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. The carapace of the waterflea *Daphnia* 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[1–3]. 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 ancestral exite (lateral lobe) that emerges from a 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[4]. Remarkably, the *vestigial*-positive region that gives rise to the *Daphnia* carapace appears to be present in *Parhyale*[5] and *Tribolium* as a small, inconspicuous protrusion. Similarly, the *vestigial*-positive developmental fields that form tergal plates in *Parhyale* appear to be present in *Daphnia*, even though *Daphnia* does not form tergal plates. Thus, rather than a novel structure resulting from gene co-option, the *Daphnia* carapace appears to have arisen from a shared, ancestral developmental field that persists in a cryptic state in other arthropod lineages. Cryptic persistence of unrecognized serially homologous developmental fields may thus be a general solution for the origin of novel structures. Our 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.

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

Results: In the water flea *Daphnia magna*, the carapace is a bivalved "cape" of exoskeleton that surrounds the animal. Shiga 2017 [1] showed that the *Daphnia* carapace emerges from the dorsal region of the head (at approximately maxilla 2), and, like insect wings, is composed of a

35 bilayered sheet of ectodermal cells and expresses and requires the "wing" genes *wingless* (*wg*), *vestigial* (*vg*) and *scalloped* (*sd*)[1]. 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[4] and Bruce and Patel 2021[6] suggests an alternative hypothesis. These analyses, drawing on over a century of morphological and embryological
40 studies as well as gene expression and loss-of-function studies, generated a model for understanding the homologies of any structure on any arthropod leg (Fig. 1). In this model, most arthropods have incorporated one or two ancestral proximal leg segments into the body wall [4,6–10] (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 body wall can be
45 distinguished by the expression of *pannier* (*pnr*) and *Iroquois* complex genes such as *araucan* (*ara*). 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. Bruce and Patel 2020[4] showed that the insect wing and *Parhyale* tergal plate are both
50 derived from ancestral exites: multi-functional lobes that emerge from proximal leg segments and that express and require "wing" genes such as *vg* and *sd*[5,11,12]. 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 suggests that
55 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[4,5] and the insect wing[4].

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*
60 *magna*, *Tribolium castaneum*, and *Parhyale hawaiensis* using in situ hybridization chain reaction (HCR) version 3.0[13,14]. A single *pnr* gene was identified in *Daphnia* which was the reciprocal best blast hit of *Drosophila*, *Tribolium*, and *Parhyale pnr* [4]. 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* [4]. *Daphnia vg* was identified
65 previously by Shiga 2017[1].

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 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[1].

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 dot on the lateral side of the leg. However, in the biramous (split) legs of *Daphnia* (Figs. 2d and 3d, S1) and *Parhyale* (Fig. S2) where an exopod emerges on the lateral side of leg segment 6, the *ara* dot is expanded to encircle the base of the exopod (see Fig. S1 for explanation of *Daphnia* leg identification).

If the *Daphnia* carapace is the exite of the 8th leg segment of a mouthpart (modified leg) on the head, this exite may still exist on the heads 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 and antenna (Fig. S3). 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 serially homologous to the 8th leg segment. The antenna *vg* domain may pattern a small shelf-like protrusion called the gena, which forms the characteristic indentation on the *Tribolium* eye. However, there is no obvious structure associated with the mouthpart *vg* domain. In *Parhyale*, *vg* patterns the flanges that protect the mouthparts, because these flanges are reduced when *vg* is knocked out (compare Figs. 1f, j in Clark-Hachtel 2020[5]). These flanges are serially homologous to the 8th leg segment, because they are bracketed by *ara* expression. Thus, rather than new, co-opted domains of *vg* expression, these *vg* head domains are ancient and conserved.

Similarly, while *Daphnia* does not form tergal plates like *Parhyale*, these exite fields still appear 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 exite fields, just like the segmentally repeating *vg*-positive *Parhyale* tergal plates. Thus, even though *Daphnia* does not form tergal plates, it still retains the regulatory architecture for making them in serially homologous positions in each body segment, which could presumably be activated in future lineages.

