

## Hypothesis

# Cryptic Persistence of Truncated Abdominal Legs in Insects Enabled Diverse Outgrowths with Novel Functions

Heather S. Bruce<sup>1\*</sup> and Nipam H. Patel<sup>1,2</sup>

<sup>1</sup> Marine Biological Laboratory, 7 MBL St. Woods Hole, MA 02543 USA

<sup>2</sup> University of Chicago, Organismal Biology & Anatomy, 1027 E 57th Street, Chicago, IL 60637 USA

\* Correspondence: hbruce@mbl.edu

**Abstract:** An iconic feature of insects is the apparent lack of legs on the abdomen, which is believed to be due to the repression of the leg-patterning gene *Distalless* (*Dll*) by abdominal Hox genes. However, in contrast to these molecular observations, it is not widely appreciated that the embryos of most insect groups do in fact form paired protrusions on most abdominal segments that appear to be homologous to the thoracic legs. However, these degenerate before hatching to form the abdominal body wall<sup>1–9</sup>. To resolve this discordance between molecular and morphological observations, the expression patterns of *pannier* and *araucan*, genes known to distinguish proximal leg segments in all arthropods<sup>10–12</sup>, are examined in embryos of the flour beetle *Tribolium castaneum*. In *Tribolium* embryos, all pregenital abdominal segments develop leg-like paired protrusions, and the stripes of *pannier* and *araucan* expression that delineate the proximal leg segments of the thorax are also expressed in the same configuration around these abdominal protrusions. This suggests that insect abdominal legs are homologous to only the proximal portion of the thoracic legs, which in insect adults forms the body wall (lateral tergum and pleura). These cryptic, truncated abdominal legs – likely inherited from their crustacean ancestors – appear to be an important wellspring for new functions in insects, such as caterpillar prolegs<sup>13,14</sup>, gills<sup>15</sup>, and structures for camouflage<sup>16</sup> and aposematic warning<sup>17</sup>.

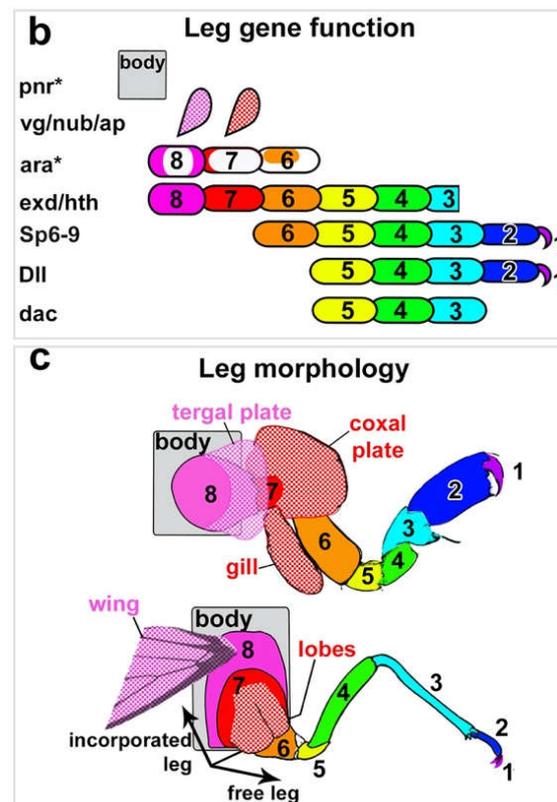
**Keywords:** arthropod; leg; evo-devo; exite

