Title: The *Daphnia* carapace and the origin of novel structures

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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 water flea *Daphnia magna* is a bivalved "cape" of exoskeleton that has been proposed to be one of many novel arthropod structures that arose through repeated co-option of genes that also pattern insect wings[2–4]. To determine whether the *Daphnia* carapace is a novel structure, we compare the expression of *pannier*, *araucan*, and *vestigial* between *Daphnia*, *Parhyale*, and *Tribolium*. Our results suggest that the *Daphnia* carapace did not arise by co-option, but instead derives from an exite (lateral 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 region that gives rise to the *Daphnia* carapace appears to be present in *Parhyale*[6] and *Tribolium* as a small, inconspicuous protrusion. Similarly, the *vestigial*-positive regions that form thoracic tergal plates in *Parhyale* appear to be present in *Daphnia*, even though *Daphnia* does not form thoracic tergal plates. 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.

Key words: novelty, carapace, exite, arthropod, leg, evo devo, *Daphnia*, *Parhyale*, *Tribolium*

Results: Many crustaceans, such as crayfish, crabs, ostracods, tadpole shrimp, and water fleas, have a carapace. Classical morphological studies have argued that the carapace emerges from the dorsal posterior of the head (at approximately maxilla 2)[7,8]. In the water flea *Daphnia magna*, the carapace is a bivalved "cape" of exoskeleton that surrounds the animal. Shiga 2017 [2] confirmed

that the *Daphnia* carapace emerges from the dorsal posterior region of the head and, like insect wings, is composed of a bilayered sheet of ectodermal cells and expresses and requires the "wing" genes *wingless* (*wg*), *vestigial* (*vg*) and *scalloped* (*sd*)[2]. They propose that the *Daphnia* carapace and other flat, lateral lobes in arthropods arose by multiple instances of co-option of wing patterning genes.

However, the analyses in Bruce and Patel 2020[5] and Bruce and Patel 2021[9] suggest an alternative hypothesis. These analyses, drawing on over a century of morphological and embryological studies as well as gene expression and loss-of-function studies, generated a model for understanding the homologies of arthropod leg and body wall structures (Fig. 1). In this model, most arthropods have incorporated one or two ancestral proximal leg segments into the body wall [5,9–13] (leg segments 7 and 8, counting from the terminal claw), but the division between "true" body wall (tergum) and the incorporated leg segments that now function as lateral body wall can be distinguished by the expression of *pannier* (*pnr*) and *Iroquois* complex genes such as *araucan* (*ara*), respectively. In the embryos of *Drosophila* (fruit fly), *Tribolium* (flour beetle) *Parhyale* (amphipod crustacean), and *Acanthoscurria* (tarantula), *ara* expression brackets the hypothesized incorporated 8th leg segment, while *pnr* is expressed in the dorsal-most tissue and marks the true body wall[9]. Bruce and Patel 2020[5] showed that the insect wing and *Parhyale* tergal plate are both derived from exites - multi-functional lobes that emerge from proximal leg segments and that are patterned by "wing" genes such as *vg* and *sd*[6,14,15]. They showed that the insect wing and *Parhyale* tergal plate emerge from the ancestral leg segment 8 that was incorporated into the body wall (Fig. 1B).

Based on this model, the morphological and molecular data in Shiga 2017 suggest that the *Daphnia* carapace did not arise by co-option, but instead represents an exite on an incorporated 8th leg segment of the head. The *Daphnia* carapace would therefore be homologous to the *Parhyale* tergal plate[5,6] and the insect wing[5].

To test the proximal-distal register of the *Daphnia* carapace, the expression of *pannier*, an *Iroquois* gene, and the wing/exite patterning gene *vg* was examined in embryos of *Daphnia magna*, *Tribolium castaneum*, and *Parhyale hawaiensis* using in situ hybridization chain reaction (HCR) version 3.0[16,17]. A single *pnr* gene was identified in *Daphnia* which was the reciprocal best blast hit of *Drosophila*, *Tribolium*, and *Parhyale pnr* [5]. A single *Iroquois* complex gene was identified in *Daphnia* which was the reciprocal best blast hit of *Drosophila*, *Tribolium*, and

Parhyale ara. This *Daphnia* gene is hereafter referred to as *ara* [5]. *Daphnia vg* was identified previously by Shiga 2017[2].

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. 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. 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 (Figs. 2 and 3). Interestingly, *ara* expression on leg segment 6 appears to mark the position of the exopod. In a uniramous *Parhyale* leg (Figs. 3e) that lacks an exopod, the leg segment 6 *ara* domain forms a circular patch on the lateral side of the leg. However, in the biramous (split) legs of *Daphnia* (Figs. 2d and 3f, S1) and *Parhyale* (Fig. S2) where an exopod emerges on the lateral side of leg segment 6, the *ara* patch is expanded, and in *Parhyale*, the patch encircles the base of the exopod (see Fig. S1 for explanation of *Daphnia* leg identification).

If the *Daphnia* carapace is the exite of the incorporated 8th leg segment of a mouthpart (modified leg) on the head, this exite may still exist 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* dorsal/proximal to the mouthparts (Fig. S3A). 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 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 (Fig. S3B, C[6]). 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 modified legs fused into a head capsule[18], 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.

Similarly, while *Daphnia* does not form thoracic tergal plates like *Parhyale*, the regulatory architecture for generating these structures still appears to exist in *Daphnia*. Shiga

2017 noted segmentally repeated dorsal domains of *wg* expression. These *wg* domains are co-expressed with *vg*. Like *Parhyale* tergal plates, this *Daphnia wg-vg* domain is ventral to *pnr* and bracketed by the two armbands of *ara*. Together, this molecular triangulation suggests that these segmentally repeating *wg-vg* domains in *Daphnia* are morphogenetic fields (a discrete set of cells capable of forming a specific organ, e.g., leg, wing, bristle, etc) that pattern exites such as thoracic tergal plates, just like the segmentally repeating *vg*-positive *Parhyale* tergal plates. Thus, even though *Daphnia* does not form tergal plates on the thorax, it still retains the regulatory architecture for making them in serially homologous positions in each thoracic segment. These thoracic exite fields could presumably be activated to form an obvious structure in future lineages.