Shiga 2017[1] 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[5]. 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 positionally (proximo-distally) homologous to the *Parhyale* tergal plate and the insect wing (Fig. 4). Many arthropods have lateral structures (carapaces, plates, gills, glands, etc), the homologies of which are frequently debated[11,15]. 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.

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[16]. 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)[1–3,17–26].

However, the work presented here, and in Bruce 2020[4] and Bruce 2021[6], provides an alternative hypothesis: these leg-associated structures (and perhaps other novel structures too) are better explained as arising at previously unrecognized serially homologous locations from existing developmental fields (for example, crustacean leg exites). In this model, developmental, morphogenetic fields can persist in a cryptic, morphologically 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 locations 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 the same proximal-distal register, but at presumably serially homologous anterior-posterior locations[27,28]. 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 developmental field may not form an obvious structure at all, as in the *vg*-positive regions of the *Tribolium* head and *Daphnia* dorsal body. Rather than de novo co-options, these developmental 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 developmental fields may therefore provide a mechanistically satisfying explanation for the origin of novel structures.

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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 [6]. **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. *pnr* (red), *ara* (green), *vg* (light blue), DAPI (grey). **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 embryos, 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. lab, labrum. abd, abdomen.

Fig. 3. Expression of *pnr* and *ara* elucidates the proximal leg segments. Dissected right half of *Parhyale* (a), *Tribolium* (c), and *Daphnia* (e) embryos. Dissected leg of *Parhyale* (b), *Tribolium* (d), and *Daphnia* (f) embryos. Large cells dorsal to *pnr* expression in a, 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[6], 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[4].

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

Animal care. Daphnia were kept in *Daphnia* culture medium[29] 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[14]. 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 legs. *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, it can be seen that the exopod and endopod of all thoracic legs each have fewer than 5 distal leg segments and no terminal claw, therefore *Daphnia* thoracic legs do not 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.

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 but is here labeled as leg segment 2 for simplicity.

Fig. S3. Expression of *vg* in *Tribolium*. *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, which are serially homologous to the wings[28], and therefore also exites. 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 maxilla (mx, arrow) and antenna (Ant). The *vg* domain in the dorsal/proximal antenna may pattern a shelf-like structure called the gena, which forms the characteristic indent in the *Tribolium* eye. However, the *vg* domain in the dorsal/proximal maxilla does not pattern any obvious outgrowth.

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[1] and Clark-Hatchel et al. 2020[5]. In *Parhyale* tergal plates, *vg* is expressed along the edge in a J-shape and *en* is expressed along the posterior edge [5]. 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. 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.

