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Author for correspondence:
Yonggang Hu
e-mail: yohu@iu.edu

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Wing serial homologues and the diversification of insect outgrowths: insights from the pupae of scarab beetles

Yonggang Hu and Armin P. Moczek

Department of Biology, Indiana University, Bloomington, IN 47405, USA

YH, 0000-0002-3438-7296; APM, 0000-0002-3478-9949

Modification of serially homologous structures is a common avenue towards functional innovation in developmental evolution, yet ancestral affinities among serial homologues may be obscured as structure-specific modifications accumulate over time. We sought to assess the degree of homology to wings of three types of body wall projections commonly observed in scarab beetles: (i) the dorsomedial support structures found on the second and third thoracic segments of pupae, (ii) the abdominal support structures found bilaterally in most abdominal segments of pupae, and (iii) the prothoracic horns which depending on species and sex may be restricted to pupae or also found in adults. We functionally investigated 14 genes within, as well as two genes outside, the canonical wing gene regulatory network to compare and contrast their role in the formation of each of the three presumed wing serial homologues. We found 11 of 14 wing genes to be functionally required for the proper formation of lateral and dorsal support structures, respectively, and nine for the formation of prothoracic horns. At the same time, we document multiple instances of divergence in gene function across our focal structures. Collectively, our results support the hypothesis that dorsal and lateral support structures as well as prothoracic horns share a developmental origin with insect wings. Our findings suggest that the morphological and underlying gene regulatory diversification of wing serial homologues across species, life stages and segments has contributed significantly to the extraordinary diversity of arthropod appendages and outgrowths.

1. Introduction

The reuse and subsequent individuation of serially homologous structures is a common avenue towards functional innovation in developmental evolution [1]. A classic example of this process is seen in the ventrally located, paired, jointed and segmentally reiterated appendages of arthropods. During arthropod evolution, ancestrally similar appendages diversified into antennae, diverse mouthparts, locomotory appendages and genitalia [2]. Yet despite this tremendous diversification, the serial homology of all appendages remains recognizable in their morphology, and is further confirmed by their shared developmental–genetic underpinnings [3,4].

By contrast, insect wings have traditionally been considered a morphological category of their own. As one of the most enigmatic evolutionary innovations, wings enabled insects to take to the sky and radiate into previously inaccessible niches. Insects typically possess two pairs of wings located on the second and third thoracic segments (T2 and T3), connected to the body wall via a complex hinge mechanism (fossil Palaeodictyoptera also possessed superficially wing-like structures on other segments; however, these winglets lacked hinges and are not considered true wings [5]; but see [6]). Thus, *bona fide* wings are restricted to T2 and T3, and their ancestral affinities to other structures did not reveal themselves until more recent work succeeded in reconciling two competing hypothesis on the evolutionary origin of wings.

Rather than deriving solely from the dorsal plate (as per the *notal extension hypothesis*) or alternatively ancestrally proximal leg branches subsequently incorporated into pleural sidewalls (as per the *exite* or *pleural extension hypothesis*), the *dual origin hypothesis* posits that both notal and pleural source tissue contribute to the formation of wings [7–15]. Because homologous tissues exist in each segment, subsequent work began to garner support for the hypothesis that homologous notal and/or pleural source tissues may have facilitated the development of other structures outside wing-bearing segments, such as the gills of mayfly nymphs [7,16], the carinated margin and pleural plate in the prothorax of *Tribolium* [8], the helmets of treehoppers [17,18] and the prothoracic horns of scarab beetles [19]. In each case, key members of the gene regulatory network (GRN) critical for the formation of *bona fide* wings are also expressed or functionally required during the formation of structures that do not resemble wings morphologically, yet appear to share a developmental origin, and thus need to be considered at least as partial wing serial homologues. Here, we seek to build and significantly expand on the role of wing serial homologues in insect diversification.

Insects possess diverse outgrowths along their body axis that lack joints, and may harbour these structures at single or multiple life stages. For example, like many beetles in the family Tenebrionidae, the red flour beetle *Tribolium* possesses gin traps, one per dorsolateral side of the first to seventh abdominal segments (A1–A7) [20,21]. Gin traps are found only in the pupal stage, consist of bifurcated, denticular outgrowths with strongly sclerotized and toothed flanges on the anterior and posterior sides, and are used as defensive structures [20,22]. Recent functional genetic analyses showed that most of the more upstream, but not downstream genes, within the hierarchy of the wing GRN are required for the correct formation of gin traps [23], and established that *Tribolium* and *Tenebrio* gin traps are partial wing serial homologues [14,24]. Partly similar pupal defensive structures exist in other insect orders (e.g. pupal gin traps in hawkmoths (Sphingidae)) as does a diverse array of projections, spikes or blade-like structures that are frequently segmentally reiterated in nymphs, larvae or pupae, though commonly lack a morphological counterpart in the adult [5]. Here, we investigate three types of non-jointed outgrowths common in the pupal stage of scarabaeine beetles such as *Onthophagus*.

Specifically, we focused on: (i) dorsomedial support structures found on T2 and T3, (ii) abdominal support structures found bilaterally on abdominal segments 3–6 (A3–A6), and (iii) the prothoracic horns which depending on species and sex may, too, be restricted to pupae or alternatively found also in adults (figure 1*a*). Dorsal and lateral support structures consist of relatively small, slender, non-jointed and sometimes pointy and curved pupal body wall projections, whereas pupal thoracic horns are typically much larger, wider and tapering projections of the anterior prothorax. While the adaptive significance of prothoracic horns has been explored in depth in the context of larval to pupal ecdysis [25,26] and sexual selection (e.g. [27]), the functional significance of pupal support structures, if any, is less clear. Both thoracic dorsal and abdominal lateral support structures are phylogenetically ubiquitous within the subfamily Scarabaeinae [28–30] and have been proposed to facilitate the correct positioning of pupae in typically underground pupation chambers and proper distancing from surrounding substrate to minimize microbial infections; however, experimental assessments of these hypotheses remain to be conducted.

In this study, we sought to determine the degree of homology of lateral and dorsal support structures to wings, and to further extend our understanding of the proposed serial homology between thoracic horns and wings originally proposed in Hu *et al*. [19]. To do so, we functionally assessed 14 genes within, as well as two genes outside, the canonical wing GRN to compare and contrast gene functions in the formation of each of these three structures in the dung beetle *Onthophagus taurus* (Scarbaeidae). We discuss our results in the context of morphological innovation through serial homologues and the role of gene network conservation and lability therein.

2. Material and methods

(a) Experimental beetles and maintenance

Adult *O. taurus* were collected, courtesy of John Allen, from Paterson Farm near Ravenswood, Western Australia, in 2018. Beetles were maintained as laboratory colonies at 24°C in sand/soil mixture under a 16/8 h (light/dark) cycle. Beetle feeding and larval preparation for injection were described previously [31,32].

(b) Identification, cloning and sequencing of *Onthophagus taurus* genes

The *O. taurus* orthologues of candidate genes were identified via reciprocal BLAST with amino acid sequences of *Tribolium* [33] and *Drosophila* [34] to *O. taurus* database (https://i5k.nal.usda.gov/). Genes were cloned and confirmed by sequencing as previously described [35]. The sequences of DNA fragments are listed in electronic supplementary material, table S2. See electronic supplementary material, methods for details.

