mune serum or 50 µl of immune serum 5 hours previously (Fig. 3). The median lethal dose (LD<sub>50</sub>) of LPS in animals treated with immune serum was significantly higher than the  $LD_{50}$  for control mice treated with nonimmune serum.

The time at which the antiserum was administered relative to the time of LPS administration was found to be of crucial importance in producing a protective effect. Mice that were injected with immune serum 3 or 6 hours prior to administration of LPS fared better than those passively immunized at the time of LPS injection or several hours after (Fig. 4). This finding suggests that endotoxin elicits cachectin/TNF production soon after its administration, and that cachectin/TNF mediates its lethal injury within a very short time. In rabbits, cachectin/TNF is produced within minutes after the intravenous administration of LPS, and peak plasma concentrations are observed after 2 hours, with a rapid decline in concentrations occurring thereafter (11). Hence, in this model, the animal is exposed to high concentrations of the hormone only briefly; it is within this interval of time that effective antibody concentrations must be present if protection is to be achieved. Presumably the necessity for prior administration of the antiserum reflects the time required for complete distribution of the antibody within the recipient animal.

These data give evidence for the role of cachectin/TNF in mediating the lethal effects of LPS. Cachectin/TNF is clearly only one of the mediators responsible for the numerous pathological effects evoked by LPS, since the passively immunized mice become febrile, and continue to appear ill and distressed. It is possible, for example, that cachectin/TNF acts in concert with other mediators (for example, interleukin-1, interferons, and lymphotoxin) in order to elicit the lethal effect of LPS.

It is important to note that mice are relatively resistant to the effects of LPS when compared to most other mammals; rabbits, for example, are approximately 1000-fold more sensitive. In LPS-sensitive species, TNF may play a more prominent role as a mediator of shock. Immunization against TNF might then be expected to yield a higher level of protection.

The potential utility of passive immunization with antisera to cachectin/TNF in animals with shock induced by septicemia (or possibly other causes) needs further exploration. An obvious corollary is the possibility that agents which affect the synthesis or binding of cachectin/TNF to its receptor might be of utility

Table 1. Protective effect of antiserum to cachectin/TNF. Female BALB/c mice (20 to 24 g) were randomly divided into six groups and injected intraperitoneally with serum from immune or nonimmune rabbits 1.5 hours before being injected with 400 µg of LPS from E. coli strain 0127:B8. Serum samples were diluted with sterile isotonic saline and injected in a final volume of 0.2 ml per mouse. LPS was also diluted in sterile saline and injected in a volume of 0.2 ml. Mortality was recorded daily, and the experiment was considered complete when no deaths were observed in any group for 3 days. The data show the number of survivors 7 days after LPS injection.

0	Serum volume injected (µl)				
Serum	10	50	200		
Immune	3/14	6/14	7/14		
Nonimmune	0/14	1/14	0/14		
Р	> 0.05	< 0.05	< 0.01		
4.004.1					

\*Chi-square test.

in this setting without compromising the host's immune system. From these studies, a better understanding of the mechanisms by which the immune system influences other tissues may emerge.

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# Morphological Novelty in the Limb Skeleton Accompanies Miniaturization in Salamanders

Abstract. Salamanders of the genus Thorius (Plethodontidae) are among the smallest tetrapods. Hypotheses of limb skeletal evolution in these vertebrates were evaluated on the basis of estimates of natural variation, comparisons of skeletal homology, and analysis of molecular phylogeny. Nine carpal arrangements occur in Thorius, more than in all twelve related genera of typically larger salamanders; six of these arrangements are unique. They represent a trend toward a decrease in the number of separate cartilages that is independent of locomotor and ecological specialization. Miniaturization may be an important source of morphological novelty, distinct from local adaptation, in vertebrates.

