

Caterpillars did not evolve from onychophorans by hybridogenesis

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The evolution and loss of distinctive larval forms in animal life cycles have produced complex patterns of similarity and difference among life-history stages and major animal lineages. One example of this similarity is the morphological forms of Onychophora (velvet worms) and the caterpillar-like larvae of some insects. Williamson [(2009) *Proc Natl Acad Sci USA* 106:15786–15790] has made the astonishing and unfounded claim that the ancestors of the velvet worms directly gave rise to insect caterpillars via hybridization and that evidence of this ancient “larval transfer” could be found in comparisons among the genomes of extant onychophorans, insects with larvae, and insects without larvae. Williamson has made a series of predictions arising from his hypothesis and urged genomicists to test them. Here, we use data already in the literature to show these predictions to be false. Hybridogenesis between distantly related animals does not explain patterns of morphological and life-history evolution in general, and the genes and genomes of animals provide strong evidence against hybridization or larval transfer between a velvet worm and an insect in particular.

hybridization | insect evolution | interphyletic crosses | larval transfer | metamorphosis

Among the most important features of animal evolution and diversity is the development of multiple phenotypes or life-history stages within an organism’s life cycle, each stage adapted to a different set of environments and ecologically independent of the others, and developmentally derived from each other by one or more dramatic metamorphoses. Many thousands of animal species have such multiphasic life histories, these species are phylogenetically widespread among major animal lineages, and many of them are ecologically important in terrestrial, freshwater, or marine communities. Familiar examples include the larval tadpole and adult frog, or the larval caterpillar and adult butterfly, while less familiar, but no less spectacular, examples fill the pages of invertebrate zoology textbooks (1).

The origin and evolution of animal larvae is broadly understood as the outcome of natural selection acting on the phenotypes of both early and late life-history stages, within constraints imposed by historical contingency and developmental processes (2, 3). These effects of adaptation and constraint in larvae and adults have produced an astonishing variety of similarities in larval form among species with different adults, differences among larvae that metamorphose into similar adults, and similarities between the larvae of some species and the adults of others (2). The earliest efforts toward such an understanding are >100 years old (4) and have matured into the subdiscipline known as larval ecology (5).

In a series of articles and books spanning >20 years and culminating in the article published in PNAS (6), Donald Williamson has “reject[ed] the Darwinian assumption that larvae and their adults evolved from a single common ancestor” and advocated the astounding alternative hypothesis “that, in animals that metamorphose, the basic types of larvae originated as adults of different lineages, i.e., larvae were transferred when,

through hybridization, their genomes were acquired by distantly related animals.” Williamson first developed this hypothesis of “larval transfer” via hybridization to explain the variation among larvae of marine crab families, as an alternative to hypotheses of convergent evolution or secondary loss of particular larval phenotypes through natural selection. He later extended this hypothesis to explain virtually all patterns of morphological variation among larval forms of aquatic and terrestrial animals. Most recently, Williamson (6) focused on superficial morphological similarities between the charismatic near-arthropods known as velvet worms (Phylum Onychophora) and the caterpillar-like larvae of some holometabolous insects and proposed that velvet worms are “the evolutionary source of caterpillars and their grub or maggot descendants” via hybridization between ancient insects and velvet worms that inserted an onychophoran-like larval form into the life cycle of the insect. Like his previous articles and books concerning larval transfer between distantly related animal clades by hybridization, Williamson’s article (6) included no evidence that could be used by a reader to choose objectively between his hypothesis and alternatives such as the very well-supported view of larvae as evolutionary specializations of the early life cycle (e.g., refs. 7–9). Instead, Williamson offered a series of more or (much) less plausible corollaries of his hypothesis that he called “a molecular biological research proposal,” which he hopes other biologists will fulfill.

Williamson (6) suggested that “many corollaries of my hypothesis are testable.” We agree and note that most of the tests have already been carried out, the results of which are readily available in the recent literature and online databases. Here, we set aside (i) the complete absence of evidence offered by Williamson in support of his hypothesis, (ii) his apparent determination to ignore the enormous errors in current understanding of inheritance, gene expression, cell fate specification, morphogenesis, and other phenomena that are implied by his hypothesis, and (iii) the abundant empirical evidence for the evolution and loss of larval forms by natural selection. Instead, we focus on Williamson’s molecular genetic predictions concerning genome size and content in insects, velvet worms, and several marine taxa, and we point out the readily available data that show those predictions to be easily rejected.