(c) dsRNA design, synthesis and injection

For each of the designed dsRNA, we executed a bioinformatic search of the whole genome of *O. taurus* using the BlastN algorithm to ensure that identical sequences in loci other than the targeted genes are less than 21mers. dsRNA was generated as described [36]. Injection was carried out within 6 days after larvae moulted to the last larval instar (i.e. the third larval instar, L3) as described before [37]. Phenotypic penetrance was calculated as: number of pupae showing phenotype/total number of pupated individuals × 100%. See electronic supplementary material, methods for details.

(d) Quantitative PCR

Quantitative PCR (qPCR) was used to examine RNAi efficiency of *casp*, and to quantify the relative expression level of *casp* in whole pupae, pupal lateral support structures and, as a negative control, the pupal ventral abdomen which is thought to be developmentally unrelated to true wings and their serial homologues. The qPCR was conducted on a LightCycler 480 detection system (Roche). The $2^{-\Delta\Delta Ct}$ (Ct represents the cycle threshold) method was used to measure the relative expression level [38]. Statistical comparison between samples were derived from three biological replicates. Each biological sample was assayed in triplicate. See electronic supplementary material, methods for details.

(e) Image processing

Images of phenotypes were taken through a stereoscope (Leica MZ-16, USA) equipped with a digital camera (Scion, USA) and Image J (NIH). The brightness and contrast of images were

adjusted across the entire image and cartoon illustrations were designed with Adobe Photoshop CC 2017 (Adobe).

3. Results and discussion

We sought to assess the serial homology to wings of three types of body wall projections commonly observed in scarab beetles: dorsal support structures, lateral support structures and prothoracic horns (figure 1*a*). To do so, we assessed the functional significance of essential components of the wing GRN in the formation of these presumed wing serial homologues. We structured our investigation hierarchically, beginning with the wing selector gene *vestigial* (*vg*), followed by several wing patterning genes involved in axis initiation and differentiation along the anterior/posterior (A/P), dorsal/ventral (D/V) and finally the proximal/distal (P/D) axes. We concluded our investigation with the functional assessment of two genes outside the canonical wing GRN to explore the potential significance of (i) genes secondarily co-opted into the GRN underlying the development of wing serial homologues and (ii) regional specification genes in the diversification of wing serial homologues.

(a) Phenotypic validation

In total, we assessed the function of 16 genes, including 14 genes within as well as two genes (*caspar* (*casp*) and *pannier* (*pnr*)) outside the canonical wing GRN. Previous work has provided detailed descriptions of the *Drosophila* null mutant or knockdown phenotypes for each wing GRN member as well as for *pnr* [39–54]. Corresponding descriptions for most wing genes are also available from *Tribolium* [23,55,56]. In *O. taurus*, RNAi-mediated downregulation of each of the canonical wing genes led to defects in wing formation (electronic supplementary material, figure S1) matching the corresponding wing defects described in both *Drosophila* and *Tribolium*. Likewise, pnr^{RNAi} yielded phenotypes similar to those described for *Drosophila* [51]. Taken together, these observations validate the efficacy of RNAi targeting wing GRN members and *pnr* in *O. taurus*. However, because no externally visible phenotype of *casp* null mutations has been reported in *Drosophila* so far, we used a qPCR approach to estimate the change of *casp* expression level after $casp^{RNAi}$ in *O. taurus*. We found *casp* expression decreased by at least 75% depending on tissue (electronic supplementary material, figure S2, Student's *t*-test, $p < 0.01$), indicating an efficient knockdown of *casp*. In addition, a bioinformatic search of the *O. taurus* genome found that the maximum of identical sequences for each dsRNA in loci other than the targeted genes are consistently less than 21mers, suggesting that off-target effects are an unlikely explanation for any of the phenotypes we observed.

(b) The wing selector gene *vg* is universally required for the development of wing serial homologues

To determine the involvement of the wing GRN in the development of lateral and dorsal support structures, we first investigated the possible function of the wing selector gene *vg*, which is widely used as a wing marker gene across insect species [8,12,14,19,23,24,57,58]. We found that RNAi targeting *vg* induced severe defects in both lateral and dorsal support structures (figure 1*b*), in addition to the large reduction or entire elimination of prothoracic horns and wings reported previously [19]. These findings support the hypothesis that wings and prothoracic horns may share a developmental origin with dorsal and lateral support structures.

(c) Anterior/posterior patterning of wing serial homologues

We first selected *engrailed* (*en*), *smoothened* (*smo*), *cubitus interruptus* (*ci*) as well as *decapentaplegic* (*dpp*) and its target genes *optomotor blind* (*omb*) and *spalt* (*sal*), all of which are known to play critical roles in the initiation and differentiation of the wing A/P axis. Specifically, in the *Drosophila* wing imaginal disc, posterior compartment cells express the selector gene *en* [41,59], while the anterior cells are marked by *ci* [48]. The short-range signalling molecule Hedgehog (Hh) is secreted from posterior cells, which upon binding to its receptor Patched (Ptc) activates the intracellular transducer Smo and in turn induces expression of *dpp* in a thin stripe of anterior cells adjacent to the posterior compartment [40,43,45]. Dpp in contrast functions as a long-range morphogen along the A/P boundary, activating or repressing the transcription of its target genes such as *omb* and *sal* in a concentration-dependent manner [49,60,61]. Lastly, *abrupt* (*ab*) acts downstream of *omb* to initiate development of the fifth longitudinal wing vein in *Drosophila* [53], in addition to an essential and conserved role in early wing development in both *Drosophila* and *Tribolium* [56].

Here, *O. taurus en* and its paralogue *invected* (*inv*) were knocked down simultaneously by RNAi to avoid functional compensation. $en + inv^{RNAi}$ did not cause detectable changes in the sizes of lateral and dorsal support structures, or prothoracic horns (figure 1*c*; electronic supplementary material, figure S3c). However, we did observe a consistent change in the orientation of dorsal support structures, resulting in the distal area to be more curved (figure 1*c*). By contrast, ci^{RNAi} led to the marked reduction of lateral support structures and prothoracic horns, but not dorsal support structures (figure 1*d*), suggesting that lateral support structures may be located in the anterior compartment of their corresponding segments (i.e. the compartment anterior to the region expressing *en*). Intriguingly, smo^{RNAi} resulted in two *opposite* phenotypes: prothoracic horns, as previously reported, were elongated [62], whereas both lateral and dorsal support structures were eliminated almost entirely (figure 1*e*).

Next, to evaluate the involvement of Dpp signalling, we selected multiple components of this pathway, including *dpp* itself as well as its targets *omb* and *sal*. We found that RNAi targeting *dpp*, *omb* or *sal* resulted in distinct sets of phenotypes. Specifically, dpp^{RNAi} greatly reduced the sizes of prothoracic horns and dorsal support structures, though less so for lateral support structures (figure 1*f*; electronic supplementary material, figure S3g), whereas omb^{RNAi} eliminated nearly the entire dorsal and lateral support structures, but did not affect the development of prothoracic horns (figure 1*h*; electronic supplementary material, figure S3d). sal^{RNAi} reduced dorsal support structures to a degree similar to that of dpp^{RNAi} (figure 1*g*) yet resulted in an enlargement of lateral support structures, including a duplication of lateral support structures in a subset of individuals (arrow in figure 1*g*), but without yielding any obvious defects in prothoracic horns (electronic supplementary material,

