TRAIT DEVELOPMENT

Beetle horns evolved from wing serial homologs

Yonggang Hu*, David M. Linz, Armin P. Moczek*

Understanding how novel complex traits originate is a foundational challenge in evolutionary biology. We investigated the origin of prothoracic horns in scarabaeine beetles, one of the most pronounced examples of secondary sexual traits in the animal kingdom. We show that prothoracic horns derive from bilateral source tissues; that diverse wing genes are functionally required for instructing this process; and that, in the absence of Hox input, prothoracic horn primordia transform to contribute to ectopic wings. Once induced, however, the transcriptional profile of prothoracic horns diverges markedly from that of wings and other wing serial homologs. Our results substantiate the serial homology between prothoracic horns and insects wings and suggest that other insect innovations may derive similarly from wing serial homologs and the concomitant establishment of structure-specific transcriptional landscapes.

How novel complex traits originate is a fundamental yet largely unresolved question in evolutionary biology (*1*, *2*). The most commonly used definition of novelty entails the absence of homology to ancestral traits (*3*). This definition, however, is increasingly difficult to reconcile with empirical findings across diverse taxa, which emphasize the differential repurposing of conserved developmental modules outside their traditional developmental context as a dominant route to innovation (*4*). How, and to what degree, evolutionary novelties may emerge from the confines of homology thus remains largely unknown. In this work, we investigated the origin of the prothoracic horns of scarabaeine beetles, a classic example of evolutionary innovation, from homologs of tissues and associated gene networks that instruct the formation of insect wings in adjacent segments.

The origin of insect wings has fueled a century-long debate. Insect wings were postulated to have arisen either as extensions of the dorsal plate (tergum, terga) of thoracic segments or, alternatively, from ancient proximal leg segments and their associated branches—structures in existence before the origin of insects and since absorbed into the side wall (pleuron, pleura) of segments (*5*). These competing hypotheses became united in the dual origin hypothesis, which posits that bona fide wings (on the second and third thoracic segments, T2 and T3) are composite structures with contributions from both tergal and pleural sources (*6*–*15*). Work in *Tribolium* and *Tenebrio* beetles has further shown that segmentally reiterated (i.e., serially homologous) tergal and pleural source tissues can also be found as distinct and morphologically diversified structures in nonwinged segments; for example, the tergal serial homolog facilitated the formation of a bilateral, edgelike structure along the prothoracic segment (T1), known as the carinated margin, as well as pupal-specific defensive structures found in abdominal segments, known as gin traps (*7*, *8*, *16*, *17*). These findings support the notion that the presence of two distinct sets of wing serial homologs per segment reflects the ancestral condition of thoracic and abdominal segments and that at least some lineages succeeded in using wing serial homologs outside T2 and T3 to evolve structures other than wings.

To determine whether prothoracic horns of scarabaeine beetles derive from tergal wing serial homologs, we first assessed the function of wing patterning genes during prothoracic horn formation. Specifically, we assessed the wing selector gene *vestigial* (*vg*); two genes critical to the early patterning of wing formation, *apterous* (*ap*) and *nubbin* (*nub*); as well as *cubitus interruptus* (*ci*) and *dishevelled* (*dsh*), key members of the hedgehog and wingless signaling pathways, respectively. Further, we examined the function of *abrupt* (*ab*), which, at least in *Drosophila* and *Tribolium*, is similarly critical for bona fide wing formation (*18*). We executed our approach in three *Onthophagus* species (*O. sagittarius*, *O. taurus*, and *O. binodis*), which reflect much of the diversity of prothoracic horn formation found in scarabaeine beetles (fig. S2) (*19*).

