M. rivulata*, is very restricted. The only hypothesis we may initially support is that the two species have shared a long common history. The common ancestor of the genealogical lines, which led to M. rivulata and M. caspica, is dated in the Upper Miocene/Lower Pliocene (from 7.2 Myr ago to 5.4 Myr ago). Because the lack of relevant paleontological data and geological events inhibit making a more accurate dating, further study is necessary, which will have to include more specimens of the three Mediterranean Mauremys species.

AKNOWLEDGMENTS

This research was partially supported by the postgraduate program "Management of marine and terrestrial biological resources" of the University of Crete and partially by the "Program for the support of young scientists" (PENED) of the Greek Ministry of Development. Many thanks to the reviewers for helpful comments on an earlier draft of this manuscript.

REFERENCES

- Avise, J. C., Arnold, J., Ball, R. M., Bermingham, E., Lamb, T., Neigel, J. E., Reeb, C. A. & Saundersk, N. C. (1987). Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology* and Systematics 18, 489-522.
- Avise, J. C., Bowen, B. W., Lamb, T., Meylan, A. B. & Bermingham, E. (1992). Mitochondrial DNA evolution at a turtle's pace: Evidence for low genetic variability and reduced microevolutionary rate in the Testudines. *Molecular Biology and Evolution* 9, 457-473.
- Bergounioux, F. M. (1955). Chelonia. In *Traité de Paléontologie*. 5, 487-544. Piveteau J. (Ed.), Paris.
- Boulenger, G. A. (1889). Catalogue of the Chelonians, Rhynchocephalians, and Crocodiles in the British Museum (Natural History). London (British Museum).
- Broin, F. (1977). Contribution a l'étude des Chéloniens. Chéloniens continentaux du Crétacé supérieur et du Tertiaire de France. Mémoires du Muséum National d'Histoire Naturelle 38,1-366.
- Busack, S. D. & Ernst, C. H. (1980). Variation in the Mediterranean populations of *Mauremys* Gray 1869 (Reptilia, Testudines, Emydidae). *Annals of the Carnegie Museum* 49, 251-264.
- Caccone, A., Milinkovitch, M. C., Sbordoni, V. & Powell,
 J. R. (1997). Mitochondrial DNA rates and biogeography in European newts (Genus Euproctus). Systematic Biology 46, 126-144.
- Engelmann, W. E., Fritzsche, J., Gunther, R. & Obst, F. J. (1993). Lurche und Kriechtiere Europas. Neumann Verlag Radebeul.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: A maximum likelihood approach. Journal of Molecular Evolution 17, 368-376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783-791.
- Fitch, W. M. (1976). Molecular evolutionary clocks. In Molecular Evolution, 160-178. Ayala, F. J. (Ed.). Sinauer, Sunderland.
- Fritz, U. (2001). Handbuch der Reptilien und Amphibien Europas. Band 3/111A Schildkröten (Testudines) 1. AULA-Verlag.
- Fritz, U. & Wischuf, T. (1997). Zur Systematik westasiatisch-südosteuropäischer Bachschildkröten (Gattung Mauremys) (Reptilia: Testudines: Bataguridae). Zoologische Abhandlungen, Staatliches Museum für Tierkunde, Dresden 49, 223-260.
- Gaffney, E. S. & Meylan, P. A. (1988). A phylogeny of turtles. In *The phylogeny and classification of the tetrapods. Vol.1, Amphibians, Reptiles and Birds.* Benton, M. J. (Ed.). Oxford, Clarendon Press.
- Gasith, A. & Sidis, I. (1983). The distribution and nature of the habitat of the Caspian terrapin *Mauremys caspica rivulata* (Testudines: Emydinae) in Israel. *Israel Journal of Zoology* **32**, 91-102.

- Haskell, A. & Porkas, M. A. (1994). Nonlethal blood and muscle tissue collection from Redbelly Turtles for genetic studies. *Herpetological Review* 25, 11-12.
- Hillis, D. M., and Huelsenbeck, J. P. (1992). Signal, noise and reliability in phylogenetic analyses. *Journal of Heredity* 83, 189-195.
- Hillis, D. M., Mable, B. K. & Moritz, C. (1996). Applications of molecular systematics: the state of the field and a look to the future. In *Molecular Systematics.* 515-543. Hillis, D. M., Moritz, C. & Mable, B. K. (Eds). Sunderland: Sinauer Accociates.
- Hirayama, R. (1984). Cladistic analysis of batagurine turtles (Batagurinae: Emydidae: Testudinoidea): a preliminary result. Studia Geologica Salmanticencia vol. especial 1, 141-157.
- Huelsenbeck, J. P. & Crandall, K. A. (1997). Phylogeny estimation and hypothesis testing using maximum likelihood. Annual Review of Ecology and Systematics 28, 437-466.
- Irwin, D. M., Kocher, T. D. & Wilson, A. C. (1991). Evolution of the cytochrome b gene of mammals. Journal of Molecular Evolution 32, 128-144.
- Iverson, J. B. (1992). A revised checklist with distribution maps of the turtles of the world. Earlham College, Green Nature Books. Florida, USA.
- Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal* of Molecular Evolution 16, 111-120.
- Kotsakis, T. (1980). Révision des tortues (Emydidae, Testudinidae, Trionychidae) du Plio-Pléistocène de Valdarno supérieur (Toscane, Italie). *Quaternaria* 22, 11-37.
- Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* 17, 1244-1245.
- Lamb, T. & Lydeard, C. (1994). A molecular phylogeny of the gopher tortoises, with comments on familial relationships within Testudinoidea. *Molecular Phylogenetics and Evolution* 3, 283-291.
- Lamb, T., Lydeard, C., Walker, R. B. & Gibbons, J. W. (1994). Molecular systematics of map turtles (*Graptemys*): A comparison of mitochondrial restriction site versus sequencing data. *Systematic Biology* 43, 543-559.
- Lenk, P., Fritz, U., Joger, U. & Wink, M. (1999). Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis* (Linnaeus 1758). *Molecular Ecology* 8, 1911-1922.
- Lenk, P., Joger, U., Fritz, U., Heidrich, P. & Wink, M. (1998). Phylogenetic patterns in the mitochondrial cytochrome b gene of the European pond turtle (Emys orbicularis): first results. In Proceedings of the Emys Symposium Dresden 96. Mertensiella 10, 159-175. Fritz, U., Joger, U., Podloucky, R. & Servan, J. (Eds).
- Lenk, P., Kalyabina, S., Wink, M. & Joger, U. (2001). Evolutionary relationships among the true vipers (Reptilia: Viperidae) inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* 19, 94-104.