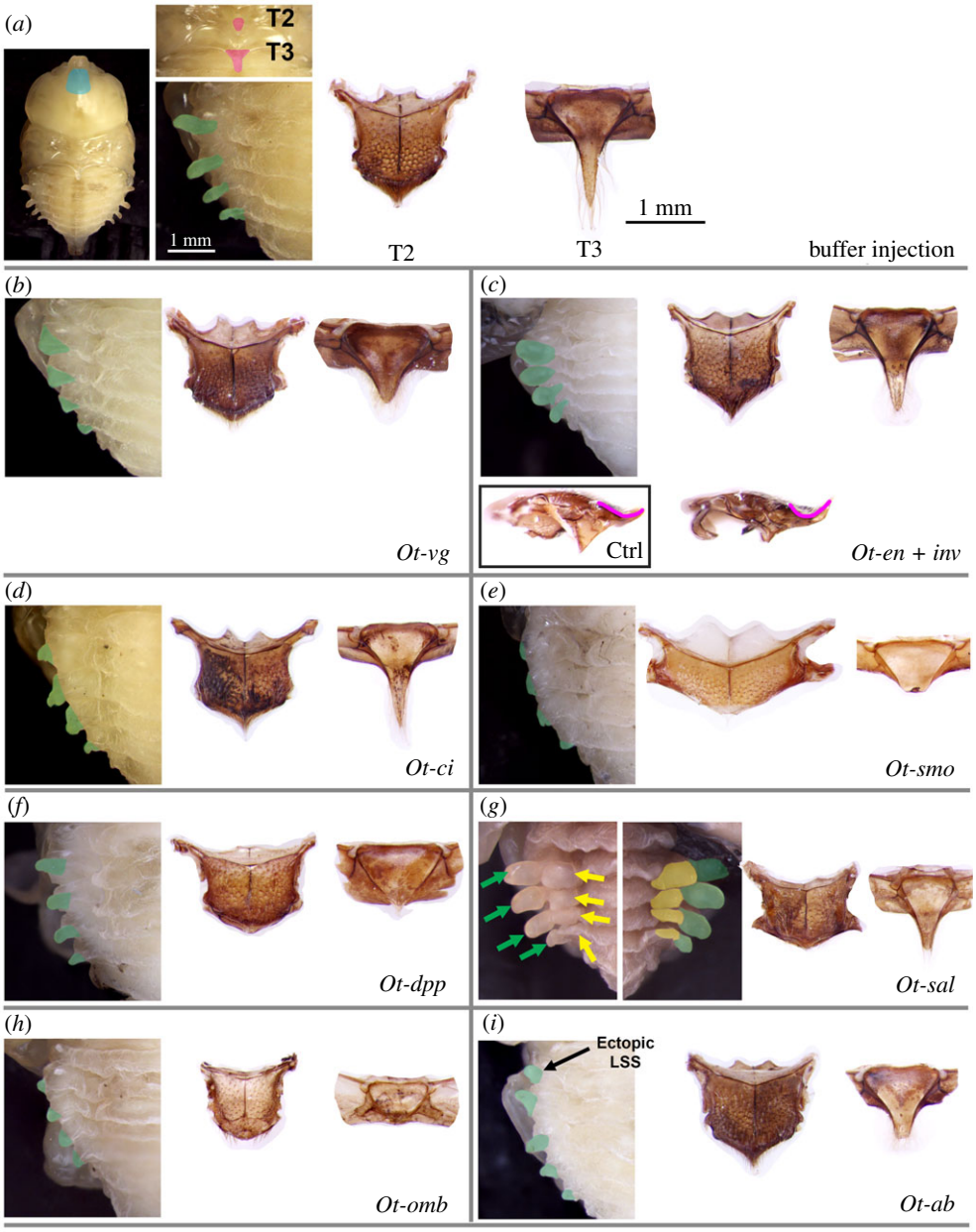

Figure 1. Functions of the wing selector gene *vg* and wing A/P axis patterning genes in the formation of different non-jointed outgrowths. (*a*) The buffer-injected individuals presenting the normal pupal prothoracic horn (cyan), lateral support structures (green) and dorsal support structures on T2 and T3 (magenta) as well as adult meso- and metanotum. (*b–i*) RNAi phenotypes of wing patterning genes *vg* (*b*), *en* + *inv* (*c*), *ci* (*d*), *smo* (*e*), *dpp* (*f*), *sal* (*g*), *omb* (*h*) and *ab* (*i*). In each grid, the panels from left to right show the phenotypes of pupal lateral support structures (green) and adult dorsal support structures on meso- and metanotum, respectively. The bottom panels in (*c*) show the lateral view of the adult dorsal support structure on the mesonotum of negative control (Ctrl) and *en* + *inv*^{RNAi} individuals (outlined with magenta line). In *sal*^{RNAi} individuals (*g*), the enlarged and duplicated lateral support structures are indicated with arrows and pseudo-colours. The arrows in (*i*) mark the formation of ectopic lateral support structures (LSS) in one more anterior segment. The images of the pupal and adult phenotypes are adjusted at the same magnification, respectively. (Online version in colour.)

figure S3e). Lastly, in addition to the previously documented reduction in prothoracic horns size [19], *ab*^{RNAi} caused a moderate reduction of dorsal and lateral support structures (figure 1*i*) yet, unexpectedly, also induced ectopic lateral support structures in the second abdominal segment (A2) which normally lacks such outgrowths (arrow in figure 1*i*). Because Hox gene manipulations are commonly able to transform segment identity, we speculate that *ab* might interact with regional Hox genes to repress the formation of lateral support structures in A2. Taken together, these results suggest that most wing A/P axis patterning genes are required for the proper formation of both dorsal and lateral support structures as well as prothoracic horns, but may functionally diverge in their specific roles.

(d) Dorso/ventral patterning of wing serial homologues

We next assessed three patterning genes critical for D/V axis patterning of wings, *apterous* (*ap*), *Serrate* (*Ser*) and *dishevelled* (*dsh*, a critical transducer of Wingless signal). As a compartment selector gene, *ap* is expressed in the dorsal compartment of wing discs, which directs the activation of *Ser* (ligand of Notch receptor) at the margin and in turn activates the expression of *wg* [39,42,44,47,50]. Wg specifies the presumptive wing margin and acts as a long-range morphogen in patterning the D/V axis of the wing [46]. The *Onthophagus* genome possesses two *ap* orthologues, *apA* and *apB* [19]. In order to avoid potential functional compensation, both *ap* orthologues were knocked down simultaneously. RNAi against *apA* + *B* or *dsh* resulted

