

# Body plan innovation in treehoppers through the evolution of an extra wing-like appendage

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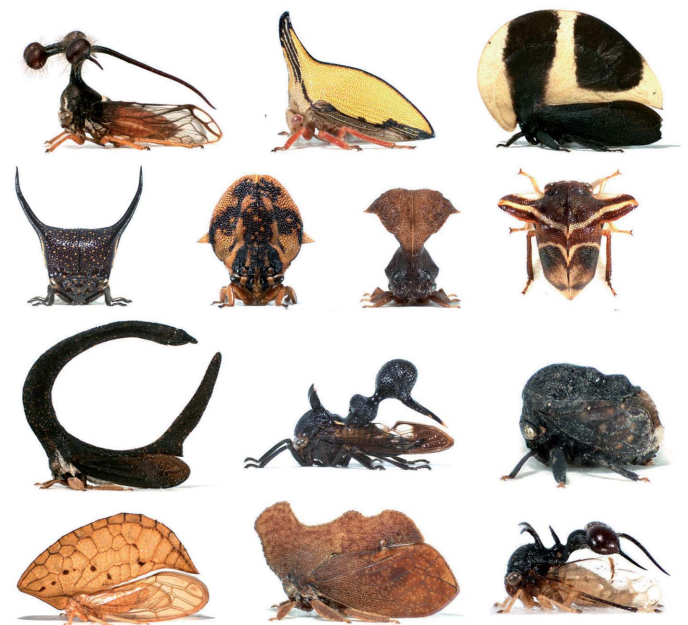
**Body plans, which characterize the anatomical organization of animal groups of high taxonomic rank<sup>1</sup>, often evolve by the reduction or loss of appendages (limbs in vertebrates and legs and wings in insects, for example). In contrast, the addition of new features is extremely rare and is thought to be heavily constrained, although the nature of the constraints remains elusive<sup>2–4</sup>. Here we show that the treehopper (Membracidae) ‘helmet’ is actually an appendage, a wing serial homologue on the first thoracic segment. This innovation in the insect body plan is an unprecedented situation in 250 Myr of insect evolution. We provide evidence suggesting that the helmet arose by escaping the ancestral repression of wing formation imparted by a member of the *Hox* gene family, which sculpts the number and pattern of appendages along the body axis<sup>5–8</sup>. Moreover, we propose that the exceptional morphological diversification of the helmet was possible because, in contrast to the wings, it escaped the stringent functional requirements imposed by flight. This example illustrates how complex morphological structures can arise by the expression of ancestral developmental potentials and fuel the morphological diversification of an evolutionary lineage.**

Treehoppers, a small group of hemipteran insects related to cicadas<sup>9</sup>, have evolved a peculiar morphological structure known as the helmet. It expands dorsally over most of the body length and has diversified to extremes within the family, conveying most of the treehoppers' shape diversity (Fig. 1). The various forms, colours and textures of the helmet may mimic natural elements ranging from thorns or seeds to animal droppings or aggressive ants<sup>10,11</sup>. Without their helmets, treehoppers are very similar to cicadas (Supplementary Fig. 1b). The helmet is exclusively shared by all treehopper species, indicating that it appeared very early in the treehoppers' evolutionary lineage (Supplementary Fig. 1a). This evolutionary pattern prompted us to investigate how the helmet evolved.

The anatomical nature and evolutionary origin of the helmet remain controversial. Although most studies consider the helmet to be merely an expansion of the pronotum, that is, an enlarged dorsal face (tergite) of the first thoracic segment<sup>12</sup> (T1), it has been suggested<sup>13</sup> that it could be a T1 appendage, a statement rejected by later workers<sup>12,14</sup>. The key feature to discriminate between a simple outgrowth and an actual appendage is the presence of a jointed articulation, making the structure movable relative to the rest of the body. We found that the helmet has some elastic mobility, for instance in *Publilia modesta*, one of the treehopper species we examined in this study (Supplementary Movie 1), suggesting that it is connected to the body through flexible attachments. Indeed, histological sections revealed that the helmet is bilaterally attached to the segment by a complex articulation (Fig. 2d–g). The attachment points consist of thin, non-sclerotized (that is, flexible) cuticle flanked by thicker, sclerotized cuticle (Fig. 2f). This configuration of flexible and hard cuticle (Fig. 2f, g, insets) defines cuticular joints that connect appendages to the body<sup>15</sup>, and is typically found at the attachment points of T2 and T3 wings (Fig. 2g). Because the helmet is

