Biogeography of *Neurergus strauchii barani* Öz, 1994 and *N. s. strauchii* (Steindachner, 1887) (Amphibia: Salamandridae) assessed using morphological and molecular data

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Abstract. Five new locations of *N. strauchii* are reported from Turkey, closing the gap between the two subspecies *N. s. barani* and *N. s. strauchii*. A molecular analysis based on 829 base pairs from two mitochondrial ribosomal genes (12S and 16S rRNA), together with geographical data from the area concerned, indicate all new populations found are very closely related to the mitochondrial sequences from specimens of *N. s. strauchii* from its type locality and suggest the river Euphrates might have acted as a natural barrier separating the only populations of *N. s. barani* known to date from all other populations of *N. s. strauchii*. A morphological analysis of all *N. strauchii* populations sampled for this study indicates that belly patterns are not a good diagnostic character to differentiate between *N. s. barani* and *N. s. strauchii*, suggesting the only reliable morphological taxonomic character that allows their identification is the different number of yellow spots in adult specimens, which is significantly higher in *N. s. strauchii*. The analyses also indicate that the number of spots is similar between sub-adult and adult *N. s. barani* but significantly different between sub-adult and adult specimens of *N. s. strauchii*, suggesting there is also a difference between both subspecies in increase in the number of spots during maturation.

Listed by IUCN as vulnerable and protected by the Bern Convention (1979), our findings indicate *N. s. strauchii* is more widely distributed than previously thought, while *N. s. barani* would be restricted to just a few localities. Disturbance of its prime habitat, mountain brooks, and uncontrolled illegal collection, especially of *N. s. barani,* are still the main threats to the Anatolia newt throughout its known distribution range.

Introduction

The genus *Neurergus* is represented by two species in Turkey: *Neurergus strauchii* (Steindachner, 1887) and *N. crocatus* Cope, 1862 (Baran and Öz, 1986). The nominate subspecies *N. s. strauchii* (Steindachner, 1887) is known from the lake Van area (Schmidtler and Schmidtler, 1970). In 1994, the subspecies *N. s. barani* Öz, 1994 was described from the mountains near the city of Malatya (Öz, 1994).This subspecies is characterized by scarcity of the dorsal yellow spots and the continuity of the ventral orange line from the gular region to the tip of the tail (Öz, 1994). Moreover, *N. s. barani* larvae show more characteristics of pond-type larvae compared to the larvae of *N. s. strauchii* (Steinfartz, 1995). Both subspecies are separated by a gap of approximately 300 km, from which no specimens are known. A genetic study using mitochondrial gene sequences, allozymes and three plasma protein loci from all known species of *Neurergus* ratified the subspecific status of *N. s. barani* from the genetic point of view and suggested this subspecies split from *N. s. strauchii* approximately 3 mya (Steinfartz et al., 2002).

The main aim of this study was to determine the possible presence of populations of *N. strauchii* in the area between the two previously known areas (populations 1, 6 and 7 for *N. s. barani* and 2 and 8 for *N. s. strauchii* in fig. 1), to collect data on their morphology and ecology and to assess the level of molecular and morphological differentiation among them.