## 1. Introduction

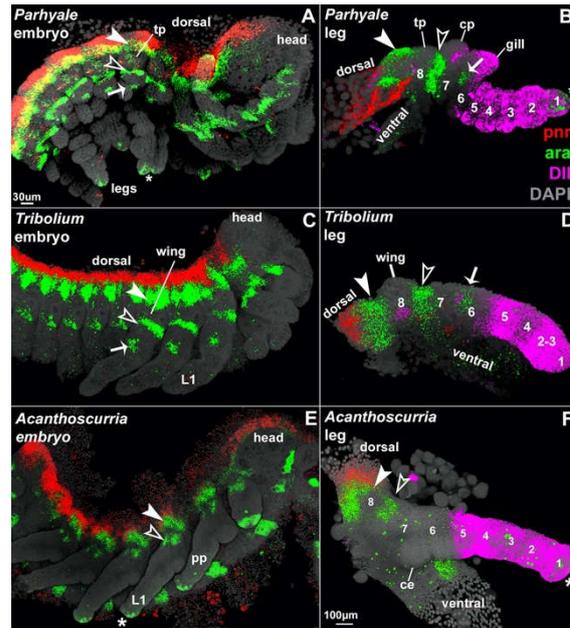
Insects are the most successful group of animals on the planet, due in part to the plethora of outgrowths that decorate their bodies with functions including flight, camouflage, and respiration. An iconic feature of the insect body plan is the presence of six walking legs, which gives the group its name, Hexapoda. It is commonly assumed in molecular and evo devo circles that insects lack legs on the pre-genital abdomen, except for the pleuropod on the first abdominal segment in embryos of certain insect groups<sup>18–22</sup>. In insect groups where larvae or adults have abdominal appendages, such as caterpillars or male sepsid flies, it has been proposed that these abdominal legs arose by re-evolution of the leg program<sup>19,21,23</sup>. By contrast, crustaceans (i.e. non-insect pancrustaceans), from which insects evolved<sup>24</sup>, generally have a pair of legs on all abdominal segments. The loss of these ancestral abdominal legs in the insect lineage is thought to have evolved when posterior Hox genes such as *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abdA*) gained the ability to suppress the leg-patterning gene *Distalless* (*Dll*) in the insect abdomen<sup>18,25</sup>.

However, morphologists since 1844<sup>1</sup> have noted that, in the embryos of most insect groups, a pair of nubs forms on most abdominal segments which appear to be homologous to the thoracic legs<sup>1–9</sup>. These abdominal nubs flatten into the body wall to form the abdominal body wall (lateral tergum, pleura, and coxosternites) before the embryo hatches. But how could abdominal legs form in insects when *Dll* is suppressed by Hox genes?

By comparing a century of previous morphological work with the expression and function of several leg- and wing-patterning genes between insects, crustaceans, and arachnids – representing three of the four main groups of arthropods – Bruce and Patel 2020<sup>10</sup>, 2021<sup>11</sup>, and 2022<sup>12</sup> concluded that arthropods ancestrally have a total of 8 leg segments, but many arthropods have incorporated proximal leg segments into the body wall (Fig. 1). Insects, for example, have incorporated proximal leg segments 7 and 8, which now form the body wall (pleura and lateral tergum, respectively)<sup>9</sup>, resulting in the familiar six (free) leg segments of insects: pretarsus/claw (1), tarsus (2), tibia (3), femur (4), trochanter (5), and coxa (6). In the embryos of all arthropods examined to date, representing three of the four major living arthropod groups – *Drosophila melanogaster* (fruit fly; insect)<sup>26–29</sup>, *Tribolium castaneum* (flour beetle; insect)<sup>10–12</sup>, *Parhyale hawaiensis* (beach scud; “crustacean”)<sup>10–12</sup>, *Daphnia magna* (water flea; “crustacean”)<sup>12</sup>, and *Acanthoscurria geniculata* (tarantula; chelicerate)<sup>11</sup> – the Iroquois complex gene *araucan* (*ara*) is expressed in two stripes that bracket the incorporated 8th leg segment, and the GATA factor *pannier* (*pnr*) is expressed in the dorsal-most tissue and marks the *bona fide* body wall that is not leg-derived (Figs. 1, 2). Thus, in contrast to other leg patterning genes<sup>30</sup>, the expression patterns of *pnr* and *ara* are highly conserved across arthropods. As such, they can be used to identify proximal leg segments even if the leg segments now function as body wall.