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The origin of novel morphological design is a primary focus of evolutionary morphology. One trend that may promote morphological novelty is phylogenetic decrease in body size, or miniaturization. Unique morphological arrangements are a common feature of dwarfed invertebrates (1, 2), many of which represent "entirely new types of organization" (2). In vertebrates, a frequent association between miniaturization and morphological novelty has been documented in many taxa, including teleost fishes (3), anuran (4) and urodele (5-7)amphibians, and squamate (8-10) and amphisbaenid (11) reptiles. Size de-

crease also has been implicated as a critical factor in the evolution of higher taxa such as frogs and salamanders (5), lizards (8), and snakes (10).

Lungless salamanders (Plethodontidae) provide some of the best examples of miniaturization among vertebrates. Decreased body size has evolved in several lineages; one, the Mexican genus Thorius, comprises the smallest extant tailed tetrapods (6, 7, 12). In this report I analyze the consequences of miniaturization in Thorius for forelimb skeletal morphology. The analysis is based on quantitative estimates of natural variation in limb osteology, on a comparison of skeletal unit homology, and on an electrophoretically derived molecular phylogeny. It reveals the following: (i) miniaturization of the genus as a whole correlates with the appearance of several unique and fundamental rearrangements of the carpal (wrist) skeleton; (ii) this variation represents a trend toward a decrease in the number of separate cartilages in the forelimb; (iii) at least one instance of evolutionary reversal—an increase in the number of separate units probably occurred; and (iv) in no instance is a novel carpal arrangement associated with any apparent locomotor or ecological shift.

*Thorius* is one of 13 genera in the tribe Bolitoglossini, which is a group of more than 150 species that has radiated extensively throughout the New World tropics (13, 14). This genus is composed of at least 15 species of arboreal or terrestrial salamanders that are endemic to montane forests of southern Mexico. Eight valid species are described formally; the remaining seven species are informally designated T. sp. A, T. sp. B, and so forth, pending formal description (Table 1) (15). All species are tiny: mean standard length (snout to posterior end of vent) in adult males ranges from 18.9 mm in T. pennatulus to 27.8 mm in T. narisovalis; adult females average 1 to 3 mm

Table 1. Frequency (%) of carpal arrangements and right-left asymmetry in population samples of adult *Thorius*. Frequencies were calculated separately for right and left sides and averaged to yield a single value. *Thorius* sp. A and E are arboreal; the remaining species are terrestrial.

Species	n*	Arrangement				Asym-
		I	II	III	IV through IX†	metry (%)
T. pennatulus	20, 19	46.2	51.2	2.7		36.8
T. macdougalli	18, 18	85.0	15.0			16.7
T. minutissimus	17, 18	75.0	16.7	8.4		35.3
T. schmidti	18, 18	83.3	11.1	5.6		33.3
T. narisovalis	20, 19	87.3	7.8	2.5	2.5 (VI)	15.8
T. troglodytes	19, 19	92.1	2.7	2.7	2.7 (IX)	11.1
T. pulmonaris	11, 11	91.0	9.1			18.2
T. sp. A	2, 2	50.0	50.0			
T. sp. B	19, 18	51.2	48.9			61.1
T. sp. C	20, 20	87.5	10.0	2.5		29.4
T. sp. D	19, 20	76.9	12.9	7.8	2.5 (VII)	26.3
T. sp. E	4, 4	25.0	75.0			
T. sp. F	17, 18	80.1	17.0		3.0 (IV)	37.5
<i>T</i> . sp. G	18, 18	94.5	2.8		2.8 (VIII)	11.1

\*Right and left sides, respectively. The smaller value represents the sample size for asymmetry estimates, except in *T. troglodytes* (n = 18), *T.* sp. C (n = 17), and *T.* sp. F (n = 16). \*Arrangement V was seen in only 1 of 12 juvenile specimens of *T. schmidti* (both sides).