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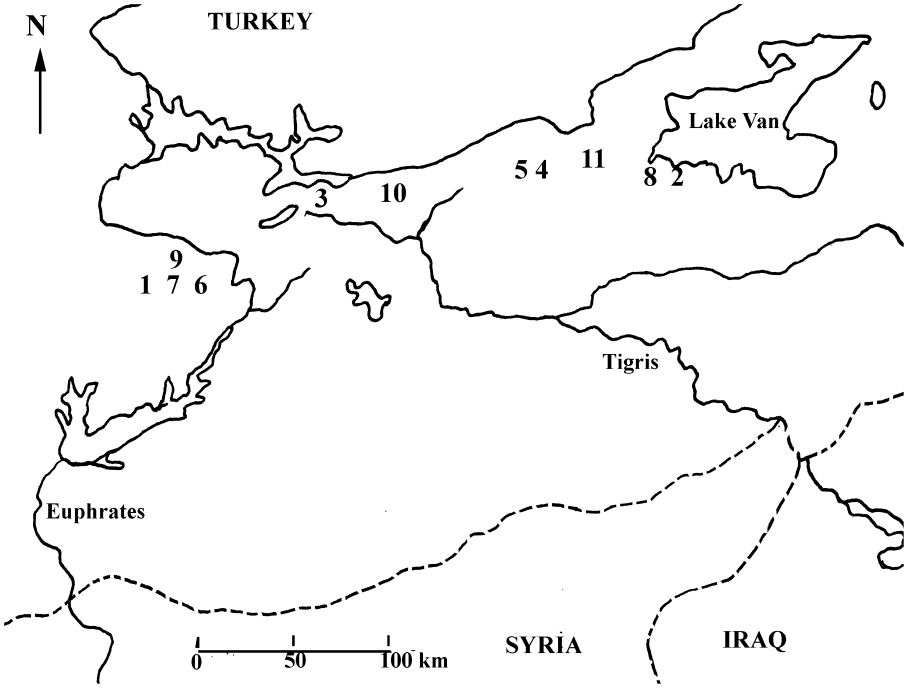

Figure 1. Distribution of *Neurergus strauchii* in Turkey. Numbers refer to the locations in Tables, Figures and the text.

Materials and methods

Four field trips to Turkey were undertaken in the period comprised between April–May 2000, 2001, 2003 and 2005.

Morphology

Snout vent and tail lengths were measured to the nearest 1 mm. The salamanders were weighed to the nearest 0.1 g. Because sex determination based on external characters was not possible in animals with a total length smaller than 13 cm, these animals were considered sub-adults. The ventral and dorsal pattern of each animal was photographed and the number of yellow spots on one half of the head, body and tail was counted. For comparison of the number of spots between sub-adults from localities 2 and 8, captive bred sub-adults of *N. s. barani* and *N. s. strauchii* respectively were included in our analyses.

Environmental parameters

The water temperature was measured to the nearest 0.1°C. Water samples were examined for their pH, GH and KH values using standard colorimetric tests (Tetra, Melle, Germany).

Molecular data

Two mitochondrial genes (16s rRNA and 12S rRNA) were sequenced from 17 specimens of *Neurergus strauchii* belonging to seven different populations. Specimen data are given in table 1 and localities shown in fig. 1. Genomic DNA was extracted from tissue samples following standard protocols described elsewhere (Carranza et al., 1999; 2000). Primers used in both amplification and sequencing were 12Sa and 12Sb (Kocher et al., 1989) for the 12S rRNA gene, and 16Sar and 16Sbr (Palumbi, 1996) for the 16Sr-RNA gene. DNA sequences were aligned using ClustalX (Thompson et al., 1997) with default parameters. Although some gaps were postulated in order to resolve length differences in the 12S rRNA and 16S rRNA gene fragments, all positions could be unambiguously aligned and therefore included in the analyses.

Two methods of phylogenetic analysis were employed. These were: Maximum likelihood (ML) and Maximum-parsimony (MP). Modeltest v. 3.06 (Posada and Crandall, 1998) was used to select the most appropriate model of sequence evolution for the ML analysis under the Akaike Information Criterion. This was the Tamura-Nei model (TrN) taking into account the number of invariable sites (I) and the shape of the Gamma distribution (G). For the MP analyses, apart from an unweighted analysis (transitions (ts) = transversions (tv)) an analysis taking into account the observed ts/tv ratio (ts = 1; tv = 6) was also carried out.

Both ML and MP analyses were performed in PAUP* v. 4.0b10 (Swofford, 1998) and included heuristic searches involving tree bisection and reconnection (TBR) branch swapping with 100 random stepwise additions of taxa. Gaps were included as a fifth state. Reliability of the MP and ML trees was assessed by bootstrap analysis (Felsenstein, 1985), involving 1000 replications.

Table 1. Details of material and sequences used in the present study. Specimen codes identify each individual sequenced (fig. 3) and its locality (fig. 1).