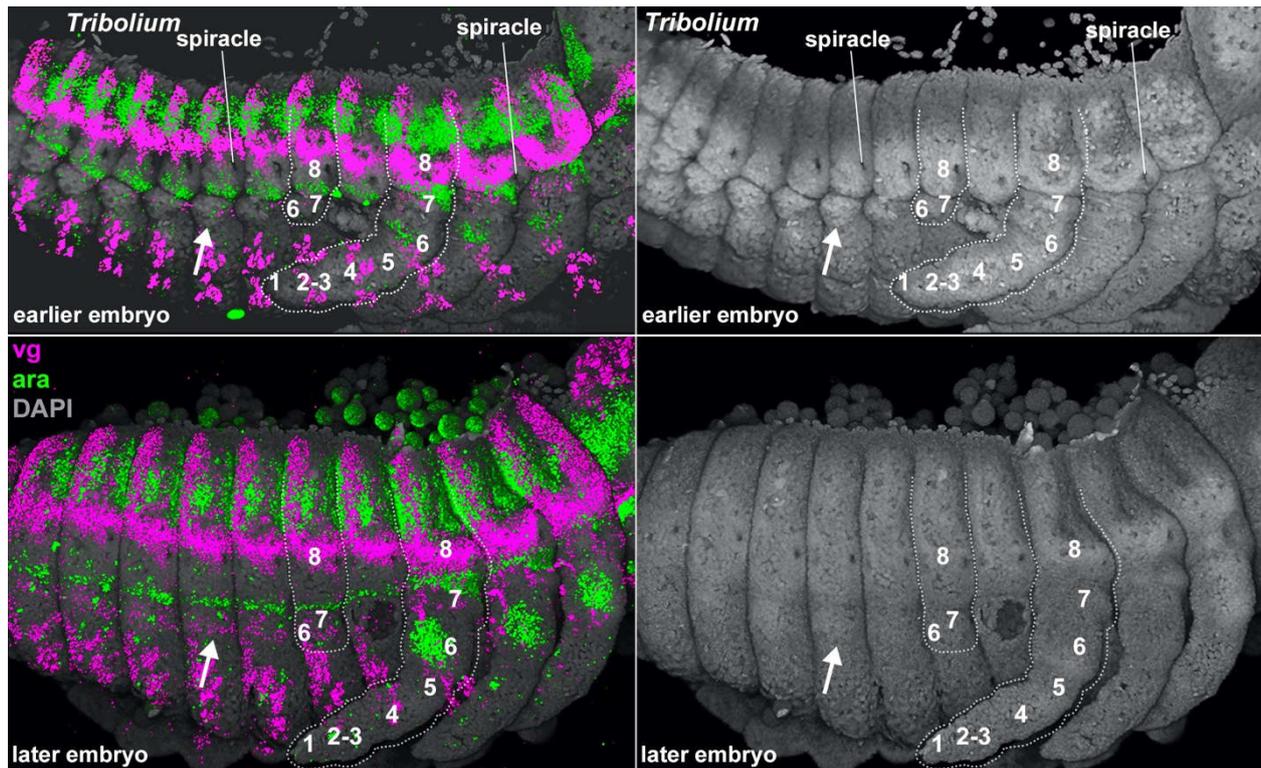
**Figure 1.** Leg segment alignment of arthropod legs based on expression and function of leg genes. From Bruce 2020 and 2021.



**Figure 2.** In all arthropods examined, *araucan* (*ara*) brackets the incorporated 8th leg segment, and *pannier* (*pnr*) is expressed in the dorsal-most tissue and marks the true body wall. A, B. Parhyale, crustacean. C, D. Tribolium, insect. E, F. Acanthoscurria, chelicerate. From Bruce 2021.

## 2. Results

In *Tribolium* embryos, we found that all pregenital abdominal segments develop leg-like paired protrusions. In situ Hybridization Chain Reaction (HCR)<sup>31,32</sup> reveals that the stripes of *pannier* and *araucan* expression that delineate the proximal leg segments of the thorax are expressed in the same configuration around these abdominal protrusions (Figs. 2, 3) as follows. In both the thorax and abdomen, *pnr* is expressed in the dorsal-most tissue, and this dorsal stripe of *pnr* is adjacent to two stripes of *ara* expression. The region bracketed by *ara* is highly similar between the thorax and abdomen: two armbands of *ara* surround one spiracle<sup>10,12,33</sup> along with one eave-like protrusion (paranotal lobe), which is marked with *vestigial* and will later form a tergal plate or a wing. This configuration of gene expression and morphological structures is a hallmark of leg segment 8, which in adult insects forms the body wall (lateral tergum)<sup>10–12,33,34</sup>.