Fig. 1. Nine carpal arrangements in *Thorius*. All are drawn as right limbs (dorsal views), although some rare variants were seen on the left side only. Arrangements III through VIII are unique to *Thorius* among bolitoglossine genera. In the primitive plethodontid arrangement, from which these nine arrangements were derived, all eight labeled carpal units are separate. Cartilage is stippled, except for that in the carpals, which are left unshaded. Abbreviations: u, ulnare; i, intermedium; r. radiale; c, centrale; cl, centrale 1; dl-2, distal carpal 1-2; d3, distal carpal 3; d4, distal carpal 4.

larger than males in most species (6). Comparable standard length values for *Nyctanolis pernix*, a salamander species that is believed to retain a morphology ancestral to all extant bolitoglossines, are 55 mm (males) and 68 mm (females) (16); species in related genera typically are even larger (13).

Limb skeletal variation in Thorius is extensive among species, within species, and even within individuals. Fourteen species surveyed display nine arrangements of skeletal elements in the carpus alone (6, 17) (Fig. 1). Each of these arrangements contains from four to seven separate anatomical units due to the variable presence of nine combinations of fused adjacent cartilages (18). As many as four arrangements occur in populations of each of several species (Table 1). The frequency of right-left asymmetry of carpal arrangement in a given specimen exceeds 25 percent in seven species and 60 percent in one species. Many arrangements that co-occur in a particular population-indeed, in some individuals-are as different from each other as arrangements that distinguish some plethodontid species or even genera (14, 19, 20).

The nine carpal arrangements bring to 12 the total number described in all 13 bolitoglossine genera, which typically include salamanders of significantly larger body size (6, 12, 19-21). Of these, six arrangements (III through VIII) are unique to Thorius, whereas three arrangements (I, II, and IX) occur at low frequencies in a few species of other, distantly related genera and apparently have appeared independently (6, 12, 19). Thus, 75 percent of the total number of carpal variants observed in bolitoglossine salamanders are present in Thorius, which includes at most 10 percent of the species in the group; 50 percent of the variants are found in no other genus. The presumed ancestral arrangement for bolitoglossine salamanders, which predominates in several extant genera, comprises eight separate cartilages (22). Each of the nine arrangements in Thorius, all of which appeared since the genus diverged from other extant lineages, has fewer units. This constitutes a second dominant trend-a reduction in the number of separate carpal units-that is characteristic of limb evolution within the genus.

Phylogenetic trees based on estimates of molecular differentiation provide hypotheses of historical relationship among taxa that are independent of morphology and may be used to characterize morphological trends (23). I used the results of an electrophoretic analysis of phylogenetic relationships within Thorius, together with the known distribution of carpal arrangements among species, to analyze the origins of and transformations among arrangements (Figs. 2 and 3A) (24).

1) Arrangements I and II are present in all species examined; presumably they characterized the common ancestor of all extant species. There is no explicit evidence that these arrangements, with six and five separate units, respectively, arose from the primitive arrangement via an intermediate stage with seven units. Instead, an initial reduction of at least two units at this transition is indicated.

2) Arrangements IV through VIII occur in only one species. They probably arose separately in lineages that already possessed arrangements I and II.

3) Arrangement IX, which contains seven cartilages, also evolved after arrangements I and II. It represents an increase in the number of separate cartilages and a reversal of the otherwise pervasive trend toward a decrease in the number of separate carpal units.

4) Arrangement III is present in seven species. If it arose only once, in the common ancestor of these species (that is, the ancestor of all species except T. sp. B), then it either has been lost or remains undetected in several descendant lineages. Alternatively, it may have evolved independently within Thorius as many as seven times. In either case, arrangement III evolved after arrangements I and II.

The above analysis provides details concerning the origin of arrangements that consideration of skeletal-unit homology alone does not provide. The skeletal-unit homology scheme (Fig. 3B), derived by ignoring the distribution of arrangements among species and assuming that transitions entailed a minimum number of fusions or divisions of cartilages, specifies different transitions among several arrangements. It also does not identify the evolutionary reversal involving arrangement IX and gives no indication of the possible multiple origin of arrangement III.