Shiga et al. 2017[2] 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* wings, *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. Comparisons with *Parhyale* are informative in explaining these observations. In *Parhyale* tergal plates, here proposed to be positionally homologous to insect wings and the *Daphnia* carapace, *vg* is expressed along the posterior edge in a J-shape[6]. Thus, in *Parhyale*, *vg* expression in tergal plates runs both orthogonally and parallel to *en* expression (Fig. S4). 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. 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. 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.

Discussion: Taken together, the expression and knockdown data in Shiga 2017 and 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. The *Daphnia* carapace would therefore be homologous to the *Parhyale* tergal plate and the insect wing (Fig. 4). Many arthropods have dorsal and lateral structures (carapaces, plates, gills, glands, etc), the homologies of which are frequently debated[14,19]. The work here demonstrates that the proximal-distal identity

of these structures can be elucidated using the expression of *pnr* and *Iroquois* genes such as *ara*, and further pinpointed with leg segment joint markers like *odd-skipped*, distal leg markers such as *Distal-less* (*Dll*), and exite genes such as *vg*. This simple molecular triangulation strategy, which does not require functional studies, can illuminate the homologies of long-debated structures on the legs and body wall of arthropods and determine which, if any, are truly novel.

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]. Arthropod legs and bodies are decorated with a fascinating diversity of structures, many of which have been proposed to be novel. Co-option of genetic pathways and "deep homology" has become a dominant explanation for the origin of novel structures within the field of evolutionary developmental biology (evo devo)[2–4,20–29].

However, the work presented here, and in Bruce and Patel 2020[5] and Bruce 2021[9], provides an alternative hypothesis: these leg-associated structures (and perhaps other novel structures too) arise from unrecognized serially homologous morphogenetic fields (morphogenetic fields = a discrete set of cells programmed to be capable of forming 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) in intermediate lineages and become elaborated again in later lineages (such as the *Daphnia* carapace), such that they may no longer be recognizable as the ancestral structure and appear to be novel. Fortunately, unrecognized serially homologous fields should be relatively straightforward to identify by employing experiments that transform homologous tissues into each other. For example, Hox knockout and misexpression studies cause ectopic legs or wings to form at serially homologous locations along the anterior-posterior axis[30,31]. According to this cryptic persistence model, structures would not have to be continuously present in a morphologically obvious state from ancestor to descendant in order to be homologous. Indeed, a morphogenetic field may not form an obvious structure at all, as in the *vg*-positive regions of the *Tribolium* head (Fig. S3) and *Daphnia* dorsal body. 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 apparently novel structures. Cryptic persistence of morphogenetic fields may therefore provide a mechanistically satisfying explanation for the origin of novel structures.

In fact, cryptic persistence is hinted at in the definition of crustacean exites. In crustacean morphology, exites are defined as lateral lobes that may occur on any of the three proximal leg segments (leg segments 6 – 8, "protopod") and on any post-mandibular leg along the anterior-posterior axis[14,19]. However, it is widely appreciated that not all possible exites occur on all proximal leg segments on all legs in all crustacean taxa. Exites occur variably across different crustacean taxa: they may appear in some lineages but not in others, on some legs and not others, and on some leg segments but not others [14,19,32]. These observations imply that there is some latent ability to form a structure at these locations: sometimes the pathway is activated and sometimes it is repressed, yet all these structures are considered exites, rather than repeatedly novel structures. The concept of cryptic persistence makes this implicit assumption explicit, and furthermore provides a mechanistic, molecular developmental explanation for the variable occurrence of exites.

For decades, researchers have been finding that many exciting structures on the legs and body wall of many insects express the same genes (the "wing gene network"). These were interpreted to be wing serial homologs or partial wing homologs, and have often been interpreted as evidence of gene co-option. This has contributed to a widespread narrative that co-option is a common mechanism for the generation of novel structures. However, given that crustacean exites may occur on any body segment[14,32], and that insects have incorporated two leg segments into the body wall, each with an exite[5], and all of these insect structures may be exites inherited from their crustacean ancestors. Thus, the lack of a molecular, developmental model from the ancestral crustacean group gave the appearance that insect structures had repeatedly arisen de novo, and spurring a mistaken foundation for one of the most widely discussed notions in evo devo. This is a clarion call for a diversity of research model organisms in every major clade, so that structures no longer appear to be novel simply because the ancestral state has not yet been investigated.

In addition to the phylogenetically unequal distribution of research organisms, a second reason for the dominance of the gene co-option model in explaining the origin of novel structures may be the poor naming of "master regulator". In many discussions surrounding the origin of novel structures, authors propose that a novel structure came about when an entire genetic pathway was co-opted[2,21]. The underlying assumption seems to be that this structure and its genetic pathway was not present at that location before, and that the tissue doing the co-opting is

unrelated to the tissue it is co-opting from, i.e. they are different morphogenetic fields, such as eye vs heart. The related assumption seems to be that it is relatively straightforward for a master regulator to become misexpressed in an unrelated tissue (i.e. outside of that master regulator's morphogenetic field at serially homologous locations, i.e. a tissue not previously primed with dozens of other TFs) in order to activate the entire structure in an entirely novel, non-serially homologous location. If this were true, we would expect misexpression of the heart gene tinman in the late ectoderm to form an ectopic heart or misexpression of eyeless in the late gut to form an eye. One might posit that such outcomes would not be expected, because the identities of different germ layers are extensively restricted at multiple levels (chromatin accessibility, which suites of transcription factors and their cofactors are expressed, etc). But the same is probably true for morphogenetic fields within the same germ layer, for example leg vs true body wall (tergum): each has been extensively programmed and restricted such that misexpression of even a “master” regulatory gene from the other’s pathway would not have an effect, because it would not be able to talk to or interact with the other pathway.