**Figure 3.** Segment identity of abdominal leg nubs in *Tribolium* embryos. Top: approximately stage NS14.1 (Klann 2021)<sup>43</sup>. Bottom: Stage NS15.4 (Klann 2021). Arrow points to 4<sup>th</sup> abdominal leg nub that later degenerates into the body wall. *araucan* (*ara*, green) is expressed in two stripes down the length of the embryo, one dorsal stripe and one lateral stripe, as well as a circular patch on leg segment 6 (coxa) of each thoracic leg. The two stripes bracket the proximal-most 8<sup>th</sup> leg segment that carries both the wing and the spiracle. *vestigial* (*vg*, pink) marks the future wing serial homologs: the wing, elytra, and tergal plates, as well as certain cells in the ventral nerve cord. Note that, in addition to differences in their shape and axial position, cells in the ventral nerve cord are larger and less compact than cells of the leg nub, thus the two are readily distinguished. Gray, DAPI, marks all cell nuclei.

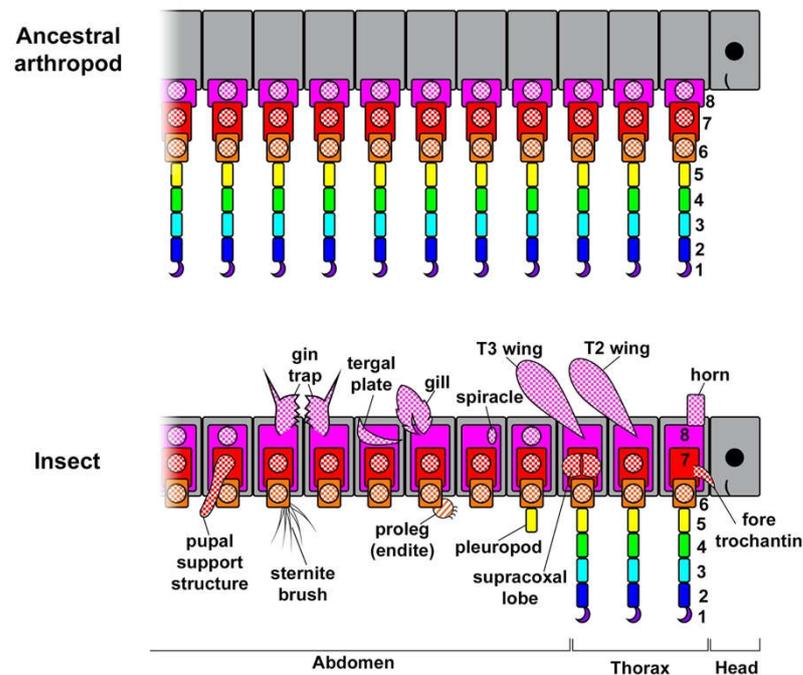
Previous experiments have shown that the insect abdomen does not express *Dll*<sup>25,35-39</sup>, which marks leg segments 1 – 5 (Fig. 1)<sup>10-12,18,38</sup>, but does express *buttonhead* (*btd*) in leg-like, paired circular domains<sup>40</sup>, which in the thoracic legs marks segments 1 – 6<sup>10-12,41,42</sup>. Based on this molecular deduction, the small abdominal protrusions ventral to leg segment 8 may represent leg segment 7 alone, or leg segments 6 and 7. Thus, rather than being completely limbless, the insect abdomen has a pair of legs on all pregenital abdominal body segments, but these abdominal legs are truncated, consisting of just the proximal two or three leg segments 6, 7, and 8 (coxa, pleura, and lateral tergum, respectively). Based on their similar positioning, embryonic development, and gene expression<sup>1-8</sup>, these abdominal leg nubs appear to be serially homologous with the proximal portions of thoracic legs.