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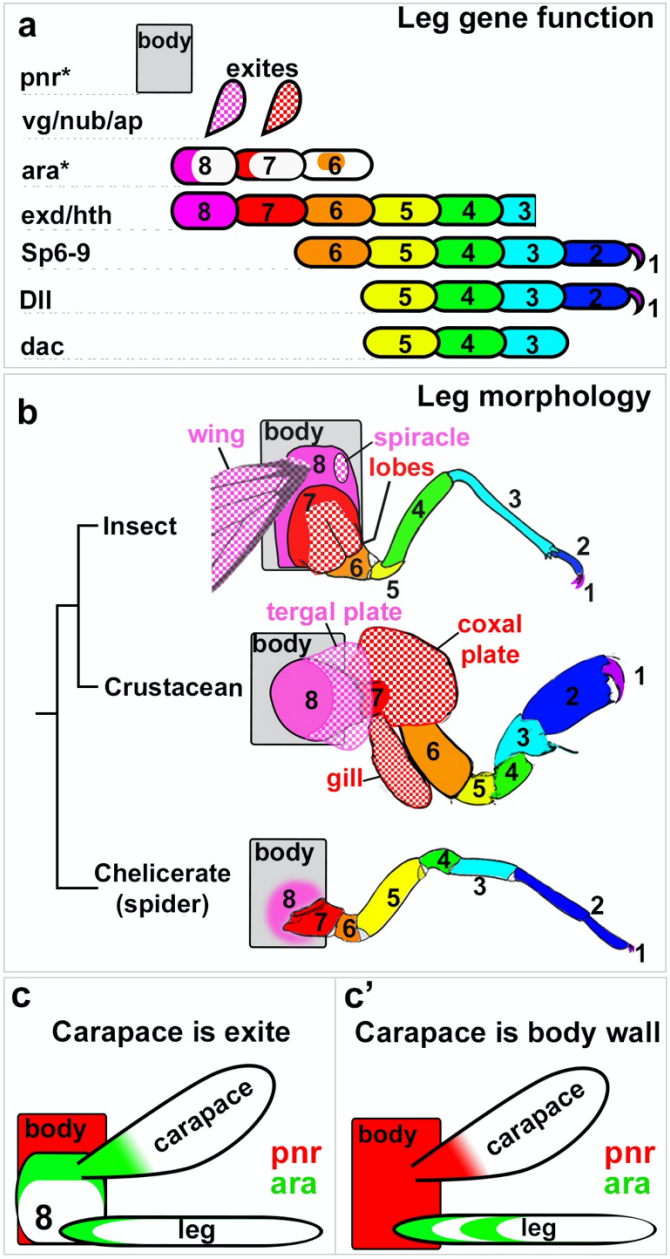
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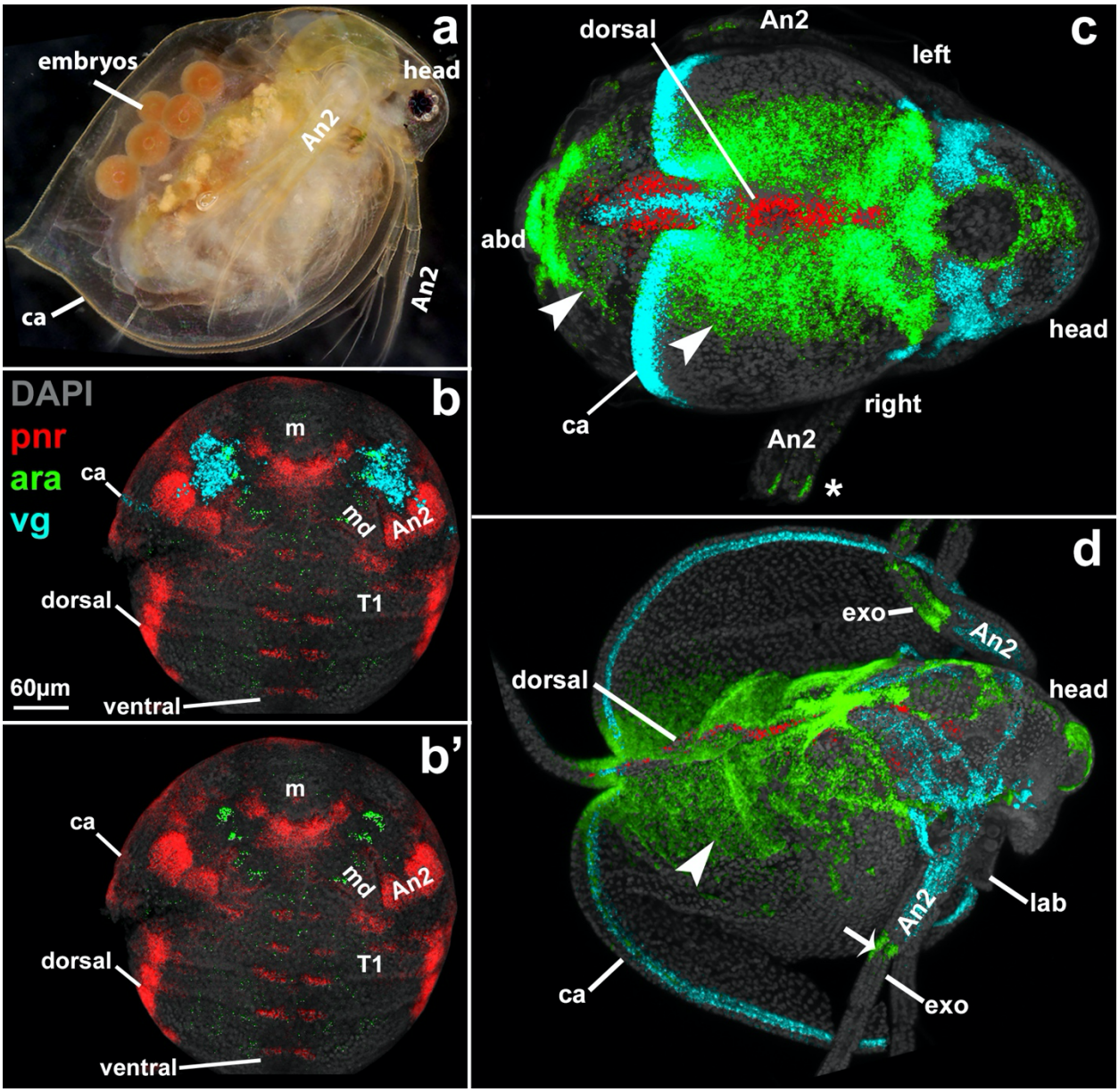
Fig. 1.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md
An2
dorsal
T1
60µm
ventral
b'
m
ca
md
An2
dorsal
T1
ventral
c
dorsal
An2
left
abd
head
ca
right
An2
*
d
exo
An2
dorsal
head
lab
An2
ca
exo