In all three beetle species examined, RNA interference (RNAi)–mediated knockdown of any of the six focal genes yielded the expected reduction of bona fide wings (on T2 and T3; see figs. S1 and S2) and—with only one exception (*nub*)—also profoundly affected the formation of prothoracic horns (Fig. 1, C to L, and figs. S3 to S5). Furthermore, we observed that hypomorphic down-regulation of *vg*, *dsh*, *ci*, *ab*, or *ap* (both paralogs simultaneously, hereafter *apA+B*), resulted in the retention of paired, bilateral vestiges of the prothoracic horn (Fig. 1 and figs. S3 and S4). Notably, bilateral prothoracic horn precursors can also be observed in wild-type individuals late in larval development (Fig. 1, N to P, and figs. S6 and S7). In addition to wings and prothoracic horns, vg^{RNAi} and, to a lesser extent, dsh^{RNAi}, $apA{+}B^{RNAi}$, ab^{RNAi}, or ci^{RNAi} also affected part of the pleural and tergal structures in T1, including the carinated margin and lateral body wall plates (Fig. 1 and fig. S5), consistent with studies in other Coleoptera (*7*, *17*).

To determine whether RNAi-mediated effects on prothoracic horn development may be an indirect consequence of wing-gene function elsewhere in the thorax, we examined the expression pattern of the wing selector gene, *vg*, using in situ hybridization chain reaction on cryosectioned thoracic tissue obtained from prepupae of *O. sagittarius* (Fig. 1M), i.e., at a stage when the larval epidermis transforms to give rise to pupal (and future adult) traits. We found that *vg* is expressed throughout developing wing tissue, carinated margin, and bilateral prothoracic horn precursors (Fig. 1, N to R, and fig. S7), thus closely paralleling the physical locations of vg^{RNAi} phenotypes.

Taken together, our results suggest (i) prothoracic horns derive from tergal bilateral source tissues that fuse to form a single medial outgrowth, (ii) wing genes are functionally required for instructing this process, and (iii) based on the specific identity of these genes, prothoracic horns may constitute partial wing serial homologs. Additional support for these conclusions can be found in previously published expression and functional analyses of four genes also known to be critical for wing formation (*patched*, *pangolin*, *homothorax*, and *decapentaplegic*) (*20*–*23*). In each case, RNAi-mediated depletion reduced or eliminated the formation of both wings and prothoracic horns as well as other wing serial homologs (*20*–*23*).

However, it remains conceivable that prothoracic horns do not derive from wing serial homologs and that, instead, the nine genes functionally implicated thus far were simply independently co-opted to instruct horn development. To begin assessing these alternative explanations, we executed two sets of experiments. First, we knocked down the Hox gene *Sex combs reduced* (*Scr*) to transform the identity of T1 to that of T2, which induces the formation of ectopic T1 wings. Specifically, we reasoned that if prothoracic horns evolved via the independent co-option of components of the wing gene network, rather than through modification of serially homologous tissues, the prothoracic horn primordia should not contribute to ectopic T1 wings. Alternatively, if horns are serially homologous to wings, ectopic wing induction should be paralleled by a commensurate reduction of horns, with the most severe T1 wing induction being paralleled by the most severe reduction of horn growth (*24*).