Taxa	Specimen code (fig. 1)	Locality	GenBank Accession Nos.		Specimen code
			12S	16S	
Euproctus asper		France	AY147258	AY147259	Steinfartz et al., 2002
Neurergus kaiseri		Zagros Mountains (Iran)	AY147250	AY147251	Steinfartz et al., 2002
Neurergus microspilotus		Quri-Qaleh SE Paveh (Iran)	AY147248	AY147249	Steinfartz et al., 2002
Neurergus crocatus		Aqrah, N. Iran (Iran)	AY147246	AY147247	Steinfartz et al., 2002
Neurergus s. barani – 1	1	Puturge SE Malatya (Turkey)	AY147244	AY147245	Steinfartz et al., 2002
Neurergus s. barani – 9	1	38°15′N; 38°37′E (Turkey)	DQ131212	DQ131195	E2706.1
Neurergus s. barani – 10	9	38°17′N; 38°35′E (Turkey)	DQ131213	DQ131196	E2706.2
Neurergus s. barani – 11	9	38°17′N; 38°35′E (Turkey)	DQ131214	DQ131197	E2706.3
Neurergus s. barani – 12	9	38°17′N; 38°35′E (Turkey)	DQ131215	DQ131198	E2706.4
Neurergus s. strauchii – 2	8	Surum, near Lake Van (Turkey)	AY147242	AY147243	Steinfartz et al., 2002
Neurergus s. strauchii – 3	3	38°34′N; 39°44′E (Turkey)	DQ131203	DQ131186	E1409.3
Neurergus s. strauchii – 4	8	38°24′N; 42°05′E (Turkey)	DQ131202	DQ131185	E1409.8
Neurergus s. strauchii – 5	4	38°44′N; 40°32′E (Turkey)	DQ131206	DQ131189	E1409.7
Neurergus s. strauchii – 6	4	38°44′N; 40°32′E (Turkey)	DQ131210	DQ131193	E1409.4
Neurergus s. strauchii – 7	4	38°44′N; 40°32′E (Turkey)	DQ131204	DQ131187	E1409.5
Neurergus s. strauchii – 8	4	38°44′N; 40°32′E (Turkey)	DQ131205	DQ131188	E1409.6
Neurergus s. strauchii – 13	11	38°41′N; 41°11′E (Turkey)	DQ131201	DQ131184	E2706.5
Neurergus s. strauchii – 14	11	38°41′N; 41°11′E (Turkey)	DQ131199	DQ131182	E2706.7
Neurergus s. strauchii – 15	11	38°41′N; 41°11′E (Turkey)	DQ131200	DQ131183	E2706.9
Neurergus s. strauchii – 16	10	38°36′N; 40°01′E (Turkey)	DQ131207	DQ131190	E2706.10
Neurergus s. strauchii – 17	10	38°36′N; 40°01′E (Turkey)	DQ131209	DQ131192	E2706.11
Neurergus s. strauchii – 18	10	38°36′N; 40°01′E (Turkey)	DQ131211	DQ131194	E2706.12
Neurergus s. strauchii – 19	10	38°36′N; 40°01′E (Turkey)	DQ131208	DQ131191	E2706.13

Results

Neurergus strauchii specimens were found at 11 localities (listed in table 2 and shown in fig. 1). The animals from localities 1, 6, 7 and 9 and animals from localities 2 and 8 were found within the known distribution ranges of *N. s. barani* and *N. s. strauchii* respectively. Localities 3-5, 10-11 are located in between the type localities of *N. s. strauchii* and *N. s. barani*. No newts were known from this area previously. These findings bridge the gap of approximately 300 km between known *N. s. strauchii* and *N. s. barani* populations. Both genetic and morphological analyses indicate that populations 1, 6, 7 and 9 belong to *N. s. barani*, while all other populations, including the newly found ones, belong to *N. s. strauchii* (populations 2-5, 8, 10-11).