A better model for the origin of apparently novel structures may be the Hox genes, where serially homologous tissues may switch between alternate identities. In this model, misexpression of “master regulators” or selector genes at previously unappreciated serially homologous locations would be *expected* to transform tissue towards the structures it patterns. Unique phenotypes may arise due to the selector gene being expressed in a different Hox or proximal-distal regulatory environment, or because the structures have not been expressed at this body position for millions of years and will thus communicate differently with the genetic pathways at this position.

Criteria should be formed to distinguish structures that have been de-repressed at a serially homologous location. For example, de-repressed serial homologs would be expected to appear in the same developmental register, to be composed of the same germ layer and similar tissue type (which in itself may be difficult to determine with certainty), and to express most of the genes in the pathway. In contrast to whole pathway co-option, single gene co-option may be plausible, and would be expected to take genes piece-meal from different pathways, like butterfly eyespots, rather than entire pathways, which would require co-opting multiple regulatory genes simultaneously.

Many lateral, leg-associated structures across arthropods may be exites. Examples include the lateral organs of arachnids, glands of pycnogonid larvae, trachea of myriapods and insects, paranota of polydesmid millipedes, abdominal gills of insects, gin traps of insects, thoracic styli of jumping bristletails, scarab beetle support structures, and oostegites of amphipod crustaceans. Rather than being repeated co-options of "wing" genes, or "partial wing homologs", these structures may simply be different kinds of exites, and the observed differences in gene expression profiles would be due to differences in the type and therefore function of the exite (i.e. respiratory gill/trachea, secretory gland, rigid plate, etc).

Morphogenetic fields are related to vestigial structures in the sense that vestigial structures are generated from morphogenetic fields. However, a morphogenetic field can exist in a dormant, repressed state where it does not form a structure at all, vestigial or otherwise, and yet it is still capable of forming a structure in the future if it becomes de-repressed. Cryptic persistence of morphogenetic fields therefore reframes the discussion to focus on the molecular networks that generate structures (or the Character Identity Networks of Wagner 2007[34]). The concept therefore encompasses vestigial structures, when the field forms a structure, but expands the idea by pointing out that not all fields generate an actual structure, and also explains why structures can re-appear at serially homologous locations. Morphogenetic fields occur at serially repeated locations, and not all of these locations will form a structure, vestigial or otherwise. But these locations should be identified and examined in order to discover dormant fields so that homologies with structures of interest may be established. Identifying all relevant serially homologous morphogenetic fields within an organism is the only way to know for sure whether a structure is expressed at a novel or a serially homologous position.

The work presented here calls into question whether gene co-option should be invoked to explain the origin of novel structures as often as it is. On a final note, it is interesting to consider that if cryptic persistence is commonplace, and most "novel" structures evolved from existing structures, then many familiar morphogenetic fields (for example, legs) may be far more evolvable, and have far more ancient origins, than currently thought.

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Author contributions

HSB conceived, designed, and performed experiments and wrote manuscript. NHP edited manuscript.

Declaration of interests

The authors declare no competing interests.

Figure legends

Fig. 1. Model of how to align all arthropod legs from Bruce 2021 [9]. **a.** A schematic of the leg structures that require each gene in chelicerates, crustaceans, insects provides a model for how to align crustacean and insect legs. Based on the function of *exd*, *hth*, *Dll*, *Sp6-9*, and *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 *pnr* and *ara* in chelicerates, crustaceans, and insects, and the function of wing/exite genes in insects and crustaceans. **b.** Morphology and proposed homologies of arthropod leg segments. Colors and patterns indicate proposed homologies. Exites (checker pattern); endites (stripe pattern). Drawings in b modified from Snodgrass 1952. Reproduced from Bruce & Patel 2021. **c-c'.** 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 (c'), then *pnr* will extend into the carapace, and the two domains of *ara* will be located ventral to the carapace.

Fig. 2. Expression of *pannier*, *araucan*, and *vestigial* in *Daphnia* embryos. **a.** *Daphnia* adult with embryos under carapace (ca), lateral view. **b, b'.** Stage 7 embryo, ventral view. Maxilla 1 and 2 not yet visible but will emerge between mandible (md) and first thoracic leg (T1) **c.** Stage 9 embryo, dorsal view. **d.** Dissected head and carapace of St 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), at the base (arrow) of the exopod (exo, on leg segment 6 in uniramous legs), and in the tip of leg segment 1 (only present in antenna 2 (An2). *vg* is expressed in the perimeter of the carapace (ca), and in the mesoderm of antenna. m, mouth. abd, abdomen. *pnr* (red), *ara* (green), *vg* (light blue), DAPI (grey).

Fig. 3. Expression of *pnr* and *ara* elucidates the proximal leg segments. Dissected right half of *Parhyale* (a), *Tribolium* (c), and *Daphnia* (e) embryos. *Daphnia* head and carapace removed to reveal trunk. Dissected legs of *Parhyale* (b), *Tribolium* (d), and *Daphnia* (f) embryos. Large cells dorsal to *pnr* expression in a and b are extra-embryonic cells that exist prior to dorsal closure. *pnr* marks the dorsal-most domain. *ara* 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); and, in crustaceans and tarantula[9], in the tip (*) of leg segment 1. Leg segment 6 domain is a dot when no exopod (exo) is present (arrow in e). Dot becomes expanded in exopod when leg is biramous (arrows in d). Note that *ara* expression in e and f in exopod does not extend to tip as it does in a and b, therefore this exopod domain is not the leg segment 1 (*) domain. Indeed, *Daphnia* thoracic legs are truncated and do not have leg segment 1, see Fig. S1 for explanation of *Daphnia* leg identification. However, *Daphnia* antenna 2 has this domain (Fig. 2d). *pnr* and *ara* are expressed in a smattering of ventral non-leg cells. tp, tergal plate. b, c, e, f from Bruce 2020 and Patel[5].