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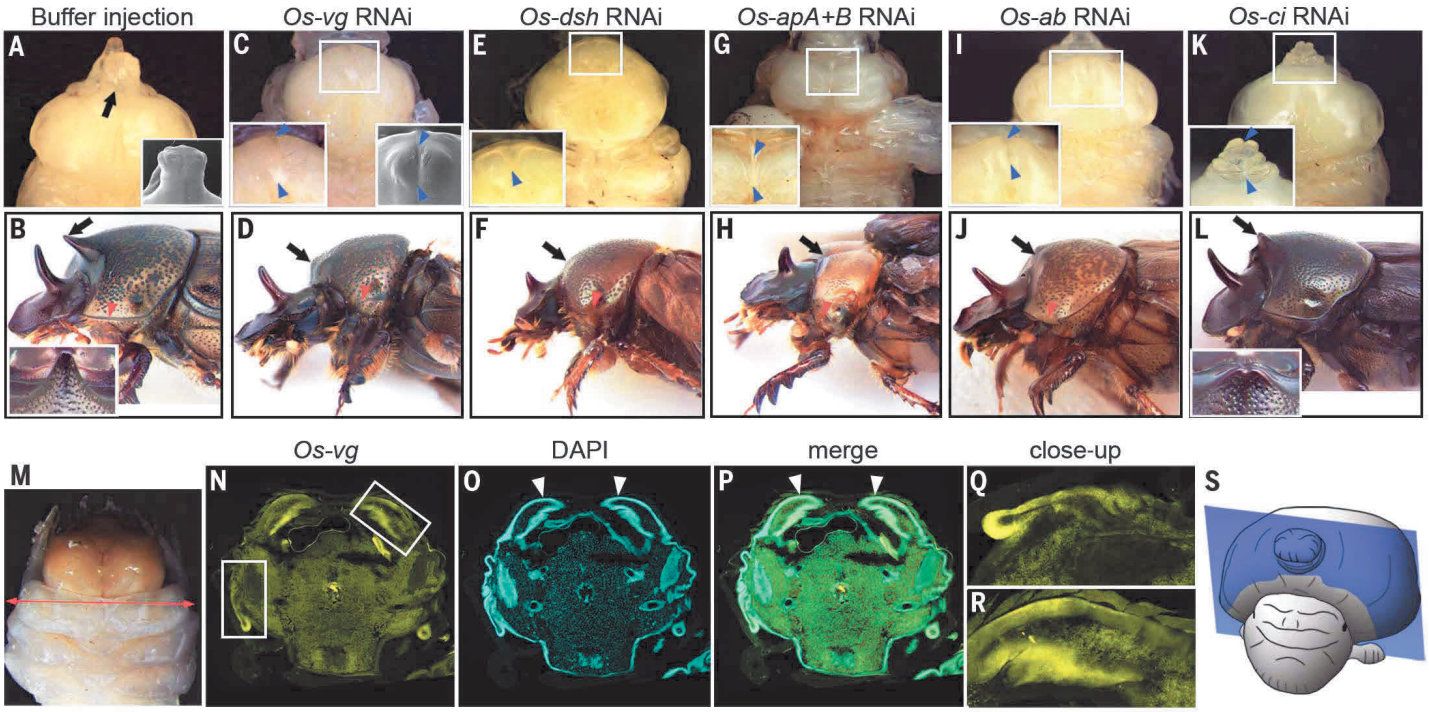

Fig. 1. Wing genes are required for prothoracic horn formation. (**A** and **B**) Buffer-injected control. The prothoracic horn [arrows in (A) and (B)] and carinated margin [red arrowhead in (B)] are indicated. Inset in (B) shows the prothoracic horn. Representative phenotypes obtained in *O. sagittarius* (*Os*) are shown in panels (C) to (L) as follows: (**C** and **D**) vg^{RNAi}; (**E** and **F**) dsh^{RNAi}; (**G** and **H**) $apA{+}B^{RNAi}$; (**I** and **J**) ab^{RNAi}; and (**K** and **L**) ci^{RNAi}. Wing gene RNAi reduces pupal prothoracic horns to paired bilateral vestiges [insets in (C), (E), (G), (I), and (K); the furrow between paired vestiges is indicated by blue arrowheads] and leads to a reduction or elimination of the adult prothoracic horns [arrows in (D), (F), (H), (J), and (L)] and defects in the carinated margin [red arrowheads in (D), (F), (H), and (J)]. Inset in (L) shows partially reduced prothoracic horn. Inset in (A) and right inset in (C) are scanning electron microscopy (SEM) images. Shown are representative phenotypes obtained in *O. sagittarius* (for *O. taurus* and *O. binodis* see fig. S3). (**M**) Prepupa illustrating the section plane in (N) through (R). (**N** to **R**) Cryosections stained with *vg* riboprobes (N) and 4′,6-diamidino-2-phenylindole (DAPI) (O) showing *vg*-positive cells in prothoracic horn primordia and carinated margin. (P) Merge of (N) and (O). Arrowheads in (O) and (P) indicate prothoracic horn primordia. (Q and R) Close-up of insets in (N) showing carinated margin and prothoracic horn primordia, respectively. (**S**) Cartoon of pupa illustrating section plane in (M) through (P).