- McDowell, S. B. (1964). Partition of the genus Clemmys and related problems in the taxonomy of the aquatic Testudinidae. Proceedings of the Zoological Society of London 143, 239-279.
- McGuire, J. A. & Heang, K. B. (2001). Phylogenetic systematics of Southeast Asian flying lizards (Iguania: Agamidae: Draco) as inferred from mitochondrial DNA sequence data. *Biological Journal of the Linnean Society* 72, 203-229.
- Melentis, J. K. (1966). Studien über fossile Vertebraten Griechenlands: 10. Clemmys caspica aus dem Pleistozän des Beckens von Megalopolis im Peloponnes (Griechenland). Annales Géologiques des Pays Helléniques 17, 169-181.
- Merkle, D. A. (1975). A taxonomic analysis of the *Clemmys* complex (Reptilia: Testudines) using starch gel electrophoresis. *Herpetologica* 31, 162-166.
- Muse, S. V. & Weir, B. S. (1992). Testing for equality of evolutionary rates. *Genetics* 132, 269-276.
- Nei, M. & Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press.
- Palumbi, S. R. (1996). Nucleic Acids II: the Polymerase Chain Reaction. In *Molecular Systematics*, 205-248.
 Hillis, D. M., Moritz, C. & Mable, B. K. (Eds). Sunderland: Sinauer Associates.
- Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817-818.
- Pough, F. H., Andrews, R. M., Cadle, J. E., Crump, M. L., Savitzky, A. H. & Wells, K. D. (1998). *Herpetology*. Prentice Hall, Upper Saddle River, New Jersey.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Press, New York.
- Schleich, H. H., Kästle, W. & Kabisch, K. (1996). Amphibians and Reptiles of North Africa. Koeltz Scientific Books, Koenigstein, Germany.
- Schneider, S., Roessli, D. & Excoffier, L. (2000). *ARLEQUIN version 2.001: A software for population genetics data analysis.* Genetics and Biometry Laboratory, University of Geneva, Switzerland.
- Shaffer, H. B., Meylan, P. & McKnight, M. L. (1997). Tests of turtle phylogeny: molecular, morphological, and paleontological approaches. *Systematic Biology* 46, 235-268.
- Sidis, I. & Gasith, A. (1985). Food habits of the Caspian terrapin (*Mauremys caspica rivulata*) in unpolluted and polluted habitats in Israel. *Journal of Herpetology* 19, 108-115.
- Siebenrock, F. (1909). Synopsis der rezenten Schildkröten, mit Berücksichtigung der in historischer Zeit ausgestorbenen Arten. Zoologische Jahrbücher 10, 427-618.
- Surget-Groba, Y., Heulin, B., Guillaume, C. P., Thorpe, R. S., Kypriyanova, L., Vogrin, N., Maslak, R., Mazzotti, S., Venczel, M., Ghira, I., Odierna, G., Leontyeva, O., Monney, J. C. & Smith, N. (2001). Intraspecific

phylogeography of *Lacerta vivipara* and the evolution of viviparity. *Molecular Phylogenetics and Evolution* **18**, 449-459.

- Swofford, D. L. (2002). PAUP 40b8a: Phylogenetic Analysis Using Parsimony (and other methods). Sunderland: Sinauer Associates.
- Swofford, D. L., Olsen, G. J., Waddel, P. J. & Hillis, D. M. (1996). Phylogenetic inference. In *Molecular* Systematics, 407-514. Hillis, D. M., Moritz, C. & Mable, B. K. (Eds). Sunderland: Sinauer.
- Taberlet, P., Fumagalli, L., Wust-Saucy, A. G. & Cosson, J. F. (1998). Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology* 7, 453-464.
- Tajima, F. (1993). Simple methods for testing the molecular evolutionary clock hypothesis. *Genetics* 135, 599-607.

- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins. D. G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. Nucleic Acids Research 24, 4876-4882.
- Tok, C. V. (1999). The taxonomy and ecology of Mauremys caspica rivulata Valenciennes, 1833 (Testudinata: Testudinidae) on Resadiye (Dacta) Peninsula. Turkish Journal of Zoology 23, 17-21.

Accepted 27.1.03

APPENDIX 1

Genetic distances (Kimura two-parameter) between the *Mauremys* haplotypes and other tax a used in the analysis. *M.r., M. rivulata; M.c., M. caspica; M.l., M. leprosa; C.a., C. aurocapitata; H.s., H. spinosa; T.m., T. marginata; E.o., E. orbicularis.*

	h l	h2	h3	h4	h5	h6	h7	h8	h9	h10	hll	C.a.	H.s.	7.m.	<i>E.o.</i>
	(M. r.)	(<i>M. r.</i>)	(M.r.)	(M.r.)	(<i>M.r.</i>)	(<i>M.r.</i>)	(<i>M.r.</i>)	(<i>M.c.</i>)	(<i>M.c.</i>)	(M.l.)	(<i>M.c.</i>)				(30)
hl (<i>M.r.</i>)															
h2 (<i>M.r.</i>)	0.24														
h3 (<i>M.r</i> .)	1.31	1.78													
h4 (<i>M</i> .r.)	0.84	1.35	0.79												
h5 (<i>M.r.</i>)	0.55	1.07	0.53	0.53											
h6 (<i>M.r</i> .)	0.28	0.80	0.26	0.53	0.26										
h7 (<i>M.r</i> .)	0.27	0.79	0.52	0.80	0.53	0.53									
h8 (<i>M.c</i> .)	3.54	3.54	3.28	3.37	3.07	3.06	3.83								
h9 (<i>M.c</i> .)	2.39	2.66	3.79	3.73	3.40	3.08	3.02	1.81							
h10 <i>(M.I.)</i>	6.07	616	6.04	5.77	5.79	5.79	6.32	7.00	6.45						
hll (<i>M.I.</i>)	5.62	5.98	5.87	5.61	5.61	5.61	5.87	6.80	6.60	0.28					
С. а.	8.76	9.04	9.03	9.33	8.94	8.58	7.77	8.39	8.19	9.72	9.46				
H. s.	10.73	11.02	11.21	10.33	10.26	10.23	9.39	9.68	10.45	11.46	11.15	11.58			
7. m.	11.62	11.64	12.40	11.48	11.41	11.38	11.17	12.26	11.20	12.75	12.95	13.6	12.46		
E. o. (30)	11.58	11.82	13.56	14.08	13.73	13.41	12.64	14.33	11.53	13.74	14.53	14.53	11.68	13.58	
E. o. (29)	11.88	12.13	13.90	14.45	14.08	13.76	12.98	14.68	11.53	14.12	14.90	14.53	11.68	13.90	0.25

SHORT NOTE

HERPETOLOGICAL JOURNAL, Vol. 14, pp. 47-49 (2004)

PRELIMINARY DATA ON REPRODUCTIVE ECOLOGY OF LACERTA LEPIDA AT A MOUNTAIN SITE IN CENTRAL SPAIN

Alfredo Salvador', José P. Veiga' and marisa Esteban²

¹Departament• de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain

²Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain

The home ranges of radio-collared eyed lizards (Lacerta lepida) were studied in the mountains of . central Spain. The home range of females and males varied from 2800-5844 m² and 1424-22106 m² respectively. Two measures of core area and home range covaried significantly with mass. Home range and 75% core area were significantly larger in males than in females, while 50% core area did not differ between sexes. Age has no significant effect on homerange size. Each male's home range overlapped with the home ranges of 2-6 males and 2-6 females. Males used 11-17 different rocks 0.5-5 m diameter as shelters during 17-25 nights. The extensive home range areas of large males may be related to maximizing access to the few reproductive females available, but costs may be high, as indicated by the mortality data.