Fig. 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. Homologous structures indicated with pink shading. Not all serially homologous structures in all animals are indicated.

Star methods

<u>Animal care</u>

Daphnia were kept in *Daphnia* culture medium[33] in cleaned pickle jars with loose-fitting glass dishes for lids 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.

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. 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 2021[17]. Embryos were removed from adults during final PBS-Tween wash prior to pre-hybridization step.

Imaging

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

Supplemental information legends

Fig. S1. *Daphnia* adult dissected thoracic legs 1 - 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. 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* thoracic legs, then the exopod and endopod are carried on leg segment 6 (basis or basipod). Counting distally from leg segment 6, the endopod of leg 1 appears to have one segment and the endopod of leg 2 has at most 3 segments. Given that a complete endopod has 5 leg segments, and no terminal claw is apparent, *Daphnia* thoracic legs do not appear to express leg segment 1. 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 Leg 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 of leg segment 8[35].

Fig. S2. *ara* expression appears to mark the position of the exopod. In a uniramous *Parhyale* T4 leg that lacks an exopod, the leg segment 6 *ara* domain (arrow) forms a dot on the lateral side of the leg. However, in a biramous second abdominal (A2) *Parhyale* leg where an exopod emerges on the lateral side of leg segment 6, the *ara* dot is expanded to encompass the base of the exopod. *ara* armband on leg segment 8 (closed arrowhead). *ara* armband on lg segment 7 (open arrowhead). *ara* expression in distal tip of leg (*). The single segment on the *Parhyale* endopod could be leg segment 1 or 2 based on leg gene knockout and expression studies (results for abdominal legs are unpublished, but from same studies as in Bruce and Patel 2020[5]) but is here labeled as leg segment 2 for simplicity.

Fig. S3. Cryptic exites in *Tribolium* and *Parhyale*. **A**. *Tribolium* embryo (lateral view). *vg* is expressed at the same register in all visible body segments. In all visible body segments, *vg* is bracketed dorsally and ventrally by *ara* expression domains. In thoracic legs T2 and T3, these *vg* expression domains pattern wings (the exites of the 8th leg segment). In the abdominal segments, *vg* patterns the gin traps (larval defense structures), which are serially homologous to the wings[31]. 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 mandible (md) and maxilla (mx),

and perhaps in the labium (homologous to crustacean mx2), which has migrated medial to the maxilla. **B**. WT Parhyale hatchling. A tergal plate (tp), coxal plate (cp), and the protrusion or flange on the head are shaded pink. **C**. When *vg* is knocked out, the tergal plate, coxal plate, and head flange are reduced (B, C, modified from Clark-Hachtel 2020[6]).

Fig. S4. Summary of expression of *hh/en* and *wg*/*vg* in *Parhyale* tergal plate, *Daphnia* carapace, and *Drosophila* wing based on Shiga et al. 2017[2] and Clark-Hatchel et al. 2020[6]. In *Parhyale* tergal plates (a), *vg* is expressed along the edge in a J-shape and *en* is expressed along the posterior edge[6]. 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). If the ventral edge of a *Parhyale* tergal plate were to expand laterally, then *en* and *wg* would be expressed orthogonally to each other, as they are in insect wings (c). d – e reproduced from Shiga et al 2017 Fig 3M[2].