Scr^{RNAi} resulted in the formation of ectopic T1 wings resembling elytra (Fig. 2, D to F), in line with results from studies in other taxa (*7*, *12*, *17*, *25*). In all three species, this induction was paralleled by a severe reduction of prothoracic horns to minute, and again paired, vestiges (Fig. 2D and figs. S8 and S9). Additionally, analysis of hypomorphic Scr^{RNAi} phenotypes showed the predicted inverse relationship between the degree to which prothoracic horn tissue was retained and the completeness of the ectopic wing induced (figs. S8 and S9).

However, such results may also be expected if *Scr* independently governs the T1-specific repression of wings and induction of thoracic horns. To assess this hypothesis, we investigated T1 wing induction while simultaneously ablating the prothoracic horn via down-regulating *pannier* (*pnr*), which patterns dorsomedial tissue identity in *Drosophila* (*26*). By itself, pnr^{RNAi} removes all dorsomedial projections, including the prothoracic horn, yet it does not affect the formation of wings on T2 and T3 nor the lateral carinated margin in T1 (Fig. 2, G to I). We reasoned that if prothoracic horn primordia contribute to ectopic T1 wings, ectopic wing size should be maximized in *Scr* single-knockdown individuals but diminished when Scr^{RNAi} is executed in a pnr^{RNAi} background that removes prothoracic horn primordia before they can be rerouted toward an ectopic wing fate. To test this hypothesis, we compared ectopic T1 wing formation in Scr^{RNAi} knockdown individuals with that of $pnr{+}Scr^{RNAi}$ double-knockdown individuals in *O. binodis*, which exhibits consistently strong RNAi phenotypes (fig. S13 and table S2). We further standardized our comparisons by controlling developmental timing of knockdowns (table S2) and by using the severity of Scr^{RNAi}-mediated mouthpart transformations and exposure of the coxal segment as a proxy for transformation severity (fig. S9). We found that ectopic wings formed in a pnr^{RNAi} background are markedly smaller in size and lack the recognizable dorsal surface traits observed in *Scr* single-knockdown individuals (Fig. 2, J to L, and figs. S9 and S10). These results provide strong support for the hypothesis that prothoracic horn primordia contribute to ectopic bilateral T1 wings.

Lastly, we sought to assess whether the presumed serial homology between prothoracic horns, wings, and other wing serial homologs, such as gin traps and their equivalents (*8*, *16*, *17*), is also reflected in unbiased, tissue-wide transcriptional profiles. We selected *O. taurus* for this approach, given the availability of a fully sequenced and annotated genome for this species. We also included in this analysis eight distinct epidermal regions, including T2 wings, four distinct T1 regions (fig. S12), and abdominal support structures from the third through sixth abdominal segment (PSS; partial wing serial homologs akin to gin traps or carinated margin, see fig. S11) (*8*, *16*, *17*, *27*). We included the dorsocentral prothorax and dorsal head epidermis, regions whose formation is, at present, considered completely unrelated to wing development. *vg* expression was detectable in all epidermal tissues examined except for the dorsal head and dorsocentral prothorax, consistent with the proposed partial serial homology between