Key words: abundancc, home range, *Lacerta lepida*, mortality, skeletochronology

The eyed lizard (*Lacerta lepida*) is a large lacertid that occurs in Mediterranean areas of south-western Europc. The natural history of this species is largely unknown (Bischoff *et al.*, 1984; Pérez-Mellado, 1998). We present here the results of a field study on home-range size carried out in the mountains of central Spain.

The study site was a deciduous oak forest (*Quercus pyrenaica*) near Navacerrada (40° 44' N, 4° 00' W), Sierra de Guadarrama, at an altitude of 1250 m. Shrubs, grasses, and rocks were also predominant at the site. For a more detailed description of cover see Salvador *et al.* (1995). We established a 1 ha plot (100 × 100m), with markers on a grid every 10 m. During 2000 and 2001 we visited the plot, five days a week, from 20 February until 15 July. We captured lizards by noosing, with a 7 m fishing pole. Capture efficiency was high and allowed us to catch all lizards on the first day they were sighted. It is likely that we captured most of the adults present in the

plot because no unmarked individuals were observed once all individuals had emerged. In the 1 ha plot, we captured seven males and six females in 2000 and four males and one female in 2001. Additional data were gathered on five males and one female captured in a nearby 3 ha plot, with markers on a grid every 20 m. This plot was less intensively sampled than the 1 ha plot.

The lizards were transported to El Ventorrillo Field Station (5 km distant by air) where they were weighed to the nearest 0.01 g on an electronic balance, and their snout-vent length (SVL) measured with a ruler to the nearest 0.5 mm. Lizards were toe-clipped for a skeletochronological study (see below). We fitted lizards with 2.5 g radio-transmitter collars (Biotrack Ltd.) and released them at the site of capture within 4 hrs of capture. Final recapture of radio-collared lizards occurred during 1-15 July, and we released them after transmitter removal.

We used a RX-8910HE (Televilt) radio receptor to locate radio-collared individuals and noted their position on a map to the nearest 1 m. Between one and three locations points were taken at each of the following time periods: 7-8 hr, 11-12 hr and 14-15 hr GMT. Home range area was measured using the convex polygon method. We used the statistical package Ranges V (Natural Environment Research Council, UK) to compute home-range areas corresponding to 50%, 75% and 100% of sightings. We define 50% and 75% values as core areas which exclude fixes furthest from arithmetic mean. The minimum number of cumulative sightings (mean+SE) that accurately estimated 50%, 75% and 100% of home-range size, using the method of Rose (1982), were, respectively, 11.3±4.4, 24.3±5.6 and 41.3±7.3 observation points. To examine the number and characteristics of night shelters used by a subset of males, we located them before daily emergence. The maximum diameter of the rocks utilized as shelter was also recorded. To analyze reproductive condition, we recaptured females several times and noted mating scars visually and the presence of eggs by palpation.

followed histological procedures We for skeletochronological estimation of age after Castanet (1978, 1987). Toes were washed for three hours in water and then decalcified in 3% nitric acid for one-two hours, according to the size of the bone. They were then rinsed with running tap water overnight. Frozen sections of 16 µm from the mid diaphysis were obtained on a cryostat freezing microtome, stained in Ehrlich's haematoxylin for 5 min and rinsed for 1 hr in tap water. The smallest transverse sections with the smallest medullar cavity and the thickest cortical bone, were selected and mounted in aqueous synthetic resin (Aquamount, Gurr) on a glass microscope slide. The age of specimens was determined by counting the number of lines of arrested growth (LAGs). Phalanges of two females captured in 2000 and recaptured in 2001 showed one additional LAG in the second year. These data show that LAGs are annually deposited in this population. Bone remodelling

Correspondence: A. Salvador, Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales, José Gutiérrez Abascal 2, 28006 Madrid, Spain. *E-mail*: mcnas500@mncn.csic.es



FIG. 1. First capture date of males (white bars) and females (black bars) of *Lacerta lepida*. Years 2000 and 2001 combined.

was obvious at the inner border of the periosteal bone. First – and even second – LAGs were partially eroded in older individuals. However, there was no evidence of LAGs totally removed by the process of endosteal resorption.

Over the two years, the first individual of the year was captured on 1 March 2000 and 26 April 2001. However, in both years, most individuals were captured for the first time during May (Fig. 1). There were no signifi-



FIG. 2. Relationship between age and size of males (black dots) and females (white dots) of *Lacerta lepida*. A, relationship between age and mass. B, relationship between age and snout-vent length.

cant differences in the date of first capture between males and females (Mann-Whitney U-Test, P=0.50).

A test of homogeneity of slopes showed that the variation of mass with age differed significantly between sexes ($F_{1,20}$ =7.78, P=0.01) (Fig. 2a). A similar result was obtained for the SVL, but in this case the difference between slopes was marginally significant ($F_{1,20}$ =3.72, P=0.07) (Fig. 2b). These results show that males and females followed a different growth trajectory, males being increasingly longer and heavier at any given age above 3-4 years.

Home-range area of four females (SVL=145-157 mm) based on 28-67 location points (mean=46 points, SE=8.2) varied between 2800-5844 m² (mean=3750 m², SE=704). Home range area of eight males (SVL=122-175 mm), based on 31-86 location points (mean=45 points, SE=6.1), varied between 1424-22106 m² (mean=11087 m², SE=2673). Mass covaried significantly with all home-range estimates but the effect of sex was only significant for 75 and 100% home range areas (Table 1).

An ANCOVA on 100% home-range area with mass and age as covariates and sex as fixed factor showed a significant effect of mass ($F_{1,11}$ =14.4, P=0.005), but no significant effects of age ($F_{1,11}$ =0.003, P=0.96) or sex ($F_{1,11}$ =1.4, P=0.27). In the 1 ha plot, each male's home range overlapped with the home ranges of 2-6 males (mean=3.7 males, SE=0.5) and 2-6 females (mean=3.3 females, SE=0.6).

Lizards spent the night under rocks 0.5-5 m in diameter. Four males (SVL=129-175 mm) used 11-17 different shelters (mean=13.5 shelters, SE=1.5) during 17-25 nights (mean=21.5 nights, SE=1.6 nights). Males used the same shelter during 2-4 consecutive nights. The same night shelter was used by the same individual and others at different times during the spring. We never observed two males together in a night shelter.

Two females (SVL=145 and 157 mm, 4 and 6 yrs old respectively) had mating scars, and later were observed to be gravid and laid eggs. Six females did not show any evidence of reproductive activity. Three of these females were small (SVL=119-122 mm), but the other three were large (SVL=145-180 mm; age= 6-10 years).

During the radiotracking period, three males were captured by predators. One of them (SVL= 173 mm) was found while being predated by a Montpellier snake (*Malpolon monspessulanus*) in 2000. Another male (SVL= 165 mm) disappeared from the study area in the same year and we were subsequently unable to detect it in the surrounding area. Thus, we supposed that it had

TABLE 1. ANCOVAs on home-range estimates with sex as factor and mass as covariate. Data for 8 males and 4 females.

