Evolution of Spinal Nerve Number in Anuran Larvae

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Abstract. We examined the number of segmental spinal nerves in 60 premetamorphic tadpoles, representing 43 species, 33 genera and 12 families of anurans. The number of spinal nerves shows a clear phylogenetic pattern, in which the primitive condition is variable, ranging from 23 to 29 pairs. Reduction of caudal spinal nerve number has occurred independently at least 7 times during anuran phylogeny. Reduction events have occurred among species within genera, among subfamilies within families, as well as at familial and higher taxonomic levels. In general, archaeobatrachian larvae have significantly more spinal nerves than neobatrachian larvae. While 2 of the 7 reduction events have occurred within the Archaeobatrachia, both of these have occurred at low taxonomic levels. In the Neobatrachia, 3 of the 5 reductions have occurred at subfamilial or higher taxonomic levels. Overall, reduced spinal nerve number correlates with tadpole body size, but not with relative tail length or with developmental stage, at least within the range of stages we examined. There is a positive correlation between number of spinal nerves and minimum time from hatching to metamorphosis, at least within the Archaeobatrachia. Species with more pairs of nerves take longer to reach metamorphosis, possibly because they have more neural tissue to produce and to resorb. Spinal nerve number per se does not appear to be associated with larval microhabitat ecology or swimming behavior. Tadpoles exhibit greater variation in spinal nerve number than adult frogs. This greater variation is tolerated presumably because the tail is an expendable organ, which is lost at metamorphosis.

Introduction

Vertebrates exhibit a great deal of variation in the number of spinal nerve roots constituting the spinal cord. Adult frogs, with their short bodies, have fewer than 12 pairs of spinal nerves, whereas snakes may have more than 500 pairs [24]. Within each major class of vertebrates, from fishes [e.g., ref. 37] to mammals [2], the number of spinal segments is a character of taxonomic significance. The primitive condition for vertebrates is the presence of a relatively large number of spinal nerves, which exit the spinal canal at more or less regular intervals along the length of the cord [2]. This condition is retained in most vertebrate groups. However, three vertebrate lineages possess a deviation from this pattern in which post sacral spinal nerves are reduced, and there is instead a cauda equina with an elongate filum terminale in the caudal region. A cauda equina and elongate filum terminale have evolved independently in some Osteichthyes, in the anuran Amphibia, and in the Mammalia [2]. In all three taxa, this derived morphology appears to result from a repatterning of metamericism in the caudal region.

In a previous study [27], we showed that the cauda equina, which typifies adult frogs, is present in larvae of some species but not others. Specifically, we showed that larvae of \textit{Xenopus laevis} possess the primitive condition of the vertebral spinal cord up to the time of metamorphosis, whereas in species of \textit{Rana} the cauda equina is present at hatching and persists throughout the tadpole stage, and the spinal cord is only slightly shortened at metamorphosis. The goals of the present study were to determine the evolution-
ary polarity of spinal cord development in anuran amphibians, to determine at what phylogenetic level developmental transformations have occurred, and to look for ecological and behavioral differences between tadpoles possessing these different morphological patterns. We report here that reduction in the number of spinal nerves in larvae has evolved many times independently among anuran amphibians. We examine the implications of these developmental-evolutionary events.

Material and Methods

We studied the segmental spinal nerves of 60 tadpoles, representing 43 species, 33 genera and 12 anuran families. All specimens came from the collection of R.W., most of which were donated by colleagues from around the world. All tadpoles were fixed in formalin and preserved in 10% neutral buffered formalin. They were staged according to Gosner [12]. Snout-vent length (SVL), total length (TOTL) and tail length (TAILL) of each specimen were measured to the nearest 0.1 mm. An effort was made to represent as many genera and families as possible with 'mature' but premetamorphic tadpoles (i.e., Gosner stages 34–40). Although this was not always possible, the few specimens outside this range were all free-living larvae whose tails had not begun to shorten (i.e., stages 25–43). Information on the minimum length of the larval period for many of the species was obtained from a literature compilation of such data by J. Just and K.N.[unpublished].

