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25. The direct study by Marinelli and Johnston (*8*) found that the $NO_3$ yield, corrected for a revised calibration factor (*11*), is less than 40%, and $NO_2$ and secondary $N_2O_5$ products have been detected in steady state (*11*) and static cell (*5, 9*) experiments.
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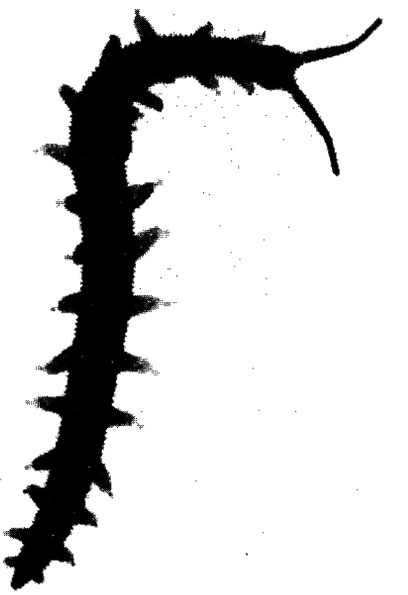

**Fig. 1.** An undescribed oviparous onychophoran species from eastern Australia.

# Evidence from 12*S* Ribosomal RNA Sequences That Onychophorans Are Modified Arthropods

J. William O. Ballard,* Gary J. Olsen, Daniel P. Faith, Wendy A. Odgers, David M. Rowell, Peter W. Atkinson

**The evolutionary relationships of the onychophorans (velvet worms) and the monophyly of the arthropods have generated considerable debate. Cladistic analyses of 12*S* ribosomal RNA sequences indicate that arthropods are monophyletic and include the onychophorans. Maximum parsimony analyses and monophyly testing within arthropods indicate that myriapods (millipedes and centipedes) form a sister group to all other assemblages, whereas crustaceans (shrimps and lobsters) plus hexapods (insects and allied groups) form a well-supported monophyletic group. Parsimony analysis further suggests that onychophorans form a sister group to chelicerates (spiders and scorpions) and crustaceans plus hexapods, but this relationship is not well supported by monophyly testing. These relationships conflict with current hypotheses of evolutionary pathways within arthropods.**

The question of whether arthropods, or jointed foot invertebrates, have a common ancestor has generated debate. Central to this discussion is the phylogenetic position of onychophorans, or velvet worms. These enigmatic invertebrates resemble slugs with legs (Fig. 1), and an early report described them as mollusks (chitons, limpets, and snails) (*1*). They have been described as the missing link between arthropods and annelids (segmented worms) because of physical similarities to both groups. Currently there are four major hypotheses of onychophoran evolutionary relationships (*2–5*) (Fig. 2). On the basis of morphological criteria, these hypotheses group myriapods (millipedes and centipedes) and hexapods (insects and allied groups) together to form the atelocerates. The morphological characteristics used by these models include the presence of anterior tentorial arms, the absence of pretarsal levator muscles, the absence of distinct appendages on the tritocephalic head segment (6), and appendage evolution (*7*).

Ultrastructural similarities between the sperm of onychophorans and euclitellates (oligochaetes, branchiobdellids, and leeches) have led to the proposition that onychophorans are more closely related to certain annelids than to arthropods (*2*) (Fig.

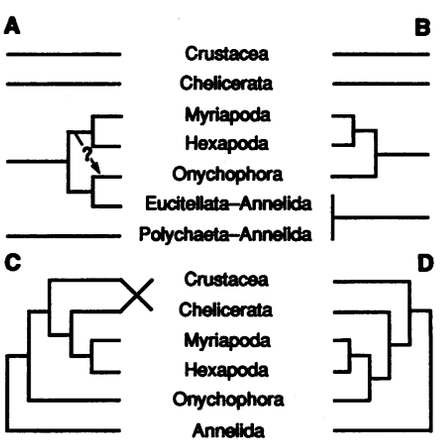


**Fig. 2.** Polyphyletic (A and B) and monophyletic (C and D) hypotheses of arthropod relationships. Polyphyletic hypotheses propose either that (**A**) onychophorans are more closely related to certain annelids than to arthropods (*2*) or (**B**) arthropodization has occurred independently at least three times (*3*). Hypotheses of arthropod monophyly propose onychophorans are either (**C**) primitive (*4*) or (**D**) closely allied to myriapods and hexapods (*5*).

