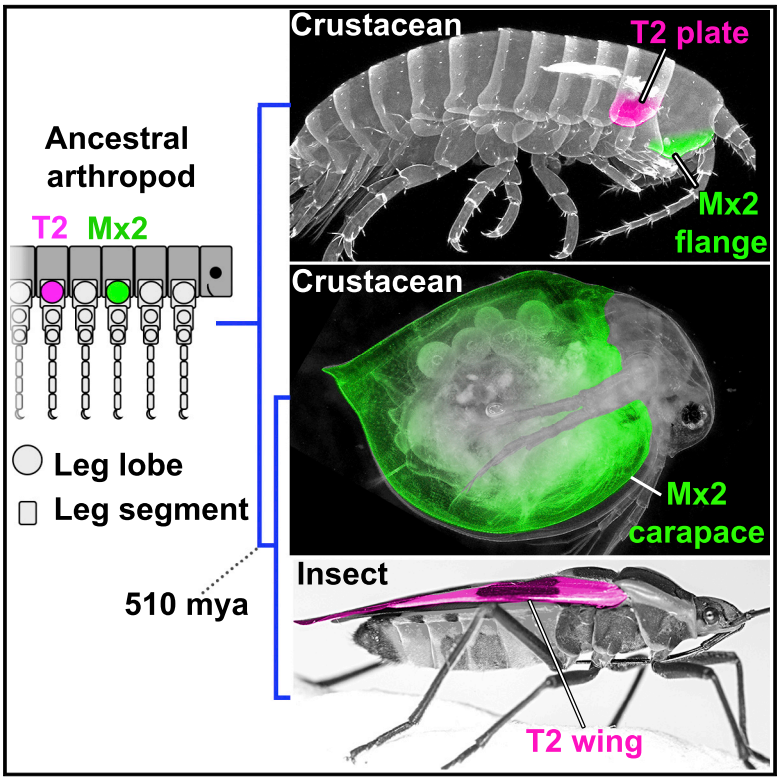
Report

Current Biology

The *Daphnia* carapace and other novel structures evolved via the cryptic persistence of serial homologs

Graphical abstract



Highlights

- The crustacean carapace evolved from an ancient, cryptic head lobe
- This ancient head lobe persists in other arthropods in a subtle or cryptic form
- The crustacean carapace is homologous to the crustacean tergal plate and insect wing
- Cryptic persistence may be a general solution for the origin of novel structures

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In brief

Bruce and Patel show that the carapace of crustaceans like *Daphnia* is homologous to the crustacean tergal plate and insect wing and evolved from an ancient, cryptic head lobe. This ancient head lobe persists in other arthropods in a subtle or cryptic form. Cryptic persistence may thus be a general solution for the origin of novel structures.

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The *Daphnia* carapace and other novel structures evolved via the cryptic persistence of serial homologs

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SUMMARY

Understanding how novel structures arise is a central question in evolution. Novel structures are often defined as structures that are not derived from (homologous to) any structure in the ancestor.[1] The carapace of the crustacean *Daphnia magna* is a bivalved "cape" of exoskeleton. Shiga et al.[2] proposed that the carapace of crustaceans like *Daphnia* and many other plate-like outgrowths in arthropods are novel structures that arose through the repeated co-option of genes like *vestigial* that also pattern insect wings.[2–4] To determine whether the *Daphnia* carapace is a novel structure, we compare previous functional work[2] with the expression of genes known to pattern the proximal leg region (*pannier*, *araucan*, and *vestigial*)[5,6] between *Daphnia*, *Parhyale*, and *Tribolium*. Our results suggest that the *Daphnia* carapace did not arise by co-option but instead derived from an exite (lateral leg lobe) that emerges from an ancestral proximal leg segment that was incorporated into the *Daphnia* body wall. The *Daphnia* carapace, therefore, appears to be homologous to the *Parhyale* tergal plate and the insect wing.[5] Remarkably, the *vestigial*-positive tissue that gives rise to the *Daphnia* carapace appears to be present in *Parhyale*[7] and *Tribolium* as a small, inconspicuous protrusion. Thus, rather than a novel structure resulting from gene co-option, the *Daphnia* carapace appears to have arisen from a shared, ancestral tissue (morphogenetic field) that persists in a cryptic state in other arthropod lineages. Cryptic persistence of unrecognized serial homologs may thus be a general solution for the origin of novel structures.

RESULTS AND DISCUSSION

Origin and identity of the crustacean carapace

Many disparate groups of crustaceans, such as crayfish, barnacles, ostracods, and tadpole shrimp, have a carapace: a fold of exoskeleton that emerges from the dorsal posterior of the head at approximately maxilla 2 and covers some portion of the trunk, which has arisen repeatedly throughout crustaceans and appears to be an ancestral feature of the group.[8–11] It performs a wide variety of functions that have contributed to the success of crustaceans, including brood care, respiration, and hydrodynamic streamlining.[8–11] In the water flea *Daphnia magna*, the carapace forms a bivalved "cape" that surrounds the animal and forms a brood chamber. Shiga et al.[2] performed RNAi knockdown in *Daphnia* embryos which showed that the carapace is patterned by the "wing" genes *wingless* (*wg*), *vestigial* (*vg*), and *scalloped* (*sd*).[2] They therefore proposed that the crustacean carapace and many other flat, lateral lobes in arthropods are novel structures that arose by repeated instances of co-option of these "wing" genes.

The crustacean carapace may be derived from an exite

However, the analyses in Bruce and Patel[5] and Bruce[6] suggest an alternative hypothesis. Drawing on over a century of morphological and embryological studies as well as gene expression and loss-of-function studies, they generated a molecular coordinate system for identifying the homologies of any arthropod ectodermal structure (Figure 1). Based on this work, it appears that most arthropods have incorporated one or two ancestral proximal leg segments into the body wall[5,6,12–17] (leg segments 7 and 8, counting from the terminal claw), but the division between "true" body wall (dorsal tergum) and the incorporated leg segments that now function as body wall (pleura) can still be distinguished by the expression of *pannier* (*pnr*) and the *Iroquois* gene *araucan* (*ara*), as follows. In the embryos of all arthropods examined to date, representing three of the four living arthropod groups—*Drosophila melanogaster* (fruit fly; insect),[18–21] *Tribolium castaneum* (flour beetle; insect), *Parhyale hawaiensis* (amphipod; crustacean), and *Acanthoscurria geniculata* (tarantula; chelicerate)—*ara* expression brackets the hypothesized incorporated 8th leg segment, whereas *pnr* is expressed in the dorsal-most tissue and marks the true body wall.[6] Thus, in contrast to other leg patterning genes,[22] the expression patterns of *pnr* and *ara* appear to be highly conserved across arthropods. Based on observations in Clark-Hachtel and Tomoyasu,[7] Bruce and Patel[5] showed that the insect wing and *Parhyale* tergal plate are both derived from an ancestral exite (a multi-functional lobe that emerges from proximal leg

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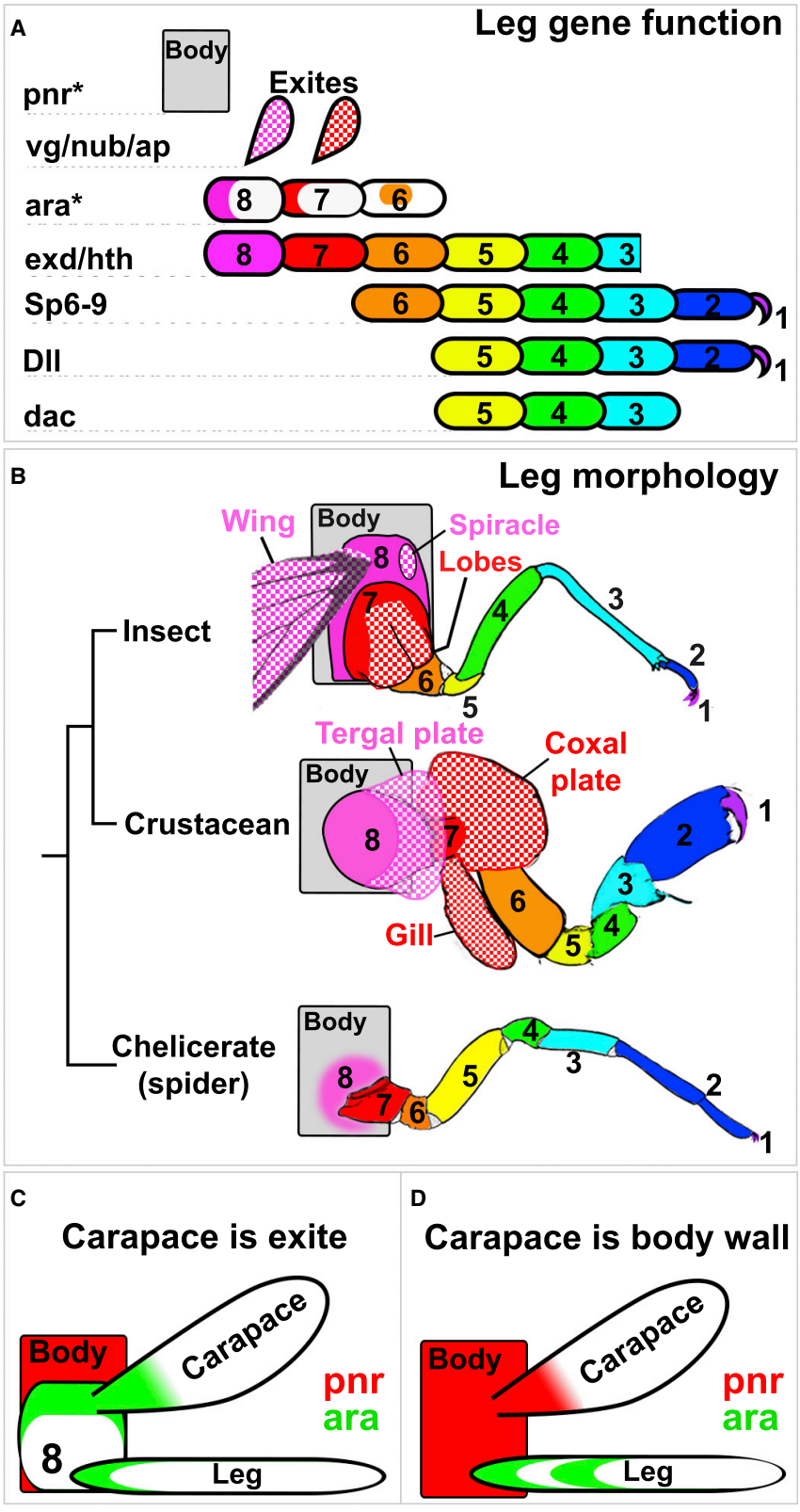