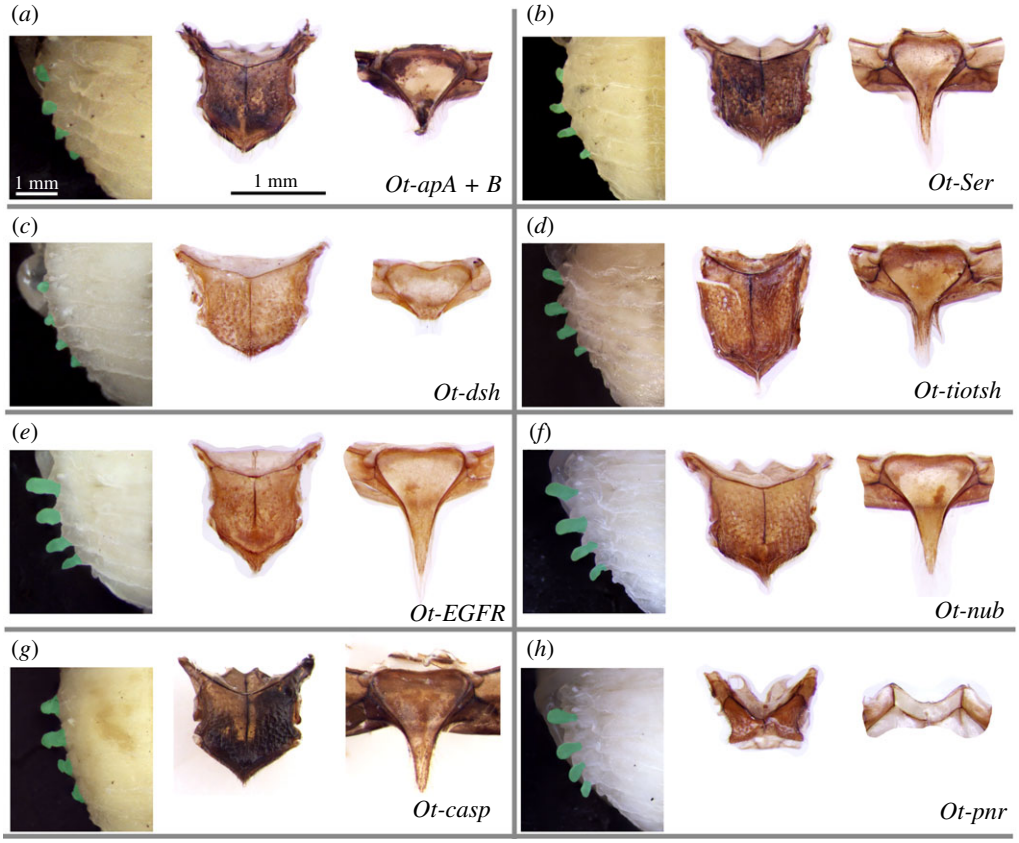

Figure 2. RNAi phenotypes of wing D/V and P/D patterning genes, as well as *pnr*, in the formation of non-jointed outgrowths. (*a–c*) RNAi phenotypes of wing D/V patterning genes *apA + B* (*a*), *Ser* (*b*) and *dsh* (*c*). (*d–f*) Phenotypes mediated by knocking down wing P/D pattering genes *tiotsh* (*d*), *EGFR* (*e*), *nub* (*f*). (*g,h*) RNAi phenotypes of gin trap-specific gene *casp* (*g*) and the regional patterning gene *pnr* (*h*). The phenotypes of pupal lateral support structures (green) and adult dorsal support structures on the mesonotum and metanotum are presented from left to right in each grid. (Online version in colour.)

in a moderate reduction of lateral support structures yet produced strong defects in dorsal support structures (figure 2*a*,*c*) and, as previously reported, in prothoracic horns [19]. In partial contrast, Ser^{RNAi} individuals presented moderately smaller lateral support structures and narrower dorsal support structures, yet no detectable defect in prothoracic horns (figure 2*b*; electronic supplementary material, figure S3f). These results support the hypothesis that similar to A/P patterning genes, most wing D/V axis patterning genes are also required for the proper formation of other wing serial homologues.

(e) Proximo/distal patterning of wing serial homologues

We focused on three genes required for P/D axis patterning during wing formation, including *tiotsh* (the single ortholog of *Drosophila tiptop* and *teashirt*), *epidermal growth factor receptor* (*EGFR*) and *nubbin* (*nub*). In the *Drosophila* wing disc, the expression of *teashirt* and *nub* presents in proximal and distal cells and guides the formation of the future notum and wing pouch, respectively [54,63]. *EGFR* is required for development of the notum by activating notum-specifying genes and indirectly controls wing outgrowth through regulation of *ap* [52]. Here, RNAi targeting *Onthophagus tiotsh* induced a slight reduction in the sizes of dorsal and lateral support structures and a mild split in the most distal region of prothoracic horns (figure 2*d* and arrowhead in electronic supplementary material, figure S3h). Unexpectedly, $EGFR^{RNAi}$ caused an elongation of prothoracic horns, yet both dorsal and lateral support structures appeared unaffected (figure 2*e*; electronic supplementary material, figure S3k). By contrast, nub^{RNAi} only resulted in a moderate reduction of wing size but failed to yield any detectable phenotypes in dorsal and lateral support structures (figure 2*f*) and, as previously reported, in prothoracic horns [19]. These results match previous findings in *Tribolium* that *nub* is not functionally required for the formation of wing serial homologues in segments other than T2 and T3 [8,14,23], even though *nub* enhancer activity is still detectable there [8,14]. These findings suggest that the formation of both pupal support structures and prothoracic horns functionally requires a subset of genes known to instruct P/D axis formation during wing development.

(f) Genes outside the wing gene regulatory network also contribute to the formation of wing serial homologues

We assessed the function of the genes *casp* and *pnr*. *casp* encodes a protein that inhibits the immune deficiency pathway in *Drosophila* and several anopheline species [64,65]. Previous work further identified *casp* as a gin trap-specific gene in *Tribolium* beetles that is not involved in the formation of wings [23]. However, because *Onthophagus* lateral support structures and *Tribolium* gin traps develop from homologous locations in their respective abdominal segments and their development is similarly dependent upon many members of the wing GRN (this study, table 1), it is reasonable to

Table 1. Phenotypic consequences of RNAi-mediated transcriptional depletion of 16 genes in the context of the formation of putative wing serial homologues in *Onthophagus* and *Tribolium*. ↑, ↓ and 0 indicate enlarged, reduced or no change in size of a given structure, respectively. # indicates changes in orientation. n.a. indicates that no functional assessment has been executed. The data collected from this study or previous studies [14,19,23,66] are indicated with black or grey symbols, respectively.

wing genes	*Onthophagus* lateral support structures	*Onthophagus* dorsal support structures	*Onthophagus* prothoracic horns	*Tribolium* gin traps	*Onthophagus* wings
vg	↓	↓	↓	↓	↓
dsh	↓	↓	↓	↓	↓
apA+B	↓	↓	↓	↓	↓
smo	↓	↓	↑	↓	↓
tiotsh	↓	↓	↓	↓	↓
dpp	↓	↓	↓	0	↓
ab	↓	↓	↓	n/a	↓
sal	↑	↓	0	↓	↓
Ser	↓	↓	0	↓	↓
omb	↓	↓	0	0	↓
ci	↓	0	↓	↓	↓
en+inv	0	0, #	0	0	↓
EGFR	0	0	↑	↓	↓
nub	0	0	0	0	↓
casp	↓	0	↓	↓	↓
pnr	0	↓	↓	n/a	0

consider both traits as homologous structures. We, therefore, sought to assess whether *casp* patterns the development of *Onthophagus* lateral support structures via $casp^{RNAi}$. We found that $casp^{RNAi}$ resulted in the incomplete formation of lateral support structures, as well as a consistent narrowing of the distal region of the prothoracic horns (figure 2*g*; electronic supplementary material, figure S3i). However, the dorsal support structures appeared unaffected (figure 2*g*). Lastly, we selected *pnr* to assess whether regional patterning genes not associated with the formation of *bona fide* wings may function in the specification of wing serial homologues in other body regions. *pnr* patterns dorsomedial tissue identity across insect orders [19,51] and we sought to assess whether the formation of dorsal support structures in the medial region of the meso- and meta-thorax is dependent upon *pnr* function. We found that pnr^{RNAi} completely eliminated dorsal support structures as well as previously documented prothoracic horns [19], but failed to disrupt the formation of lateral support structures (figure 2*h*). These findings support the hypothesis that the formation of dorsomedially located wing serial homologues is dependent upon *pnr* which has maintained its ancestral and conserved function in the patterning of dorsomedial tissues.