Rhizocephalans Have Small Genomes and Did Not Acquire Larvae by Hybridization with Other Crustaceans

The Rhizocephala are barnacles that parasitize the blood sinuses and other internal spaces of other crustaceans, especially decapod crabs (1). The sexually mature, parasitic adult stages of rhizocephalans lack such typical animal features as a digestive tract, lack arthropod characteristics such as segmentation, jointed limbs, or a molted exoskeleton, and lack typical barnacle

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features such as calcified shell plates. However, the early life histories of rhizocephalans closely resemble other crustaceans, and the planktonic larval stage that recruits to a new host and initiates a new infection is called a cypris that is indistinguishable from the bivalved cyprid larvae of other barnacles and considered to be diagnostic for this class of crustaceans (Cirripedia). Many other more typical barnacles live in close association with animal hosts (e.g., whales and turtles) as external parasites and may be somewhat modified for these close associations. Rhizocephalans are very highly modified barnacles that have become extremely specialized as endoparasites.

Williamson (6) rejects this view in favor of a hypothesis in which Rhizocephala are not barnacles but acquired a cypris by hybridization with a barnacle:

“The two contrasting concepts to explain rhizocephalans can be experimentally distinguished. If rhizocephalans are parasitic barnacles, i.e., adults that lost all barnacle morphology by reduction yet their larvae retain virtually all features of larval barnacles, their genomes should be typical of cirripedes. Alternatively, if, as I suggest, rhizocephalans are not arthropods but acquired arthropod larvae by hybrid transfer, at least three genomes should be detected. Those that code for nauplius and cypris larvae should be similar to those in cirripedes, while the third ‘adult’ genome should differ distinctively from that of cirripedes.”

Notably, Williamson (6) does not propose how barnacles themselves acquired the distinctive cyprid form or explain what the taxonomic affinities of Rhizocephala might be if they are not barnacles. The adaptive significance of the cypris is an interesting unresolved question, but molecular phylogeneticists have successfully answered the second question: rhizocephalans are indeed a clade of barnacles. Phylogenies based on genes that each are expressed in both larvae and adults of rhizocephalans and other crustaceans (e.g., ref. 10) unambiguously support rhizocephalans as the close sister group to the thoracican barnacles (including familiar acorn barnacles and stalked gooseneck barnacles). The sister group to the Rhizocephala + Thoracica is a clade of barnacles called the Acrothoracica with typical forms (including long cirrus-shaped legs for suspension feeding by adult females) but unusual lifestyles (females burrow into coral and mollusk shells and have brief liaisons with short-lived dwarf males attached to the female) (11). Molecular phylogenies of barnacles and related crustaceans show no evidence of multiple genomes with different phylogenetic histories as proposed by Williamson. Indeed, the genomes of rhizocephalans could not contain a rhizocephalan “adult” genome plus two independent crustacean “larval” genomes of the size characteristic of barnacles and other crustaceans because rhizocephalan genomes are smaller than other barnacle genomes and smaller than most other crustacean genomes. Genome sizes or *C* values (total mass of DNA per haploid genome) are known for thousands of animal species mainly from studies using cytological methods including Feulgen densitometry and flow cytometry (12). A single known genome size from a sacculinid rhizocephalan (*C* = 0.67 pg) (13) is smaller than all other known barnacle genomes (0.74–2.60 pg) (12), rather than larger as predicted by Williamson’s hypothesis.

Holometabolous Insects Have Small Genomes Relative to Insects Without Larvae

Major insect taxa vary widely in the complexity of their life cycles. Understanding the evolution of multiphasic life cycles with specialized larvae in some insect groups but not in others has a rich empirical and theoretical history (e.g., refs. 14 and 15). Williamson (6) rejects the results of this deep and productive

research tradition and speculates instead that insect orders that include larvae arose not by natural selection but by hybridogenesis. On the basis of this speculation, he predicts that insect genomes will vary in size as a consequence of hybridization with onychophorans that introduced larvae resembling velvet worms (and genomes of these donors) into some insect life cycles but not others:

“If insects acquired larvae by hybrid transfer, the total base pairs of DNA of exopterygote insects that lack larvae will be smaller than those of endopterygote (holometabolous) species that have both larvae and pupae.”

Williamson (6) does not explain why such a hybridization should have added the onychophoran morphology to the early part of the insect life cycle before sexual maturity rather than to the mature adult part of the life cycle. Nevertheless, the prediction regarding genome size differences can be tested and has been rejected. Genome sizes (12) are relatively large (rather than small as predicted by Williamson) in the orders Blattodea (*C* = 0.23–5.15 pg), Heteroptera (0.18–6.15 pg), Odonata (0.37–2.20 pg), Orthoptera (1.55–16.93 pg), and Phasmatodea (1.95–8.00 pg) that lack caterpillar-like larvae. Genome size tends to be smaller in holometabolous Coleoptera (0.16–5.02 pg), Diptera (0.09–1.52 pg), Hymenoptera (0.16–0.77 pg), and Lepidoptera (0.29–1.94 pg), but the ranges broadly overlap those for the other orders.