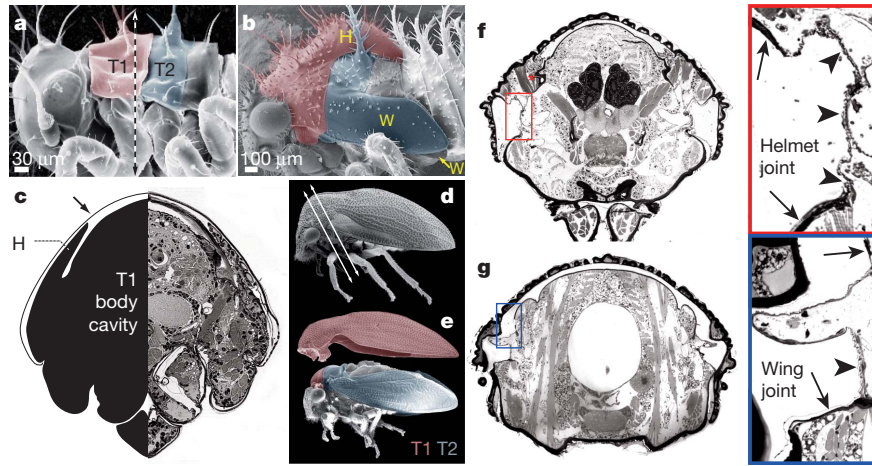
attached to T1 by jointed articulations, it follows that it is a T1 dorsal appendage, a situation completely unexpected in extant insects. The treehoppers' helmet is therefore distinct from the thoracic expansions that evolved in other insect lineages, for instance in horn beetles<sup>16</sup> or in various other hemipterans (Supplementary Fig. 2a–c), which are cuticular projections and not articulated appendages. The conclusion that the helmet is a bona fide appendage does not exclude the hypothesis that, from an evolutionary perspective, the helmet initially arose from cuticular expansions. In this gradualist picture, the prothoracic outgrowths observed in some hemipterans might represent evolutionary forerunners of the treehoppers' helmet.

The presence of an extra dorsal appendage in treehoppers represented a rare opportunity to address how this type of body plan innovation emerged: either *de novo* or through the redeployment of an existing developmental program. Unlike most appendages, which are obviously paired, the helmet appears externally as a single structure both in adult and nymphal stages (Fig. 2b, e). To trace the developmental origin of the helmet, we sectioned first-instar nymphs and found that the helmet originates from two bilateral primordia, which later fuse along the dorsal midline (Fig. 2a, c and Supplementary Figs 3 and 4). The helmet is therefore a T1 dorsal appendage with a bilateral origin. Because the only known dorsal thoracic appendages in insects are wings (on T2 and T3), we explored the possibility that the helmet is



**Figure 1 | Morphological diversity in treehoppers is conveyed by the helmet.** Representative sample of neotropical treehopper (Membracidae) species (see Supplementary Table 1 for species names).

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**Figure 2 | The helmet is a T1 dorsal appendage with a bilateral origin.**