(g) The role of the wing gene regulatory network in the diversification of insect outgrowths

In this study, we functionally investigated 14 genes within the wing GRN to assess their role in the formation of three presumed wing serial homologues: paired lateral support structures, dorsal support structures and the prothoracic horns. Of those 14 genes, we found the same 10 genes to be functionally required for the proper formation of lateral and dorsal support structures, respectively (table 1). In addition, we found *en* to be functionally necessary for the patterning of orientation of dorsal support structures, whereas *ci* is involved in the development of lateral support structures. Moreover, nine of 14 genes are required for the formation of prothoracic horns (table 1 and figure 3). Finally, seven of our 14 focal wing genes are functionally required for all three presumed wing serial homologues (table 1). Collectively, these data support the hypothesis that all three structures share a developmental origin with insect wings and may, therefore, qualify as wing serial homologues.

However, given that both dorsal support structures and traditional fore- and hindwings are found on the same segments (T2 and T3), it may not be as straightforward to

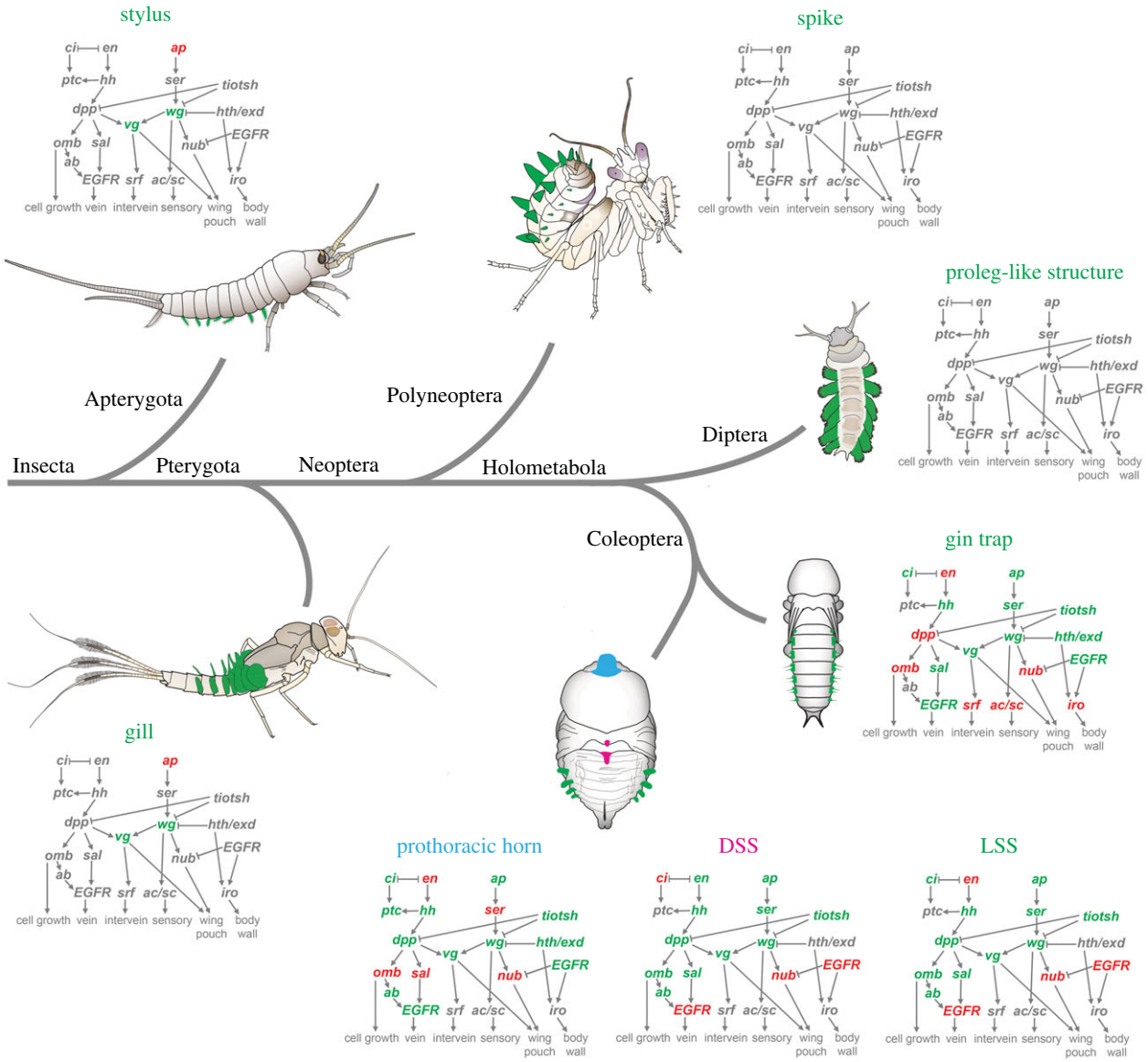

Figure 3. Examples of various, non-wing outgrowths across insects that may have relied on the wing GRN in their origin and diversification. The *Drosophila* wing GRN is adapted from previous work [55]. Within the GRN, genes that are required, not required, or not yet tested for a given structure are marked in green, red or grey, respectively. Four taxa are shown for whom expression and/or gene-function data are available (anticlockwise from top left): the styli of bristletails *Pedetontus unimaculatus* (Archeognatha, [7]), the tracheal gills of mayfly nymphs *Ephoron eophilum* (Ephemeroptera, [7]), the pupal outgrowths of *Onthophagus* dung beetles (prothoracic horn; DSS, dorsal support structures; LSS, lateral support structures, this study) and the gin traps of the red flour beetle *T. castaneum* (Coleoptera, [23]). In addition, two examples of taxa are shown that can be hypothesized based on morphology to similarly derive from wing serial homologues, but for which functional or expression data remain to be collected: the bilateral proleg-like projections of the mountain midge (Diptera) and the abdominal spikes of flower mantids *Pseudocreobotra wahlbergii* (Mantodea). (Online version in colour.)

simply view them as serial homologues of each other. The standard definition of serial homologues normally presumes their existence on separate body regions such as segments. However, serially reiterated structures can also exist on a much narrower spatial scale, such as the eye spots of butterflies, which appear serially reiterated on the surface of the same wing [67,68]. A potential resolution may emerge when we consider that in *Drosophila*, both the wing and the notum develop from cell populations contained within the same wing imaginal disc, which in its entirety is characterized by the expression of *vg* during embryogenesis [57,58]. While *Onthophagus* beetles lack wing imaginal discs and instead form wings late in larval development through epidermal outbuddings, notal differentiation in T2 and T3 may be characterized by *vg* expression or function restricted in the lateral and posterior area similar to that in various insect species [7,10,14]. If so, this may help explain how the wing GRN may be similarly involved in the formation of *bona fide* wings and dorsal support structures in the same segment.

Alternatively, wing GRN genes may have become secondarily co-opted into the posterior meso- and metanotum to guide the formation of dorsal support structures. The location of dorsal support structures in T2 and T3 exhibits at least some correspondence to the scutellum portion of the notum, i.e. a component of the dorsal body wall. Previous work showed that the distal wing and the proximal body wall share parts of a developmental GRN (see [69] for review), including the core wing marker gene *vg*. This calls into question whether all *vg*-positive cells are necessarily related to wings or wing serial homologues. Most previous studies concerning the origin of insect wings consider *vg* a reliable wing selector

gene. However, an alternative explanation is that *vg* and possibly other wing related genes could be co-opted into the notal region and subsequently instruct evolutionary changes within the scutellar region in some lineages. If correct, only a subset of *vg*-dependent tissue ought to be considered homologous or serially homologous to wings. A similar mechanism has been proposed in the cladoceran crustacean *Daphnia magna*, in which the core *wg-vg* wing module expressed in the posterior part of the dorsal head facilitates the formation of a flat shield that covers the dorsal part of the body in many crustaceans [70]. This raises the possibility that repeated co-option of a conserved gene regulatory module across body regions might provide a general route towards facilitating the emergence of diverse outgrowths in different life stages across hemi-, holo- and ametabolous hexapods. For example, Niwa *et al*. [7] document the expression of both *vg* and *wg* in the gills of mayfly nymphs and styli of bristletails, respectively, while the expression of several wing patterning genes is also detectable in the primordia of helmets of hemimetabolous treehoppers [17,18]. Similar co-option events may, therefore, also underlie other conspicuous outgrowths, such as the abdominal spikes in flower mantids or the proleg-like projections in mountain midges (figure 3), suggesting promising opportunities for comparative studies in the future.