Holometabolous Insect Genomes Have Relatively Few Genes and Did Not Acquire Onychophoran Genes by Hybridization

In addition to variation in the total size of the genome (or *C* value), major animal lineages (including insects) vary considerably in the gene content of their genomes. The sources of gene number variation and the processes that favor duplication and loss of genes are diverse and include some instances of lateral gene transfer (16). Williamson (6) ignores these diverse mechanisms in favor of hybridogenesis:

“Genome sequences are known for the fruitfly, *Drosophila melanogaster*, the honeybee, *Apis mellifera*, the malarial mosquito, *Anopheles gambiae*, the red flour beetle, *Tribolium castaneum*, and the silkworm, *Bombyx mori*: holometabolous species, with marked metamorphoses. I predict that an earwigfly (Mercoptera Meropeidae), an ear wig (Dermaptera), a cockroach (Diptoptera), or a locust (Orthoptera) will have not necessarily fewer chromosomes but will have fewer base pairs of protein-coding chromosomal DNA than have these holometabolans.”

The remarkable growth of the catalog of whole-genome sequences for animals already includes enough insect species to test and reject this prediction. Whole-genome sequences and gene content are known for all five of these model holometabolous insect species (17–19). Contrary to Williamson’s prediction (6), the gene content of these genomes is low rather than high: they range in number from 10,157 genes (*A. mellifera*) to 16,404 genes (*T. castaneum*). All five of these genomes have fewer genes than do complete fish, bird, or mammal genomes from species in which the developmental sequence lacks a caterpillar or any other distinctive larval form (18). Although there are no complete whole-genome sequences yet available from other insect orders for comparison, all five of these holometabolous insect genomes contain fewer genes than do the draft whole-genome sequences for two other arthropods, the tick *Ixodes scapularis* (GenBank accession no. ABJB000000000; 20,467 genes) and the branchiopod crustacean *Daphnia pulex* (wFleaBase.org; 30,939 genes), and some are smaller than the provisional gene content

(12,082 genes) estimated from expressed sequence tags of the pea aphid *Acyrtosiphon pisum* (20).

Holometabolous Insect Genomes Do Not Contain an Onychophoran-Like Genome Acquired by Hybridization

Evolutionary biologists have often (and sometimes loudly) debated whether major evolutionary innovations in phenotypes are driven mainly by the evolution of regulatory sequences that modify the functional expression of genes at different times of the life cycle and in different parts of the body, or mainly by divergence of the nucleotide sequence and organization of protein-coding genes themselves (e.g., refs. 21 and 22). A necessary feature of Williamson's hypothesis (6) is the inheritance of a complete cassette of key genes whose expression determines the phenotype of the added larval form and that encode gene products different from those of paralogous loci already in the recipient genome whose expression determines the adult phenotype. This assumption and its corollary prediction put Williamson firmly if unwittingly on the far end of the structuralist side of this important debate. He wrote:

“Also the genome of an onychophoran that resembles extant species will be found in insects with caterpillar or maggot-like larvae.”

Whole-genome sequence analysis will form one important part of the basis for addressing this general question regarding the molecular mechanisms of adaptive phenotypic evolution. An incisive test of Williamson's specific prediction (6) will depend on the results of a whole-genome sequence analysis of a velvet worm. The best available evidence comes from molecular phylogenetic analyses, which Williamson's hypothesis predicts should sometimes show close relationships between onychophoran and holometabolous insect genes (for orthologues transferred from the former to the latter by hybridization) and sometimes show close relationships between hemimetabolous and holometabolous insects (for orthologues shared only among insects). All of the available phylogenetic tests strongly reject this prediction. In the best (and most recent) test, Roeding et al. (23) sampled 149 genes (11,168 codons) from the genome of a scorpion and aligned those sequences to orthologues from the genomes of 2 onychophorans and 17 insects, plus 12 crustaceans and 21 other chelicerates and myriapods. These data represent a substantial proportion ($\approx 1\%$ in total) of the 10,000–16,000 holometabolous insect protein-coding genes. Roeding et al. (23) used this very large sequence alignment to estimate phylogenetic relationships. They found no evidence for close similarity of any onychophoran genes to genes from holometabolous insects to the exclusion of other insects (or crustaceans). Instead, they found extraordinarily strong phylogenetic support for a clade of all insect genes, closely related to branchiopod crustaceans, with Onychophora (including all velvet worm genes) as the sister group to the true arthropods.