Figure 1. Model of how to align all arthropod legs

(A) A schematic of the leg structures patterned by each gene in chelicerates, crustaceans, and insects provides a model for how to align arthropod legs. Based on the function of *extradenticle* (*exd*), *homothorax* (*hth*), *Distalless* (*Dll*), *Sp6-9*, and *dachshund* (*dac*), the six distal leg segments (leg segment 1 through leg segment 6) of chelicerates, crustaceans, and insects correspond with each other in a one-to-one fashion. The alignment of the two proximal leg segments is based on expression of *pannier* (*pnr*), *araucan* (*ara*), and *odd-skipped* in chelicerates, crustaceans, and insects, and the function of wing/exite genes in insects and crustaceans. From Bruce and Patel.[5]

(B) Morphology and proposed homologies of arthropod leg segments. Colors and patterns indicate proposed homologies. Exites (checker pattern). Insect and spider drawings were modified from Snodgrass.[29] Panel modified from Bruce and Patel.[5]

(C and D) Based on the above model, predictions can be made about the expression of *pnr* (red) and *ara* (green) in the *Daphnia* carapace. If the carapace is an exite on an incorporated 8th leg segment (C), *pnr* will be expressed in a narrow stripe dorsal to the carapace, and the *ara* domain adjacent to *pnr* will extend into the carapace. If the carapace is an outgrowth of the body wall (D), then *pnr* will extend into the carapace, and the two domains of *ara* will be located ventral to the carapace.

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segments[23] and that is patterned by "wing" genes such as *vg* and *sd*[7,24,25]) that emerges from the ancestral leg segment 8 that was incorporated into the body wall (Figure 1).[26–28]

Based on this previous work, the morphological and molecular data in Shiga et al.[2] suggest that the *Daphnia* carapace did not arise by co-option but instead is derived from an ancient exite on an incorporated 8th leg segment of the head. The *Daphnia* carapace would therefore be homologous to the *Parhyale* tergal plate[5,7] and the insect wing,[5] in the sense that all three derive from plate-like exites that emerge from the incorporated 8th leg segment.

To test the proximal-distal register of the *Daphnia* carapace, the expression of *pnr*, *ara*, and *vg* was examined in embryos of *Daphnia magna*, *Tribolium castaneum*, and *Parhyale hawaiensis* using *in situ* hybridization chain reaction (HCR) version 3.0.[30,31] A single *pnr* gene was identified in *Daphnia* (Figure S1). A single *Iroquois* gene with closest homology to *ara/caupolican/iro2* was identified in *Daphnia* on NCBI (Figure S1). This *Daphnia* gene is hereafter referred to as *ara*.[5] *Daphnia vg* was identified previously by Shiga et al.[2]

The *Daphnia* carapace is an exite of the head

If the *Daphnia* carapace is the exite of the incorporated 8th leg segment, then our model predicts that *pnr* will be expressed in a narrow stripe dorsal to the carapace, and the *ara* domain adjacent to *pnr* will extend into the carapace (Figure 1C). Alternatively, if the carapace is a dorsal, non-leg-derived structure, then *pnr* expression should extend into the carapace, and the two domains of *ara* will be located ventral to the carapace (Figure 1D). In either case, *vg* will be expressed along the edge of the carapace.[2]

Consistent with the hypothesis that the carapace is an exite on leg segment 8, *Daphnia vg* is expressed along the edge of the carapace, *pnr* is restricted to a narrow, dorsal stripe above the carapace, and the *ara* domain adjacent to *pnr* extends into the carapace (Figures 2 and 3).

The head exite persists in arthropods without a carapace

If the *Daphnia* carapace is the exite of the incorporated 8th leg segment of a mouthpart (modified leg) on the head, this exite may be present on the head appendages of arthropods that do not form a carapace. In support of this hypothesis, *vg* is expressed in the head of *Tribolium* and *Parhyale* dorsal/proximal to the mouthparts (Figures S3A and S3B). This *vg* domain is bracketed by *ara* expression, just like the insect wing, the *Parhyale* tergal plate, and the *Daphnia* carapace. This region is therefore presumably homologous to the 8th leg segment. Notably, there is no obvious structure associated with the *Tribolium* mouthpart *vg* domain. In *Parhyale*, *vg* patterns the flange-like protrusion that protects the mouthparts because the flange is reduced when *vg* is knocked out (Figures S3C and S3D).[7] This flange emerges from the incorporated 8th leg segment because it is bracketed by *ara* expression. Given that the arthropod head is composed of several body segments compacted into a contiguous unit,[33] the head flange likely represents several adjacent exites. Thus, rather than new, co-opted domains of *vg* expression, these *vg* head domains are ancient and conserved.

Carapaces, plates, and wings are homologous as exites

The RNAi knockdown data in Shiga et al.,[2] together with the expression data presented here, suggest that the *Daphnia* carapace evolved by posterior expansion of the exite of the incorporated 8th leg segment of maxilla 2 (Figure S4). The *Daphnia* carapace would therefore be homologous to the *Parhyale* tergal plate and the insect wing, in the sense that all three derive from plate-like exites that emerge from the incorporated 8th leg segment. Notably, similar tergal plates can be found in neighboring lineages such as cephalocarids ("tergopleurae"),[34] remipedes ("pleurotergites"),[35] and silverfish ("paranotal lobes"), and like *Parhyale*, these plates may also emerge from an incorporated 8th leg segment (Figure 4). If so, then insect wings and the *Daphnia* carapace would be homologous to structures in neighboring lineages, situating them in a homologous sequence rather than being *de novo* structures. This sequence demonstrates how the same exite program deployed at various serially homologous positions along the anterior-posterior axis can become expanded in different lineages to form what appear to be novel structures.

Similarly, the work presented here may shed light on the origin and homologies of crustacean carapaces in general. The carapace is present in some but not all crustacean lineages, but it has been proposed to be homologous across crustaceans.[8–11] This suggests that all crustaceans have a latent ability to make a carapace. If the *Daphnia* carapace is derived from a cryptic exite, then the carapaces of crustaceans in general may derive from exites. This would explain the latent ability of crustaceans to form carapaces. Thus, rather than repeated co-option of "wing" genes as proposed by Shiga et al.,[2] the emergence of carapaces in various lineages may represent repeated expansions of existing structures.

Co-option versus cryptic persistence

Understanding how novel structures arise is a central question in evolution. Novelties are often defined as structures that are not homologous to any structure in the ancestor nor to any other structure in the same organism.[1] Co-option of genetic pathways and "deep homology" have become a dominant explanation for the origin of novel structures within the field of evolutionary developmental biology (evo devo).[2–4,37–46] For example, arthropod legs and bodies are decorated with a fascinating diversity of structures, including carapaces, plates, knobs, gills, horns, helmets, and so on.[4,24,47] Several of these have been proposed to be novel, the result of co-option events where components of the insect wing patterning network become expressed (perhaps at non-homologous positions) where they generate a *de novo* structure.[2,38,39,41,42,45,48–50]

However, the work presented here, and in Bruce and Patel[5] and Bruce,[6] provides an alternative hypothesis: these leg-associated structures (and perhaps other novel structures too) arise from unrecognized, serially homologous morphogenetic fields (morphogenetic field = a discrete set of cells programmed to form a specific organ, e.g., leg, wing, bristle, etc.). In this model, morphogenetic fields can persist in a cryptic, unrecognizable form (such as the *Parhyale* head flange; Figures 4 and S3) in intermediate lineages and become elaborated again in later lineages (such as the *Daphnia* carapace; Figure 4), such that they may no longer be recognizable as the ancestral

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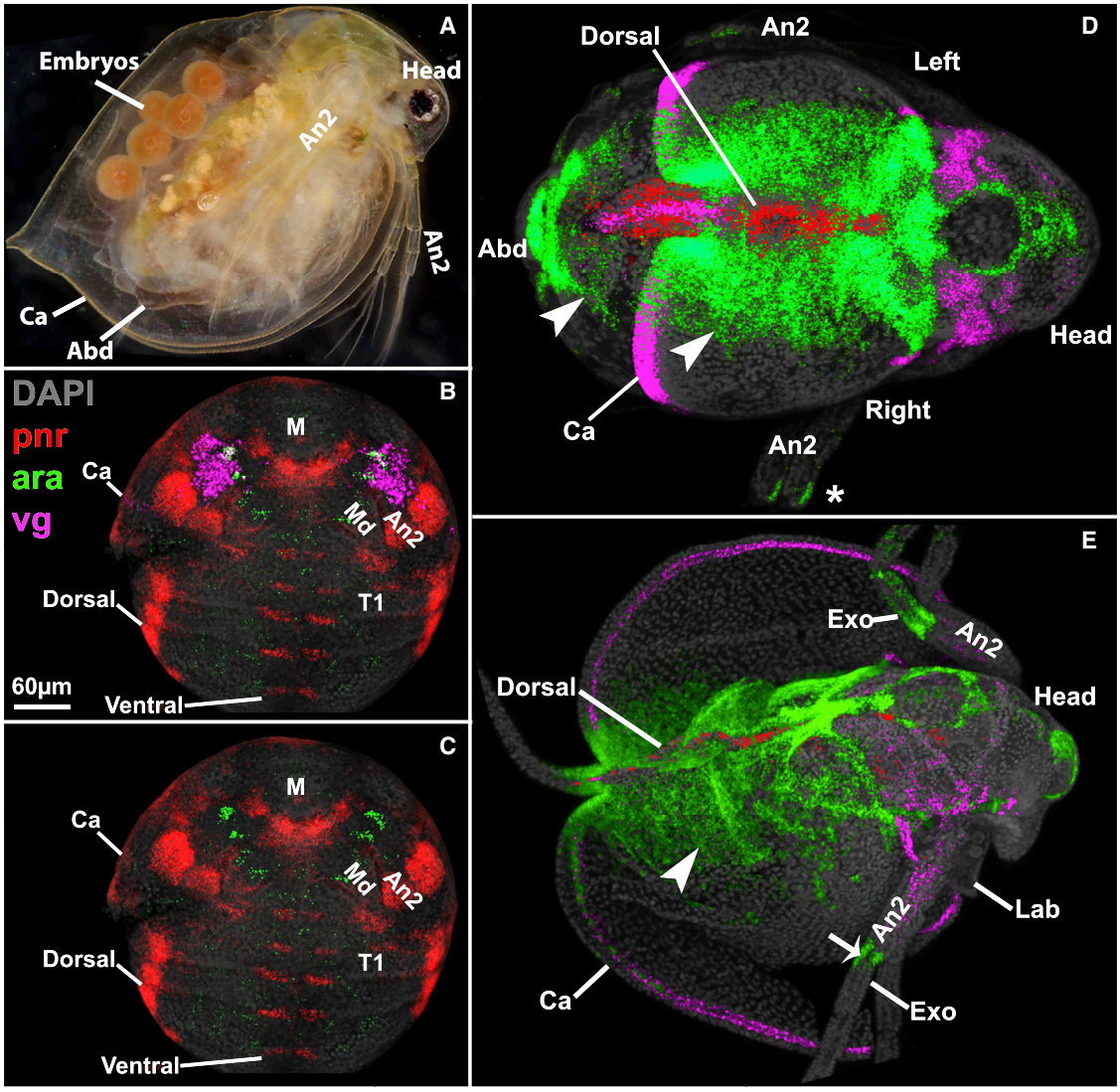