(h) Evolutionary lability of the wing gene regulatory network and the diversification of insect outgrowths

Even though our study identified diverse components of the wing GRN as required for proper development regardless of the specific identity of a given wing serial homologue, several important differences also emerged.

First, wing serial homologues diverged in their reliance on key members of the canonical wing GRN (figure 3). For instance, *Ser* was required only for the development of dorsal and lateral support structures, while $EGFR^{RNAi}$ appeared to enhance prothoracic horn formation but left dorsal and lateral support structures unaffected, whereas *dpp* and its target genes *sal* and *omb* affected both dorsal and lateral support structures, but in distinctly different ways. One explanation of this diversity may reside in differential input of local Hox genes along the body axis, which among diverse other functions are tasked to inhibit wing formation in normally wingless segments, or to confer the differentiation between forewings and hindwings [24,71–73]. Specifically, in *Drosophila*, the development of halteres, a highly reduced and specialized hindwing that functions as a balance organ, is shaped by the Hox gene *Ultrabithorax* (*Ubx*) which regulates genes at several levels of the wing patterning hierarchy [74,75]. Therefore, various wing serial homologues, including the focal structures of this study, could similarly have diversified through modification in their regulatory interactions with regional Hox genes. Likewise, interactions with segment-specific Hox genes may determine the segment-specific presence/absence of a given wing serial homologue in different taxa, such as between *Tribolium* gin traps (located on A1–A7) and *Onthophagus* lateral support structures (A3–A6). More generally, many of the genes studied here and the pathways they are part of function essentially as genetic switches, deeply conserved in their switch architecture but evolutionarily deconstrained in precisely which target genes they switch on or off. It, therefore, may be expected that signatures of serial homology be most obvious in master regulators followed by second-order switches but not necessarily the precise developmental functions regulated by them.

Furthermore, lateral support structures and prothoracic horns may only represent *partial* wing serial homologues since a growing body of evidence points towards a dual origin of insect wings (i.e. a combined contribution of the tergal plate of thoracic segments and ancestrally proximal leg branches) [7–15]. Indeed, a wing-like GRN exists in both tergal and pleural-derived structures across insects and even crustacean species [7,8,10,14,15,19,23]. Therefore, partial wing serial homologues existing at different locations may correspondingly rely on *partial* wing GRNs. For example, *ap*, which is strongly expressed in the dorsal region of the segment corresponding to the future tergum, is not detected in the mayfly gill or bristletail stylus primordium [7]. Therefore, both structures may only be reflective of pleural wing serial homology. By contrast, the functional requirement of *ap* for the formation of lateral support structures, prothoracic horns and *Tribolium* gin traps indicate that all three structures correspond to tergal wing serial homologues of wings. Collectively, our results thus suggest that the morphological diversification of wing serial homologues—both across species and along the body axis of the same taxon—is accompanied by both significant conservation and lability in the underlying wing GRN.

4. Conclusion

Collectively, our findings suggest that the morphological and underlying gene regulatory diversification of wing serial homologues across species, life stages and segments has contributed significantly to the extraordinary diversity of appendages and outgrowths so common in arthropods. Even though expression and functional significance of the wing selector gene *vg* as well as that of diverse members of the core wing GRN were found to be conserved across homologues, we also detected key divergences at multiple levels within the GRN hierarchy. This interplay between conservation and divergence illustrates how the modular nature of GRNs serves to scaffold development and evolution of novel and diverse morphologies.

Data accessibility. All data are available in the main text or the electronic supplementary material.

Authors' contributions. Y.H. and A.P.M. conceived and designed the research; Y.H. performed the research and analysed the data, Y.H. and A.P.M. wrote the manuscript. Both authors gave final approval for publication and agree to be held accountable for the work performed therein.

Competing interests. All authors declare that they have no competing interests.