Specimens were cleared in trypsin, buffered with 30% saturated borax [36] and peripheral nerves were stained with Sudan black B [26]. Segmental spinal nerves were counted and camera lucida drawings (fig. 1) were made under a dissecting stereomicroscope. Spinal nerves were identified by their point of exit from the spinal cord. All caudal nerves clearly possessing spinal roots were counted for each specimen. Counting becomes more difficult as one moves caudally and the nerve roots become progressively smaller. In all tadpoles, a plexus of nerves located posterior to the root of the most posterior spinal nerve innervates the caudalmost myotomes (fig. 1). This plexus is derived from the peripheral branches of several more rostral spinal nerves. Thus, there is no longer a pattern of one nerve to one myotome in the region posterior to the root of the last spinal nerve.

Evolutionary changes in the number of spinal nerves were studied by analyzing changes in spinal nerve number along the branches of a phylogenetic tree. The branching order of our tree follows that of Duellman and Trueb [9], except that we follow Cannatella [5] in considering the Pelobatidae to be the sister group of the Pipidae. At lower taxonomic levels, we follow Cannatella and Trueb [6] for relationships within the Pipidae; Hedges [14], W.E. Duellman [pers. commun.] and D.C. Cannatella [pers. commun.] for relationships within the Hylidae, and Hillis and Davis [16] for relationships within the Ranidae. For phylogenetic analysis, the average number of spinal nerves for each species, rounded to the nearest integer, was used. Evolutionary changes in this character were partitioned along the branches of the tree using the parsimony method of Farris [10]. This analysis uses A. truei as the outgroup. The same results would be obtained by using any non-anuran amphibian species as the outgroup, because all anurans have more spinal nerves than A. truei. We did not use Felsenstein's [11] method for phylogenetic analysis because minimum development times were not available for all species.

Mann-Whitney U tests (alpha = 0.05, two-tailed) were used to compare spinal nerve number, developmental stage and time, and the various measures of body size and tail length between groups. Spearman's rank correlation was used to test for significant correlations among these variables within and between groups. Species
### Table 1. Family, genus, species, number of specimens (n), number of spinal nerves, Gosner stage, SVL, TOTL, species code, genus code, family code and minimum development time for each species included in this study

<table>
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<tr>
<th>Family</th>
<th>Species</th>
<th>n</th>
<th>Spinal nerves</th>
<th>Gosner stage</th>
<th>SVL mm</th>
<th>TOTL mm</th>
<th>Species code</th>
<th>Genus code</th>
<th>Family code</th>
<th>Minimum development time, days</th>
<th>Reference</th>
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<td>90*</td>
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<td>36</td>
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<td>3</td>
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<td>34</td>
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<td>54.3</td>
<td>22</td>
<td>18</td>
<td>8</td>
<td>28</td>
<td>20</td>
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</tbody>
</table>

For species represented by more than 1 specimen, species means are given. Mean number of spinal nerves and mean Gosner stage are rounded to the nearest integer.

* Minimum development time reported for the genus.
means only were used to support phylogenetic inferences made from statistical tests. Data from all specimens were used whenever this resulted in more conservative tests of the hypotheses that size or stage alone accounted for the observed variation. The data were analyzed by testing the significance of mean differences: (1) between the two traditionally recognized subordinal groups, the Archaeobatrachia (Ascaphidae, Discoglossidae, Rhinophrynidae, Pipidae, and Pelobatidae) and the Neobatrachia (all other families) [9], and (2) between the species with primitive spinal nerve numbers (≥23 pairs of spinal nerves) and species with derived spinal nerve numbers (≤22 pairs of spinal nerves). All size measurements presented in the text are means (mm) ± one standard error of the mean.