Figure 2. Expression of *pannier* (*pnr*), *araucan* (*ara*), and *vestigial* (*vg*) in *Daphnia* embryos

(A) *Daphnia* adult with embryos under carapace (Ca), lateral view.

(B and C) Stage 7 embryo,[32] ventral view.

(D) Stage 9 embryo, dorsal view.

(E) Dissected head and carapace of Stage 11 embryo, dorsal view, showing *ara* and *pnr* expression without trunk underneath. *pnr* marks the dorsal-most domain. *ara* is expressed in four domains: in a dorsal region adjacent to *pnr* (closed arrowhead), in a second domain on leg segment 7 (not visible here), on leg segment 6 (arrow; in *Daphnia*, leg segment 6 is identifiable by the presence of the exopod, Exo), and in the tip of the leg (visible in antenna 2, An2). *vg* is expressed in the perimeter of the carapace (Ca), and in the mesoderm of antenna. M, mouth. Md, mandible. Abd, abdomen. *pnr* (red), *ara* (green), *vg* (pink), DAPI (gray). *Daphnia* staging based on Mittmann et al.[32]

See also Figures S1 and S2.

structure and appear to be novel. Structures would not have to be continuously present in a morphologically obvious state from ancestor to descendant to be considered homologous. Indeed, a morphogenetic field may not form an obvious structure at all, as in the *vg*-positive regions of the *Tribolium* head (Figure S3). Rather than *de novo* co-options, these morphogenetic fields are always there, persisting in a dormant, truncated, or highly modified state and de-repressed in various lineages to form what look like novel structures. One prediction of this model is that "novel" structures will form at molecularly predetermined and predictable locations rather than at random locations.

Cryptic persistence may explain many novel structures

Kukalová-Peck[26] first proposed that insect wings may derive from crustacean exites, leading to several studies investigating this possibility.[51–53] Insect evo-devo researchers then began noticing that many insect body wall outgrowths shared the same regulatory pathway with wings, and these outgrowths were proposed to have arisen by numerous co-option

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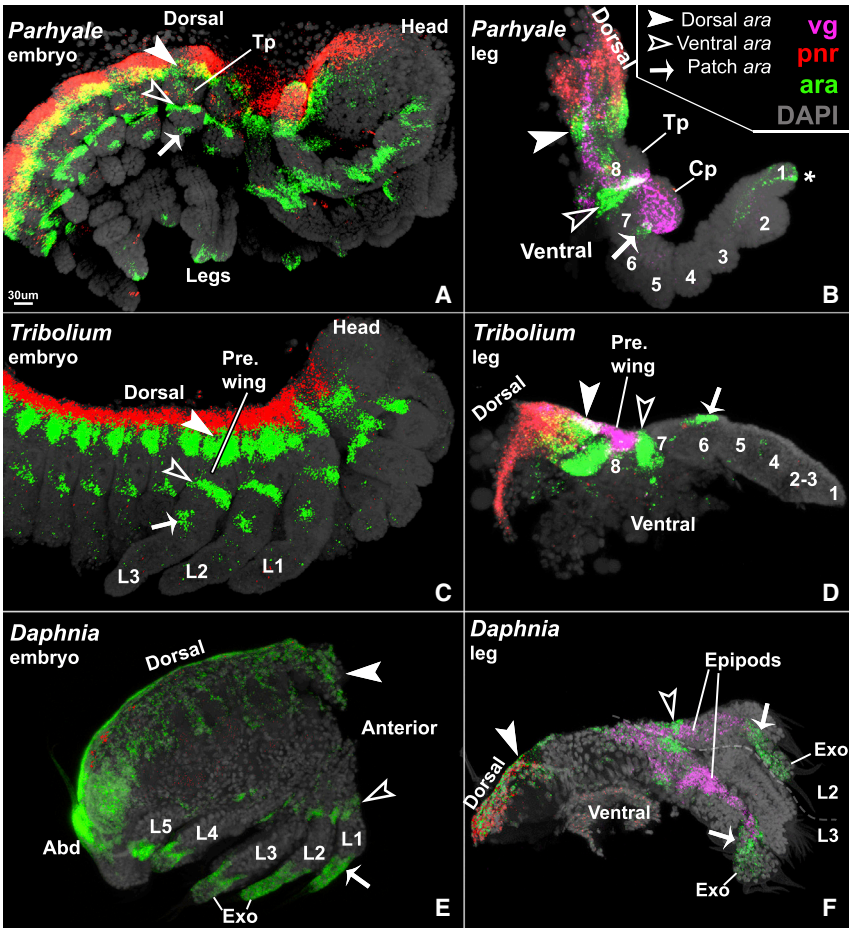

Figure 3. Expression of *pannier* (*pnr*) and *araucan* (*ara*) elucidates the proximal leg segments

Dissected right half of *Parhyale* (A), *Tribolium* (C), and *Daphnia* (E) embryos. *Daphnia* head and carapace were removed to reveal trunk. Dissected legs of *Parhyale* (B), *Tribolium* (D), and *Daphnia* (F) embryos. *Daphnia* legs are phyllopodous, so individual leg segments cannot be counted. *pnr* (red) marks the dorsal-most domain. *ara* (green) is expressed in four domains: a dorsal region adjacent to *pnr* (closed arrowhead); a second region on leg segment 7 (open arrowhead); a third region on leg segment 6 (arrow; in *Daphnia*, leg segment 6 is identifiable by the presence of the exopod, Exo); and a fourth region, in crustaceans and tarantula,[6] in the tip (*) of leg segment 1. *vestigial* (*vg*, pink) is expressed along the edge of exites, including the coxal plate (Cp) on leg segment 7, the tergal plate (Tp) on leg segment 8, the presumptive wing (Pre.wing), and the epipods of *Daphnia*. (A) and (C) are from Bruce and Patel.[5] See also Figures S1 and S2.

events.[2,38,39,41,42,45,48–50] However, this previous work centered on insects and was not situated within the crustacean morphology literature. In 2018, Bruce and Patel[54] proposed that, like insect wings, other insect outgrowths may also be derived from crustacean exites, including the *Oncopeltus* supracoxal lobe, the thoracic stylus of jumping bristletails, beetle gin traps, and insect abdominal gills. In contrast to co-option, Bruce and Patel[54] suggested that insect outgrowths arise from ancient, conserved exite fields via a cryptic persistence mechanism. Given that most arthropods appear to have incorporated the proximal leg into the body wall[5,6] and that exites evolved prior to the divergence of modern arthropods[6,55] and occur on any and all body segments,[24] many arthropod outgrowths may in fact be derived from exites.[6,51–54,56–58] It is, therefore, not surprising that insects have ectodermal outgrowths on any and every body segment; it is expected. We propose that exite fields are poised on leg segments 6–8 of every leg on every body segment in all arthropods, ready to be de-repressed/activated under the right circumstances and then molded into new shapes and functions by evolution.

Cryptic persistence of morphogenetic fields may provide a mechanistically satisfying explanation for the origin of novel structures in general. Many unexpected structures may be related and far more ancient and evolvable than currently believed, which has deep implications for how we assume genetic networks evolve.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Imaging
  - Gene phylogenies

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.06.073.

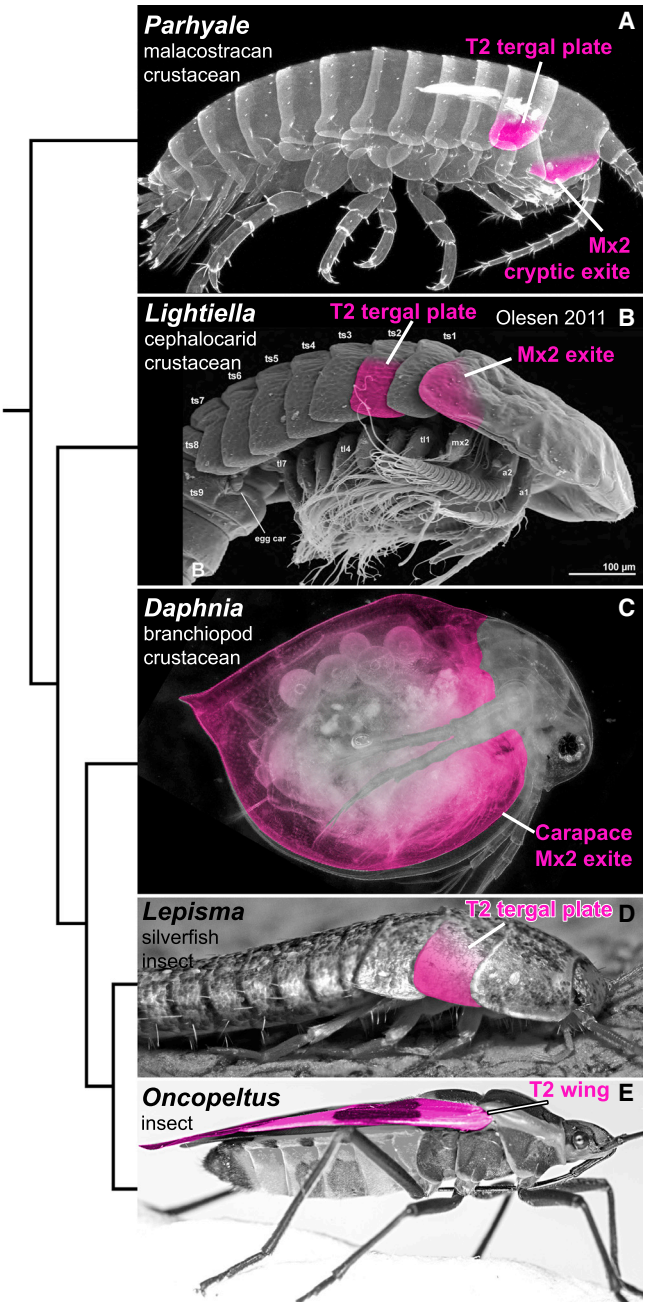

Figure 4. The Daphnia carapace is hypothesized to be the exite of the incorporated 8th leg segment, homologous to the *Parhyale* tergal plate and insect wing