	50% core area		75%	core area	100% area		
	F	Р	F	Р	F	Р	
Mass	6.65	0.003	17.8	0.002	30.0	< 0.001	
Sex	2.34	0.16	6.0	0.03	7.0	0.027	

been captured by an unidentified predator. The head of a third male (SVL=155 mm) was found near its transmitter on top of a 15 m boulder in 2001, suggesting that it had been killed by a bird of prey. None of the females were killed by predators.

At the study site, eyed lizards emerge from hibemation later than the smaller lizard Psammodromus algirus, which is active from February (Veiga & Salvador, 2001). It is possible that eyed lizards that emerge early risk predation exposure during long basking periods because of low temperatures. Late emergence from hibernation leads to a short annual period of activity of no more than six months. Thus, time available for growth is relatively short, which may account for the small size of adults in comparison to those from lower altitudes in central parts of the Iberian Peninsula (Castilla & Castanet, 1986; Mateo & Castanet, 1994). The low number of reproductive females also indicates that females do not reproduce every year. This result suggests that females are frequently unable to accumulate enough reserves for reproduction during the relatively short annual activity period.

The lack of differences in 50% core area between males and females seems to indicate that this area would mainly be related to fulfilling energetic requirements. In fact, 50% core area accounted only for 12.6 ± 1.9 (mean±SE) percent of the total home range. The larger home ranges of eyed lizard males may be related to the need to maximize access to females, imposing costs of reproduction on males, but there are few field data sets available (Perry & Garland, 2002). Large male eyed lizard have extensive home-range areas and this may be related to maximizing access to the few reproductive females available, but costs may be high, as indicated by the mortality data.

Acknowledgements. The "El Ventorrillo" Field Station of the Museo Nacional de Ciencias Naturales provided logistical support. The research was supported by DGESIC projects PB97-1245 and BOS2001-0533, and 07M-0109-00CAM. Permission for this study was granted by Agencia de Medio Ambiente, Comunidad de Madrid (10/184331.7/99 and 10/209806.7/00).

REFERENCES

- Bischoff, W., Cheylan, M. & Böhme, W. (1984). Lacerta lepida Daudin, 1802 - Perleidechse. In Handbuch der Reptilien und Amphibien Europas. Vol. 2, 181-210.
 Böhme, W. (Ed.). Wiesbaden: Aula Verlag.
- Castanet, J. (1978). Les marques de croissance osseuse comme indicateurs de l'âge chez les lézards. *Acta Zoologica* **59**, 35-48.
- Castanet, J. (1987). La squelettochronologie chez les Reptiles. 111 Aplication. Annales Sciences Naturelles, Zoologie 8, 157-172.
- Castilla, A. & Castanet, J. (1986). Growth, age and longevity of *Lacerta lepida* (Lacertidae, Lacertilia) assessed by skeletochronology. In *Studies in Herpetology*, 331-336. Rocek, Z. (Ed.). Prague: Charles University.
- Mateo, J. A. & Castanet, J. (1994). Reproductive strategies in three Spanish populations of the ocellated lizard, *Lacerta lepida* (Sauria, Lacertidae). *Acta Oecologica* 15, 215-229.
- Pérez-Mellado, V. (1998). Lacerta lepida Daudin, 1802.
 In Reptiles. Fauna Ibérica. Vol. 10, 198-207. Salvador,
 A. (Coord.). Madrid: Museo Nacional de Ciencias Naturales.
- Perry, G. & Garland Jr., T. (2002). Lizard home ranges revisited: effects of sex, body size, diet, habitat, and phylogeny. *Ecology* 83, 1870-1885.
- Rose, B. (1982). Lizard home range: methodology and functions. *Journal of Herpetology* **16**, 259-269.
- Salvador, A., Martín, J. & López, P. (1995). Tail loss reduces home range size and access to females in male lizards, *Psammodromus algirus. Behavioral Ecology* 6, 382-387.
- Veiga, J. P. & Salvador, A. (2001). Individual consistency in emergence date, a trait affecting mating success in the lizard *Psammodromus algirus*. *Herpetologica* 57, 99-104.

Accepted: 1.5.03

FORUM

HERPETOLOGICAL JOURNAL, Vol. 14, pp. 51-53 (2004)

TORTOISE SYSTEMATICS: A CRITIQUE OF A RECENT PAPER BY VAN DER KUYL *ET AL*. (2002)

JARMO PERÄLÄ

Ecological Genetics Research Unit, Department of Ecology and Systematics, University of Helsinki, Finland

Key words: nomenclature, taxonomy, Testudines, phylogeny

An article by van der Kuyl et al. (2002) on tortoise systematics published recently in Molecular Phylogenetics and Evolution [MPE] demands critical discussion. My written criticism, submitted initially for publication in MPE's Letters to the Editor section, was rejected by the editor on the grounds that "...it mainly criticizes formal taxonomic practices of van der Kuyl et al. (2002) rather than providing noteworthy reinterpretations of molecular evolutionary issues". Thus, it seems that MPE is prepared to publish papers on phylogenetic issues in zoology even if such works fail to comply with the International Code of Zoological Nomenclature (ICZN, 1999) [henceforth the Code]. Consequently, and also because of the independently unverifiable origins of the materials used, it is currently almost impossible to interpret the results as published in van der Kuyl et al. (2002). In other words, the paper in question does not allow reinterpretation of evolutionary issues because of the unorthodox nomenclature and presentation used. For this critique, names of taxa as used by van der Kuyl et al. (2002) are indicated with quotation marks ["..."].

General comments. The authors (van der Kuyl et al., 2002) used mitochondrial 12S rRNA gene sequences to test variation and phylogenetic relationships among Mediterranean and Central Asian tortoises, that is, the genus Testudo Linnaeus 1758 sensu lato (in the sense of Lapparent de Broin, 2000, 2001; among others). Sampled testudinid taxa also included representatives of Geochelone Fitzinger 1835, Chelonoidis Fitzinger 1835, and Indotestudo Lindholm 1929. Maximum likelihood and neighbour-joining analyses yielded close to identical tree topologies. Two major lineages within "Testudo" comprise (a) "T. graeca", "T. marginata" and "T. kleinmanni" and, (b)" T. hermanni", "T. horsfieldii" and "Indotestudo elongata", according to the authors. Van der Kuyl et al. (2002) state that maximum parsimony analysis supports the first clade but not the second. However, the published phylogenies pertaining to testudinids - apart from their monophyly - generally

have very low bootstrap support and are thus largely unresolved, a point the authors fail to address. Haplotype variation is said to be greater in "*T. graeca*" than in "*T. hermanni*". Despite the interesting scope of the article, it seems likely that the reviewers of this particular paper were molecular biologists exclusively, rather than taxonomists, and that no experts in chelonian systematics were consulted before acceptance and publication.