Results

Table 1 gives the SVL, TOTL, TAILL, Gosner stage, minimum development time and number of spinal nerves for all 60 individuals examined in this study. Spinal nerve number ranged from 17 to 29, with the highest number occurring in Discoglossus pictus (Discoglossidae) and in 1 specimen of Xenopus laevis (Pipidae) and the lowest in Microhyla heymonsi (Microhylidae), Osteopilus brunneus (Hylidae) and in

Fig. 2. Variation in number of spinal nerves within the Anura: among species (A), among genera (B), and among families (C). Taxa are ordered following Duellman and Trueb [9] with the more derived taxa having higher numbers. Vertical lines indicate the range within a taxon. The number above a point or line equals the sample size for that taxon wherever there is more than 1 specimen. Species, genus and family codes for each taxon are given in Table 1.
Fig. 3. The relationship between number of spinal nerves and: snout-vent length (A), total length (B), tail length (C), relative tail length (D), and developmental stage (E) [12]. Closed circles refer to archaeobatrachian species; open circles refer to neobatrachian species. Each data point represents 1 specimen, although statistics were computed using species means. Overall, there is a significant correlation of the size variables with spinal nerve number, which is predominately due to the neobatrachian frogs. See text for explanation. Relative tail length does not correlate with spinal nerve number within or between groups. Developmental stage is negatively correlated with spinal nerve number only in the Archaeobatrachia, but this is likely an artifact of sampling.

A specimen of Scaphiopus holbrookii (Pelobatidae). There was some variation in spinal nerve number at intrafamilial, intrageneric and intraspecific levels (fig. 2).

Before examining the data for evolutionary trends, we ask how spinal nerve number relates to tadpole size, shape, and developmental stage. For the sample of all 60 specimens, there is a positive correlation of tadpole size with the number of spinal nerves (fig. 3A-C). Although the correlations are significant for SVL (p < 0.01), TOTL (p < 0.01) and TAILL (P < 0.02), the coefficients are low (all r < 0.4). For the archaeobatra-
size appears to be due to larval body size. In a stepwise regression, TAILL after SVL did not explain significantly more variation than SVL alone (additional $\tau^2$ due to TAILL after SVL = 0.001, $p > 0.05$).

The correlation between number of spinal nerves and relative tail length (TAILL/TOTL, fig. 3D) was not significant for the sample overall ($p = 0.47$), or for the Archaeobatrachia and Neobatrachia separately (all $p > 0.1$). Thus, a disproportionately long tail in anuran larvae does not of itself reflect a high number of spinal nerves.

For all specimens, there is no correlation of spinal nerve number with developmental stage ($p = 0.09$, fig. 3E). There is a negative correlation ($p = 0.02$) of spinal nerve number with developmental stage when all specimens of archaeobatrachian larvae are included in the analysis, but the correlation is not sustained when the specimens are pooled by species ($p = 0.06$). It is likely that this correlation is an artifact of representing some archaeobatrachian species with early developmental stages (fig. 3E). There is no correlation between spinal nerve number and developmental stage for the Neobatrachia ($p = 0.49$ by specimen, $p = 0.26$ by species).

The inferred evolutionary changes in spinal nerve number are shown in figure 4. Almost all of the taxa in the suborder Archaeobatrachia retain the primitive number of 23-29 spinal nerves. In archaeobatrachian larvae, the mean number of spinal nerves (24.4 ± 0.9) is significantly higher ($p = 0.01$) than in neobatrachian larvae (21.9 ± 0.6). This difference is not due to size or stage differences between the two groups. In mean size and developmental stage, our samples of archaeobatrachian and neobatrachian tadpoles are not significantly different ($p > 0.3$ for all Mann-Whitney U tests).

Reduction of spinal nerve number has occurred at least twice in the Archaeobatrachia. The first reduction, to 22 pairs, occurs in the common pipevine ancestor [6] of *Hymenochirus* and *Pipa*. The difference in spinal nerve number between the pipoids and other anurans could not be analyzed statistically because of the small sample size (n = 4). The second reduction, to 17 pairs, occurs within the genus *Scaphiopus*. The sample size within this lineage (n = 4) is also too small to analyze statistically.