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2A). Similarities in the embryonic development of onychophorans and atelocerates have been used as evidence for monophyly of these two groups to form the uniramians, although there is dissent over arthropod monophyly (Fig. 2, B and D). Investigators who discount the embryological evidence (*4*) have suggested that the closest extant relatives of atelocerates are the other mandibulate arthropods, the crustaceans (shrimps and lobsters) (Fig. 2C). The monophyly of this assemblage is further supported by the extraordinary similarity of the compound eye ommatidia of hexapods and crustaceans. The myriapod eyes are considered to be modified secondarily (*8*).

Of the existing numerical approaches to investigating phylogeny, maximum parsimony methods have been used most extensively (*9*). These methods minimize the amount of evolutionary change required to explain the available data. Maximum parsimony comparisons of partial cytoplasmic 18S ribosomal RNA (rRNA) sequences from chelicerates (spiders and scorpions), myriapods, crustaceans, and hexapods support the hypothesis that these arthropods are monophyletic relative to annelids and mollusks (*10, 11*). However, bootstrapping (*12*), a statistical method for obtaining an estimate of error, does not support this hypothesis (*11*). In contrast to maximum parsimony analyses, evolutionary parsimony analysis of a subset of these data does not suggest arthropod monophyly (*13*). Because there are relatively few 18S rRNA transversion positions (*11*), we sequenced a segment of the mitochondrial small ribosomal subunit, 12S rRNA (*14*), in order to investigate the phylogenetic position of onychophorans and the monophyly of arthropods. This region of 12S rRNA was chosen