Adult newts in breeding condition were found in mountain brooks in all localities with the exception of localities 4 and 5, where 14 sub-adult and four adult individuals were found on land under stones, and of locality 11 where one adult female was found on land under a stone. The number of yellow spots in juvenile and adult newts is presented in table 3. Our data shows the number of spots in *N. strauchii* follows a west-east cline, with specimens from populations 1, 6, 7 and 9 that correspond to the subspecies *N. s. barani* in the west, presenting the lower number of spots, followed by a steady increase in populations of *N. s. strauchii* 3, 10, 4, 5 and 11 with the easternmost populations 2 and 8 showing the highest number of spots (see fig. 1 and table 3).

In contrast with the situation in the adult newts, no significant differences in the number of spots were found between the sub-adults of populations 1, 2, 4 and 5 (table 3). Adults of *N. s. strauchii* (populations 2 and 8) and adults from populations 4 and 5 had a significantly higher number of spots than sub-adults from the

Table 2. Localities and their environmental characteristics at which specimens of *Neurergus strauchii* were found. Numbers represent the localities within the known distribution of *N. s. barani* (1, 6, 7, 9) and *N. s. strauchii* (2, 8) and the new localities in between the known *N. s. strauchii* and *N. s. barani* distribution ranges (3-5, 10-11).

Locality (number cf fig. 1)	Date	Altitude (m above sea level)	Water temp. (°C)	GH	KH	pH
38°15′N; 38°36′E(1)	30/4/2001	1880	17.2	6	6	9
38°15′N; 38°39′E(6)	30/4/2001	1580	15.3	7	6	8.5
38°15′N; 38°38′E(7)	30/4/2001	1618	13.9	6	6	8
38°15′N; 38°37′E(1)	13/5/2005	1715	15.1	4	4	8
38°17′N; 38°35′E(9)	14/5/2005	1223	14.5	10	10	8
39°21′N; 42°15′E(2)	4/5/2001	1765	11.0	2	2	7.5
38°24′N; 42°05′E(8)	4/5/2001	1627	10.9	7	7	8.5
38°36′N; 40°01′E(10)	15/5/2005	1353	15.5	8	8	8
38°41′N; 41°11′E(11)	16/5/2005	1459	17.3	4	4	8.5
38°34′N; 39°44′E(3)	25/4/2003	977	13.8	11	11	8.5
38°44′N; 40°32′E(4)	26/4/2003	1088	12.9	4	4	7.5
38°40′N; 40°27′E(5)	26/4/2003	1328	13.4	1	1	7

Table 3. Average number of yellow spots ± standard deviation on head, body or tail of *N. strauchii* from different populations (see Table 1 for localities); $n =$ number of animals.

Age	Subspecies	Localities	n	Head	Body	Tail
Adult	*barani*	1,6,7,9	24	5 ± 1.3	16.0 ± 4.5	12.1 ± 3.3
	strauchii	3	10	7.4 ± 1.4	24.0 ± 6.4	9.9 ± 2.0
	strauchii	10	8	7.9 ± 1.8	25.9 ± 6.5	17.6 ± 4.1
	strauchii	4,5	6	9.4 ± 1.8	30.3 ± 8.5	15.4 ± 2.8
	strauchii	11	8	11.9 ± 2.2	35.0 ± 5.0	24.0 ± 3.8
	strauchii	2,8	13	14.1 ± 3.9	44.9 ± 14.0	25.0 ± 9.1
Sub-adult	*barani*	1	8	5.8 ± 0.7	14.9 ± 3.7	8.8 ± 0.8
	strauchii	2	5	5.8 ± 0.8	13.6 ± 2.9	12.4 ± 1.1
	strauchii	4,5	14	6.1 ± 1.5	13.1 ± 2.1	10.3 ± 2.1

same populations. In *N. s. barani* (populations 1, 6 and 7) this difference was not observed.

No differences were noted between the belly patterns of *N. s. strauchii* and *N. s. barani*: both subspecies either showed a continuous or an interrupted orange ventral line. Body measurements are summarized in fig. 2. The body condition index (BCI = 10000 × W(g) / SVL(mm)) of females was borderline significantly higher than that of males (30.8 ± 4.2 and 29.0 ± 4.0 g/mm respectively, $P = 0.05$ T test). The subadult newts measured had a significantly lower BCI compared to the adults (BCI = 24.6±2.0). Males had a relatively shorter tail (SVL/TL = 1.05 ± 0.09) than females (0.99 ± 0.05, $P < 0.05$ T test). Juveniles had relatively shorter tails than adults (1.12 ± 0.10, $P < 0.001$ T test).