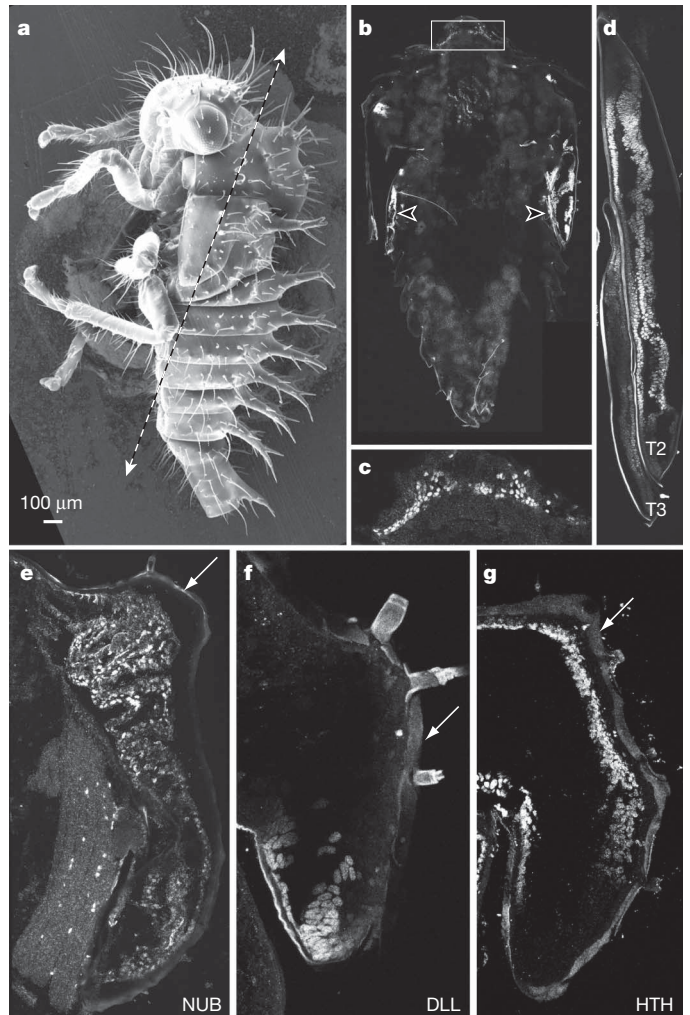
**a, b,** Scanning electronic microscopy (SEM) images of nymphal stages 1 and 5 of *Publilia* sp., showing wings (W) on T2 (in blue) and the helmet (H) on T1 (in red). **c,** Sectioned (dotted arrowed line in **a**) first nymphal stage (right) and schematic (left); the external cuticle covers the helmet primordia (arrow).

**d, e,** SEM images of intact (**d**) and dissected (**e**) *P. modesta* adults. **f, g,** Thick sections through a *P. modesta* adult thorax showing the helmet's articulation and the cuticular joints (boxes and insets) of helmet and wings (arrowheads point to thin, flexible cuticle, and arrows to thick cuticle). Muscles connect the helmet to the body (asterisk in **f**).

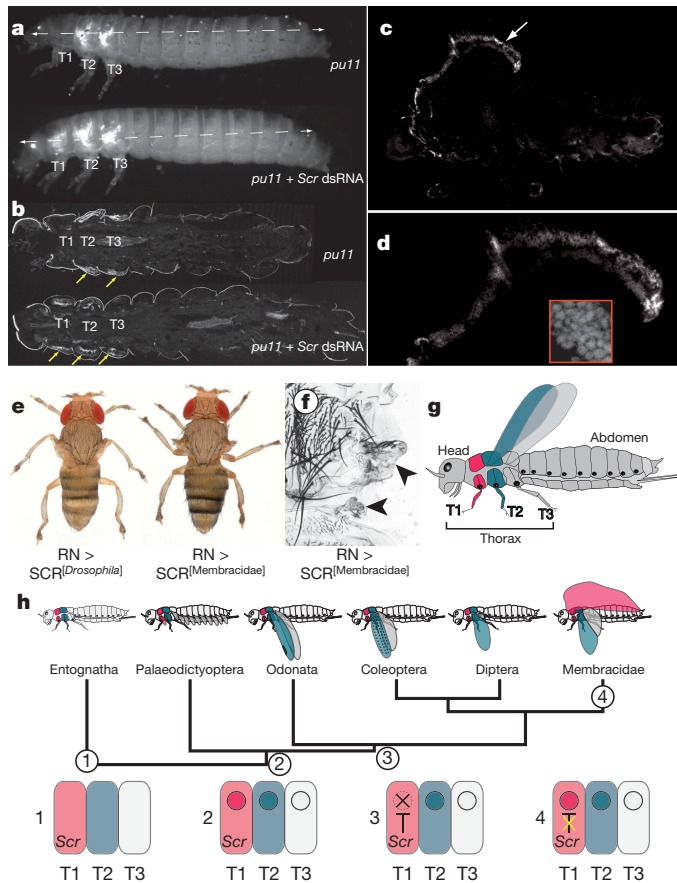
a fused pair of wing serial homologues. Consistent with this notion, the wings and the helmet share several distinct morphological features: the helmet hinge consists of flexible, non-sclerotized cuticle (Fig. 2d, f) embedding small cuticular plates reminiscent of the pteralia that characterize the wing hinge region<sup>17</sup> (Supplementary Fig. 5); both appendages consist of two layers of epithelial cells interconnected by large cuticular columns<sup>12</sup>; these layers unfold similarly on emergence, as any insect wing does (Supplementary Movie 2 and Supplementary Fig. 6); and a complex vein network covers both structures<sup>13</sup> (Supplementary Fig. 1c). All together, these anatomical observations suggest that the helmet is a fused pair of wing serial homologues.