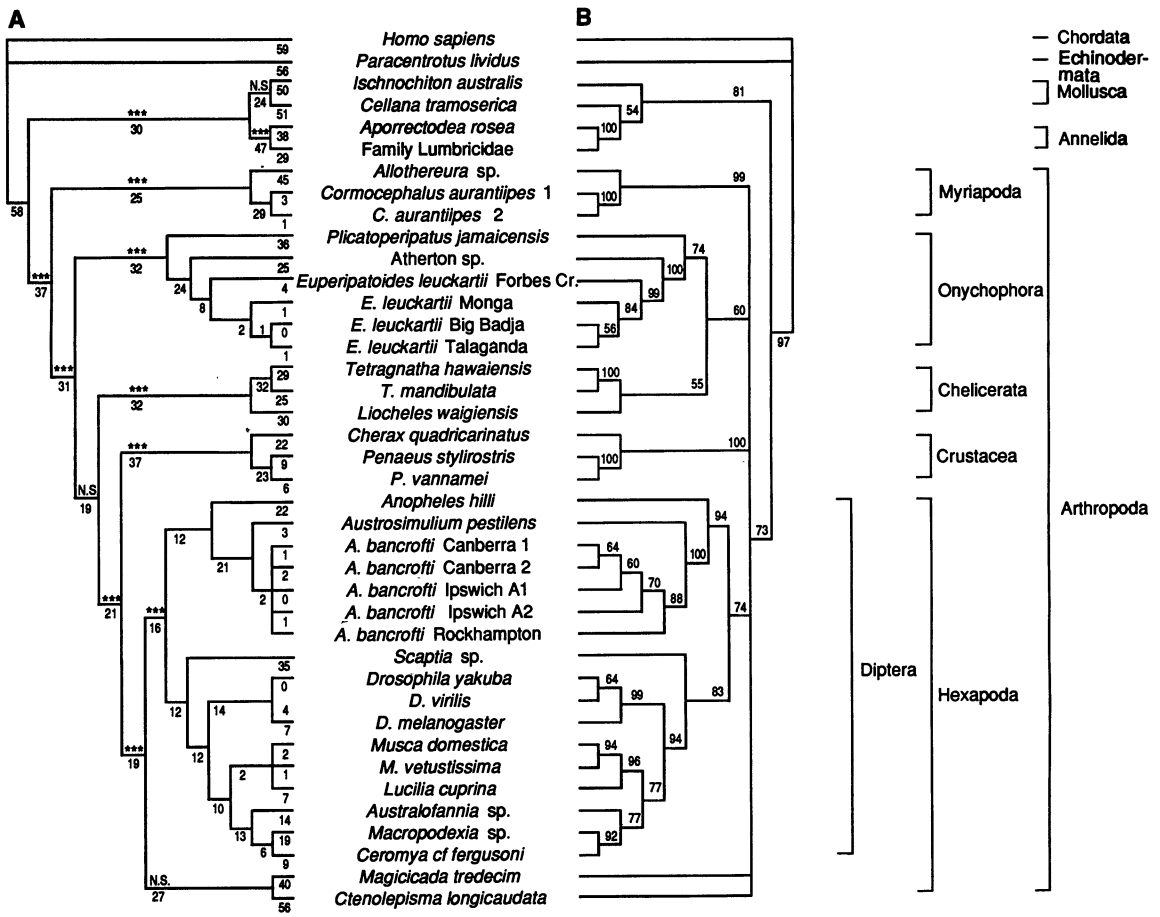


**Fig. 3.** Phylogenetic inferences generated from the 40 taxa 329 character state matrix, (245 informative sites). (**A**) A strict consensus tree, resulting from maximum parsimony analyses, constrained to contain all significantly monophyletic groups (below). Asterisks above the lines show significantly monophyletic nodes whereas numbers below the lines indicate branch lengths. This analysis generated 64 equally parsimonious trees of length 1418 steps. This is seven steps longer than the initial analyses during which PAUP found 144 parsimonious trees. Hennig86 found 128 trees of the same length. (**B**) A neighbor-joining phylogenetic tree generated by PHYLIP (*21*) using the Kimura two-parameter model with no transition transversion bias. Numbers at nodes indicate the bootstrap percentages from 2000 samples. Bootstrap values less than 50 indicate that the assemblage is not well supported and these nodes have been collapsed to yield a consensus. The series of parsimony analyses with T-PTP testing proceeds as follows. Arthropods including onychophorans are monophyletic (T-PTP = 0.05, difference 4 steps), and a hypothetical arthropod ancestor is calculated using character state optimization based on the tree topology found within arthropods. This ancestor taxon is then combined with other outgroup taxa to test monophyly of annelids and mollusks. Annelids are monophyletic (T-PTP = 0.01, 18 steps), but a parallel test constraining mollusc monophyly gives a tree 3 steps longer than the converse. In an a posteriori analysis mollusks and annelids are monophyletic (T-PTP = 0.01, 12 steps). Onychophoran and dipteran monophyly is supported when annelids are the outgroup to all arthropods (T-PTP = 0.04, 5 steps, and T-PTP = 0.03, 3 steps, respectively). Monophyly of myriapods and the remaining arthropods is subsequently supported (T-PTP = 0.01, 4 steps, and T-PTP = 0.01, 3 steps, respectively). Chelicerate and crustacean monophyly is supported in parallel (T-PTP = 0.01, 3 steps, and T-PTP = 0.01, 9 steps). Crustaceans plus hexapods are monophyletic (T-PTP = 0.03, 1 step) when myriapods are the outgroup and the ingroup consists of *C. longicaudata* and *M. tredecim* and the onychophoran, dipteran, chelicerate, and crustacean ancestors. *Ctenolepisma longicaudata* and *M. tredecim* are not significantly monophyletic in a parallel test (T-PTP = 0.11, 1 step). Subsequent parsimony analysis suggests chelicerates and crustaceans plus hexapods are monophyletic; however, T-PTP testing shows this is not significant (T-PTP = 0.31, 1 step). In a subsequent test of non-monophyly, T-PTP testing could not reject the hypothesis that onychophorans and chelicerates are monophyletic (T-PTP = 0.50, 1 step). Hexapods are monophyletic (T-PTP = 0.01, 1 step) when onychophorans and chelicerates are the outgroup to crustaceans and hexapods. Parallel testing shows monophyly of *C. longicaudata* and *M. tredecim* is not well supported (T-PTP = 0.15, 2 steps). With the crustacean ancestor as the outgroup and dipteran ancestor as the ingroup, monophyly of *M. tredecim* and *C. longicaudata* is again not significant (T-PTP = 0.69, 0 steps). Monophyly tests of major groups within arthropods were properly defined as a priori tests (*23*). However, testing monophyly of chelicerates and crustaceans plus hexapods and non-monophyly of onychophorans and chelicerates was correctly a posteriori; the hypothesis of monophyly arose as a result of cladistic analysis. The simpler a priori test initially applied here preempts the a posteriori test, as T-PTP values will always be higher for the latter.

for the analysis because previously it has been useful over a broad taxonomic range (*14, 15*).