Reduction in spinal nerve number is not a synapomorphy for the Neobatrachia. The ancestral condition for neobatrachians is retention of a large number of spinal nerves (fig. 4). Among neobatrachian taxa, variations in the number of spinal nerves occur within the genus *Caliobatrachus*. Also, the hypothesis that a larval stage with 21 spinal nerves is a secondarily reduced state is supported by the sizes of the types of the five derived taxa, with the three species of *Hylicus* having significantly fewer spinal nerves than the 21 to 29 types of the remaining species. However, the evolvability of the spine number suggests that this 21 to 29 type may be the ancestral condition for this group.
Evolution of Spinal Nerves in Tadpoles

Fig. 5. The relationship between mean number of spinal nerves and the minimum time from hatching to metamorphosis (n = 30). Closed circles refer to archaeobatrachian species; open circles refer to neobatrachian species. Data on minimum development time is taken from the literature (table 1). The relationship between the two variables is not significant for all species, for the Archaeobatrachia alone, or for the Neobatrachia alone. However, it is significant for the family Hylidae.

In a stepwise regression on the data of spinal nerve number (y) using the multiple regression line (additive model y = 23.9 + 0.98x, r² = 0.57, p < 0.05).

The relationship between mean number of spinal nerves (y) and minimum development time (x) (3D was fitted, r² = 0.47, p = 0.02) of tadpoles of different species when these variables are individually regressed, is not significant for all species (p = 0.05). There is an overall significant regression (p = 0.049 by ANCOVA).

When regressed against minimum development time, the taxa Archaeobatrachia and Neobatrachia were divided into primitive and derived groups, respectively. The derived Archaeobatrachia have 23.9 ± 0.6 (mean ± SEM) spinal nerves (n = 14), while the primitive Neobatrachia have 23.9 ± 0.4 (n = 15). This is a significant difference (t = 4.31, p = 0.001). The derived Neobatrachia, however, are not significantly different from the derived Archaeobatrachia. In conclusion, the taxa are not significantly different from each other, but the derived Neobatrachia are different from the primitive Archaeobatrachia.

Counts were performed on larval size and number of spinal nerves. The results are not significantly different from each other. However, the derived Neobatrachia tend to have larger larval sizes and a higher number of spinal nerves than the derived Archaeobatrachia.

These results suggest that the derived Neobatrachia have undergone a significant increase in size and number of spinal nerves, possibly indicating a shift towards a more aquatic lifestyle and hence a need for more efficient gill respiration.

taxa, there have been at least 5 independent reductions of spinal nerve number: (1) to 21 pairs within the genus Heleophryne; (2) to 22 pairs within the genus Allosodes; (3) to 21–17 pairs in the common ancestor of the hyline hylids; (4) to 21–17 pairs in the common ancestor of the bufonids; (5) to 22–17 pairs in the common ranid ancestor of the ranids, hyperoliids and microhylids. For the within-genus reductions (1 and 2 above), the sample sizes are too small to compare statistically. The hyline hylids have significantly fewer spinal nerves than the phyllomedusine hylids (species means, p = 0.02). The bufonids have significantly fewer spinal nerves than the leptoactylylids (species means, p = 0.01), and the ranoids (i.e., ranids, hyperoliids and microhylids) have significantly fewer spinal nerves than the bufonids (i.e., leptoactylylids, hylids and bufonids; p < 0.002). In the entire tree, the only evolutionary increase in the number of spinal nerves occurs within the genus Microhyla. The number appears to have increased secondarily from 21 to 23 spinal nerves in Microhyla petrigena.

We now compare those species that have the primitive number of spinal nerves (23–29 pairs) to those that have the derived condition (<22 pairs) independent of their superfamilial taxonomy and ask again whether differences in spinal nerve number are related to differences in size, stage or shape. Mean SVL, TOTL and TAILL of the species with the primitive pattern (n = 9) are 18.0 ± 1.5, 43.3 ± 3.5, 26.2 ± 2.0, respectively. Means for the same size measures in the derived forms are 14.5 ± 1.2, 36.4 ± 3.1 and 21.9 ± 1.9. SVL differs significantly (p = 0.03), whereas TOTL and TAILL approach significance (p = 0.07, p = 0.06). However, neither relative tail length nor developmental stage differ between groups (both p > 0.3). Once again, there is some evidence that spinal nerve number correlates with overall size, but not with relative tail length or developmental stage.