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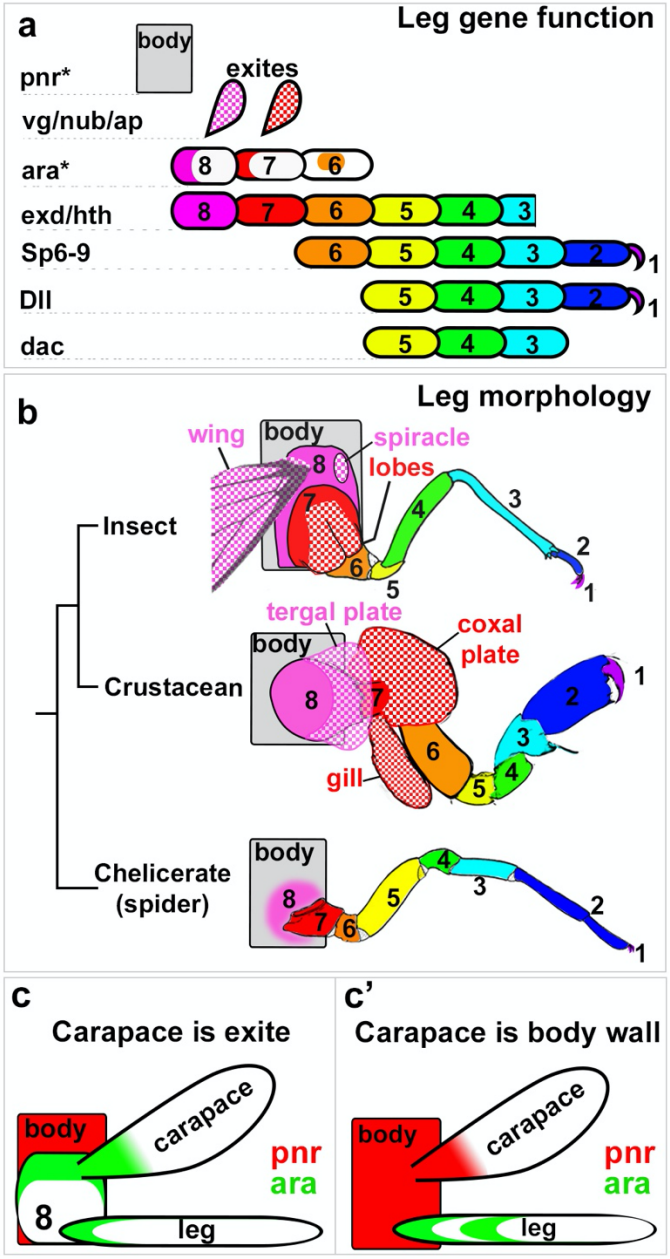
470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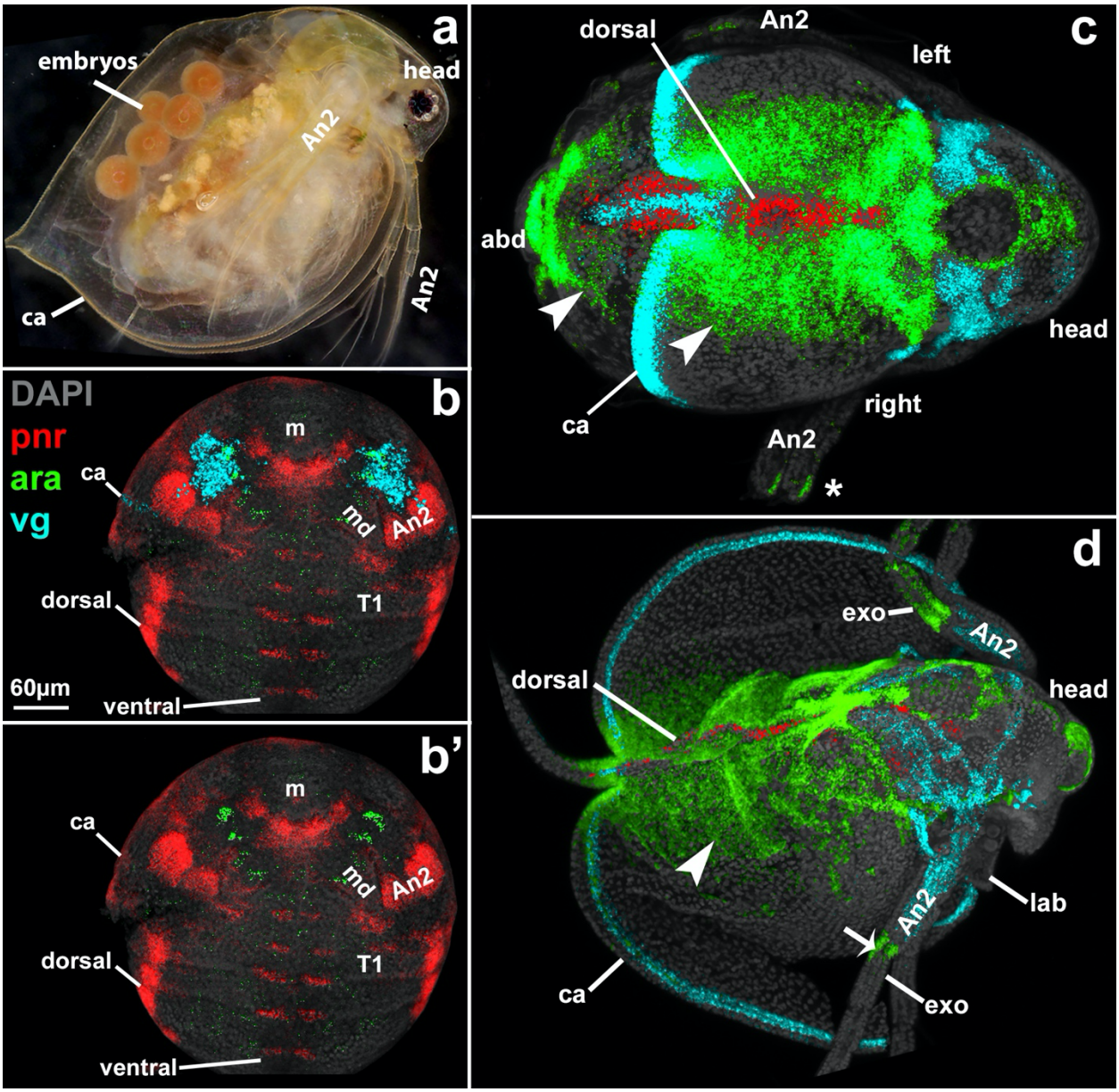
Leg gene function
body
exites
pnr*
vg/nub/ap
ara*
exd/hth
Sp6-9
Dll
dac
b
Leg morphology
wing
body
spiracle
lobes
Insect
tergal plate
coxal
plate
Crustacean
gill
Chelicerate
(spider)
c
Carapace is exite
carapace
pnr
ara
leg
c'
Carapace is body wall

Fig. 1.


a
embryos
head
An2
An2
ca
b
DAPI
pnr
ara
vg
m
ca
md
An2
dorsal
T1
60µm
ventral
b'
m
ca
md
An2
dorsal
T1
ventral
c
dorsal
An2
left
abd
head
ca
right
An2
*
d
exo
An2
dorsal
head
lab
An2
ca
exo