### 3. Discussion

The results presented here answer the question of how legs can form on the insect abdomen despite the repression of *Dll* by posterior Hox genes in the insect lineage: only the distal leg, represented by leg segments 1 – 5 (claw to trochanter), is repressed by the Hox genes; the three proximal leg segments that do not depend on *Dll* function<sup>41,42</sup>, i.e., leg segments 6 – 8, are still generated. This is consistent with previous findings that a) loss of *Dll* does not delete the entire insect leg<sup>35-39</sup>; and more importantly, b) *Dll* is not sufficient to initiate leg development<sup>41,42,44</sup>. Together, these observations indicate that leg initiation must be achieved by other, more upstream genes. Candidates that could potentially initiate the entire arthropod leg (i.e., leg segments 1 – 8, which also includes the wing) are

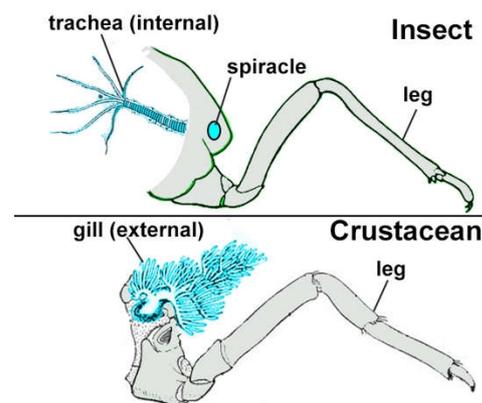
genes such as *btd*<sup>44</sup> and *Sp6-9* (*Sp1* in *Drosophila*)<sup>41,42</sup>, and the juxtaposition of dorsal *dpp* with ventral *wg*<sup>22,45,46</sup>. Notably all of these genes have similar expression in the thorax and abdomen: *btd* is expressed in leg-like, paired, circular domains in both the thorax and the abdomen of insects<sup>40</sup>, and the intersecting stripes of *dpp* and *wg* that initiate leg development in the thorax are similarly expressed in the abdomen. This lends further support to the existence of cryptic insect abdominal legs.

Why truncate these ancestral abdominal legs instead of simply deleting the whole structure? One reason is that several essential structures develop from these proximal leg segments, such as the respiratory system (the spiracle and tracheae)<sup>2,47,48</sup> as well as various exocrine glands like defensive scent glands<sup>49</sup> and oenocytes<sup>2</sup>, which perform lipid processing, pheromone secretion, and developmental signaling<sup>50</sup>. In addition to these essential structures, many other useful structures are also carried on this leg-derived abdominal body wall, including tergal plates, gin traps<sup>51</sup>, knob-like pupal support structures<sup>52</sup>, dorsal “umbilical cord”-like structures in embryos of viviparous earwigs<sup>53</sup>, rod-like sensory organs in certain hemipterans<sup>54</sup>, and larval gills<sup>15,55</sup> (Fig. 4). Furthermore, in some insect lineages, the embryonic abdominal legs do not degenerate and instead form prolegs in caterpillars<sup>14,56</sup>, sawflies<sup>13</sup>, and Dipteran watersnipes<sup>19</sup>, as well as the adult sepsid fly male sternal brushes used in courtship<sup>23</sup>. Many of these insect abdominal structures have been called novel structures, which are commonly defined as structures that are not derived from, or homologous to, any structure in the ancestor nor any other structure in the individual<sup>57</sup>. However, rather than lacking homology, all of these structures likely derived from abdominal leg exites (leg lobes like gills and tergal plates) and legs inherited from their crustacean ancestors that have persisted in a cryptic state in insect embryos<sup>10,12,58</sup>. A similar molecular approach could be used to assay for cryptic abdominal legs in the paraphyletic “entomostracan” crustaceans which, like insects, also appear to lack abdominal legs<sup>59,60</sup>.



**Figure 4.** Model of potential exite locations in arthropods. Top: Generalized arthropod ancestor indicating leg segments (colored rectangles) and regions where leg exites may potentially form (colored circles). Bottom: Generalized insect indicating leg segments and regions where leg exites may potentially form as well as examples of specific structures that are here proposed to be derived from cryptic, truncated abdominal legs. In insect: 1=claw, 2=tarsus, 3=tibia, 4=femur, 5=trochanter, 6=coxa, 7=subcoxa, 8=precoxa. Note that 7 and 8 now form lateral body wall in insects. “Proleg” here refers to Lepidopteran prolegs.