Multiple references to earlier literature in van der Kuyl et al. (2002) appear without an actual citation, as if the published inferences were those of the authors themselves [e.g., "...six species are currently recognized in the genus Testudo"]. On the other hand, several relevant references to testudinid systematics from within the past ten to twenty years (including the erection of new taxa: Chkhikvadze & Tuniyev, 1986; Weissinger, 1987; Chkhikvadze, 1988; Chkhikvadze et al., 1990; Chkhikvadze & Bakradze, 1991; Pieh, 2001a; to name a few) are omitted without explanation, or citations refer to second-hand sources (e.g. "Ernst & Barbour, 1989b", with reference to the resurrection of Indotestudo). Problematic taxa erected or resurrected over a decade ago by Martin and/or Highfield, the validity of which have been debated at least since the publication of lyerson (1992). are taken seemingly at face value by van der Kuyl et al. (2002). Generalized, and incorrect or contradictory, statements are made without empirical proof, particularly relative to geographical distributions, and supposed human introductions, of taxa. Some species group names are attached to geographical populations irrespective of their correct use and/or previously published literature [see Specifics below]. Apart from being very short (circa 400 nucleotides only), the gene sequence used is probably too conservative for inferring taxonomically meaningful variation in testudinids at species level [meaning subspecific level in the sense of the authors], because mitochondrial 12S rRNA partly fails to mirror major structural differences which would be found easily by the application of morphological methodologies (e.g. Perälä, 2002). Other - and preferably multiple - genes might be more suitable for this task. In addition, "areas of difficult alignment" (van der Kuyl et al., 2002) - which could potentially contain relevant information - were excluded from the analysis. Of quickly evolving mitochondrial gene sequences, cytochrome b (Lenk et al., 1998, 1999; Feldman & Parham, 2002) and additionally adjacent tRNAs and ND4 (Feldman & Parham, 2002) have been shown to be relatively informative at the species level in other chelonians. For example, cytochrome b was more informative than 12S rRNA regarding genetic variation in T. graeca Linnaeus 1758 from Morocco and Spain (Álvarez et al., 2000). Only subsequent analyses will show which genes, and whether mitochondrial or nuclear, are most useful in reconstructing the phylogenetic history of Mediterranean and Central Asian tortoises - a history that should be reflected in classifications.

Specifics. It is not clear why some allopatric populations classified initially under the same taxon and

Correspondence: J. Perälä, Ecological Genetics Research Unit, Department of Ecology and Systematics, PO Box 65 (Biocenter 3, Viikinkaari 1), FIN-00014 University of Helsinki, Finland. *E-mail*: jarmo.perala@helsinki.fi

with demonstrated 12S rRNA divergence were included in the phylogenetic analysis (African "T. graeca" including "T. whitei") by van der Kuyl et al. (2002), but others were not: sequences derived from some of the investigated populations of "T. hermanni" and some European "T. graeca" were excluded, while others were included. The selected outgroup taxa (*Emys* Duméril 1806 and Trachemys Agassiz 1857 [both Emydidae Rafinesque 1815]; and Cuora Gray 1855 [Geoemydidae Theobald 1868]) might additionally be suboptimal for the purpose. It is possible that these taxa are phylogenetically too distant in relation to the ingroup to be of practical use in character polarization. The use of other more closely related testudinids in the outgroup should have been the logical choice. More dramatically, the paper displays a lack of understanding of fundamental taxonomic principles, such as the fact that scientific names are permanently attached to type specimens which determine type localities and the subsequent use of nomenclature for a given population, not to mention other basic taxonomic applications as regulated by the Code (ICZN, 1999). This should be unacceptable in a paper dealing with biological systematics. It is very hard to interpret what van der Kuyl et al. (2002) actually meant by some of the taxon names (they were sometimes misspelled, such as "chelonoides" for Chelonoidis Fitzinger 1835), which appear with (though often incorrect) or without authority and date. Although (part of) the mentioned sequence data are deposited with GenBank, it does not help the interpretation of the results that no independently verifiable data are provided for the geographical origins of the samples; that is, references to voucher specimens (and their physical location) with accompanying detailed locality information. The importance of being able to simultaneously analyse morphology and DNA data derived from exactly the same specimens is emphasized by Puorto et al. (2001).

As for confusing taxonomy, there are too many incorrect applications to be mentioned point by point. However, one notable failure is that the name "*Testudo whitei* (Highfield and Martin, 1989)", used presumably for *T. whitei* Bennett 1836, is applied to North African tortoises despite the earlier inference that the type of *T. whitei* (which the authors have not examined; Ballasina, pers. comm. 2002), does not correspond morphologically to any African testudinid, nor to the specimens attributed to *T. whitei* Bennett by Highfield & Martin (1989) (Bour in David, 1994; Perälä in Ernst *et al.*, 2000). It is also noteworthy that in their Table 1 (including its legend), the authors technically introduce although probably unintentionally - three new names as follows (van der Kuyl *et al.*, 2002: 180):

(a) "*Testudo* (graeca) whitei d"..."^d Proposed new species, formerly *T. graeca*" from Algeria. The name *Testudo graeca whitei* van der Kuyl *et al.* 2002 is a primary junior homonym of *T. whitei* Bennett 1836, and thus permanently invalid (ICZN, 1999: Art. 57.1). Furthermore, it is a *nomen nudum* because no type specimen was fixed and because the new name was not

accompanied by a description (ICZN, 1999: Articles 16.4, 72.3, and 13.1.1). In this context it is relevant to note that the type locality of *Testudo graeca* Linnaeus 1758 is in Santa Cruz, Oran, Algeria by designation of Strauch (1862), and that *T. graeca* is also the type species of *Testudo* Linnaeus 1758 by designation of Bell (1828).

(b) "*Testudo graeca Sardinia* ^a"..."^a Proposed new subspecies" from Sardinia. The name *Testudo graeca sardinia* van der Kuyl *et al.* 2002 is unavailable (a *nomen nudum*) according to Articles 16.4, 72.3, and 13.1.1 (ICZN, 1999) because no holotype was designated and no description was provided.

(c) "Testudo hermanni boettgeri" from "Greece (Peloponnesus) "…"^a Proposed new subspecies." The name Testudo hermanni boettgeri van der Kuyl et al. 2002 is a primary junior homonym of T. hermanni boettgeri Mojsisovics 1889 and thus permanently invalid (ICZN 1999: Art. 57.1). It is also a nomen nudum because no type specimen was fixed and because the new name lacked a description (ICZN, 1999: Articles 16.4, 72.3, and 13.1.1).

It is also noteworthy that, the paper by van der Kuyl et al. (2002) was published later elsewhere with only cosmetic changes and with a reshuffled order of authors (Ballasina et al., 2002). Because all of the mistakes described above for the article by van der Kuyl et al. (2002) were retained in Ballasina et al. (2002), including technical introductions of new names, these names, *Testudo graeca whitei* Ballasina et al. 2002 and *Testudo graeca sardinia* Ballasina et al. 2002 (Ballasina et al., 2002: 123, Table 1 including legend) are similarly unavailable for the same reasons. Additionally, the name "*Testudo graeca ibera*" is used inexplicably for Lebanese tortoises (Ballasina et al., 2002: 123, Table 1).