475 **Fig. 2**

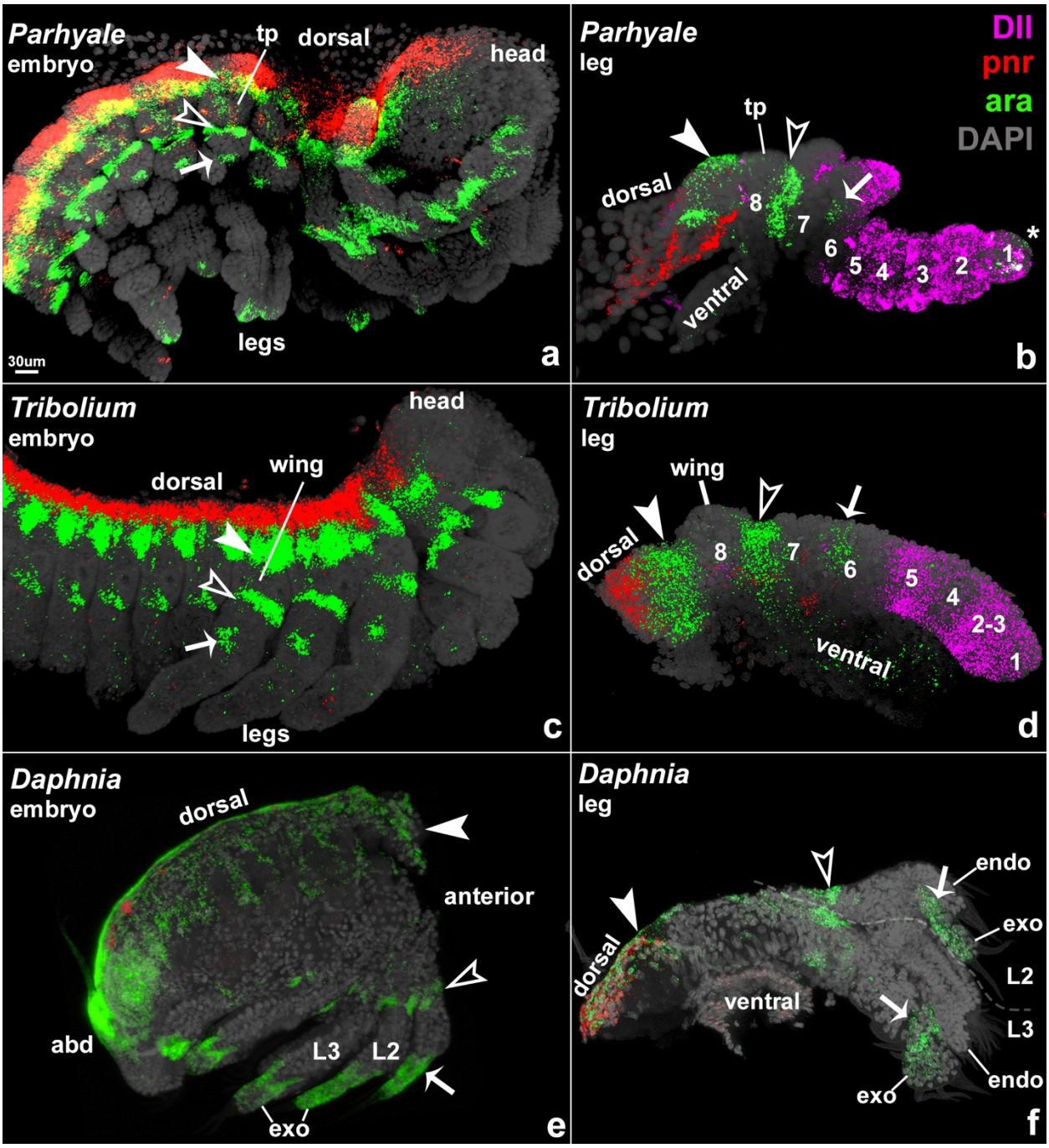

Parhyale
embryo
tp
dorsal
head
legs
30um
a
Parhyale
leg
Dll
pnr
ara
DAPI
tp
dorsal
ventral
8
7
6
5
4
3
2
1
*
b
Tribolium
embryo
head
dorsal
wing
legs
c
Tribolium
leg
wing
dorsal
ventral
8
7
6
5
4
2-3
1
d
Daphnia
embryo
dorsal
anterior
abd
L3
L2
exo
e
Daphnia
leg
endo
exo
L2
L3
endo
exo
dorsal
ventral
f