DNA from 31 terminal taxa was prepared (*16*), and the 12S rRNA region was amplified by polymerase chain reaction and sequenced (*17, 18*). Nine additional 12S rRNA sequences (*19*) were derived from the literature. We analyzed nucleotide sequence variation by maximum parsimony with the computer programs PAUP (*20*) and Hennig86 (*21*) (Fig. 3A) and by neighbor-joining analysis using PHYLIP (*22*) (Fig. 3B). In parsimony analyses, gaps introduced to improve alignment were scored as ambiguities (missing information). Although the resulting hierarchical tree structure presented evidence for monophyly of the component groups, additional testing in support of monophyly was required. In this study, monophyly or T-PTP testing (*23, 24*) and bootstrap (*12*) tests were applied to parsimony and neighbor-joining analyses, respectively.

Maximum parsimony analyses with T-PTP testing indicate that arthropods include onychophorans and are monophyletic relative to annelids and mollusks (Fig. 3A). This proposal is supported by neighbor-joining analysis with bootstrapping (Fig. 3B). Annelids were chosen as an appropriate outgroup to evaluate arthropod relationships in subsequent analyses with T-PTP testing (*25*). Parsimony analyses suggest that onychophorans form a sister group to chelicerates and crustaceans plus hexapods, but T-PTP testing shows the available 12S rRNA data cannot significantly resolve this trichotomy (Fig. 3A). In comparison to these results, neighbor-joining analysis suggests that onychophorans and chelicerates are sister taxa (Fig. 3B). These data imply that onychophorans are a highly specialized assemblage, neither a primitive "missing link" nor an appropriate outgroup for analyzing arthropod phylogenetic relationships. Parsimony analyses with T-PTP testing further indicate that myriapods represent the earliest arthropod branch and are the sister group to the remaining taxa including a monophyletic crustacean plus hexapod assemblage. This suggests that the diverse eye structures of myriapods are primitive and not derived (*8*) and that the morphological criteria used to unite the atelocerates result from convergent evolution and are not shared derived characters (*6*). Phylogenetic analyses indicating that myriapods are basal to the remainder of the arthropoda suggest that the assemblage is older than previously thought. A myriapod-like fossil recently described from marine deposits in the Middle Cambrian superficially resembles *Portalia* and *Redoubtia* from the Burgess Shale (*26*). The later fossils were originally described as holothurians (sea cucumbers) (*27*) but a more recent evaluation concluded their relationships are problematic (*28*).

Data obtained from 18S rRNA (*10, 11*) support some of the evolutionary relationships proposed in our reconstructed parsimony analyses. Field and co-workers (*10*) reported that the myriapod occupied an unexpectedly deep position in their tree; however, they had difficulty placing it because of the long branch length. Maximum parsimony analyses with additional 18S rRNA sequences (*11*) suggested that myriapods and chelicerates are the sister group to crustaceans plus hexapods. Although this tree was not well supported, no trees within 1% of the most parsimonious recognized a monophyletic myriapod plus hexapod assemblage (*11*). Parsimony analyses of both 12S rRNA and 18S rRNA sequence data cast doubt on the monophyly of atelocerates.

Within the major assemblages, general congruence with independently derived phylogenies provides additional support for our reconstructed tree (Fig. 3A). Within onychophorans, *Plicatoperipatus jamaicensis* was the sole member of the family Peripatidae sequenced. This family is the sister group to the family Peripatopsidae. Within Peripatopsidae, the unnamed Atherton species is electrophoretically (*29*) and morphologically (*30*) distinct from *Euperipatoides*. Phylogenetic inferences generated from 18S rRNA analyses support monophyly of the chelicerates (*11*) and the crustaceans used in this study (*31*). There is also high congruence between 12S rRNA and morphologically derived trees within dipteran hexapods (*32*). Hexapod phylogenetic relationships are complicated by the accumulation of nucleotide substitutions in the 12S rRNA of the thysanuran *Ctenolepisma longicaudata*. The reconstructed tree clusters *C. longicaudata* and the hemipteran, *Magicicada tredecim*. However, T-PTP tests do not support monophyly of this clade. Additional sequences will be required to resolve relationships between these hexapod orders.

These data demonstrate that 12S rRNA sequence data can resolve arthropod relationships over a broad taxonomic range. Some further corroboration of the significant monophyletic groups is found using 18S rRNA sequence data and electrophoretic and morphological characters within arthropod assemblages. We propose that arthropods include onychophorans and are monophyletic. Moreover, we cannot find support for monophyly of uniramians or atelocerates and suggest phylogenetic relationships within arthropods should be reassessed. Our reconstructed tree represents a new framework for arthropod evolutionary pathways (*33*).