The present criticism is a serious one because it is essential to address the matter sooner rather than later from the points of view of stable nomenclature, general editorial practice regarding evolutionary journals, and before mistakes are adopted into subsequent literature, or into conservation and animal-welfare policies. The latter policies might potentially legitimize introductions or translocations of individuals with identical molecular make-ups in the sense of van der Kuyl et al. (2002) - but without known origins or parentage [confiscations, captive stock] - into wild populations. This could have catastrophic effects on the genetic authenticity, as well as (due to ecological incompatibilities or pathology) for the general well-being of tortoise populations in the Mediterranean (Anonymous, 2001; Pieh, 2001b; Perälä, 2001), a region still certainly harbouring as yet formally undescribed tortoise diversity (Perälä, 2002).

Acknowledgements. I thank an anonymous reviewer for his comments which enhanced the final version of this critique.

Editor's Note: Van der Kuyl *et al.* were offered the opportunity to reply to Perälä's critique but declined to do so.

REFERENCES

- Álvarez, Y., Mateo, J. A., Andreu, A. C., Díaz-Paniagua, C., Dietz, A. & Bautista, J. M. (2000). Mitochondrial DNA haplotyping of *Testudo graeca* on both continental sides of the Straits of Gibraltar. *Journal of Heredity* 91, 39-41.
- Anonymous (2001). DGHT-AG Schildkröten beschliesst cinstimmig Resolution gegen unverantwortliche Auswilderung. *Elaphe* 9, 37-38.
- Ballasina, D. L. Ph., van der Kuyl, A. C., Dekker, J. T., Maas, J., Willemsen, R. E. & Goudsmit, J. (2002). Phylogenetic relationships among the species of the genus *Testudo* (Testudines: Testudinidae) inferred from mitochondrial 12S rRNA gene sequences. In *Chelonii* 3, 117-129: Actes du Congrès International sur le genre Testudo - 7 au 10 Mars 2001. B. Fertard and B. Culorier (Eds.). Gonfaron, France: Editions SOPTOM.
- Bell, T. (1828). Characters of the order, families, and genera of the Testudinata. *Zoological Journal* **3**, 513-516.
- Chkhikvadze, V. M. (1988). Taxonomic status of modern land tortoise of Middle Asia and Kazakhstan [sic]. News of the Academy of Sciences, Georgian S.S.R., Biological Series 14, 110-113. (In Russian)
- Chkhikvadze, V. M., Amiranashvili, N. G. & Ataev, C. H. (1990). New subspecies of tortoise from the southwestern Turkmenistan [sic]. News of the Science Academy of Turkmen S.S.R., Biological Series 1, 72-75. (In Russian)
- Chkhikvadze, V. M. & Bakradze, M. A. (1991). On the systematic position of the Recent land turtle from the Araxes Valley [sic]. *Trudy Tbilissk* 305, 59-63: University of Tbilisi. (In Russian)
- Chkhikvadze, V. M. & Tuniyev, B. (1986). On the taxonomic status of modern land tortoise of the western Transcaucasus [sic]. Bulletin of the Academy of Sciences of the Georgian S.S.R. 123, 617-620. (In Russian)
- David, P. (1994). Liste des reptiles actuels du monde. I. Chelonii. *Dumerilia* 1, 1-127.
- Ernst, C. H., Altenburg, R. G. M. & Barbour, R. W. (2000). *Turtles of The World*: World Biodiversity Database CD-ROM Series. University of Amsterdam: Expert Center for Taxonomic Identification & Paris: UNESCO-Publishing.
- Feldman, C. R. & Parham, J. F. (2002). Molecular phylogenetics of Emydine turtles: Taxonomic revision and the evolution of shell kinesis. *Molecular Phylogenetics and Evolution* 22, 388-398.
- Highfield, A. C. & Martin, J. (1989). Testudo whitei Bennett, 1836. New light on an old carapace – Gilbert White's Selborne tortoise re-discovered. Journal of Chelonian Herpetology 1, 13-22.
- ICZN (1999). International Code of Zoological Nomenclature. Fourth Edition adopted by the International Union of Biological Sciences. London: ITZN.

- Iverson, J. B. (1992). A Revised Checklist with Distribution Maps of the Turtles of the World. Richmond, Indiana: Privately published.
- Lapparent de Broin, F. de, (2000). Les chéloniens de Sansan. In La faune miocène de Sansan et son environnement. Ginsburg, L. (Ed.). Mémoires du Muséum National d'Histoire Naturelle 183, 219-261.
- Lapparent de Broin, F. de, (2001). The European turtle fauna from Triassic to the present. *Dumerilia* 4, 155-217.
- Lenk, P., Fritz, U., Joger, U. & Wink, M. (1999). Mitochondrial phylogeography of the European pond turtle, *Emys orbicularis* (Linnaeus 1758). *Molecular Ecology* 8, 1911-1922.
- Lenk, P., Joger, U., Fritz, U., Heidrich P. & Wink, M. (1998). Phylogeographic patterns in the mitochondrial cytochrome b gene of the European pond turtle (*Emys orbicularis*): first results. In Mertensiella 10, 159-175: Proceedings of the EMYS Symposium Dresden 96. Fritz, U., Joger, U., Podloucky, R. & Servan, J. (Eds.). Rheinbach: DGHT.
- Perälä, J. (2001). A new species of *Testudo* (Testudines: Testudinidae) from the Middle East, with implications for conservation. *Journal of Herpetology* 35, 567-582
- Perälä, J. (2002). The genus Testudo Linnaeus, 1758 sensu lato (Testudines: Testudinidae): Phylogeny, Taxonomy, Conservation. PhD thesis, University of Bristol.
- Pieh, A. (2001a). Testudo graeca soussensis, eine neue Unterart der Maurischen Landschildkröte aus dem Sousstal (Südwest-Marokko). Salamandra 36, 209-222.
- Pieh, A. (2001b). Eine Resolution gegen unverantwortliche Auswilderung von Schildkröten. *Radiata* 10, 18.
- Puorto, G., Da Graça Salomão, M., Theakston, R. D. G., Thorpe, R. S., Warrel, D. A. & Wüster, W. (2001). Combining mitochondrial DNA sequences and morphological data to infer species boundaries: phylogeography of lanceheaded pitvipers in the Brazilian Atlantic forest, and the status of *Bothrops* pradoi (Squamata: Serpentes: Viperidae). Journal of Evolutionary Biology 14, 527-538.
- Strauch, A. (1862). Chelonologische Studien mit besonderer Beziehung auf die Schildkrötensammlung der kaiserlichen Akademie für Wissenschaften zu St. Petersburg. Mémoires de l'Académie Impériale des Sciences de St.-Pétersbourg, VII^e Série 5(7), 1-196 + 1 pl.
- Van der Kuyl, A. C., Ballasina, D. L. Ph., Dekker, J. T., Maas, J., Willemsen, R. E. & Goudsmit, J. (2002). Phylogenetic relationships among the species of the genus Testudo [sic] (Testudines: Testudinidae) inferred from mitochondrial 12S rRNA gene sequences. *Molecular Phylogenetics and Evolution* 22, 174-183.
- Weissinger, H. (1987). *Testudo graeca anamurensis* ssp. nov. aus Kleinasien. ÖGH-Nachrichten 10/11, 14-18.