Because small tadpoles tend to reach metamorphosis faster than large tadpoles, a last variable that we examined was the minimum time from hatching to metamorphosis in relation to spinal nerve number (fig. 5). Information on minimum development time was available for 30 of 43 species we examined (10 Archaeobatrachia, 20 Neobatrachia). Spinal nerve number is not significantly correlated with development time, overall or within the Archaeobatrachia or the Neobatrachia. Mean development time is not significantly higher for our sample of Archaeobatrachia (115 ± 42 days) than for the Neobatrachia (144 ± 49 days, p = 0.48), or between tadpoles with primitive (156 ± 58 days) versus derived (144 ± 49 days) spinal nerve numbers (p = 0.18). However, there was a significant correlation between development time and spinal nerve number in the Hylidae (p < 0.025), the family with the most variation in spinal nerve number, and the family that is represented by the largest number of species.

Discussion

Our analysis of spinal nerve number in anuran larvalvae reveals several clear phylogenetic patterns, which are largely independent of larval size and developmental stage. Most obvious is the general direction of
evolution, which has been toward reduction in spinal nerve number. The primitive condition for anuran larvae is the presence of a variable number of spinal nerves, ranging from 23 to 29 pairs. In our sample of 43 anuran species, we find only 1 case of an increase in spinal nerve number (Microhyla petrigena). In contrast, reduction of spinal nerve number has evolved independently at least 7 times, twice in the Archaeobatrachia and 5 times in the Neobatrachia.

There is more variation in spinal nerve number within families than within genera, and more variation within genera than within species, but there is some variation in spinal nerve number at all taxonomic levels (fig. 2). It is interesting that the amount of variation in spinal nerve number differs among anuran lineages. At the intrageneric and intraspecific levels, the archaeobatrachian families exhibit greater variation in spinal nerve number than the neobatrachian families (fig. 2B, C). At least some archaeobatrachians continuously add new caudal segments throughout their larval period [17, 29, 30, 38]. Given this indeterminate pattern of neural and myotomal growth, it seems likely that the number of spinal nerves in archaeobatrachian tadpoles could be altered by environmental factors in the same way as temperature influences vertebral number in fishes [23]. The lower intrageneric and intraspecific variation in spinal nerve number among Neobatrachians may reflect their indeterminant form of caudal growth. The number of spinal nerves and myotomes is fixed earlier in neobatrachian development, presumably by the time of hatching. Thus, these larvae should be less responsive to environmentally induced variation in spinal nerve number. These observations are consistent with the fact that tadpole size correlates with spinal nerve number in the pipoids and bufonoids but not in the ranoids.

Among families (fig. 2A), three patterns of variation are found. Archaeobatrachian families, except the Pelobatidae, have relatively large numbers of spinal nerves, but with little intrafamilial variability. Bufonoid families have greater intrafamilial variability in spinal nerve number. Lastly, ranoid families have relatively few spinal nerves and low variability in spinal nerve number at all taxonomic levels. These three patterns reflect the taxonomic levels at which reductions have occurred in the three different superfamilial lineages (fig. 4). Reduction events occur only within genera among the archaeobatrachians, but at the generic, subfamilial and familial levels in the bufonoids. In the ranoids, the single reduction occurs at the superfamilial level.

The obvious question, of course, is: Why has reduction of the caudal spinal cord occurred so many different times in anuran phylogeny? Our hypothesis is that reduction of the caudal spinal cord in anuran larvae is associated with the shift from indeterminate to determinate growth, as well as with a reduction in the time it takes tadpoles to reach metamorphosis. During development, neural tissue is both the slowest to proliferate during ontogeny [44] and the slowest to be resorbed during metamorphosis [4]. Production and resorption of more spinal nerves may thus impose limits on minimum development time in species with indeterminate growth.

Individual cases from both the Archaeobatrachia and Neobatrachia illustrate the relationship between development time and spinal cord development. For example, species of the genus Scaphiopus have fewer spinal nerves than any other Archaeobatrachia that we studied (fig. 2A). Scaphiopus species also have the shortest development time of all species that we studied (table 1). We note here that the pelobatids have been considered by some authors to be a separate superfamilial grade intermediate between the Archaeobatrachia and the Neobatrachia. However, regardless of their superfamilial status, they must have evolved a reduced spinal nerve number independently because the primitive number of spinal nerves is retained by less derived pelobatid species (i.e., Leptobrachium montanum, fig. 4).