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16. DNA was prepared from individuals using the method of S. Kidd, T. J. Lockett, and M. W. Young [*Cell* **34**, 421 (1983)] or as follows. Individual specimens were homogenized in 500 μl of 0.1 M tris, 0.1 M EDTA (pH 9) followed by a 1-hour incubation at 65°C with 1/2 volume of 2% SDS. Homogenates were incubated on ice for 30 min with 1/6 volume of 8 M potassium acetate. Centrifugation was used to remove cell debris. The supernatant was extracted with phenol and incubated at 37°C with ribonuclease (5 μg/ml for 20 min) and then with Proteinase K (25 μg/ml for 20 min). DNA was extracted with Gene-Clean (Bio 101 Inc.).
17. Initial PCR conditions were 10 to 100 ng of DNA, 0.9 units of *Taq* polymerase, 4 mM of each primer, 25 mM tris-HCL, 125 mM KCL, 0.1% gelatin, 3.1 to 8.75 mM $MgCl_2$, and 2.5 mM of each NTP. The reaction was overlaid with mineral oil and cycled 30 to 45 times (92° to 94°C for 30 s, 43° to 55°C for 2 min, and 72°C for 0 to 30 s). The two primer sets were L1091 with H1478 (*14*) and 12*Sai* with 12*Sbi* (*15*). Single-stranded DNA was purified according to the lambda-exonuclease technique [R. G. Higuchi and H. Ochman, *Nucleic Acids Res.* **17**, 5865 (1989)] and purified and sequenced [M. Kreitman, in *NATO ASI Series*, vol. 57, *Molecular Techniques in Taxonomy*, G. M. Hewitt, A. W. B. Johnson, J. P. W. Young, Eds. (Springer-Verlag, Berlin, 1991), pp. 357–367]. To identify each unique sequence, species names are followed by collection site and an integer, if required.
18. Organism identifications: Mollusks, *Cellana tramoserica* and chiton *Ischnochiton australis* by G. Clarke and D. Colgan (Australian Museum) (GenBank accession numbers L02388 and L02389); annelids, *Aporrectodea rosea* and a juvenile also in family Lumbricidae by G. Dyne (National Forest Inventory, Canberra) (L02392 and L02403); myriapods, *Allothereura* sp. and taxa within *Cormocephalus aurantiipes* by J. M. Waldock (Western Australian Museum) (L02376,

L02379, and L02402); Australian onychophorans by D.M.R. (L02377, L02378, L02380, L02395, and L02414) and *Plicatoperipatus jamaicensis* by P. D. N. Herbert (Department of Zoology, University of Guelph, Canada) (L02410); chelicerate, *Liocheles waigiensis* by R. Moran (CSIRO) (L02397) and crustacean, *Cherax quadricarinatus* by R. Bedding (CSIRO) (L02396); hexapods, *Macropodexia* sp., *Scaptia* sp., *Ceromya cf. fergusoni*, and *Australofannia* sp. by D. H. Colless (CSIRO) (L02384, L02393, L02387, and L02401); *Lucilia cuprina*, *Musca domestica*, and *M. vetustissima* by P. Cranston (CSIRO) (L02391, L02409, and L02400); *Anopheles hilli* by P. Sweeney (Australian Army) (L02382); taxa within *Austrosimulium* by J.W.O.B. (L02383, L02385, L02386, L02390, L02398, and L02399); *Drosophila melanogaster* by W.A.O. (L02394); and *Ctenolepisma longicaudata* by J. A. L. Watson (CSIRO) (L02381).