Similar tergal plates can be found in cephalocarids and silverfish and may also emerge from an incorporated 8th leg segment. Structures that are proposed to be homologous as exites on the 8th leg segment of max2/labium and thoracic segment 2 (T2) are indicated with pink shading. Max2/labium of insect is not shaded because it is unclear whether any exite-like structure forms here. Phylogeny based on Lozano-Fernandez et al.[36] *Oncopeltus* image credit: Aaron Pomerantz. See also Figures S3 and S4.

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AUTHOR CONTRIBUTIONS

H.S.B. conceived, designed, and performed the experiments and wrote the manuscript. N.H.P. edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

1. Müller, G.B., and Wagner, G.P. (1991). Novelty in evolution: restructuring the concept. Annu. Rev. Ecol. Syst. *22*, 229–256.
2. Shiga, Y., Kato, Y., Aragane-Nomura, Y., Haraguchi, T., Saridaki, T., Watanabe, H., Iguchi, T., Yamagata, H., and 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. Preprint at bioRxiv. https://doi.org/10.1101/160010.
3. Hu, Y., Schmitt-Engel, C., Schwirz, J., Stroehlein, N., Richter, T., Majumdar, U., and Bucher, G. (2018). A morphological novelty evolved by co-option of a reduced gene regulatory network and gene recruitment in a beetle. Proc. Biol. Sci. *285*, 20181373–20181379.
4. Fisher, C.R., Wegrzyn, J.L., and Jockusch, E.L. (2020). Co-option of wing-patterning genes underlies the evolution of the treehopper helmet. Nat. Ecol. Evol. *4*, 250–260.
5. Bruce, H.S., and Patel, N.H. (2020). Knockout of crustacean leg patterning genes suggests that insect wings and body walls evolved from ancient leg segments. Nat. Ecol. Evol. *4*, 1703–1712.
6. Bruce, H.S. (2021). How to align arthropod leg segments. Preprint at bioRxiv. https://doi.org/10.1101/2021.01.20.427514.
7. Clark-Hachtel, C.M., and 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.
8. Calman, W.T. (1909). A Treatise on Zoology. Part VII. Appendiculata. Third Fascicle Crustacea, R Lankester., ed. (Adam and Charles Black).
9. Fryer, G. (1996). The carapace of the branchiopod Crustacea. Phil. Trans. R. Soc. Lond. B *351*, 1703–1712.
10. Olesen, J. (2013). The crustacean carapace. In Functional Morphology and Diversity (Oxford University Press), pp. 103–139.
11. Newman, W.A., and Knight, M.D. (1984). The carapace and crustacean evolution—a rebuttal. J. Crustac. Biol. *4*, 682–687.
12. Snodgrass, R.E. (1927). Morphology and Mechanism of the Insect Thorax (Smithsonian Institution).
13. Kobayashi, Y., Niikura, K., Oosawa, Y., and Takami, Y. (2013). Embryonic development of Carabus insulicola (Insecta, Coleoptera, Carabidae) with special reference to external morphology and tangible evidence for the subcoxal theory. J. Morphol. *274*, 1323–1352.
14. Matsuda, R. (1970). Morphology and evolution of the insect thorax. Mem. Entomol. Soc. Can. *102*, 5–431.
15. Tiegs, O.W. (1940). The embryology and affinities of the symphyla based on a study of Hanseniella agilis. J. Cell Sci. *s2*, 1–208.
16. Ewing, H.E. (1928). The Legs and Leg-Bearing Segments of Some Primitive Arthropod Groups, with Notes on Leg-Segmentation in the Arachnida (Smithsonian Institution), pp. 1–41.

17. Deuve, T. (2018). What is the epipleurite? A contribution to the subcoxal theory as applied to the insect abdomen. Ann. Soc. Entomol. Fr. *54*, 1–26.
18. Ikmi, A., Netter, S., and Coen, D. (2008). Prepatterning the Drosophila notum: the three genes of the iroquois complex play intrinsically distinct roles. Dev. Biol. *317*, 634–648.
19. Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., Simpson, P., and Morata, G. (2000). Generation of medial and lateral dorsal body domains by the pannier gene of Drosophila. Development *127*, 3971–3980.
20. Diez del Corral, R., Aroca, P., Gómez-Skarmeta, J.L., Cavodeassi, F., and Modolell, J. (1999). The Iroquois homeodomain proteins are required to specify body wall identity in Drosophila. Genes Dev. *13*, 1754–1761.
21. Cavodeassi, F., Diez del Corral, R., Campuzano, S., and Domínguez, M. (1999). Compartments and organising boundaries in the Drosophila eye: the role of the homeodomain Iroquois proteins. Development *126*, 4933–4942.
22. Abzhanov, A., and Kaufman, T.C. (2000). Homologs of Drosophila appendage genes in the patterning of arthropod limbs. Dev. Biol. *227*, 673–689.
23. Maas, A., Haug, C., Haug, J.T., and Olesen, J. (2009). Early crustacean evolution and the appearance of epipodites and gills. Arthropod Syst. Phylogeny *67*, 255–273.
24. Boxshall, G.A., and Jaume, D. (2009). Exopodites, epipodites and gills in Crustaceans. Arthropod Syst. Phylogeny *67*, 229–254.
25. Ruiz-Losada, M., Blom-Dahl, D., Córdoba, S., and Estella, C. (2018). Specification and patterning of Drosophila appendages. J. Dev. Biol. *6*, 17.
26. Kukalová-Peck, J. (1983). Origin of the insect wing and wing articulation from the arthropodan leg. Can. J. Zool. *61*, 1618–1669.
27. Ohde, T., Mito, T., and Niimi, T. (2022). A hemimetabolous wing development suggests the wing origin from lateral tergum of a wingless ancestor. Nat. Commun. *13*, 979.
28. Tomoyasu, Y. (2021). What crustaceans can tell us about the evolution of insect wings and other morphologically novel structures. Curr. Opin. Genet. Dev. *69*, 48–55.
29. Snodgrass, R.E. (1952). A Textbook of Arthropod Anatomy (Cornell University Press).
30. Choi, H.M.T., Schwarzkopf, M., Fornace, M.E., Acharya, A., Artavanis, G., Stegmaier, J., Cunha, A., and Pierce, N.A. (2018). Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. Development *145*. dev165753–dev165122.
31. Bruce, H.S., Jerz, G., Kelly, S., McCarthy, J., Pomerantz, A., Senevirathne, G., Sherrard, A., Sun, D.A., Wolff, C., and Patel, N.H. (2021). Hybridization chain reaction (HCR) in situ protocol. protocols.io. https://doi.org/10.17504/protocols.io.bunznvf6.
32. Mittmann, B., Ungerer, P., Klann, M., Stollewerk, A., and Wolff, C. (2014). Development and staging of the water flea Daphnia magna (Straus, 1820; Cladocera, Daphniidae) based on morphological landmarks. EvoDevo *5*, 12.
33. Ortega-Hernández, J., and Budd, G.E. (2016). The nature of non-appendicular anterior paired projections in Palaeozoic total-group Euarthropoda. Arthropod Struct. Dev. *45*, 185–199.
34. Olesen, J., Haug, J.T., Maas, A., and Waloszek, D. (2011). External morphology of Lightiella monniotae (Crustacea, Cephalocarida) in the light of Cambrian 'Orsten' crustaceans. Arthropod Struct. Dev. *40*, 449–478.
35. Koenemann, S., Schram, F.R., Hönemann, M., and Iliffe, T.M. (2007). Phylogenetic analysis of Remipedia (Crustacea). Organ. Divers. Evol. *7*, 33–51.
36. Lozano-Fernandez, J., Giacomelli, M., Fleming, J.F., Chen, A., Vinther, J., Thomsen, P.F., Glenner, H., Palero, F., Legg, D.A., Iliffe, T.M., et al. (2019). Pancrustacean evolution illuminated by taxon-rich genomic-scale data sets with an expanded remipede sampling. Genome Biol. Evol. *11*, 2055–2070.
37. Davidson, E.H., and Erwin, D.H. (2006). Gene regulatory networks and the evolution of animal body plans. Science *311*, 796–800.
38. Bowsher, J.H., and Nijhout, H.F. (2009). Partial co-option of the appendage patterning pathway in the development of abdominal appendages in the sepsid fly Themira biloba. Dev. Genes Evol. *219*, 577–587.
39. Monteiro, A. (2012). Gene regulatory networks reused to build novel traits: co-option of an eye-related gene regulatory network in eye-like organs and red wing patches on insect wings is suggested by optix expression. BioEssays *34*, 181–186.
40. Lee, P.N., Callaerts, P., De Couet, H.G., and Martindale, M.Q. (2003). Cephalopod Hox genes and the origin of morphological novelties. Nature *424*, 1061–1065.
41. Emlen, D.J., Corley Lavine, L., and Ewen-Campen, B. (2007). On the origin and evolutionary diversification of beetle horns. Proc. Natl. Acad. Sci. USA *104* (*Suppl 1*), 8661–8668.
42. Moczek, A.P. (2009). On the origins of novelty and diversity in development and evolution: a case study on beetle horns. Cold Spring Harb. Symp. Quant. Biol. *74*, 289–296.
43. Shubin, N., Tabin, C., and Carroll, S. (2009). Deep homology and the origins of evolutionary novelty. Nature *457*, 818–823.
44. Shubin, N., Tabin, C., and Carroll, S. (1997). Fossils, genes and the evolution of animal limbs. Nature *388*, 639–648.
45. Glassford, W.J., Johnson, W.C., Dall, N.R., Smith, S.J., Liu, Y., Boll, W., Noll, M., and Rebeiz, M. (2015). Co-option of an ancestral hox-regulated network underlies a recently evolved morphological novelty. Dev. Cell *34*, 520–531.
46. True, J.R., and Carroll, S.B. (2002). Gene co-option in physiological and morphological evolution. Annu. Rev. Cell Dev. Biol. *18*, 53–80.
47. Boxshall, G.A. (2004). The evolution of arthropod limbs. Biol. Rev. Camb. Philos. Soc. *79*, 253–300.
48. Fisher, C.R., Kratovil, J.D., Angelini, D.R., and Jockusch, E.L. (2021). Out from under the wing: reconceptualizing the insect wing gene regulatory network as a versatile, general module for body-wall lobes in arthropods. Proc. Biol. Sci. *288*, 20211808.
49. Hu, Y., Linz, D.M., and Moczek, A.P. (2019). Beetle horns evolved from wing serial homologs. Science *366*, 1004–1007.
50. Hu, Y., and Moczek, A.P. (2021). Wing serial homologues and the diversification of insect outgrowths: insights from the pupae of scarab beetles. Proc. Biol. Sci. *288*, 20202828.
51. Averof, M., and Cohen, S.M. (1997). Evolutionary origin of insect wings from ancestral gills. Nature *385*, 627–630.
52. Damen, W.G.M., Saridaki, T., and Averof, M. (2002). Diverse adaptations of an ancestral gill: a common evolutionary origin for wings, breathing organs, and spinnerets. Curr. Biol. *12*, 1711–1716.
53. Franch-Marro, X., Martín, N., Averof, M., and Casanova, J. (2006). Association of tracheal placodes with leg primordia in Drosophila and implications for the origin of insect tracheal systems. Development *133*, 785–790.
54. Bruce, H.S., and Patel, N.H. (2018). Insect wings and body wall evolved from ancient leg segments. Preprint at bioRxiv. https://doi.org/10.1101/244541.
55. Liu, Y., Edgecombe, G.D., Schmidt, M., Bond, A.D., Melzer, R.R., Zhai, D., Mai, H., Zhang, M., and Hou, X. (2021). Exites in Cambrian arthropods and homology of arthropod limb branches. Nat. Commun. *12*, 4619.
56. Grillo, M., Casanova, J., and Averof, M. (2014). Development: a deep breath for endocrine organ evolution. Curr. Biol. *24*, R38–R40.
57. Sánchez-Higueras, C., Sotillos, S., and Castelli-Gair Hombría, J. (2014). Common origin of insect trachea and endocrine organs from a segmentally repeated precursor. Curr. Biol. *24*, 76–81.
58. Hanna, L., and Popadić, A. (2020). A hemipteran insect reveals new genetic mechanisms and evolutionary insights into tracheal system development. Proc. Natl. Acad. Sci. USA *117*, 4252–4261.
59. Huelsenbeck, J.P., and Ronquist, F. (2001). MRBAYES: bayesian inference of phylogenetic trees. Bioinformatics *17*, 754–755.

60. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods *9*, 671–675.

61. Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. *7*, 539.

62. Darriba, D., Taboada, G.L., Doallo, R., and Posada, D. (2011). ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics *27*, 1164–1165.

63. Cho, Y., Jonas-Closs, R.A., Yampolsky, L.Y., Kirschner, M.W., and Peshkin, L. (2022). Intelligent high-throughput intervention testing platform in Daphnia. Aging Cell *21*, e13571.

64. Klüttgen, B., Dülmer, U., Engels, M., and Ratte, H.T. (1994). ADaM, an artificial freshwater for the culture of zooplankton. Water Res. *28*, 743–746.

65. Clark-Hachtel, C.M., Linz, D.M., and 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*, 16951–16956.

66. Klann, M., Schacht, M.I., Benton, M.A., and Stollewerk, A. (2021). Functional analysis of sense organ specification in the Tribolium castaneum larva reveals divergent mechanisms in insects. BMC Biol. *19*, 22.

67. Browne, W.E., Price, A.L., Gerberding, M., and Patel, N.H. (2005). Stages of embryonic development in the amphipod crustacean. Parhyale hawaiensis. genesis *42*, 124–149.

68. Gillis, W.Q., Bowerman, B.A., and Schneider, S.Q. (2008). The evolution of protostome GATA factors: molecular phylogenetics, synteny, and intron/exon structure reveal orthologous relationships. BMC Evol. Biol. *8*, 112.

69. Kerner, P., Ikmi, A., Coen, D., and Vervoort, M. (2009). Evolutionary history of the iroquois/Irx genes in metazoans. BMC Evol. Biol. *9*, 74.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
UltraPure glycerol	Invitrogen	Catalog number 15514029
32% aqueous paraformaldehyde	Electron Microscopy Sciences	Catalog number 15714-S
RGComplete	Reed Mariculture	https://reefnutrition.com/
Sylgard 184	Dow Corning	Material Number 1673921
Experimental models: Organisms/strains		
Daphnia magna, strain IL-MI-8 from pond in Jerusalem, Israel	Leonid Peshkin, Harvard Medical School	N/A
Parhyale hawaiensis, strain Chicago-F	Nipam Patel, Marine Biological Laboratory	N/A
Tribolium castaneum	Yoshinori Tomoyasu, Miami University	N/A
Oligonucleotides		
Daphnia magna pannier	GenBank: XP_032779631.1	Molecular Instruments Lot Number: PRG133
Daphnia magna araucan	GenBank: KZS08873.1	Molecular Instruments Lot Number: PRG134
Daphnia magna vestigial	GenBank: AB465512	Molecular Instruments Lot Number: PRG132
Tribolium castaneum pannier	GenBank: XM_008202266.2	Molecular Instruments Lot Number: PRD736
Tribolium castaneum araucan	GenBank: XM_008194186.2	Molecular Instruments Lot Number: PRD737
Tribolium castaneum vestigial	GenBank: XM_008201106.2	Molecular Instruments Lot Number: PRD232
Parhyale hawaiensis pannier	GenBank: MT103930.1	Molecular Instruments Lot Number: PRD223
Parhyale hawaiensis araucan	GenBank: MT103931.1	Molecular Instruments Lot Number: PRD221
Parhyale hawaiensis vestigial	GenBank: MG703506.1; B4 initiator adapter, 20 probe pairs (40 sequences)	See Table S1 in supplemental information
Software and algorithms		
Mr. Bayes plug-in for Geneious	Huelsenbeck and Ronquist[59]	https://www.geneious.com/features/phylogenetic-tree-building/
Geneious free version	Geneious Prime 2019	https://www.geneious.com/
FigTree v1.4.4	Rambaut, A. FigTree v1.3.1. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh.	http://tree.bio.ed.ac.uk/software/figtree/
ImageJ	Schneider et al.[60]	https://imagej.nih.gov/ij/
Clustal Omega	Sievers et al.[61]	https://www.ebi.ac.uk/Tools/msa/clustalo/
ProtTest 3.4.2	Darriba et al.[62]	https://github.com/ddarriba/prottest3
Zen Black 2.3	Zeiss	https://www.zeiss.com/corporate/int/home.html
FIJI 2.1.10	ImageJ	https://imagej.nih.gov/ij/download.html
Photoshop 2020	Adobe	https://www.adobe.com/products/photoshop.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Heather Bruce (hbruce@mbl.edu).

Materials availability

This study did not generate new unique reagents.

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Data and code availability

- Accession numbers are listed in the key resources table. Microscopy data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

For all species, embryos were collected from multiple females on multiple days and sex ratio is presumably 50:50.

Daphnia magna

Daphnia magna (from Leonid Peshkin, Harvard Medical School;[63] strain IL-MI-8 from pond in Jerusalem, Israel) were kept in *Daphnia* culture medium[64] in cleaned pickle jars with loose-fitting glass dishes for lids on a windowsill and fed daily with 3 – 6 drops of RGComplete (reefnutrition.com) depending on population size. To reduce overcrowding and resting egg production, all but the largest *Daphnia* were removed once every two weeks by pouring through two nets into a Tupperware, the first net with a 2mm pore size to catch the largest *Daphnia*, the second net with a fine pore size such that no hatchlings went through to the Tupperware. The 2mm net was then placed upside down over the mouth of the pickle jar and the water in the Tupperware was poured through the 2mm net, releasing the largest *Daphnia* back into the pickle jar.

Tribolium castaneum

Tribolium castaneum (from Yoshinori Tomoyasu[65])were kept at 30 °C with 70% humidity in whole wheat flour plus 5% baker's yeast powder in glass mason jars with a paper towel lid. Embryos used in this study are approximately Stage NS 15.[66]

Parhyale hawaiensis

Parhyale hawaiensis (Chicago-F strain) were kept at room temperature in plastic tanks with artificial seawater (Instant Ocean), crushed coral, an aeration stone, and a biofilter (AquaClear Powerhead 20, AquaClear Quick Filter, aquarium sponge pad), and fed carrots, kelp granules, and shrimp pellets. Embryos used in this study are approximately Stage 22.[67]

METHOD DETAILS

Fixation

From jars culled as above, the largest *Daphnia* were coaxed with light to one area of the pickle jar and removed with a plastic pipette with tip cut off to a Sylgard 184 (Dow Corning) dish. Water was removed from Sylgard dish to immobilize *Daphnia. Daphnia* with embryos were picked up gently with forceps and placed in a medicine cup with culture medium. Once all animals with embryos had been gathered, animals were transferred to a 1.5mL Eppendorf tube, water removed with pipette, then animals were fixed for 1-2 hours by adding 3.2% aqueous paraformaldehyde (Electron Microscopy Sciences) in *Daphnia* culture medium. Less fixation time seems to reduce background fluorescence in the 488 channel. Fixed animals were washed 3x5min with PBS-Tween then dehydrated stepwise into methanol, then stored at -20C.