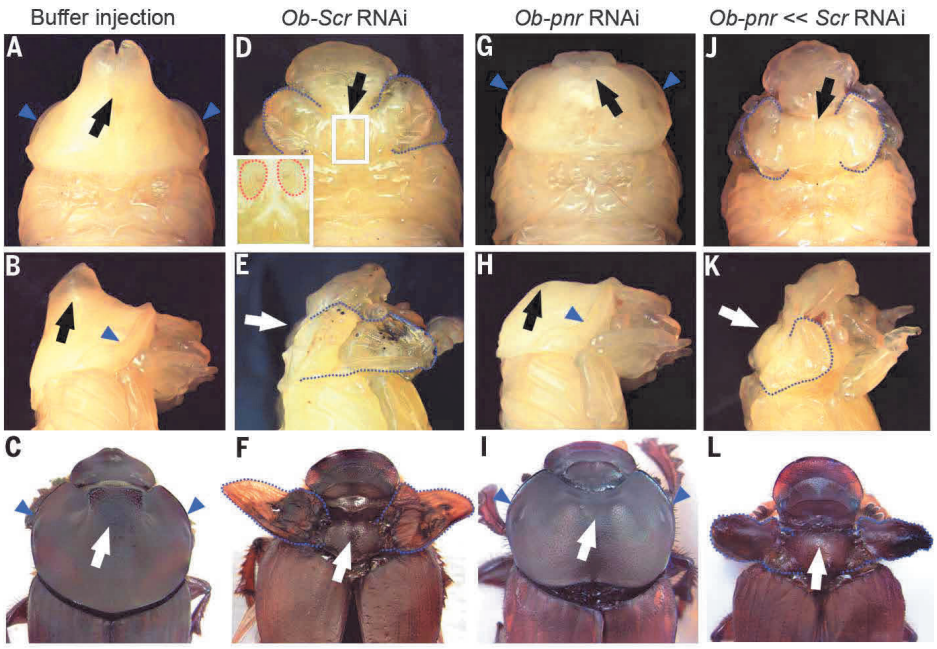

Fig. 2. The prothoracic horn contributes to ectopically induced prothoracic wings. (**A** to **C**) Buffer injected control. The prothoracic horn [arrows in (A) to (C)] and carinated margin [blue arrowheads in (A) to (C)] are indicated. (**D** to **F**) Pupal and adult phenotypes of Scr^{RNAi}. Scr^{RNAi} reduces pupal prothoracic horn [arrows in (D) to (F)] resulting in small, paired horn vestiges [inset in (D)] and also induces large ectopic prothoracic wings [blue outlines in (D) to (F)]. (**G** to **I**) pnr^{RNAi}. pnr^{RNAi} removes prothoracic horns [arrows in (G) to (I)] without affecting the carinated margin [blue arrowheads in (G) to (I)]. (**J** to **L**) pnr^{RNAi} followed by Scr^{RNAi}. Sequential knockdown of *pnr* followed by *Scr* removes prothoracic horns [arrows in (J) to (L)] and results in only partial induction of ectopic prothoracic wings [blue outlines in (J) to (L)]. Across all images, the prothoracic horn and carinated margin are indicated by arrows and blue arrowheads, respectively. The ectopic wings are outlined with dotted lines.

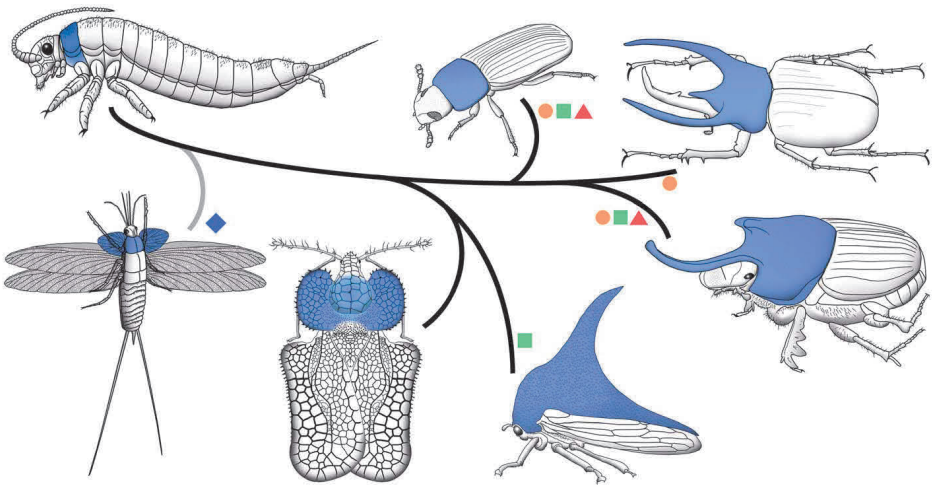

Fig. 3. Diversification of prothoracic morphology among various extant and extinct insect lineages. Shapes indicate lineages where paleontological evidence (blue diamond) (*11*), expression data (green squares) (*7*, *29*), functional data (orange circles) (*7*, *28*), or transformation (via Hox manipulation) data (red triangles) (*7*) are available to support the hypothesis of wing serial homology. Insect lineages shown are (clockwise from top left) a hypothetical apterygote insect ancestor, Tenebrionidae, Dynastinae, Scarabaeinae, Membracidae, Tingidae, and Palaeodictyoptera.