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References

1. Carroll SB, Grenier JK, Weatherbee SD. 2005 *From DNA to diversity: molecular genetics and the evolution of animal design*. Malden, MA: Blackwell.
2. Snodgrass RE. 1935 *Principles of insect morphology*. New York, NY: McGraw Hill.
3. Casares F, Mann RS. 2001 The ground state of the ventral appendage in *Drosophila*. *Science* **293**, 1477–1480. (doi:10.1126/science.1062542)
4. Angelini DR, Kaufman TC. 2005 Insect appendages and comparative ontogenetics. *Dev. Biol.* **286**, 57–77. (doi:10.1016/j.ydbio.2005.07.006)
5. Grimaldi DA, Engel MS. 2005 *Evolution of the insects*. Cambridge, UK: Cambridge University Press.
6. Kukalová-Peck J. 2008 Phylogeny of higher taxa in Insecta: finding synapomorphies in the extant fauna and separating them from homoplasies. *Evol. Biol.* **35**, 4–51. (doi:10.1007/s11692-007-9013-4)
7. Niwa N, Akimoto-Kato A, Niimi T, Tojo K, Machida R, Hayashi S. 2010 Evolutionary origin of the insect wing via integration of two developmental modules. *Evol. Dev.* **12**, 168–176. (doi:10.1111/j.1525-142X.2010.00402.x)
8. Clark-Hachtel CM, Linz DM, Tomoyasu Y. 2013 Insights into insect wing origin provided by functional analysis of vestigial in the red flour beetle, *Tribolium castaneum*. *Proc. Natl Acad. Sci. USA* **110**, 16 951–16 956. (doi:10.1073/pnas.1304332110)
9. Clark-Hachtel CM, Moe MR, Tomoyasu Y. 2018 Detailed analysis of the prothoracic tissues transforming into wings in the cephalothorax mutants of the Tribolium beetle. *Arthropod. Struct. Dev.* **47**, 352–361. (doi:10.1016/j.asd.2018.06.005)
10. Medved V, Marden JH, Fescemyer HW, Der JP, Liu J, Mahfooz N, Popadić A. 2015 Origin and diversification of wings: insights from a neopteran insect. *Proc. Natl Acad. Sci. USA* **112**, 15 946–15 951. (doi:10.1073/pnas.1509517112)
11. Elias-Neto M, Belles X. 2016 Tergal and pleural structures contribute to the formation of ectopic prothoracic wings in cockroaches. *Open Sci.* **3**, 160347. (doi:10.1098/rsos.160347)
12. Prokop J, Pecharová M, Nel A, Hörnschemeyer T, Krzemińska E, Krzemiński W, Engel MS. 2017 Paleozoic nymphal wing pads support dual model of insect wing origins. *Curr. Biol.* **27**, 263–269. (doi:10.1016/j.cub.2016.11.021)
13. Requena D, Álvarez JA, Gabilondo H, Loker R, Mann RS, Estella C. 2017 Origins and specification of the *Drosophila* wing. *Curr. Biol.* **27**, 3826–3836; e5. (doi:10.1016/j.cub.2017.11.023)
14. Linz DM, Tomoyasu Y. 2018 Dual evolutionary origin of insect wings supported by an investigation of the abdominal wing serial homologs in *Tribolium*. *Proc. Natl Acad. Sci. USA* **115**, E658–E667. (doi:10.1073/pnas.1711128115)
15. Clark-Hachtel CM, Tomoyasu Y. 2020 Two sets of candidate crustacean wing homologues and their implication for the origin of insect wings. *Nat. Ecol. Evol.* **4**, 1694–1702. (doi:10.1038/s41559-020-1257-8)
16. Almudi I *et al.* 2020 Genomic adaptations to aquatic and aerial life in mayflies and the origin of insect wings. *Nat. Commun.* **11**, 2631. (doi:10.1038/s41467-020-16284-8)
17. Prud'homme B, Minervino C, Hocine M, Cande JD, Aouane A, Dufour HD, Kassner VA, Gompel N. 2011 Body plan innovation in treehoppers through the evolution of an extra wing-like appendage. *Nature* **473**, 83–86. (doi:10.1038/nature09977)
18. Fisher CR, Wegrzyn JL, Jockusch EL. 2020 Co-option of wing-patterning genes underlies the evolution of the treehopper helmet. *Nat. Ecol. Evol.* **4**, 250–260. (doi:10.1038/s41559-019-1054-4)
19. Hu Y, Linz DM, Moczek AP. 2019 Beetle horns evolved from wing serial homologs. *Science* **366**, 1004–1007. (doi:10.1126/science.aaw2980)
20. Hinton HE. 1946 The 'gin-traps' of some beetle pupae; a protective device which appears to be unknown. *Trans. R. Entomol. Soc. Lond.* **97**, 473–496. (doi:10.1111/j.1365-2311.1946.tb00273.x)
21. Wilson MCL. 1971 The morphology and mechanism of the pupal gin-traps of *Tenebrio molitor* L. (Coleoptera. *Tenebrionidae)*. *J. Stored Prod. Res.* **7**, 21–30. (doi:10.1016/0022-474X(71)90034-8)
22. Eisner T, Eisner M. 1992 Operation and defensive role of 'gin traps' in a coccinellid pupa (*Cycloneda sanguinea*). *Psyche J. Entomol.* **99**, 265–273. (doi:10.1155/1992/54859)
23. Hu Y, Schmitt-Engel C, Schwirz J, Stroehlein N, Richter T, Majumdar U, Bucher G. 2018 A morphological novelty evolved by co-option of a reduced gene regulatory network and gene recruitment in a beetle. *Proc. R. Soc. B* **285**, 20181373. (doi:10.1098/rspb.2018.1373)
24. Ohde T, Yaginuma T, Niimi T. 2013 Insect morphological diversification through the modification of wing serial homologs. *Science* **340**, 495–498. (doi:10.1126/science.1234219)
25. Moczek AP. 2006 Integrating micro- and macroevolution of development through the study of horned beetles. *Heredity* **97**, 168–178. (doi:10.1038/sj.hdy.6800871)
26. Moczek AP, Cruickshank TE, Shelby A. 2006 When ontogeny reveals what phylogeny hides: gain and loss of horns during development and evolution of horned beetles. *Evolution* **60**, 2329–2341. (doi:10.1111/j.0014-3820.2006.tb01868.x)
27. Madewell R, Moczek AP. 2006 Horn possession reduces maneuverability in the horn-polyphenic beetle, *Onthophagus nigriventris*. *J. Insect. Sci.* **6**, 1–10. (doi:10.1673/2006_06_21.1)
28. Burmeister F. 1930 Die BrutfuÈrsorge und das Bauprinzip der Gatting Onthophagus Latreille. *Z. Morphol. Tiere* **16**, 559–647. (doi:10.1007/BF00407269)
29. Ballerio A. 1999 Revision of the genus *Pterorthochroaetes* first contribution (Coleoptera: Scarabaeoidea: Ceratocanthidae). *Folia Heyrovskyana* **7**, 221–228.
30. Grebennikov VV, Ballerio A, Scholtz CH. 2002 Larva and pupa of *Cyphopisthes descarpentriesi* Paulian (Coleoptera: Scarabaeoidea: Ceratocanthidae) and their phylogenetic implications. *Austral. J. Entomol.* **41**, 367–374. (doi:10.1046/j.1440-6055.2002.00307.x)
31. Moczek AP, Nagy LM. 2005 Diverse developmental mechanisms contribute to different levels of diversity in horned beetles. *Evol. Dev.* **7**, 175–185. (doi:10.1111/j.1525-142X.2005.05020.x)
32. Kijimoto T, Moczek AP, Andrews J. 2012 Diversification of doublesex function underlies morph-, sex-, and species-specific development of beetle horns. *Proc. Natl Acad. Sci. USA* **109**, 20 526–20 531. (doi:10.1073/pnas.1118589109)
33. Herndon N *et al.* 2020 Enhanced genome assembly and a new official gene set for *Tribolium castaneum*. *BMC Genomics* **21**, 47. (doi:10.1186/s12864-019-6394-6)
34. Thurmond J *et al.* 2019 FlyBase 2.0: the next generation. *Nucleic Acids Res.* **47**, D759–D765. (doi:10.1093/nar/gky1003)
35. Linz DM, Hu Y, Moczek AP. 2019 The origins of novelty from within the confines of homology: the developmental evolution of the digging tibia of dung beetles. *Proc. R. Soc. B* **286**, 20182427. (doi:10.1098/rspb.2018.2427)
36. Philip BN, Tomoyasu Y. 2011 Gene knockdown analysis by double-stranded RNA injection. In *Molecular methods for evolutionary genetics* (eds V Orgogozo, MV Rockman), pp. 471–497. Totowa, NJ: Humana Press.
37. Moczek AP, Rose DJ. 2009 Differential recruitment of limb patterning genes during development and diversification of beetle horns. *Proc. Natl Acad. Sci. USA* **106**, 8992–8997. (doi:10.1073/pnas.0809668106)
38. Livak KJ, Schmittgen TD. 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408. (doi:10.1006/meth.2001.1262)
39. Cohen B, McGuffin ME, Pfeifle C, Segal D, Cohen SM. 1992 apterous, a gene required for imaginal disc development in *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. *Genes Dev.* **6**, 715–729. (doi:10.1101/gad.6.5.715)
40. Tabata T, Eaton S, Kornberg TB. 1992 The *Drosophila* hedgehog gene is expressed specifically in posterior compartment cells and is a target of engrailed regulation. *Genes Dev.* **6**, 2635–2645. (doi:10.1101/gad.6.12b.2635)
41. Tabata T, Schwartz C, Gustavson E, Ali Z, Kornberg TB. 1995 Creating a *Drosophila* wing de novo, the role of engrailed, and the compartment border hypothesis. *Development* **121**, 3359–3369.
42. Diaz-Benjumea FJ, Cohen SM. 1993 Interaction between dorsal and ventral cells in the imaginal

disc directs wing development in *Drosophila*. *Cell* **75**, 741–752. (doi:10.1016/0092-8674(93)90494-B)