An unexpected aspect of this result that bears on Williamson's hypothesis (6) is its implications for the evolution of larval forms. If insects are highly derived crustaceans closely related to water fleas, brine shrimp, and other Branchiopoda with the characteristic crustacean nauplius larval form, then this phylogeny suggests that the nauplius was lost in the lineage leading to the first insects followed by the gain of a new holometabolous life cycle with a distinctive caterpillar (or maggot or grub) larva.

Onychophorans Have Genomes Larger Than Those of Holometabolous Insects and Did Not Hybridize with Insects

In addition to the predictions above concerning genome size, genome content, and phylogenetic relationships among insects that are or are not descended from an ancient hybridization event with a velvet worm, Williamson's hypothesis (6) can be

tested on the basis of the relative sizes of the genomes of the hypothesized donor and recipient lineages. He wrote:

“Onychophoran genomes will be smaller than those of holometabolous insects.”

This prediction is easily rejected. The genome size database (12) includes C values for the velvet worms *Epiperipatus biolleyi* ($C = 4.43$ pg) and *Euperipatoides kanangrensis* (6.88 pg). The latter is larger than all reported C values for holometabolous insects and larger than almost all other insect C values. Like the genome size data for rhizocephalan barnacles, these published C values for velvet worms are not merely inconsistent with Williamson's hypothesis (6) but directly contradict its simplest predictions.

Tunicates Have Genome Sizes Similar to Larvaceans and Did Not Acquire Tadpole Larvae by Hybridization with Larvaceans

Although Williamson (6) did not refer to any of the genomic data noted above for arthropods and velvet worms, he did refer to some genome size data for another group (the Urochordata or invertebrate chordates) that includes an analogous similarity between the larval form of one taxon (the tunicates or sea squirts) and the adult form of another (the larvaceans). The evolution of life histories among Urochordata, and the larval or adult expression of traits such as the notochord and dorsal nerve cord that they share in common with vertebrates, forms the basis for a rich and highly productive area of empirical research and scholarship (24) that Williamson dismisses in favor of hybridogenesis:

“Urochordates, comprising tunicates and larvaceans, present a comparable case. Larvaceans are tadpoles throughout life. Garstang (6) regarded larvaceans as persistent tunicate larvae, and, if so, their genomes would resemble those of tunicates. However, if larvaceans provided the evolutionary source of marine tadpole larvae, their genomes would be smaller and included in those of adult tunicates. The genome of the larvacean *Oikopleura dioica* is approximately one-third that of the tunicate *Ciona intestinalis*, consistent with my thesis (22, 23).”

Four known genome sizes for Urochordata are publicly available in consecutive entries in the animal genome size database (12). Inexplicably, Williamson (6) selectively refers to only half of these data and claims they are consistent with his prediction, but the prediction is easily shown to be false merely by including the other two data. In contrast to the relatively large genomes of the tunicates *Ciona intestinalis* ($C = 0.20$ pg) (6) and *Ascidia atra* (0.16 pg), the genome of a third tunicate *Phallusia mammillata* is actually smaller (0.06 pg) (25) than that of the larvacean *Oikopleura dioica* (0.07 pg).

Specific Tests of Larval Transfer by Genetic Analysis of Experimental Hybrids Have Rejected Its Predictions

Williamson (6) wrote:

“Many biologists appear unwilling to consider that animals with metamorphosing larva descended from hybrids, who evolved in separate lineages before interspecific, interfamilial, or even interphyletic hybridization.”

Williamson (6) ended his article, like some of his previous publications, by appealing to the research community of evolutionary geneticists to take his hypothesis and predictions seriously by putting them to the test:

“I urge genomicists to test my larval transfer hypothesis by analysis of genomes (*i*) of animals with larvae, (*ii*) of

related animals without larvae, and (iii) of relatives of the proposed adult source of the larval form.”

His claim (not really a prediction) that his peers are unwilling to consider his hypothesis is also false. One of us made a good-faith effort to take seriously Williamson's (26) astonishing claim of experimental laboratory hybridizations between eggs of a tunicate and sperm of a sea urchin that produced offspring with sea-urchin-like larval and adult phenotypes.

Characterization of both the maternal mitochondrial genome and the biparental nuclear genome of these offspring revealed only sea urchin genes and genomes and found no evidence of tunicate genes and genomes (27). In later books and essays,

Williamson ignored the probative value of this result (e.g., ref. 28) or dismissed it as a failed “search for sea-squirt DNA” (29).

Williamson's (6, 26) hypothesis of animal life-cycle evolution via hybridogenesis has changed over time but has failed to flourish among other biologists after >20 years in the literature because it can be readily falsified by empirical tests, and because Williamson and his colleagues have not acknowledged those tests as crucial failures of the hypothesis (30). We readily acknowledge the importance of hybridization and other forms of lateral gene transfer in evolution, but there is so far no evidence of a role for these processes in the evolution and loss of animal larvae.

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