If the wings and the helmet are serial homologues, then their development must rely on a shared genetic program. We therefore searched for shared molecular signatures of wing and helmet development. A scant handful of transcription factors, including Nubbin<sup>18</sup>, mark wing developmental fate and allow for discrimination between wing and other appendage precursors<sup>19</sup>. We monitored the spatial deployment of Nubbin using a cross-reactive antibody<sup>19</sup> and detected Nubbin expression during nymphal stages in the developing wings, as expected given its evolutionary conservation<sup>19</sup> (Fig. 3a–d). Remarkably, Nubbin is found in the developing helmet also and its expression parallels that of the wings (Fig. 3b, c, e). Two other genes involved in the proximo-distal axis specification of appendages, *Distal-less* (*Dll*) and *homothorax* (*hth*), are also expressed in the developing helmet, and their distribution determines the helmet proximo-distal axis, from the hinge region to its posterior tip (Fig. 3f, g). These results suggest that the helmet and the wings share the same genetic program for their development, supporting the proposition that the treehopper's helmet is a T1 wing serial homologue.

The finding that treehoppers have evolved a T1 dorsal appendage is surprising in that all other extant winged insects have dorsal appendages restricted to T2 and T3 (ref. 20). This prompted the question of how the insect body plan has been modified in treehoppers. The fossil record indicates that the insect body plan progressively evolved some 350 Myr ago from one in which all segments bore wings or wing-like appendages to one in which the wings are confined to T2 and T3 (ref. 21). This transition was sculpted by *Hox* genes<sup>5</sup>, which evolved the ability to repress wing formation in the abdominal segments and T1. *Hox* gene repression of wing formation has been maintained for 250 Myr of insect evolution. In particular, *Sex combs reduced* (*Scr*) represses wing formation on T1 (Fig. 4e, left) through the repression of wing-growth and -patterning genes<sup>5,22–24</sup>. For instance, when *Scr* is knocked down in *Tribolium*<sup>25</sup> (Coleoptera), ectopic wing primordia that express Nubbin



**Figure 3 | Wing-patterning genes are expressed in the developing helmet.** **a,** *P. modesta* nymph (stage 4) showing the section plane of **b**. **b–e,** *P. modesta* stage-4 (**b, c**) and stage-5 (**d, e**) nymphs stained with an anti-NUB antibody. Sections reveal wing (**b**, arrowheads; **d**) and helmet (**c, e**) expressions. **f, g,** Sagittal sections stained with anti-DLL (**f**) and anti-HTH (**g**) antibodies; the bright outline surrounding the specimen is the auto-fluorescent cuticle (arrows in **e–g**). Specimens in **e–g** are at different nymphal stages.



**Figure 4 | *Scr* and the evolution of T1 appendages.** **a, b**, Fifth-instar *Tribolium*  $Cx^{dpt}$  (ref. 22) larvae express the wing marker *pu11*-GFP (**a**, top panel), and Nubbin (arrows in **b**, top panel) in T2 and T3. When *Scr* is downregulated, ectopic wing primordia expressing Nubbin form on T1 (**a, b**, lower panels). **c, d**, Anti-SCR antibody staining on a *P. modesta* nymph (stage 5) sagittal section showing expression in T1, including the helmet (arrow in **c**; inset in **d** shows the nuclear distribution of the protein). **e, f**, Overexpression of *Drosophila* (**e**, left) and treehopper (**e**, right; **f**) *Scr* in fly imaginal discs abolishes wing and haltere formation (arrowheads in **f**). **g**, Generic body plan of a winged insect. **h**, Evolution of the regulatory link between *Scr* and the dorsal appendage development programme, from no link ancestrally (1, 2), to a repression (3) and the secondary loss in treehoppers (4).

form on T1 (Fig. 4a, b). This result shows that *Scr* prevents T1 wing formation through the repression of, at least, *nubbin* expression.