Fig. 2

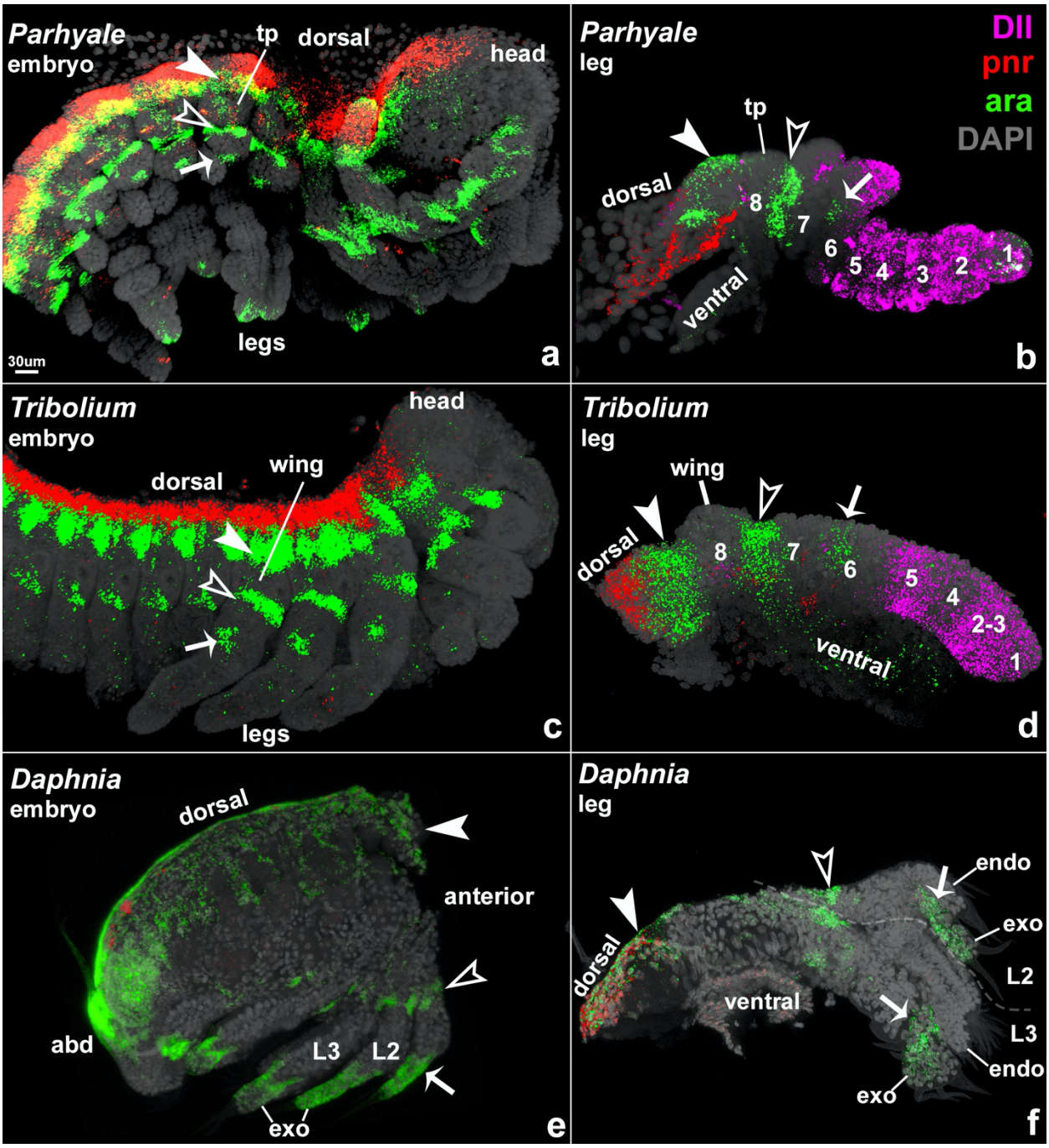

Parhyale
embryo
tp
dorsal
head
legs
30um
a
Parhyale
leg
Dll
pnr
ara
DAPI
tp
dorsal
ventral
8
7
6
5
4
3
2
1
b
Tribolium
embryo
head
dorsal
wing
legs
c
Tribolium
leg
wing
dorsal
ventral
8
7
6
5
4
2-3
1
d
Daphnia
embryo
dorsal
anterior
abd
L3
L2
exo
e
Daphnia
leg
endo
exo
L2
L3
endo
exo
dorsal
ventral
f