A total of 829 base pairs (474 bp of the 12S rRNA and 355 of the 16S rRNA) were included in the phylogenetic analyses. Of these, 99 were variable and 48 parsimony-informative. The phylogenetic analyses are presented in fig. 3. All three independent phylogenetic analyses gave identical results and indicate that all new populations of *N. strauchii* sampled for this study are very closely related with the mtDNA sequences of *N. s. strauchii* from locality 8 (fig. 1; Steinfartz et al., 2002). The samples of *N. s. barani* from localities 1 and 9 (fig. 1) are part of the *N. strauchii* assemblage (bootstrap value 100) but at the same time represent an independent lineage separated from all other samples of *N. strauchii* analyzed by 9-11 nucleotide differences (1-1.4% uncorrected genetic divergence).

The other three species of *Neurergus* included in the analyses (*N. kaiseri* Schmidt, 1956*, N. microspilotus* Nesterov, 1916, and *N. crocatus*) form a not very well-supported group

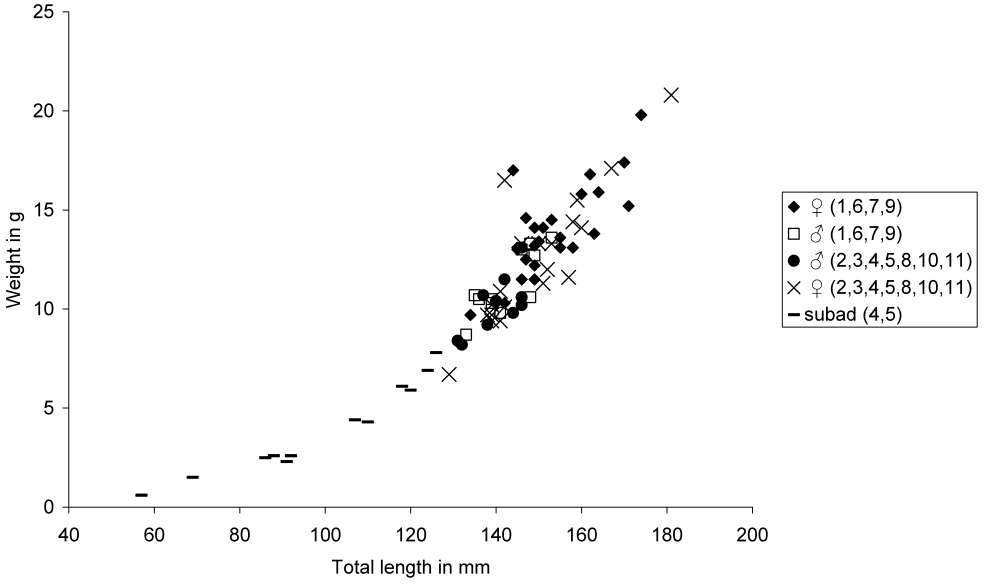

Figure 2. The total length and weight of *Neurergus strauchii* specimens from different locations (in parentheses, see also Fig. 1 and Tables 1 and 2).

(bootstrap values 68, 68 and 97 in the ML (TrN+I+G), MP (ts = tv), Mp (ts = 1; tv = 6) and analyses respectively), while the clade formed by *N. microspilotus* and *N. kaiseri* is recovered only in the ML (TrN+I+G) and MP (Ts = 1, Tv = 6) analyses (see fig. 3).

Discussion

The finding of five new populations of *N. strauchii* (populations 3, 4, 5, 10, 11) and posterior morphological and molecular analyses show that the distribution range of *N. s. strauchii* is much larger than it was previously thought, extending a further approximately 300 km to the west and closing the gap between *N. s. strauchii* and *N. s. barani*. Our data, together with a geographical analysis of the area, suggest that the River Euphrates might be acting as a natural barrier to gene-flow between both subspecies. The results agree with Steinfartz et al. (2002), who also found a relatively high level of genetic differentiation at both mitochondrial (12S and 16S rRNA) and nuclear levels between *N. s. barani* (populations 1 and 9 in our study) and the easternmost population of *N. s. strauchii* (population 8 in our study).