In situ HCR

In situ HCR performed as in Bruce et al.[31] In brief, embryos were rehydrated from methanol to 1xPBS with 0.1% Tween 20 (PTw), permeabilized in an SDS detergent solution (1% SDS, 0.5% Tween 20, 50mM Tris-HCL pH7.5, 1mM EDTA pH8.0, 150mM NaCl) for 30min, pre-hybridized for 30 minutes, then hybridized overnight at 37°C. For the hairpin chain reaction, fluorescent hairpins were heated for 90 seconds at 95°C, then cooled for 10 minutes. Embryos were incubated in pre-amplification solution for 30 minutes, cooled hairpins were added, and embryos incubated overnight at room temperature. Embryos were then incubated in 50% glycerol (UltraPure, Invitrogen) in 1x PBS with 0.1mg/mL of DAPI (4′,6-diamidino-2-phenylindole) for several hours and then moved to 70% glycerol. HCR probe sequences available from Molecular Instruments in key resources table.

QUANTIFICATION AND STATISTICAL ANALYSIS

Imaging

Embryos were imaged with Zeiss LSM880 confocal with Zen Black software. Image processing done with Fiji-ImageJ.[60] Fiji "Image Calculator > Subtract" method was used to remove high background from yolk autofluorescence. Figures processed using Adobe Photoshop 2020.

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CellPress

Gene phylogenies

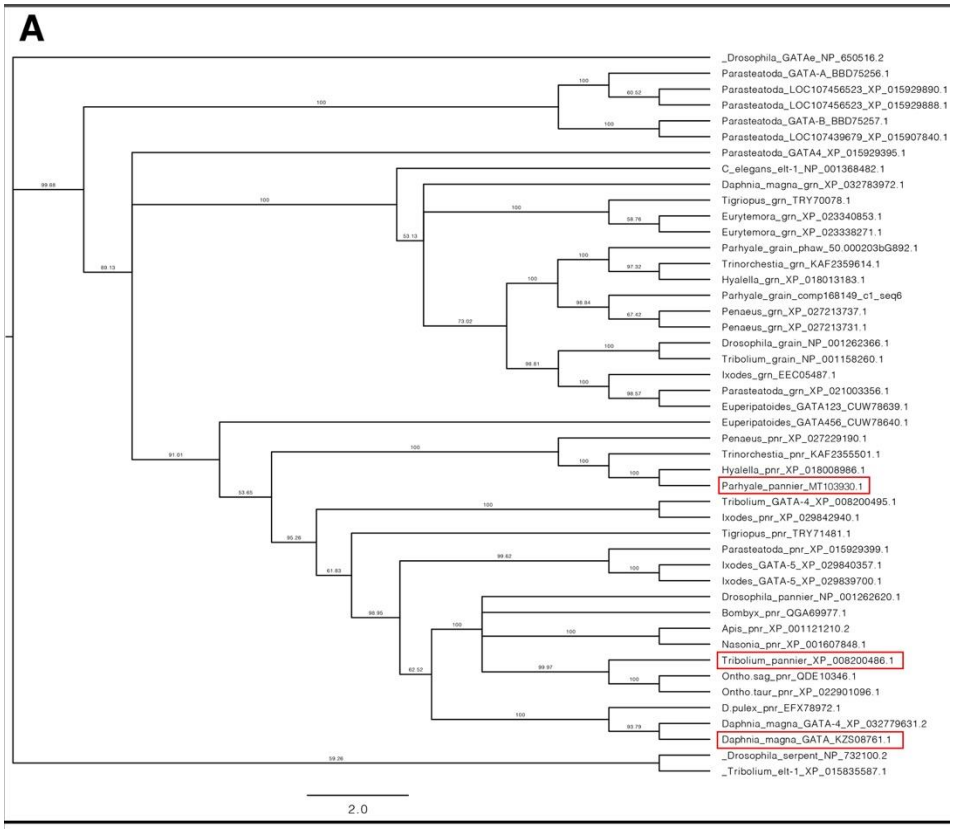
Pannier and *araucan* sequences in *Parhyale*, *Tribolium*, and *Daphnia* were first identified by reciprocal best blast and then confirmed with phylogenies. Protein sequences obtained from NCBI. Phylogeny generated with Geneious free version (https://www.geneious.com/) using Clustal Omega (Sievers, et al.[61]), ProtTest 3.4.2 (https://github.com/ddarriba/prottest3, Darriba et al.[62]), and the Mr. Bayes plug-in for Geneious (Huelsenbeck and Ronquist 2001[59]). Mr. Bayes was run with default parameters (chain length 1,100,000, burn-in 100,00, subsampling frequency 200, and unconstrained branch lengths), C. elegans elt-1 (GATA123) as outgroup for GATA456[68] and mouse Mohawk as outgroup for Iroquois,[69] and JTT+INV+G parameters as identified in ProtTest. Consensus tree generated from Sorted Topologies using Geneious Consensus Tree Builder and re-rooted with FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Current Biology, Volume *32*

Supplemental Information

The *Daphnia* carapace and other novel structures evolved via the cryptic persistence of serial homologs

Heather S. Bruce and Nipam H. Patel


A
_Drosophila_GATAe_NP_650516.2
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Parasteatoda_GATA-B_BBD75257.1
Parasteatoda_LOC107439679_XP_015907840.1
Parasteatoda_GATA4_XP_015929395.1
C_elegans_elt-1_NP_001368482.1
Daphnia_magna_grn_XP_032783972.1
Tigriopus_grn_TRY70078.1
Eurytemora_grn_XP_023340853.1
Eurytemora_grn_XP_023338271.1
Parhyale_grain_phaw_50.000203bG892.1
Trinorchestia_grn_KAF2359614.1
Hyalella_grn_XP_018013183.1
Parhyale_grain_comp168149_c1_seq6
Penaeus_grn_XP_027213737.1
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Drosophila_grain_NP_001262366.1
Tribolium_grain_NP_001158260.1
Ixodes_grn_EEC05487.1
Parasteatoda_grn_XP_021003356.1
Euperipatoides_GATA123_CUW78639.1
Euperipatoides_GATA456_CUW78640.1
Penaeus_pnr_XP_027229190.1
Trinorchestia_pnr_KAF2355501.1
Hyalella_pnr_XP_018008986.1
Parhyale_pannier_MT103930.1
Tribolium_GATA-4_XP_008200495.1
Ixodes_pnr_XP_029842940.1
Tigriopus_pnr_TRY71481.1
Parasteatoda_pnr_XP_015929399.1
Ixodes_GATA-5_XP_029840357.1
Ixodes_GATA-5_XP_029839700.1
Drosophila_pannier_NP_001262620.1
Bombyx_pnr_QGA69977.1
Apis_pnr_XP_001121210.2
Nasonia_pnr_XP_001607848.1
Tribolium_pannier_XP_008200486.1
Ontho.sag_pnr_QDE10346.1
Ontho.taur_pnr_XP_022901096.1
D.pulex_pnr_EFX78972.1
Daphnia_magna_GATA-4_XP_032779631.2
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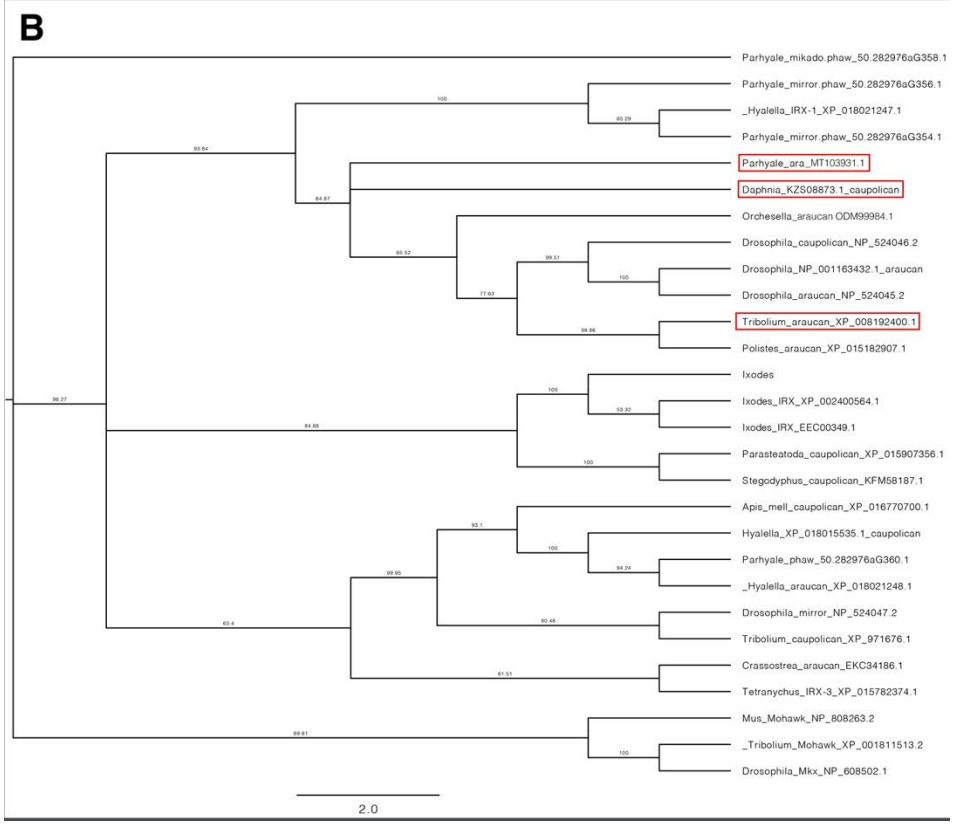
_Tribolium_elt-1_XP_015835587.1
2.0


B
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Parhyale_mirror.phaw_50.282976aG356.1
_Hyalella_IRX-1_XP_018021247.1
Parhyale_mirror.phaw_50.282976aG354.1
Parhyale_ara_MT103931.1
Daphnia_KZS08873.1_caupolican
Orchesella_araucan ODM99984.1
Drosophila_caupolican_NP_524046.2
Drosophila_NP_001163432.1_araucan
Drosophila_araucan_NP_524045.2
Tribolium_araucan_XP_008192400.1
Polistes_araucan_XP_015182907.1
Ixodes
Ixodes_IRX_XP_002400564.1
Ixodes_IRX_EEC00349.1
Parasteatoda_caupolican_XP_015907356.1
Stegodyphus_caupolican_KFM58187.1
Apis_mell_caupolican_XP_016770700.1
Hyalella_XP_018015535.1_caupolican
Parhyale_phaw_50.282976aG360.1
_Hyalella_araucan_XP_018021248.1
Drosophila_mirror_NP_524047.2
Tribolium_caupolican_XP_971676.1
Crassostrea_araucan_EKC34186.1
Tetranychus_IRX-3_XP_015782374.1
Mus_Mohawk_NP_808263.2
_Tribolium_Mohawk_XP_001811513.2
Drosophila_Mkx_NP_608502.1
2.0

Figure S1. Phylogenies for GATA (A) and Iroquois (B) proteins, related to Figures 2 and 3. *Pannier* and *araucan* in *Parhyale*, *Tribolium*, and *Daphnia* first established by reciprocal best blast and then confirmed with phylogenies.