Because of the particular distribution of the carpal arrangements among species examined-two in all species, six in a single species-virtually any dendrogram clustering these species will yield the same general results concerning the evolution of forelimb patterns. A single exception concerns the likelihood of independent derivation of arrangement III in different species, which will vary according to branching scheme. Thus, general observations based on molecular data concerning the origins of and transFig. 2. Dendrogram linking 15 species of Thorius, based on unweighted pair-group method analysis of Nei's D (15, 24). The distribution of the nine carpal arrangements among 14 species is indicated: data are unavailable for T. dubitus. Circles denote species that have arrangement III.

Fig. 3. (A) Character transitions that result when the distribution of carpal arrangements among species is superimposed on the molecular phylogeny (Fig. 2). (B) Character transitions derived from consideration of skeletal-unit homology. Arabic numerals indicate the number of separate cartilages in each arrangement; PPA, primitive plethodontid arrangement (23).



formations among most carpal arrangements are not a function of either the clustering algorithm or the genetic distance measure used. Similarly, the phenomenon of extensive limb skeletal variation is independent of hypotheses concerning the specific historical transformations among arrangements.

There is no obvious functional explanation for the evolution of several unique arrangements of the limb skeleton in Thorius (and common intraspecific and within-individual variation) or of the associated, pervasive trend toward reduction in the number of separate carpal units. A decrease in the number of carpal units brought about by fusion of adjacent cartilages has evolved independently in several genera of plethodontid salamanders in both temperate North America and the New World tropics (12), although in no genus is the trend as pervasive as in Thorius. In Bolitoglossa, a widespread neotropical genus, carpal fusion is associated with arboreal locomotion (19, 21). Mesopodial rearrangements of similar magnitude also preceded the independent evolution of arboreality in species of the Central American genus Chiropterotriton and the temperate genus Aneides (12, 25). In Thorius, however, none of the unique carpal arrangements is associated exclusively with arboreal locomotion; all are known only in terrestrial species. Moreover, arrangements in the limited samples available of the two rare arboreal species, T. sp. A

and T. sp. E, are the same ones that predominate in all terrestrial species examined (Table 1). Extensive rearrangements of the limb skeleton are independent of any apparent locomotor or ecological specialization.

Limb skeletal variation in Thorius provides an example of miniaturization that correlates with the widespread occurrence of novel morphology. Similar changes have been described for the hindlimb (6) as well as for cranial morphology (7, 26). These changes represent a pool of alternate morphological designs that is available for subsequent evolutionary diversification and adaptation. Should morphological novelty be as common in other miniaturized taxa by comparison with larger, related forms, this would support the suggested predominant role of phylogenetic size reduction in morphological evolution and the origin of higher taxa (1). It also would justify further investigation of the mechanistic, developmental relation between miniaturization and alteration of morphogenetic patterning.

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- Specimens were prepared as cleared whole mounts differentially stained for bone and cartilage (6). Specimens examined of most species are listed in J. Hanken, Morphological and Genetic Investigations of Miniaturization in Sal-*Genetic Investigations of Miniaturization in Sat-amanders (Genus Thorius)* (University Micro-films, Ann Arbor, Mich., 1980). Additional specimens are as follows: *Thorius* sp. A, M (Mexican field tag series) 5541-5542, and *Thor-ius* sp. E, M 5097-5098, HBS (H. B. Shaffer field tag series) 2257, 2259. Specimens are deposited in the Museum of Vartabeta Zoolow. Universiin the Museum of Vertebrate Zoology, Universi-ty of California, Berkeley. Data are unavailable

for the extremely rare terrestrial species Thorius duhitu

- 18. The fusion combinations are as follows: 1, ulnare, intermedium; 2, ulnare, intermedium, cen-trale; 3, distal carpal 4, centrale; 4, distal carpal 3, distal carpal 4, centrale; 5, distal carpal 1-2, distal carpal 3, distal carpal 4, centrale; 6, distal carpal 1-2, centrale; 7, radiale, centrale 1; 8, distal carpal 1-2, distal carpal 3; and 9, distal carpal 3, distal carpal 4.
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## **Relation of Spectral Types to Oil Droplets**