Fig. 3

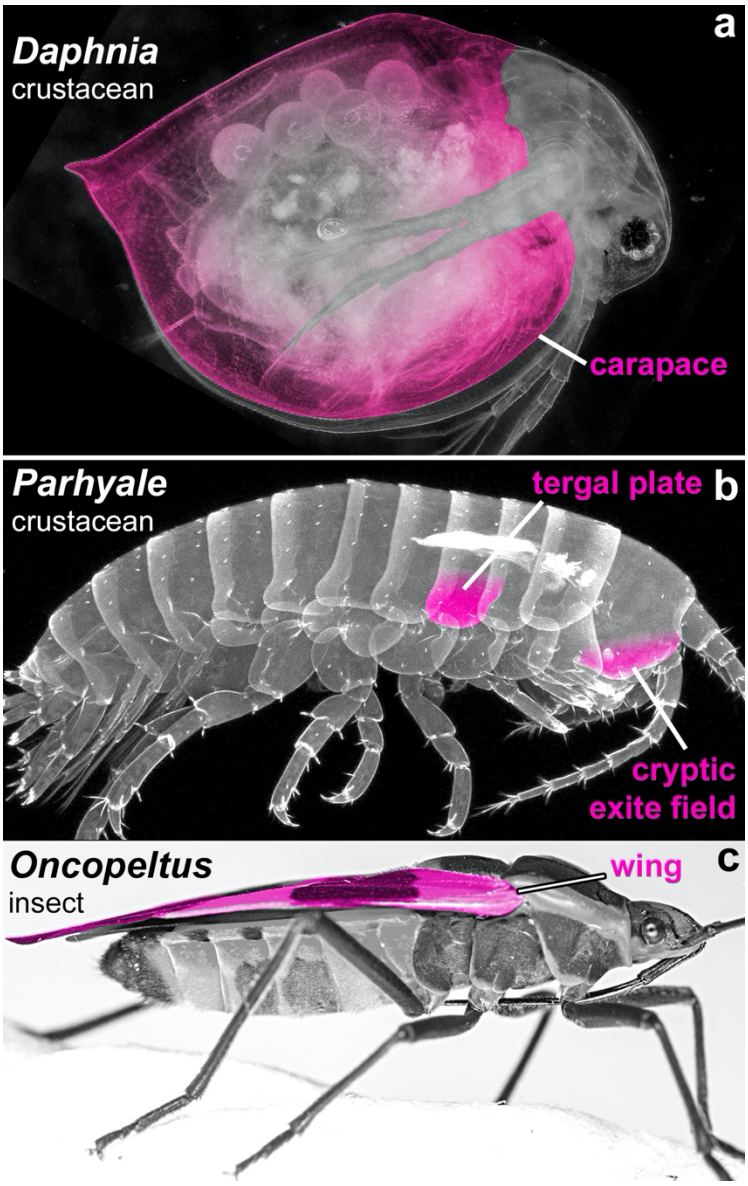

a
Daphnia
crustacean
carapace
b
Parhyale
crustacean
tergal plate
cryptic
exite field
c
Oncopeltus
insect
wing