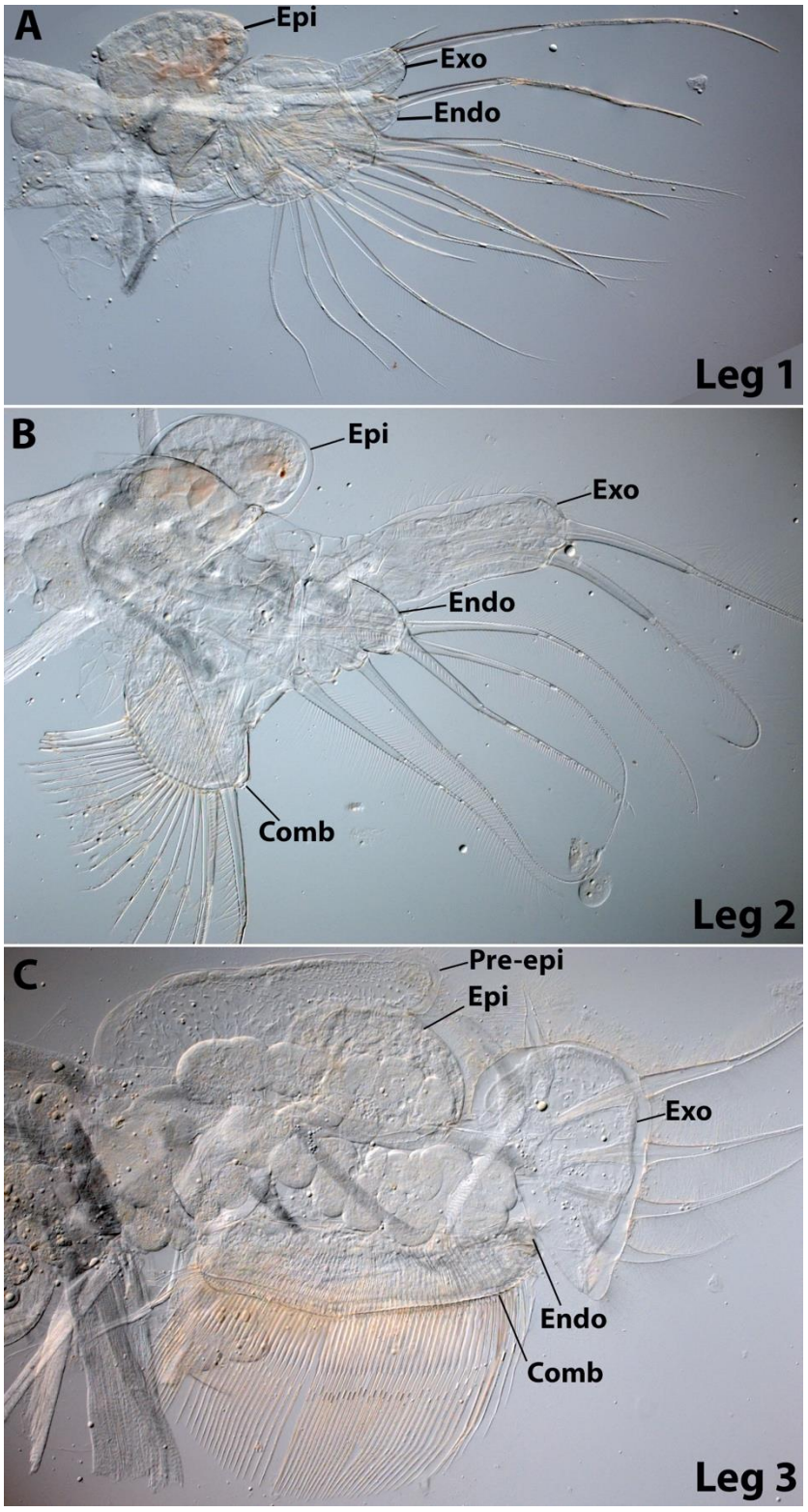
A
Epi
Exo
Endo
Leg 1
B
Epi
Exo
Endo
Comb
Leg 2
C
Pre-epi
Epi
Exo
Endo
Comb
Leg 3

Figure S2. *Daphnia* adult dissected thoracic legs–1 – 3 (A – C), related to Figures 2 and 3.
Daphnia legs are "phyllopodous", or leaf-shaped, and challenging to relate to walking legs in other arthropods such as *Parhyale* and *Tribolium*. However, a few key points facilitate comparisons (also compare *Daphnia* adult legs here to *Daphnia* embryonic legs in Figures 2 and 3). First, leg segment 6 can be identified because when crustacean legs are "biramous", i.e., split into a lateral exopod (exo) and a medial endopod (endo), such as the *Daphnia* Antenna 2 and thoracic legs, then the exopod and endopod are carried on leg segment 6 (basis or basipod). Second, each *Daphnia* thoracic leg is identified by its shape and setal patterns. For example, Leg 2 has a short comb (gnathobase) and an elongated exopod with two long setae, while Legs 3 and 4 have a wide comb and a paddle-shaped exopod with an array of six setae. Endopod in C is underneath comb and not clearly visible. Epi, epipod (a type of exite) of the coxa (leg segment 7). Pre-epi, pre-epipod (a type of exite) of leg segment 8[S1].

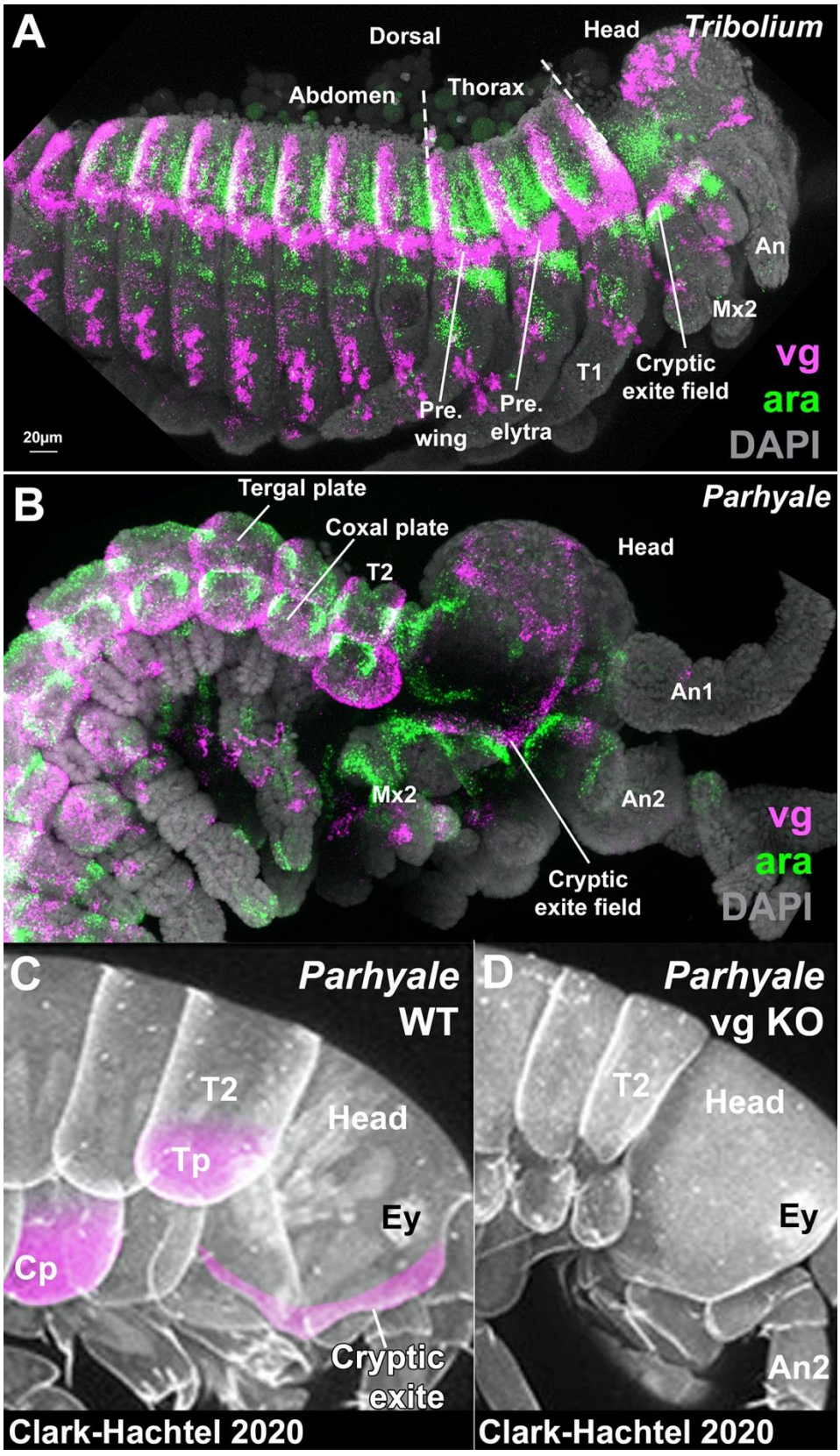
A
Tribolium
Dorsal
Head
Abdomen
Thorax
An
Mx2
T1
Cryptic exite field
Pre. wing
Pre. elytra
20µm
vg
ara
DAPI
B
Parhyale
Tergal plate
Coxal plate
T2
Head
An1
Mx2
An2
Cryptic exite field
vg
ara
DAPI
C
Parhyale WT
T2
Head
Tp
Ey
Cp
Cryptic exite
Clark-Hachtel 2020
D
Parhyale vg KO
T2
Head
Ey
An2
Clark-Hachtel 2020