The most obvious difference between *N. s. strauchii* and *N. s. barani* is the difference in the number of yellow spots between adults of both subspecies. In this study, we found a gradual increase in the number of spots in adult newts eastwards. Besides, the number of spots was the same in sub-adult and adult *N. s. barani*, whereas a marked increase in the number of spots was noticed in the *N. s. strauchii* populations for which sub-adult and adult specimens were analyzed (populations 2, 4, 5 and 8 in fig. 1; Schmidtler and Schmidtler, 1970). This finding suggests that the main phenotypic difference between both subspecies is an increase in the number of spots during maturation in *N. s. strauchii*, which is absent in *N. s. barani*. Moreover, we could not find any differences in belly patterns between *N. s. barani* and *N. s. strauchii* and therefore we conclude that this morphological character, initially suggested by Öz (1994), is of low taxonomical value and should not be taken into consideration for the differentiation between both subspecies of *N. strauchii.*

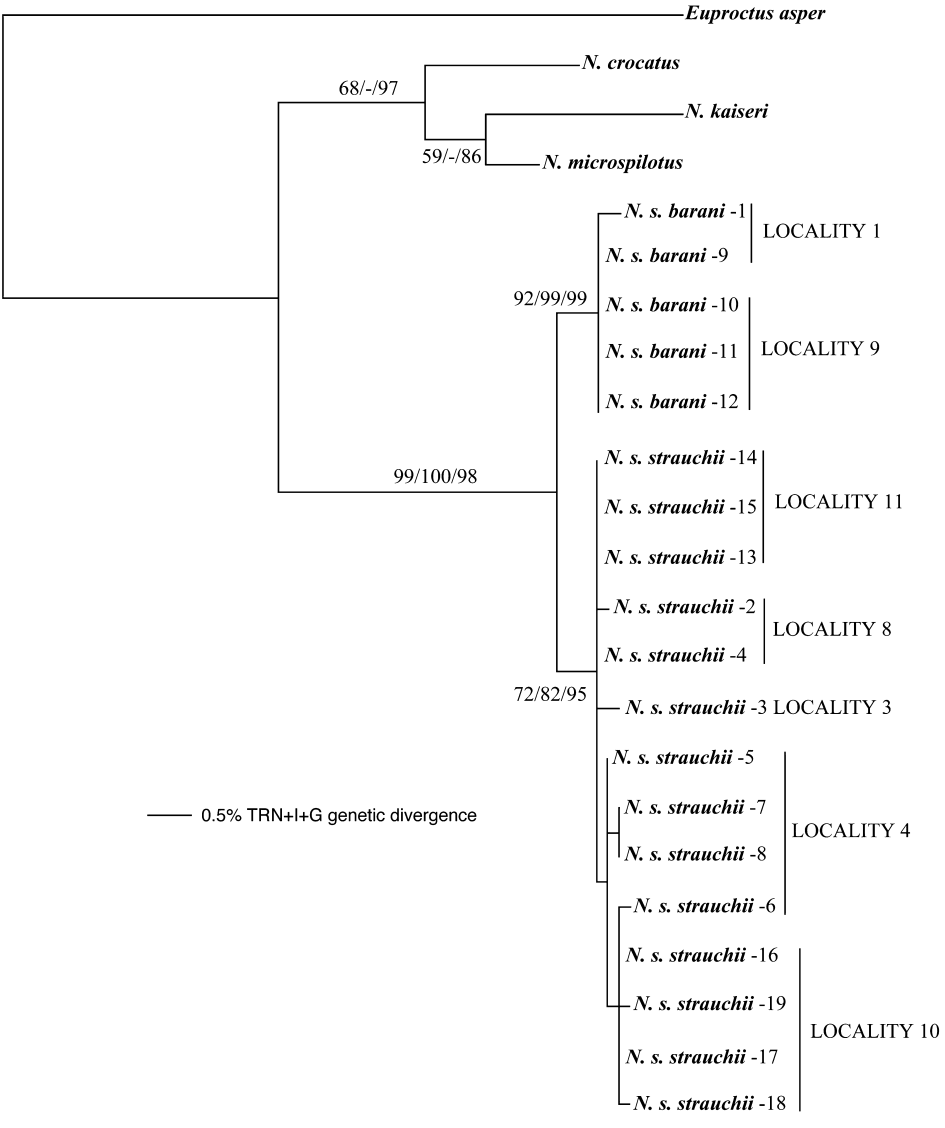

Figure 3. ML (TrN+I+G; loglikelihood = −174.97093) tree of *Neurergus* including all new populations of *N. strauchii* sampled for this study. Numbers indicate bootstrap support values for that node. Bootstrap values from left to right refer to: ML, MP (ts = tv; number of steps 76), MP (ts = 1, tv = 6, number of steps = 182). The dash indicates the topology was not supported by the MP (ts = tv) analysis.

We found fresh spawn from up to one hundred eggs at several locations (1, 9, 10) on the underside of rocks. At location 9 we also found eggs attached to the rock bottom and branches exposing the eggs to direct sunlight. All the males of *N. s. barani* at the type locality were in 2001 in courtship condition with silverblueish markings on the tails. The presence of these markings agrees with Steinfartz (1995) but contradicts Öz (1994), who suggested there are no color differences between male and female *N. s. barani*. In May 2000 on the same location, a thick layer of snow was still present and no animals were found. On the other hand, a high number of *N. s. strauchii* individuals were found in May 2000 whereas only 3 specimens were seen in May 2001. These findings suggest that, at least at this altitude and depending on weather