A conspicuous example from the Neobatrachia is the Hylidae, in which all our hyline hydids were found to have fewer spinal nerves than our phylomedusine hydids, and the hyline hydids all develop faster (table 1). However, it should be noted that there are other cases, such as the leptodactylids Leptodactylus pentadactylus [20] and Lepidobatrachus laevis [33], in which rapid development time has evolved without a reduction in the number of spinal nerves. Furthermore, development time can be reduced by other mechanisms as well, for example by reduction of total body size. Here, we suggest that selection for a short larval period may be one of many processes that has favored evolutionary reduction in spinal nerve number in larval anurans.

We have previously reported [27] a higher number of spinal nerves in Xenopus larvae than in Rana of similar size and developmental stage, and we speculated that differences in the caudal spinal cord of these two species may be related to their ecological differences. Marschall [18] was the first to point out these differences in the tadpole stage, and others [43]. Our results suggest that these differences result from micromorphological specialization patterns. If these patterns do not result from the same evolutionary adaptation, these adaptations must be made with the same physiological or morphological mechanisms, in the ways we have discussed above. This may be a general feature of the rapid metamorphosis of Osteostraci and other recent forms.

Although rapid metamorphosis occurs in many groups of higher vertebrates, it is not a universal feature. All members of the order Euteleostomi, which are aquatic or amphibious, have similar morphological features and rates of development, which suggests that they must have a similar superfamily grade. In the newts, for example, developmental rates vary from 11 days in Triturus cristatus [9] to 27 or more in Triturus cristatus [9]. However, it is not unlikely that these species have diverged further in their evolutionary history. The differences in metamorphosis among species of newts and salamanders are due to ecological differences rather than to micromorphological differences in the tadpole stage. Further studies of the micromorphology of other species of newts and salamanders may reveal these differences.
these genera may be related to differences in their ecology and behavior [see also ref. 42]. Xenopus tadpoles live midwater and scull with the tip of the tail [18] while Rana tadpoles are more benthic and have a distinctly different kinematic pattern when swimming [43]. Our sample is large enough and diverse enough to include larvae from virtually all known tadpole microhabitats. Surveying this larger sample of species does not reveal any obvious relationship between the number of spinal nerves that tadpoles have and either their ecology or swimming behavior. This point is made by examining the three species with the fewest spinal nerves. Microhyla heymonsi is a funnel-mouthed tadpole that feeds in a head-up posture at the water surface and hovers in the water column by rapidly oscillating its long, filamentous tail tip [15]. Osteopilus brunneus has an extremely attenuate tail. This arboreal larva rarely swims and, when forced to do so, swims poorly [22]. Scaphiopus holbrooki is a generalized benthic pond larva with a tail shape and swimming behavior like Rana [R.W., unpublished observations].

Although the number of caudal spinal nerves per se does not reflect obvious differences in the way of life of tadpoles, this does not mean that caudal spinal cord morphology in tadpoles is devoid of functional implications. Other neural characters, such as the presence or absence of motor neurons in the posterior half of the spinal cord, may be associated with differences in tadpole locomotor behavior and ecology.

Our analysis shows that spinal nerve number is an evolutionarily plastic character that has changed many times within the Anura, as it has within vertebrates as a whole. Whereas all adult anurans possess similarly abbreviated spinal cords, there is great phylogenetic diversity in the developmental pathway by which this adult morphology is reached. Adult anurans, in fact, possess the most highly reduced spinal cords known among tetrapods, with little variation in the number of spinal segments. There are only 10 or 11 paired spinal nerves in postmetamorphic anurans [9]. Our data show far greater diversity in premetamorphic stages. In tadpoles, unlike all other vertebrates, the tail is a temporary structure that is resorbed at metamorphosis. The greater morphological diversity in larvae may be tolerated because the tadpole tail is not constrained to develop into a functional adult organ. Therefore, anuran larvae exhibit more variation in the caudal spinal cord than any other vertebrates, including anuran adults.

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