19. Additional sequences were from *Homo sapiens* [S. Anderson *et al.*, *Nature* **290**, 457 (1981)], *Paracentrotus lividus* [P. Cantatore, M. Noberti, M. N. Gadaleta, C. Saccone, *J. Biol. Chem.* **264**, 10965 (1989)], *Tetragnatha hawaiensis* and *T. mandibulata* [H. B. Croom, R. G. Gillespie, S. R. Palumbi, *J. Arachnol.* **19**, 210 (1991)], *Penaeus stylirostris* and *P. vannamei* [R. Palumbi and J. Benzie, *Mol. Mar. Biol. Biotechnol.* **1**, 27 (1991)], *Drosophila yakuba* [D. O. Clary and D. R. Wolstenholme, *J. Mol. Evol.* **22**, 252 (1985)], *D. virilis* [*ibid.* **25**, 116 (1987)], and *Magicicada tredecim* (*15*).
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25. Morphological and developmental evidence suggest annelids are the appropriate outgroup to arthropods [P. Weygold, *Z. Zool. Syst. Evolutionsforsch.* **24**, 19 (1986); P. A. Meglitsch and F. R. Schram, *Invertebrate Zoology* (Oxford Univ. Press, Oxford, ed. 3, 1991), p. 596]. However, because annelids plus mollusks were shown to be monophyletic by T-PTP testing and the sister group to arthropods using 18*S* rRNA sequence data [M. T. Ghislin, in *Oxford Surveys in Evolutionary Biology* (Oxford Univ. Press, Oxford, 1988), pp. 66–95; (*10*)], analysis was also run with annelids plus mollusks as the outgroup to arthropods; this further corroborated myriapods as basal to chelicerates and onychophorans.
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# Predator-Induced Phenotypical Change in Body Morphology in Crucian Carp

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**In a field experiment where the presence or absence of piscivorous pike (*Esox lucius*) in ponds was manipulated, the morphology of crucian carp (*Carassius carassius*) diverged, such that individuals became deeper bodied in pond sections with pike. A laboratory experiment confirmed that the presence of this predator induced a change in body morphology in the carp. Estimation of prey vulnerability to predation by pike, a gape-limited predator, revealed that this increase in body depth resulted in crucian carp reaching a size that provided refuge from predation. However, this change in morphology incurs a cost through an increase in drag when the carp are swimming. Because crucian carp are limited by resources in the absence of piscivores and by the substantial cost of the defensive morph in their presence, phenotypic plasticity should be the optimal strategy for this species.**

Various morphological structures in prey organisms function as efficient adaptations against predation (*1*), and these morphological defenses could be either constitutive or environmentally induced. The evolution and maintenance of inducible defenses is favored when the defense incurs a fitness cost, when predation intensity varies temporally or spatially, and when prey have reliable cues for predator detection (*2*, *3*). Predator-induced morphological defenses occur in a number of invertebrates, mainly aquatic taxa (*2*). Waterborne cues from predators or chemicals released by injured conspecifics trigger the development of defenses that reduce predation rates (*2*, *4*). However, induced defenses have been shown to incur a fitness cost through a reduction of growth or reproduction or both (*2*, *4*, *5*). Here, we report on a predator-induced change in body morphology in a vertebrate, the freshwater fish crucian carp (*Carassius carassius*).

Crucian carp are extremely vulnerable to predation (*6*, *7*). In lakes with piscivores, especially pike *Esox lucius*, crucian carp populations consist of a small number of large individuals (*6*). However, without piscivores, crucian carp form dense populations of small individuals (*6–8*). The body morphologies of monospecific pond populations and multispecies lake populations differ, with lake individuals much deeper bodied. The two morphs originally were considered as separate species (*Cyprinus vulgaris* and *C. gibelio*); however, in the early 1800s it was shown by transplant experiments that these two species were one (*9*). The presence of two morphs has previously been considered a result of differences in resource levels; however, we show that increased body depth can also be an inducible morphological defense that reduces the risk of predation.

For part of a study evaluating the effects of trophic structure on freshwater communities, we divided into halves two small, eutrophic ponds (Severin's and Mats' ponds, 0.1 ha each) with monospecific crucian carp populations and introduced pike into one half (*10*). After 12 weeks, crucian carp had diverged in body shape; in pond sections with pike, carp tended to have a deeper body (Fig. 1). Given this result, we hypothesized that the change in body morphology could be a result of several things: (i) selective predation, (ii) an increase in resource availability, or (iii) a predator-induced phenotypic modification of body shape. The small variance in body depth and the absence of overlap between treatments (Fig. 1) suggested no polymorphism with regard to this trait in the original population; thus, selective predation on genetically determined morphs could not account for the increase in body depth.

High resource availability may be responsible for the shift in morphology, as suggested by a study in Finland where crucian carp increased in body depth when introduced at a low density of 187 fish per hectare to a fishless pond (*8*). In our ponds, the reduction of the crucian carp density by pike permitted an increase in the density of large, cladoceran zooplankton (*11*). This increase in food availability in the pike section could account for the differences in the carp body depth. However, in another experiment we transplanted crucian carp from a pond with a

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