Figure S3. Cryptic exites in *Tribolium* and *Parhyale*, related to Figures 3 and 4.

vg and *ara* are expressed in the same register in all visible body segments: *vg* is bracketed dorsally and ventrally by *ara* expression domains. (A). *Tribolium* embryo (lateral view). In insect thoracic legs T2 and T3, these *vg* expression domains pattern the presumptive (pre.) elytra and wings (the exites of the 8th leg segment). In the abdominal segments, *vg* patterns the gin traps (pupal defense structures), which are serially homologous to wings[S2]. In the head, as in the other body segments, *vg* is expressed in the same register and is bracketed by *ara*. Here, *vg* is expressed in the dorsal/proximal antenna (An), mandible (md) and maxilla (mx), and perhaps in the labium (homologous to crustacean mx2), which has migrated medial to the maxilla. (B). *Parhyale* embryo (lateral view). Note that *ara* expression brackets all tergal plates on leg segment 8, but the tergal plates have extended outward and ventrally, such that *vg* appears to overlap the ventral armband of *ara*. In the head, as in the other body segments, *vg* is expressed in the same register and is bracketed by *ara*. Here, *vg* is expressed in the dorsal/proximal Antenna 1 and 2 (An1, An2), mandible, maxilla 1, and perhaps maxilla2 (mx2). (C). WT Parhyale hatchling. A tergal plate (tp), coxal plate (cp), and the protrusion or flange on the head are shaded pink. (D). When *vg* is knocked out, the tergal plate, coxal plate, and head flange are reduced (C, D modified from Clark-Hachtel 2020[S3]).

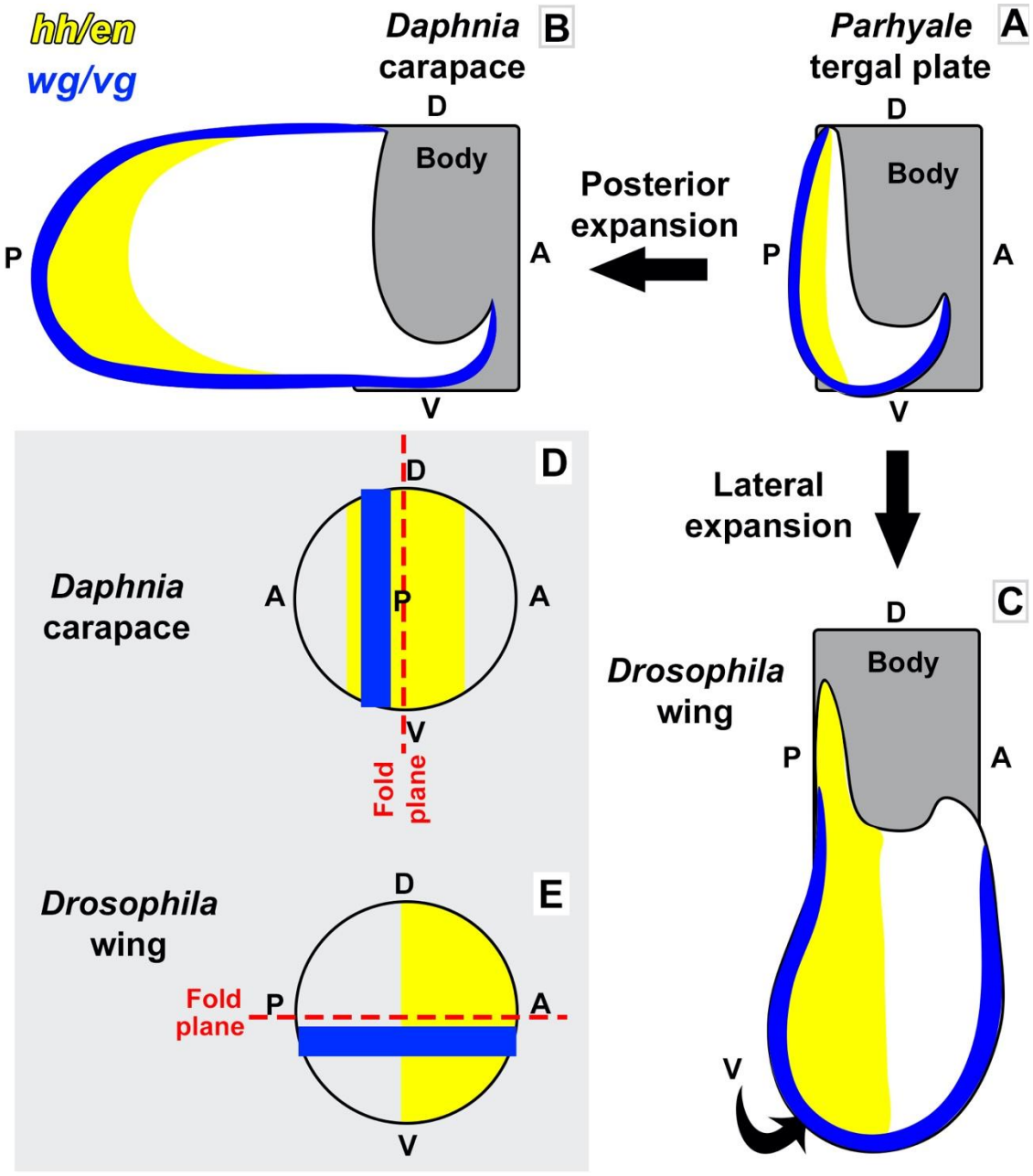
hh/en
wg/vg
Daphnia
carapace
B
D
Body
P
A
V
Posterior
expansion
Parhyale
tergal plate
A
D
Body
P
A
V
Lateral
expansion
C
D
Drosophila
wing
Body
P
A
V
D
Daphnia
carapace
A
P
A
V
Fold
plane
E
Drosophila
wing
Fold
plane
P
A
D
V

Figure S4. Summary of expression of *hh/en* and *wg/vg* in *Parhyale* tergal plate, *Daphnia* carapace, and *Drosophila* wing, related to Figure 4.

Shiga et al. 2017[S4] note that the expression domains of *hedgehog* (*hh*)/*engrailed* (*en*) and *wg* in *Drosophila* wings and the *Daphnia* carapace are not oriented as expected if the two are homologous: in *Drosophila* wing discs, *hh/en* and *wg* are expressed orthogonally to each other, while in the *Daphnia* carapace, *hh/en* and *wg/vg* are expressed parallel to each other (discussed in Tomoyasu 2021[S5]). Comparisons with *Parhyale* are informative in explaining these observations. In *Parhyale* tergal plates (A), *vg* is expressed along the posterior edge in a J-shape[S3]. Thus, in *Parhyale*, *vg* expression in tergal plates runs both orthogonally and parallel to *en* expression. If the posterior edge of a *Parhyale* tergal plate expanded posteriorly, then *en* and *wg* would be expressed in parallel in this expanded structure, as they are in the *Daphnia* carapace (B). On the other hand, if the ventral edge of a *Parhyale* tergal plate were to expand ventrally, then *en* and *wg* would be expressed orthogonally to each other, as they are in insect wings (C). Thus, the difference in the expression axes of *wg* and *en* between *Drosophila* and *Daphnia* may be a function of the direction in which the exite expanded, rather than a lack of homology. D – E reproduced from Shiga et al 2017 Fig 3M[S4].

Parhyale hawaiensis vestigial	Accession: MG703506.1 B4 initiator adapter 20 probe pairs (40 sequences)	CCTCAACCTACCTCCAACaaTTGAATTCAACAAACTGGAACAAAC AGCTTTAAACATTGCGGTCAGAGTTatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaTGGGAAGGGATTTTTATTTCATAAA GGTCAGTAATTTCGCTCTTTATTGCatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaTTCTTTGGTTTCGCGTTCCCTCAGA GTCCAAATTACAGGCAGAAATGCCAatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaGTTTTTATAAGTAAAACAAGTTAAT AGCTTTAAAACGTGCTTTCCCCAAGatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaATCAGAGATTGCGTGGAGCCGGGAA GACATCTGCCTATGATGCAAATTTAatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaAATATACGGCCGTGTGATGTGGTTT CTAGCTGCAGAATACGACATGACGTatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaCTGCCGACGTGAAGCAATTGATGAC GTATGCTGTAATATCCGTTAGTTGGatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaTTTACTGTCTTTAATCTATTTTATC GCTTCTACTAGCCGCAGTGTTAACTatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaGTCTTTGGGACCCTCCTGCTGCACT TTTATGTTGTTTTTAAAACCAGTAGatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaTAGTGGCTGGAGGAGTAGCTGGAGT GACTCCAGCCCAGCAGCCACAGGAGatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaAGAAGGCCTCGTGGTAGCGACCCCA GCCCGTGGGCAGCTGCCAGGTCCCCatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaCGGGACGGCCGGTAAGGCCGGTATC GTCGGGCTTGGTCATAGTCATCTGTatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaCCGTACTGCTGGTACGGCCGGGGCG GCTAAGGACGGTTGCAGGCCGAGGCatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaCGTGAGCCATGCTGCGGTGGCTGTA AGGACGACATGGCTGCTTGGTAGACatTCTCACCATATTCGCTTC CCTCAACCTACCTCCAACaaAGGGTCATGGTGTGCGGAGTTCTGC

Table S1. In situ HCR probe sequences used for *Parhyale vestigial*, related to STAR methods.

Ordered as 50pmol o-pool from IDT. Accession: MG703506.1, B4 initiator adapter, 20 probe pairs (40 sequences).

References

S1. Hansen, H.J. (1925). Studies on Arthropoda II. (Copenhagen: Gyldendalske Boghandel).

S2. Linz, D.M., and Tomoyasu, Y. (2018). Dual evolutionary origin of insect wings supported by an investigation of the abdominal wing serial homologs in Tribolium. *115*, E658–E667.

S3. Clark-Hachtel, C.M., and 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.

S4. Shiga, Y., Kato, Y., Aragane-Nomura, Y., Haraguchi, T., Saridaki, T., Watanabe, H., Iguchi, T., Yamagata, H., and 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, https://doi.org/10.1101/160010

S5. Tomoyasu, Y. (2021). What crustaceans can tell us about the evolution of insect wings and other morphologically novel structures. Current Opinion in Genetics & Development *69*, 48–55.