Fig. 3

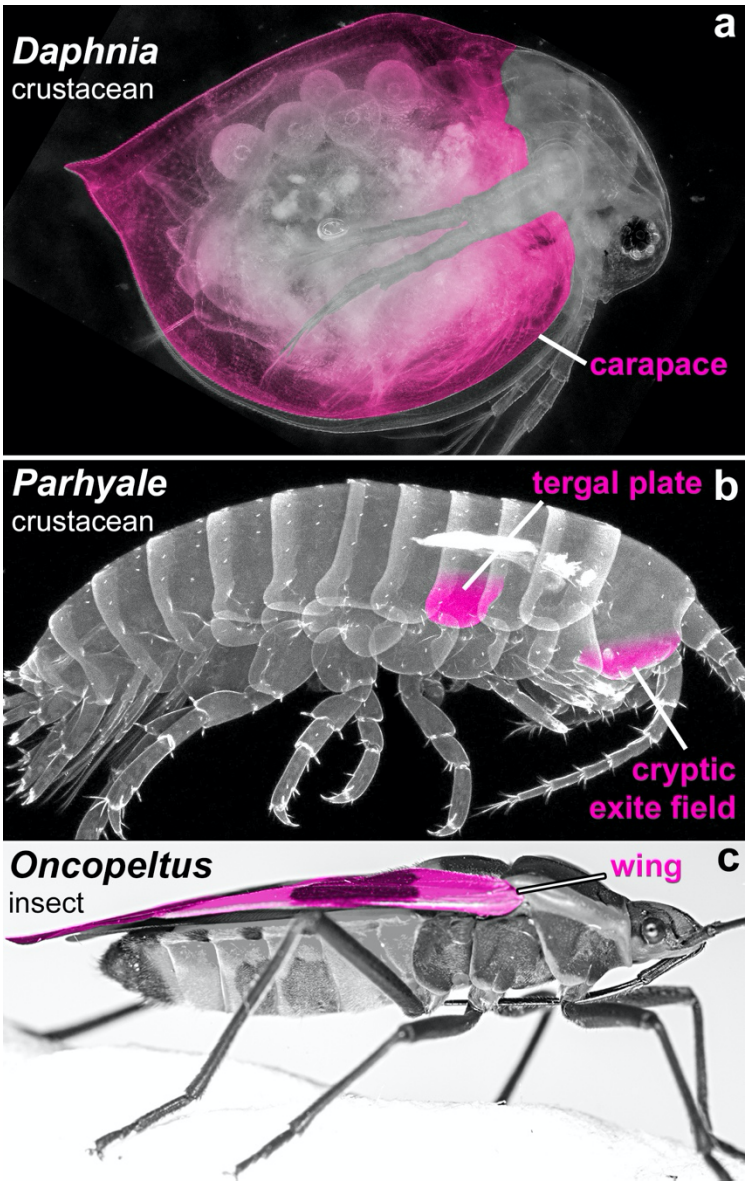

a
Daphnia
crustacean
carapace
b
Parhyale
crustacean
tergal plate
cryptic
exite field
c
Oncopeltus
insect
wing