Accepted: 22.10.03

BOOK REVIEWS

The Snakes of Trinidad & Tobago. Hans E. Boos (2001). 270 pp. Texas A & M University Press, College Station, Texas. £36.50 (cloth).

I read most of this book while flying to England to resume a study I have been doing on the ecology of grass snakes (Natrix natrix) and I must confess that Hans Boos caused me to wonder briefly if I had chosen to go in the wrong direction this time. By tropical standards, the snake fauna of Trinidad and Tobago is not spectacularly rich - after all, these are islands - but it is huge compared to that of England, and certainly large enough to more than stir my interest. According to Boos, Trinidad has 44 species of snakes, four of them venomous, and Tobago 21, none venomous, for a total fauna on the two islands of 47 species. Several of these species are rare, however, and there also are a number of disputed records of other species, all thoroughly discussed and analyzed by Boos. Most of the species found on Tobago also occur on Trinidad, but each island has one endemic species (Erythrolamprus ocellatus on Tobago and Leptophis stimsoni on Trinidad); each also has an endemic subspecies of different wider-ranging species. In total, the fauna of the two islands is represented by seven Families: Typhlopidae, Leptotyphlopidae, Aniliidae, Boidae, Colubridae, Elapidae, and Viperidae. Boos introduces and describes this fauna, discusses various myths about snakes, and pleads for appreciation of snakes and for their conservation.

The book is comprised of six sections: a long introduction; a series of individual species accounts; a chapter on snakebite; two brief appendices; a glossary; and a lengthy list of references. The introduction consists of descriptions of Trinidad and Tobago (geography, climate, topography, etc.), a brief summary of snake biology, and a detailed history of herpetology in Trinidad and Tobago, naturally focused on snakes. This review of history makes up the bulk of the introduction and it is very comprehensive. It will appeal to those with a specialist interest in the area, but it is a long haul for the more generalist reader – I doubt that anyone who has had even the slightest involvement with the study of snakes in Trinidad and Tobago is not mentioned. That said, I found a theme or two amid all the detail. One such theme that impressed me was how difficult it has been to construct a list of species of snakes that genuinely occur on Trinidad or Tobago. Boos gives a good account of how historical confusions arise, plaguing efforts to enumerate species, and lists the repeated errors that have been made, including his own. I also detected a note of faint exasperation at the taxonomic name changes that Boos has had to deal with in assessing this fauna. On page 30 of the introduction, for example, he comments on an "avalanche of papers" and "reshuffling of genera" with "little new being discovered". One thing is clear, however: Boos himself has been in the thick of herpetological work on Trinidad and Tobago and has

had the direct acquaintance of many of the other main players. This gives the book not only a strong personal touch, but authority.

One thing that we are losing with the current trend towards standardizing common names (e.g. CSESN, 2000) is local colour. For example, on Vancouver Island, where I live, reddish specimens of the Northwestern Garter Snake (Thamnophis ordinoides) are sometimes called by the wonderfully evocative name of "Red Racer". There apparently is little fear of loss of this kind of thing on Trinidad and Tobago, though, where the various species of snakes have amazing, and usually multiple, common names. My favourite is "Ground Puppy" for Leptotyphlops albifrons, but other memorable ones include "Beauty of the Road" for Liophis melanotus and "Brother Death" for Lachesis muta, Boos lists 27 local names for the latter species, many of them variations on the same word or words; five to ten local names for the same species does not seem to be unusual. Many of these names are derived from French Creole (e.g. "Mapepire Balsain" for Bothrops atrox).

Boos presents a separate account for each species known to occur on Trinidad or Tobago. Some of these are fairly brief, especially if not much is known about the species, but some accounts are very long indeed (e.g. 17 pages on the anaconda). The longer accounts are devoted to those species that are either well studied or spectacular in some way (e.g. giant snakes or venomous snakes). The subheadings in each species account include: Specimens; Description; Range; Local Names; and Natural History. Many accounts are accompanied by black-and-white photographs, with a separate set of colour plates in the middle of the book. Boos exhaustively reviews any contentious issues about taxonomy or distribution and writes a great deal about folklore, again especially with respect to the more spectacular species. However, he is not short on stories about other kinds of snakes, either; curiously, for example, several species are reputed to attack and beat pregnant women. Boos also does a good job of tying some of these myths to folklore from other parts of the world. Evidently, as in various other parts of the world, there is a general belief in Trinidad that all species of snakes are venomous and dangerous, so that any snake tends to be killed on sight; Boos attempts to dispel these myths and to allay fears. There is plenty of basic information about natural history of snakes in these species accounts, but, despite the sheer barrage of stories in places, I found the "tall tales" aspect of this book most fascinating.

The section on snakebite is typically exhaustive and again replete with anecdotes, including those about various antidotes for snake venom. Boos provides a useful summary of venomous snakes and the venom apparatus, factors influencing the severity of snakebite, and the nature and action of snake venoms. Although he does note in passing the possibility of adverse reactions of patients to antivenins, I was surprised that this issue did not receive a bit more emphasis. The two appendices cover, respectively, "The Belgian Black Stone" (an alleged cure for snakebite) and how to identify large black snakes in Trinidad and Tobago. This somewhat quirky selection of topics extends to the final glossary, a series of tersely, and sometimes poorly, defined terms.

There are a few obvious typographical errors. For example, on p. 161, Fig. 44 is misidentified as Fig. 43. The toad's "parotid" glands on p. 192 should be "parotoid" glands. Boos also repeatedly uses the rather outmoded term "ovoviviparous" in reference to livebearing snakes, rather than the contemporary "viviparous". Then again, maybe "ovoviviparous" better fits the historical tone of much of this book, which several times took me back to Ditmars' day (no doubt aided by the fairly frequent reference to the man himself). Boos' writing is pretty dense in places and I'm not sure I'd recommend it as bedtime (or aeroplane) reading. However, he did make me feel like visiting Trinidad to look for snakes. If I ever do so, his book will be a most valuable reference and definitely worth re-reading.

REFERENCES

Committee on Standard English and Scientific Names (CSESN - Brian I. Crother, Chair). (2000). Scientific and standard English names of amphibians and reptiles of North America north of Mexico, with comments regarding confidence in our understanding. Society for the Study of Amphibians and Reptiles, Herpetological Circular No. 29.

Patrick T. Gregory University of Victoria

Amphibians and Reptiles of Baja California including its Pacific Islands and the Islands in the Sea of Cortés. (2002) L. Lee Grismer. 413 pp. University of California Press, Berkeley. £66.00 (cloth).