43. Basler K, Struhl G. 1994 Compartment boundaries and the control of *Drosophila* limb pattern by hedgehog protein. *Nature* **368**, 208–214. (doi:10.1038/368208a0)
44. Blair SS, Brower DL, Thomas JB, Zavortink M. 1994 The role of apterous in the control of dorsoventral compartmentalization and PS integrin gene expression in the developing wing of *Drosophila*. *Development* **120**, 1805–1815.
45. Tabata T, Kornberg TB. 1994 Hedgehog is a signaling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* **76**, 89–102. (doi:10.1016/0092-8674(94)90175-9)
46. Williams JA, Paddock SW, Vorwerk K, Carroll SB. 1994 Organization of wing formation and induction of a wing-patterning gene at the dorsal/ventral compartment boundary. *Nature* **368**, 299–305. (doi:10.1038/368299a0)
47. de Celis JF, Garcia-Bellido A, Bray SJ. 1996 Activation and function of Notch at the dorsal–ventral boundary of the wing imaginal disc. *Development* **122**, 359–369.
48. Domínguez M, Brunner M, Hafen E, Basler K. 1996 Sending and receiving the hedgehog signal: control by the *Drosophila* Gli protein Cubitus interruptus. *Science* **272**, 1621–1625. (doi:10.1126/science.272.5268.1621)
49. Lecuit T, Brook WJ, Ng M, Calleja M, Sun H, Cohen SM. 1996 Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. *Nature* **381**, 387–393. (doi:10.1038/381387a0)
50. Neumann CJ, Cohen SM. 1996 A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the *Drosophila* wing. *Development* **122**, 3477–3485.
51. Calleja M, Herranz H, Estella C, Casal J, Lawrence P, Simpson P, Morata G. 2000 Generation of medial and lateral dorsal body domains by the pannier gene of *Drosophila*. *Development* **127**, 3971–3980.
52. Wang S-H, Simcox A, Campbell G. 2000 Dual role for *Drosophila* epidermal growth factor receptor signaling in early wing disc development. *Genes Dev.* **14**, 2271–2276. (doi:10.1101/gad.827000)
53. Cook O, Biehs B, Bier E. 2004 . brinker and optomotor-blind act coordinately to initiate development of the L5 wing vein primordium in *Drosophila*. *Development* **131**, 2113–2124. (doi:10.1242/dev.01100)
54. Zirin JD, Mann RS. 2007 Nubbin and Teashirt mark barriers to clonal growth along the proximal–distal axis of the *Drosophila* wing. *Dev. Biol.* **304**, 745–758. (doi:10.1016/j.ydbio.2007.01.025)
55. Tomoyasu Y, Arakane Y, Kramer KJ, Denell RE. 2009 Repeated co-options of exoskeleton formation during wing-to-elytron evolution in beetles. *Curr. Biol.* **19**, 2057–2065. (doi:10.1016/j.cub.2009.11.014)
56. Ravisankar P, Lai Y-T, Sambrani N, Tomoyasu Y. 2016 Comparative developmental analysis of *Drosophila* and *Tribolium* reveals conserved and diverged roles of abrupt in insect wing evolution. *Dev. Biol.* **409**, 518–529. (doi:10.1016/j.ydbio.2015.12.006)
57. Williams JA, Bell JB, Carroll SB. 1991 Control of *Drosophila* wing and haltere development by the nuclear vestigial gene product. *Genes Dev.* **5**, 2481–2495. (doi:10.1101/gad.5.12b.2481)
58. Halder G, Polaczyk P, Kraus ME, Hudson A, Kim J, Laughon A, Carroll S. 1998 The Vestigial and Scalloped proteins act together to directly regulate wing-specific gene expression in *Drosophila*. *Genes Dev.* **12**, 3900–3909. (doi:10.1101/gad.12.24.3900)
59. Morata G, Lawrence PA. 1975 Control of compartment development by the engrailed gene in *Drosophila*. *Nature* **255**, 614–617. (doi:10.1038/255614a0)
60. Nellen D, Burke R, Struhl G, Basler K. 1996 Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357–368. (doi:10.1016/S0092-8674(00)81114-9)
61. Tsuneizumi K, Nakayama T, Kamoshida Y, Kornberg TB, Christian JL, Tabata T. 1997 Daughters against dpp modulates dpp organizing activity in *Drosophila* wing development. *Nature* **389**, 627–631. (doi:10.1038/39362)
62. Kijimoto T, Moczek AP. 2016 Hedgehog signaling enables nutrition-responsive inhibition of an alternative morph in a polyphenic beetle. *Proc. Natl Acad. Sci. USA* **113**, 5982–5987. (doi:10.1073/pnas.1601505113)
63. Cifuentes FJ, García-Bellido A. 1997 Proximo–distal specification in the wing disc of *Drosophila* by the nubbin gene. *Proc. Natl Acad. Sci. USA* **94**, 11 405–11 410. (doi:10.1073/pnas.94.21.11405)
64. Kim M, Lee JH, Lee SY, Kim E, Chung J. 2006 Caspar, a suppressor of antibacterial immunity in *Drosophila*. *Proc. Natl Acad. Sci. USA* **103**, 16 358–16 363. (doi:10.1073/pnas.0603238103)
65. Garver LS, Dong Y, Dimopoulos G. 2009 Caspar controls resistance to *Plasmodium falciparum* in diverse anopheline species. *PLoS Pathog.* **5**, e1000335. (doi:10.1371/journal.ppat.1000335)
66. Crabtree JR, Macagno ALM, Moczek AP, Rohner PT, Hu Y. 2020 Notch signaling patterns head horn shape in the bull-headed dung beetle *Onthophagus taurus*. *Dev. Genes Evol.* **230**, 213–225. (doi:10.1007/s00427-020-00645-w)
67. Saenko SV, Marialva MS, Beldade P. 2011 Involvement of the conserved Hox gene Antennapedia in the development and evolution of a novel trait. *EvoDevo* **2**, 9. (doi:10.1186/2041-9139-2-9)
68. Abbasi R, Marcus JM. 2015 Colour pattern homology and evolution in Vanessa butterflies (Nymphalidae: Nymphalini): eyespot characters. *J. Evol. Biol.* **28**, 2009–2026. (doi:10.1111/jeb.12716)
69. Ruiz-Losada M, Blom-Dahl D, Córdoba S, Estella C. 2018 Specification and patterning of *Drosophila* appendages. *J. Dev. Biol.* **6**, 17. (doi:10.3390/jdb6030017)
70. Shiga Y, Kato Y, Aragane-Nomura Y, Haraguchi T, Saridaki T, Watanabe H, Iguchi T, Yamagata H, Averof M. 2017 Repeated co-option of a conserved gene regulatory module underpins the evolution of the crustacean carapace, insect wings and other flat outgrowths. *bioRxiv* 160010. (doi:10.1101/160010)
71. Carroll SB, Weatherbee SD, Langeland JA. 1995 Homeotic genes and the regulation and evolution of insect wing number. *Nature* **375**, 58–61. (doi:10.1038/375058a0)
72. Tomoyasu Y, Wheeler SR, Denell RE. 2005 Ultrabithorax is required for membranous wing identity in the beetle *Tribolium castaneum*. *Nature* **433**, 643–647. (doi:10.1038/nature03272)
73. Tomoyasu Y. 2017 Ultrabithorax and the evolution of insect forewing/hindwing differentiation. *Curr. Opin. Insect Sci.* **19**, 8–15. (doi:10.1016/j.cois.2016.10.007)
74. Weatherbee SD, Halder G, Kim J, Hudson A, Carroll SB. 1998 Ultrabithorax regulates genes at several levels of the wing-patterning hierarchy to shape the development of the *Drosophila* haltere. *Genes Dev.* **12**, 1474–1482. (doi:10.1101/gad.12.10.1474)
75. Pavlopoulos A, Akam M. 2011 Hox gene Ultrabithorax regulates distinct sets of target genes at successive stages of *Drosophila* haltere morphogenesis. *Proc. Natl Acad. Sci. USA* **108**, 2855–2860. (doi:10.1073/pnas.1015077108)