If insect abdominal legs were inherited from their crustacean ancestors, then the functional structures on these legs may also have been inherited from crustaceans<sup>10,61</sup>. Insect tracheae may be internalized crustacean gills (Fig. 5)<sup>44,62</sup>; insect wings, tergal plates, helmets, horns, and other ectodermal outgrowths likely evolved from crustacean plate-type outgrowths<sup>10,12,63</sup>; and insect secretory glands (salivary, endocrine, exocrine, etc.) may have evolved from similar glands in crustaceans<sup>49,62,64</sup>. Surprisingly, respiratory organs and secretory glands can be homeotically transformed into each other<sup>49,62,64</sup> and plate-type outgrowths arise from the same tissue as respiratory organs<sup>65</sup>, therefore all three types of structures may have arisen from a common embryonic exite-like structure on the lateral side of the proximal 8<sup>th</sup> leg segment<sup>10–12</sup> that was inherited from the ancestor of all arthropods. Future studies may determine whether and how the different functional types of exites can be interconverted in nature.



**Figure 5.** Similarity of internal insect tracheae and external crustacean gills. Modified from Snodgrass 1935 and Boxshall 2009.

Notably, multiple exites may emerge from one leg segment in crustaceans, like the anterior and posterior gills (arthrobranchs) of decapods<sup>34,66,67</sup>, and these multiple exites may even have different functions, such as the protective plate, respiratory gill, and brood-care lobe (oostegite) on leg segment 7 (coxa) of amphipod crustaceans like *Parhyale*<sup>34,65,67</sup>. Therefore, it is unsurprising if insects also have multiple exites with divergent functions emerging from the same leg segment, like the wing and spiracle that emerge from leg segment 8 that now forms body wall (lateral tergum)<sup>2,10,33</sup>. It will be interesting to determine whether each leg segment is limited to a set number of exites at restricted locations, or if any number of exites can arise in any location of the leg segment. In the latter case, it may be difficult to track the homology of individual exites within a leg segment over large phylogenetic distances.

This perspective of ancient homology plus divergence, rather than concepts like “partial homology”, explains why structures that have clearly different functions, such as wings and gills, often share some genes but not others: they are anciently homologous as exites, but not as wings, horns, tracheae, etc.<sup>1,15,52,68–72</sup>. Similarly, it is likely that familiar genes such as *vestigial*, *tracheless*, *ventral veins lacking*, *blistered*, and *apterous* confer specific functions and shapes to exites rather than positional identity<sup>44,63,73–75</sup>. While useful for determining whether a structure is derived from an exite, these and other exite-specifying genes are probably less informative for determining positional homology between different arthropods<sup>44,63,73,74</sup>, in contrast to the well-conserved proximal-distal positional markers *pnr* and *ara*, along with joint markers like *odd-skipped*<sup>10–12</sup>.

The above perspective also provides an alternative interpretation of other insect abdominal structures, for example the posterior lobe on the genitalia of male *Drosophila* flies. The posterior lobe has been proposed as a novel structure that resulted when spiracle genes became co-opted into an unrelated structure, the genitalia<sup>76</sup>. However, given that genitals appear to be serially homologous to legs<sup>77–80</sup>, and respiratory structures like spiracles/tracheae are likely derived from the leg, then perhaps the genital “leg” program

retains the ability to activate the spiracle/tracheae program. Given that respiratory structures need not be internal (crustacean gills are external lobes and the *Drosophila* larval posterior spiracle is external), it is plausible that the posterior lobe is an external spiracular structure. Rather than arising through the co-option of genes by an unrelated tissue, the posterior lobe may be the result of de-repression or re-activation of a serial homolog. This hypothesis would be supported if the posterior lobe emerges from the proximal-lateral side of the genital “leg” and if Iroquois genes like *ara* are expressed dorsal and ventral to the lobe.

In summary, the retention of proximal leg segments in the insect abdomen for essential functions like respiration and secretion appears to have allowed the non-essential plate-like outgrowths to become elaborated into new, useful structures like gin traps and camouflage. Thus, cryptic, truncated abdominal legs appear to serve as an important well-spring of new structures and functions in insects.

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