prothoracic horns, PSS, carinated margin, and wings (fig. S12C). Expanding this approach to include 41 genes known to be functionally required for some aspect of wing formation, clusters T2 wings together with the carinated margin and pupal support structures, as expected. However, this aproach reveals significant transcriptional divergence of prothoracic horn tissue from other wing serial homologs (fig. S12D). Lastly, transcriptome-wide clustering of all 4191 differentially expressed transcripts shows no distinct clustering patterns corresponding to wing relatedness among any of the tissues examined. Combining the results of our functional analysis detailed above with these unbiased RNA sequencing (RNA-seq) findings raises the possibility that prothoracic horn formation may indeed be instructed by a core gene network, serially homologous to that also used in bona fide wings. However, on the level of gene networks, such serial homology may be restricted to core regulators and the early stages of tissue specification.

Our results help advance the current debate surrounding the origin of morphological novelty in insects and in animal evolution more broadly. First, our findings may help explain why the prothorax of insects has emerged as a hotspot of evolutionary innovation. Morphological elaborations have evolved independently in diverse insect orders, including the wing-like elaborations of extinct Paleodictyoptera, the lateral, leaflike outgrowths of lace bugs (Tingidae, Hemiptera), the helmets of treehoppers (Membracidae, Hemiptera), or the posteriorly projecting outgrowths of tetrigid grasshoppers (Tetrigidae, Orthoptera). Our analyses of gene function, gene expression, and Hox gene–mediated transformation support the hypothesis that scarabaeine prothoracic horns evolved from bilateral, partial wing serial homologs, whereas our transcriptomic profiling documents the simultaneous establishment of structure-specific transcriptional landscapes. Taken together, our findings raise the possibility that other insect innovations may have similarly evolved by using wing serial homologs as developmental-genetic starting points around which transcriptional repertoires became established, and through these diversified networks are now able to support differentiation events specific to each trait. Paleontological data, gene expression, and functional analyses across diverse taxa are beginning to accumulate support for such a scenario (Fig. 3) (*7*, *11*, *28*, *29*).

More generally, our results contribute to a growing call for reexamining the usefulness of defining morphological novelty through the absence of homology. According to this view

novelty begins where homology ends, yet exactly where homologous relationships cease has become increasingly difficult to delineate, as findings in evolutionary developmental biology have forced a revision of homology away from a binary designation and toward a more layered understanding of homologous relationships, resulting in the emergence of concepts such as "deep" or "partial homology" (*4*). In contrast, others have advocated disconnecting novelty and homology entirely (*30*). According to this perspective, as evolutionary biology is fundamentally positioned within a framework of descent with modification, everything new must ultimately emerge from the old, and it may therefore be most productive to follow a dichotomy originally proposed by Wilkins (*31*) that divides evolutionary novelties into operational types—those whose evolutionary and developmental origins we can trace and those whose precursors and ancestral affinities we have yet to discover. Here we show that a textbook example of evolutionary novelty, the prothoracic horns of beetles, derives partly from wing serial homologs, whose existence predates the origin of insects. On one side, this may cause us to question whether prothoracic horns should still be considered an evolutionary novelty. Alternatively, our results may serve to illustrate how substantial morphological innovation, rather than somehow emerging in the absence of homology, may instead be initiated through it.

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SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/366/6468/1004/suppl/DC1
Materials and Methods
Figs. S1 to S13
Tables S1 to S3
References (*32*–*43*)

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Where do horns come from?

One of the most pronounced examples of a sexually selected trait is the prothoracic horns of scarab beetles, which, in the most extreme cases, can be nearly half as long as the length of the beetle. It is fairly easy to understand how selection might have shaped these horns, but understanding how development shaped them from a hornless ancestor is a much more complex proposition. Hu *et al.* show that these horns are generated from wing homologs and argue that many other insect traits may have followed similar transcriptional paths (see the Perspective by Nijhout).

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