conditions, the mating season starts approximately at the end of April beginning of May for both subspecies. Besides, several large larvae were found at the type locality of *N. s. barani*, suggesting a late spawning in the previous year with an extended period of larval development. This finding of large larvae metamorphosing in their second year is in line with the findings of Schmidtler (1994) who describes finding a fresh spawn of *N. s. strauchii* on the 5th of June 1976 near Bitlis and large larvae, together with gravid females of *N. s. barani,* at the type locality in June 1993.

The streams in which *N. strauchii* was found were all slightly alkaline. The water was soft to moderately hard. However, these data were collected during periods of high rainfall. Probably, these values can be strongly influenced by heavy rains or periods of prolonged drought.

Upon manipulation, one sub-adult animal showed a defensive posture. This defensive behaviour was similar to observations made by Brodie et al. (1992).

N. strauchii is a strictly protected species (appendix II) by the Convention on the Conservation of European Wildlife and Natural Habitats (also known as Bern Convention), which was ratified by Turkey on the 2nd of May 1984. In Resolution No. 6 (1998) of the Standing Committee, *N. strauchii* is listed as a species requiring specific habitat conservation measures. The status of *N. strauchii* in Turkey is not clear, although IUCN (2004) lists them as Vulnerable. We found *N. strauchii* to be locally common, provided a suitable habitat is present. The inaccessibility of the area promotes the conservation of this species. Disturbance of prime *N. strauchii* habitat, mountain brooks, was noticed on several occasions: road construction works, tapping of sources and household sewage. Especially the situation at the type locality of *N. strauchii barani* is worrying: in May 2005, preparations for road enlargement at the terra typica of this subspecies were noticed, endangering the prime breeding habitat. Besides, adult newts have been illegally collected during the breeding season by animal traders/dealers and sold in Europe, which might put heavy pressure on some populations (for example the type locality of *N. s. barani*). These threats might lead to local extinction of *N. strauchii* throughout its known range. There is currently no legal protection status for any amphibian species in Turkey. The new Nature Conservation Law, however, is in preparation and it will include the concept of "protected species" (Güven Eken, pers. comm.). It is likely that all subspecies of *N. strauchii* will be listed as protected species. *N. s. barani* may receive stronger protection through the designation of the Kubbe Dagi as a protected area (Eken, pers. com.).

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