Fig. 4

485

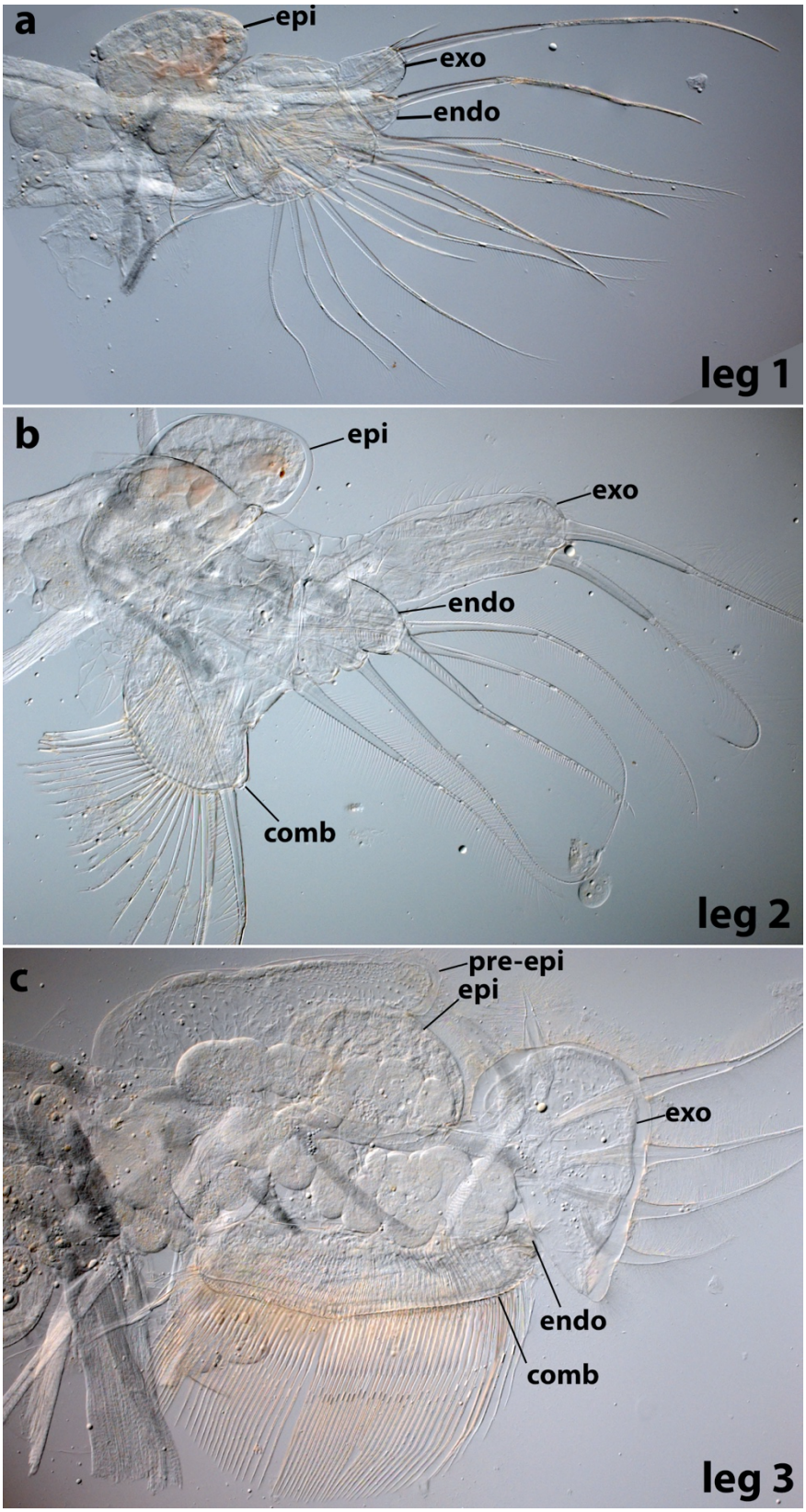

a
epi
exo
endo
leg 1
b
epi
exo
endo
comb
leg 2
c
pre-epi
epi
exo
endo
comb
leg 3

Fig. S1

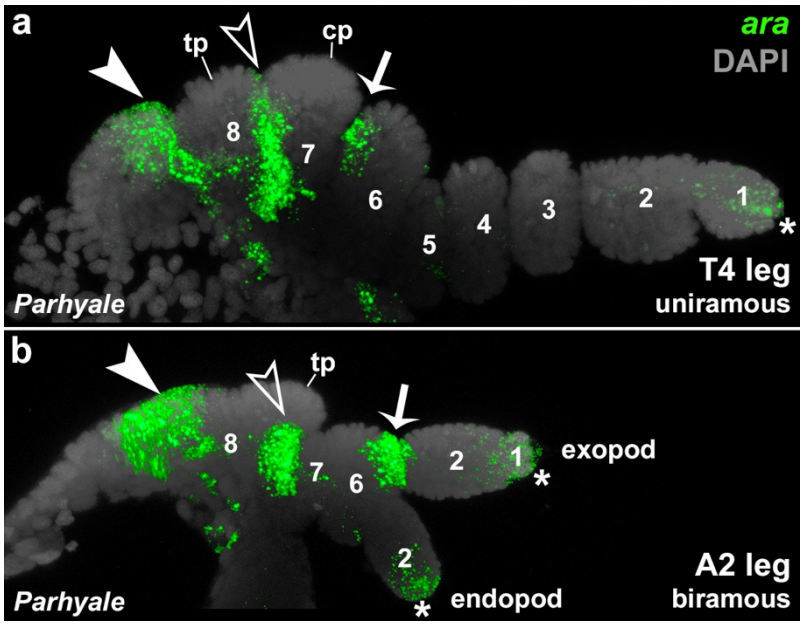

a
ara
DAPI
tp
cp
8
7
6
5
4
3
2
1
*
T4 leg
uniramous
Parhyale
b
tp
8
7
6
2
1
exopod
*
2
endopod
*
A2 leg
biramous
Parhyale

Fig. S2

490

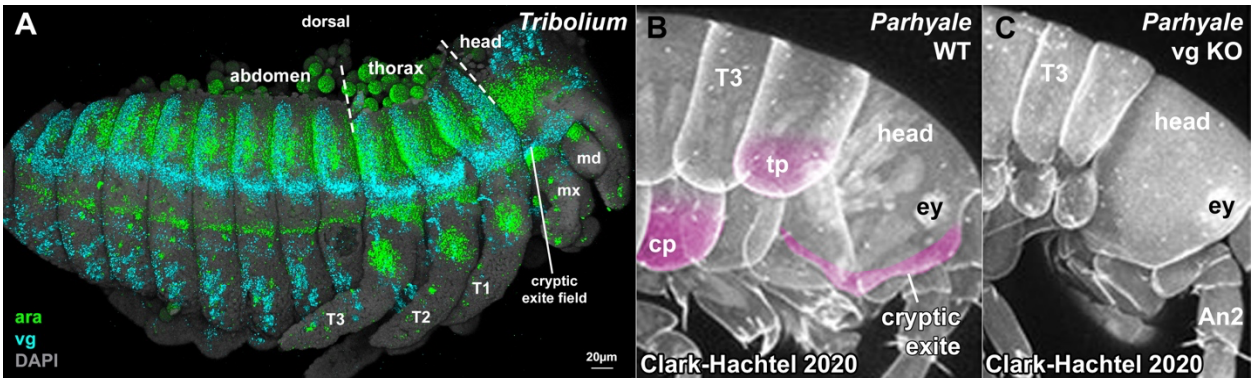

A
dorsal
Tribolium
head
abdomen
thorax
md
mx
cryptic
exite field
T1
T2
T3
ara
vg
DAPI
20µm
B
Parhyale
WT
T3
head
tp
ey
cp
cryptic
exite
Clark-Hachtel 2020
C
Parhyale
vg KO
T3
head
ey
An2
Clark-Hachtel 2020

Fig. S3

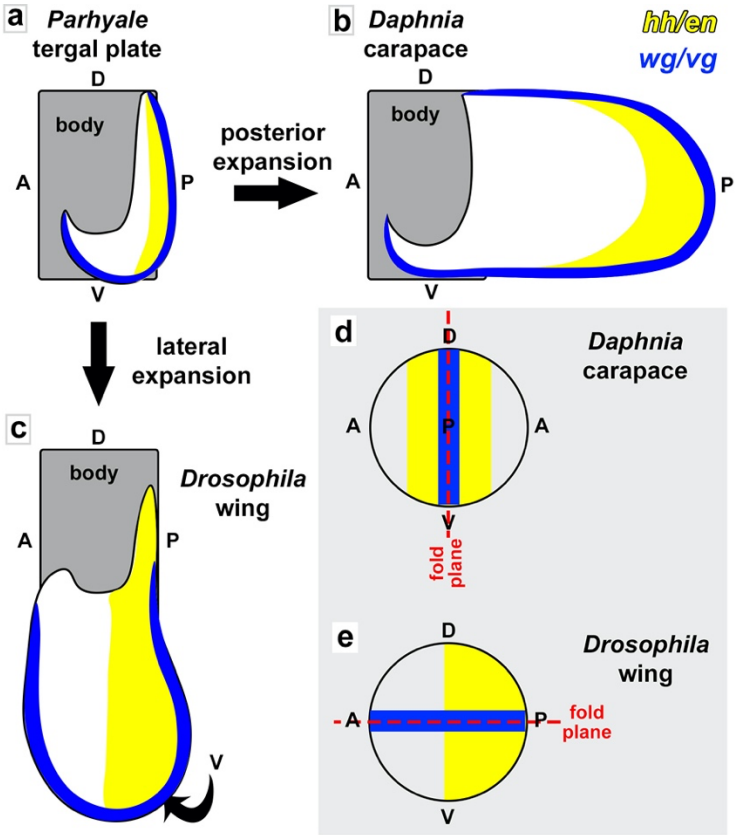

a
Parhyale
tergal plate
D
body
A
P
V
posterior
expansion
b
Daphnia
carapace
hh/en
wg/vg
D
body
A
P
V
lateral
expansion
c
D
body
Drosophila
wing
A
P
V
d
D
Daphnia
carapace
A
P
A
V
fold
plane
e
D
Drosophila
wing
A
P
fold
plane
V

Fig. S4