The front cover of this striking book is sufficient to fire the imagination of most red-blooded herpetologists. It features a blue-headed lizard (*Pterosaurus thalassinus*) basking on a red rock which overlooks a steep, vegetated slope leading down to a sea coloured, in part, the most unlikely shade of green. The spectacular photography does not end there. Indeed it seems that each section's title page is inhabited by a quite unlikely creature, the colours of which are exceeded only by the beauty of its panoramic background.

The book begins with a brief introduction by Harry W. Greene and continues with an equally brief preface. There then follows a well-written general introduction containing (importantly) clear maps of the peninsula and its islands, as well as photographs of the diverse variety of its vegetation types. There are also several useful reference tables. By page 40, however, the book gets around to the part which many armchair explorers will want to see – the species accounts. This section has its own introduction (basically "How to use this book") and a taxonomic key. Each group (salamanders, frogs and

toads, turtles and tortoises, lizards, and snakes) family and genus is described with a concise paragraph and each species account has details of identification, relationships and taxonomy, distribution, description, geographic variation, natural history, and additional remarks. There is also a small photograph of a specimen of each species and a distribution map. I would like to criticise the size of the photographs (8 × 5 cm) but in fact they are generally very clear and if any larger would have reduced the amount of space for the informative text. After all, this is not a field guide and the text descriptions seem precise, especially with additional reference to the section's introduction and 11-page glossary.

I turned eagerly to the account for *Bufo punctatus* (the red-spotted toad), a species evocative of Baja (which I remember from night-driving around the southern part of the peninsula during the international meeting in La Paz, 2000 – it was fortunate that there had been rain!) and was pleased to see it treated to a comprehensive account including a description of its mating call (true for all the vocal amphibians) and tadpole, as well as a comment that, in parts of the peninsula, its viscera is sometimes used to treat rattlesnake bites!

Looking through the extensive section on lizards, it is again tempting to wish for larger photographs, but each species' "Identification" text refers to unique characters which can often be seen in the well-chosen pictures used. Added to this, many species have disjunct distributions and can be identified by location! More than one photograph is provided for extremely variable species and the distribution maps for species (e.g. the zebratailed lizard, Callisaurus draconoides) with distinguishable subspecies clearly show the position(s) of any intergrade zones. For those species (of any group) which look essentially identical without the finest examination, larger photographs would in any case be of no help!

It is good to see, for each species, text on its natural history, as this is sadly lacking in some similar books. For species where the natural history is poorly known, the author says so, and does give any information which is available.

At the end of the book are appendices covering insular species, a (welcome) taxonomic key in Spanish and a list of cited references, as well as an index.

In short, this is an exhaustive and fascinating book (preferably to be perused at leisure over a bottle of *Modelo Negro*) which could only have been produced by an author with extensive first-hand experience of the animals and habitats within its pages. Absolutely essential for anyone with an interest in the herpetofauna of Lower California. In fact, if I must find a bad word to say about it, it would be that it cannot be slipped into the pocket so as to be referred to whilst chasing a disappearing night-snake through a field of vicious cacti...

John W. Wilkinson *The Open University*

THE HERPETOLOGICAL JOURNAL

INSTRUCTIONS TO AUTHORS (revised January 2004)

- 1. The Herpetological Journal publishes a range of features concerned with reptile and amphibian biology. These include: Full Papers (no length limit); Reviews and Mini-reviews (generally solicited by a member of the editorial board); Short Notes; controversies, under Forum (details available from the Editor); and Book Reviews. Faunistic lists, letters and results of general surveys are not published unless they shed light on herpetological problems of wider significance. Authors should bear in mind that the Herpetological Journal is read by a wide range of herpetologists from different scientific disciplines. The work should therefore appeal to a general herpetological audience and have a solid grounding in natural history.
- 2. Two copies of all submissions, and illustrations, should be sent to the Scientific Editor, together with a computer diskette (IBM formatted) containing the text and, if possible, figures. Alternatively, submission by e-mail is possible please contact the Scientific Editor for information. All papers will be subject to peer review by at least two referees. Authors are invited to suggest the names of up to three referees, although the editor may choose alternative referees to those suggested. Papers will be judged on the basis of the reports supplied by referees, scientific rigor, and the degree of general interest in the subject matter. The Editor's decision will be final
- 3. Authors should consult a recent issue of the Journal regarding style. Papers should be concise with the minimum number of tables and illustrations. They should be written in English and spelling should be that of the *Oxford English Dictionary*. Papers should be typed or produced on a goodquality printer, and double-spaced with wide margins all round. The journal is typeset direct from the author's electronic text, so all manuscripts should be prepared using a word processor (preferably on a PC-compatible microcomputer). If figures are prepared using computer graphics, they should be supplied separately and NOT embedded in the text of the word processor file. Preferred formats are MS Word for Windows (text) and MS Excel, Bitmap, TIFF, Windows Metafiles (.wmf, emf) or JPEG files (graphics).
- 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.
- Tables are numbered in a rabie numerals, e.g. TABLE I: 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 metrie 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 cate of young red-eared terrapins (*Pseudemys scripta* elegans) in the laboratory. British Journal of Herpetology 2, 206–210.
 - Dunson, W. A. (1969a). Reptiltan salt glands. In *Exocrine glands*, 83=101. Botelho, S. Y., Brooks, E. P. and Shelley, W. B. (Eds). Philadelphia: University of Pennsylvania Press.
 - Dunson, W. A. (1969b). Electrolyte electrolyte electrolyte salt gland of the Galapagos marine iguana. *American Journal of Physiology* **216**, 995–1002
- 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. Twenty-five offprints OR a PDF file of the paper are provided free of charge. Further copies (minimum of twenty-five) may be purchased provided that they are ordered at the time the proofs are returned.
- 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.

THE HERPETOLOGICAL JOURNAL

Volume 14, Number 1 2004

CONTENTS

Full papers

Bias in estimation of newt population size: a field study at five ponds using drift fences, pitfalls and funnel traps	K. WEDDELING, M. HACHTEL, U. SANDER & D. TARKHNISHVILI	1
Annual cycle of nutritional organ mass in a temperate zone anuran, <i>Rana chensinensis</i> , from northern China	Xin Lu	9
Feeding ecology of <i>Vipera latastei</i> in northern Portugal: ontogenetic shifts, prey size and seasonal variations	J. C. Brito	13
Reassessment of the validity and diagnosis of the pitviper Trimeresurus venustus Vogel, 1991	A. MALHOTRA & R. S. THORPE	21
The inter- and intraspecific status of Aegean <i>Mauremys rivulata</i> (Chelonia, Bataguridae) as inferred by mitochondrial DNA sequences	G. Mantziou, N. Poulakakis, P. Lymberakis, E. Valakos & M. Mylonas	35
Short Note		
Preliminary data on reproductive ecology of Lacerta lepida at a mountain site in central Spain	A. SALVADOR, J. P. VEIGA & M. ESTEBAN	47
Forum		
Tortoise systematics: a critique of a recent paper by van der Kuyl <i>et al</i> .	J. PERÄLÄ	51
Book Reviews		55

Herpetological Journal vol. 13, no. 4 was published on 12 May 2004