### in Cones of Turtle Retina

Abstract. The spectral sensitivities and color of oil droplets of cone photoreceptors in the retina of the red-eared turtle (Pseudemys scripta elegans) were investigated by intracellular recording and injections of Lucifer yellow dye. Six morphological types of cones could be distinguished by the color of the oil droplets located in the outermost inner segments. Single cones containing either red or pale green oil droplets were sensitive to red light, cones with yellow oil droplets to green, and cones with clear oil droplets to blue. Contrary to previous reports, both principal and accessory members of double cones were sensitive to red, and no diffusion of dye was detected between the two apposed members. Thus, the oil droplets provide a reliable morphological basis for further investigation of the neuroanatomical networks underlying the processing of color information in the vertebrate retina.

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Cone photoreceptors in the turtle retina have characteristic morphological organelles, that is, colored oil droplets. In the red-eared turtle, absorption spectra of the visual pigments measured by microspectrophotometry (MSP) (1) were correlated with the color of oil droplets: cones with either red or orange (principal member of double cones) oil droplets contained a red-absorbing visual pigment, cones with yellow oil droplets and the accessory member of double cones contained a green-absorbing visual pigment, and cones with colorless oil droplets contained a blue-absorbing visual pigment. These three distinct spectral types of cones were confirmed by intracellular recordings (2, 3). Thus, the gil droplets were used to identify the spectral types of cones in anatomical studies (4-7). A recent MSP study (8), however, has called into question data on the oil droplet-visual pigment combinations in the red-eared turtle. Intracellular stainings with Lucifer yellow (9) has shown

that two-thirds of the cones with colorless oil droplets were actually red-sensitive. In addition, the accessory members of double cones in Reeves' turtle were not green-sensitive (10), as would be expected from earlier reports. Because color information processing in the vertebrate retina has frequently been studied in red-eared turtles. I have reexamined the question of the color of the oil droplets in the three spectral types of cones. Through the use of intracellular staining with Lucifer yellow, I have confirmed some of the previous oil dropletspectral sensitivity combinations, but new evidence suggests that some earlier identification (4-7) needs to be reevaluated.

Eyecup preparations of the lightadapted red-eared turtle, Pseudemys scripta elegans, (carapace length, 18 to 22 cm) were used. Cones located about 2 mm dorsal to the visual streak were investigated. Details of the experimental procedures were as described elsewhere (10, 11). A total of 215 cells, whose somata were located at the outer nuclear layer, were recorded from intracellularly and filled with Lucifer yellow CH. About half of the filled cells were excluded by morphological inspection-42 displaced bipolar cells and 50 cones from which the dye had leaked. Thus, definite morphological identification was obtained in 123 cones in which pairing of spectral sensitivity and color of the oil droplet was investigated (Table 1).

In a whole-mounted isolated turtle retina, photoreceptors were of seven morphological types (4, 9): rods with no oil droplet and six types of cones containing different colored oil droplets (Fig. 1). Single cones contained either red, pale green (or fluorescent) (9), yellow, or clear oil droplets. Double cones were the apposition of two dissimilar cones formed as a figure theta, with the principal member containing an orange oil droplet and the accessory member having none.

Spectral sensitivities (11) were obtained from 85 single cones. Sixty cones contained a red oil droplet, the most distinctive type because of the dense red carotinoid (1) that absorbed the yellow fluorescence emitted from the Lucifer yellow. A fluorescent ring was seen in the flat-mounted preparation (Fig. 1A). These cones had a peak spectral sensitivity at 620 nm (Fig. 2A). For wavelengths longer than 620 nm, each spectral sensitivity curve agreed with the calculated absorption spectrum of the red-absorbing visual pigment and red oil droplet combination (12). Large variations were seen for the shorter wavelengths (<540