The expression of Nubbin in the developing treehoppers' helmet led us to propose that this structure evolved because *Scr* no longer exerted its ancestral repressive effect on wing formation, and we devised several possibilities that would account for this situation. First, *Scr* expression might be excluded from the helmet. We found, however, that *Scr* is expressed in the entire developing helmet (Fig. 4c, d), which is a priori incompatible with the T1-appendage-repressive function of *Scr* that is required until eclosion<sup>23,25</sup>. Next we considered that in treehoppers *Scr* might have lost the ability to repress dorsal appendage development. We tested this possibility by ectopically expressing treehopper *Scr* in *Drosophila*. Ectopic expression of *Drosophila Scr*<sup>5</sup> in fly wing and haltere precursors blocks their development (Fig. 4e, left). Similar ectopic expression of treehopper *Scr* results in identical phenotypes (Fig. 4e, right, and Fig. 4f). This result suggests that in treehoppers *Scr* is still capable of repressing T1 dorsal appendage development. All together, these results indicate that the evolution of the helmet is not due to a change in *Scr* expression or function, but rather to some genetic changes that occurred downstream of *Scr*. We propose that in treehoppers the wing developmental program, which involves *nubbin*, has

become unresponsive to *Scr* repression, possibly through selective regulatory changes downstream of *Scr* (Fig. 4g, h).

The distribution of wings along the body axis in insects seems particularly stable, as the only modifications in 250 Myr of evolution have been occasional losses or reductions<sup>20</sup>. This body plan stability could be attributed to intrinsic developmental constraints that would prevent the evolution of extra appendages<sup>4,26</sup>. Alternatively, it is conceivable that insects with extra sets of appendages do appear but are immediately counterselected. Identifying which type of constraint—developmental versus selective—limits the evolution of body plan has been a long-standing question<sup>3</sup> that is difficult to address experimentally. Our results show that treehoppers have evolved a T1 dorsal appendage, thereby departing from the typical winged-insect body plan, by expressing a developmental potential that had been maintained under the repression of a *Hox* gene for 250 Myr. This argues that the constraint preventing extra dorsal appendage formation in insects is not developmental but rather selective. We submit that morphological innovations can arise from the deployment of existing but silenced developmental potentials, therefore requiring not so much the evolution of new genetic material but instead the expression of these potentials.

The breadth of morphological diversity in helmets that has evolved in less than 40 Myr (and C. Dietrich, personal communication) is unusual for an appendage. The pace of appendage evolution is generally slow, probably because of the strong selective pressure associated with their role in locomotion. This is particularly true for the wings<sup>28</sup>, and we speculate that, initially alleviated from functional requirements, the recently evolved helmet was free to explore the morphological space through changes in its developmental program. A reminiscent pattern of appendage diversification on relaxed selection is observed for beetle elytra, which diverted from their primary flight function and have evolved all sorts of cuticular expansions, sculptures and glands<sup>20</sup> (Supplementary Fig. 7). More generally, these examples illustrate how a structure or an organ relieved from its original function (for instance by duplication or disuse), is “left to the free play of the various laws of growth”<sup>29</sup> and provides a new substrate for morphological diversification.

## METHODS SUMMARY

**Specimen collection.** We collected *P. modesta* specimens in Wisconsin (USA). **Cloning and *Drosophila* genetics.** UAS-*Scr* (*Drosophila* and treehopper) constructs were generated with standard cloning techniques and inserted at the same genomic position, preventing differences in transgenes activity due to position effects. *SCR* and *Nubbin* coding sequence alignments are shown in Supplementary Figs 8 and 9, respectively. *P. modesta Scr* and *nubbin* GenBank accession numbers are JF342360 and JF342361, respectively. **Immunocytochemistry.** We used the following antibodies: anti-*SCR* (a gift from D. Andrews), anti-Nubbin (a gift from M. Averof), anti-DLL (a gift from S. Carroll) and anti-HTH (a gift from A. Salzberg). For full details, see Supplementary Methods.

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Author Contributions** B.P. and N.G. conceived the project and designed the experiments; B.P., H.D.D., N.G. and V.A.K. collected *Publilia* specimens; B.P., C.M., J.D.C. and V.A.K. performed cloning; C.M., J.D.C., M.H. and N.G. did the immunostaining; and J.D.C., B.P. and N.G. carried out fly transgenesis and genetic experiments. A.A. made the histological sections, which were analysed by B.P. and N.G. N.G. and M.H. shot images and movies. N.G. made the anatomical dissections and observations. All authors participated in data analysis. B.P. and N.G. wrote the manuscript.

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