Fig. 4

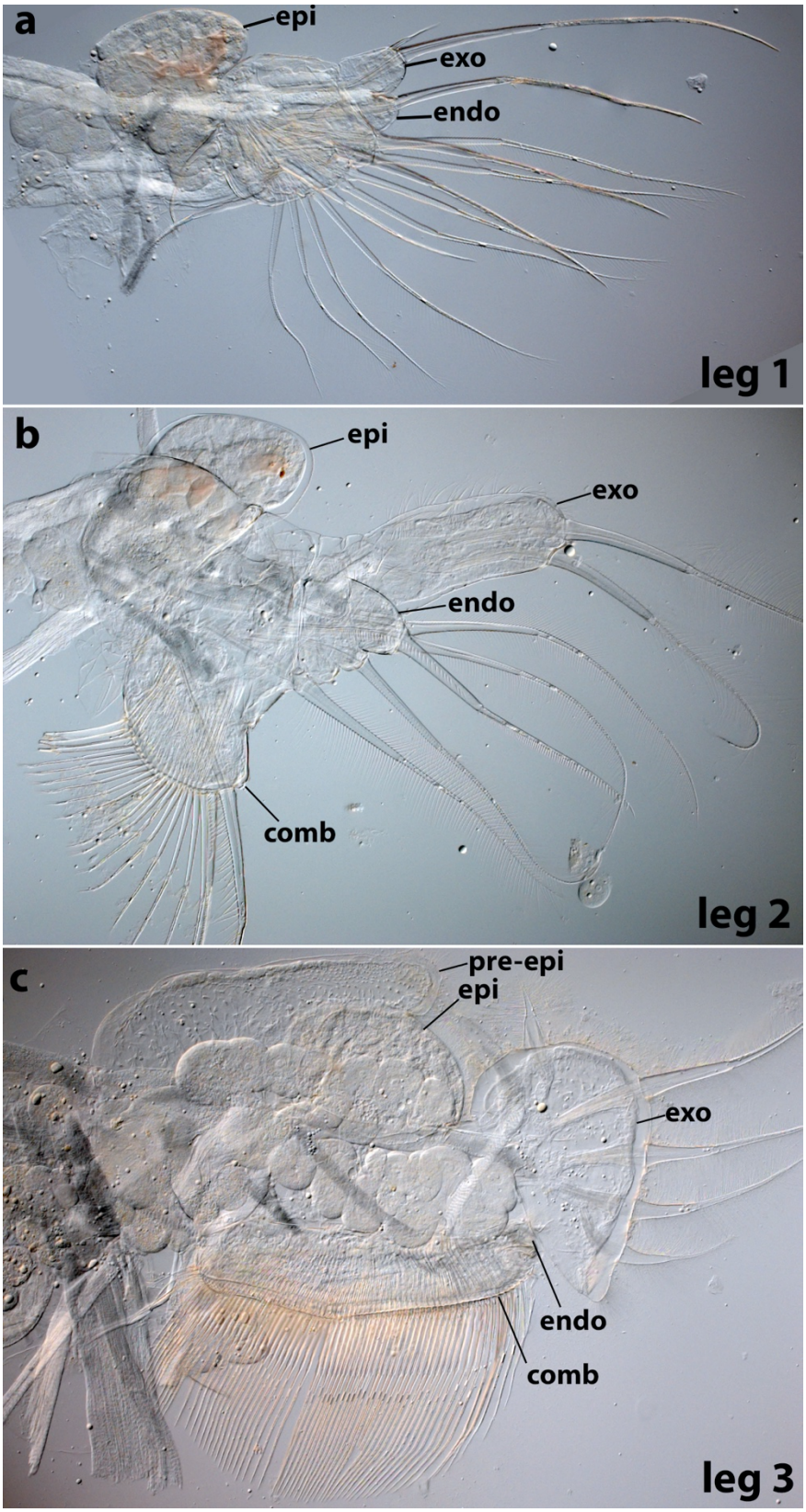

a
epi
exo
endo
leg 1
b
epi
exo
endo
comb
leg 2
c
pre-epi
epi
exo
endo
comb
leg 3

Fig. S1

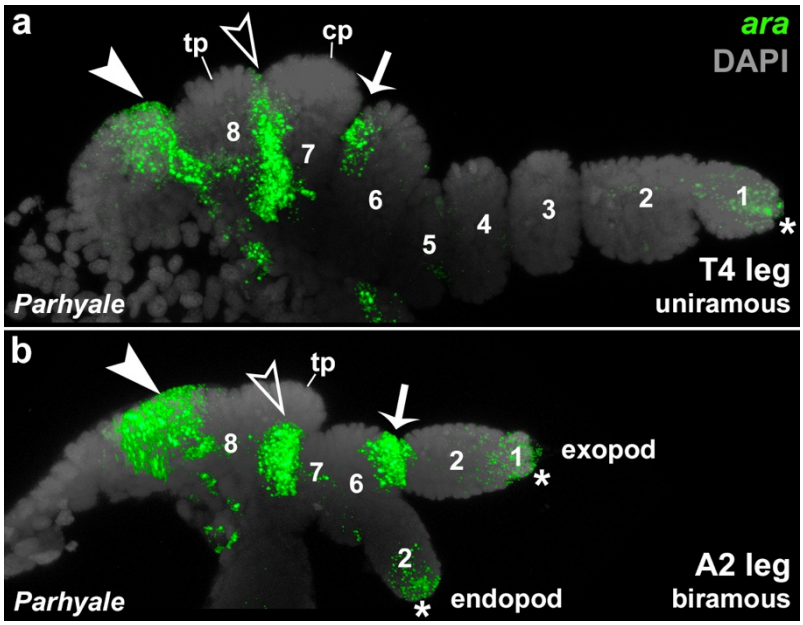

a
ara
DAPI
tp
cp
8
7
6
5
4
3
2
1
*
T4 leg
uniramous
Parhyale
b
tp
8
7
6
2
1
exopod
*
2
endopod
*
A2 leg
biramous
Parhyale

Fig. S2

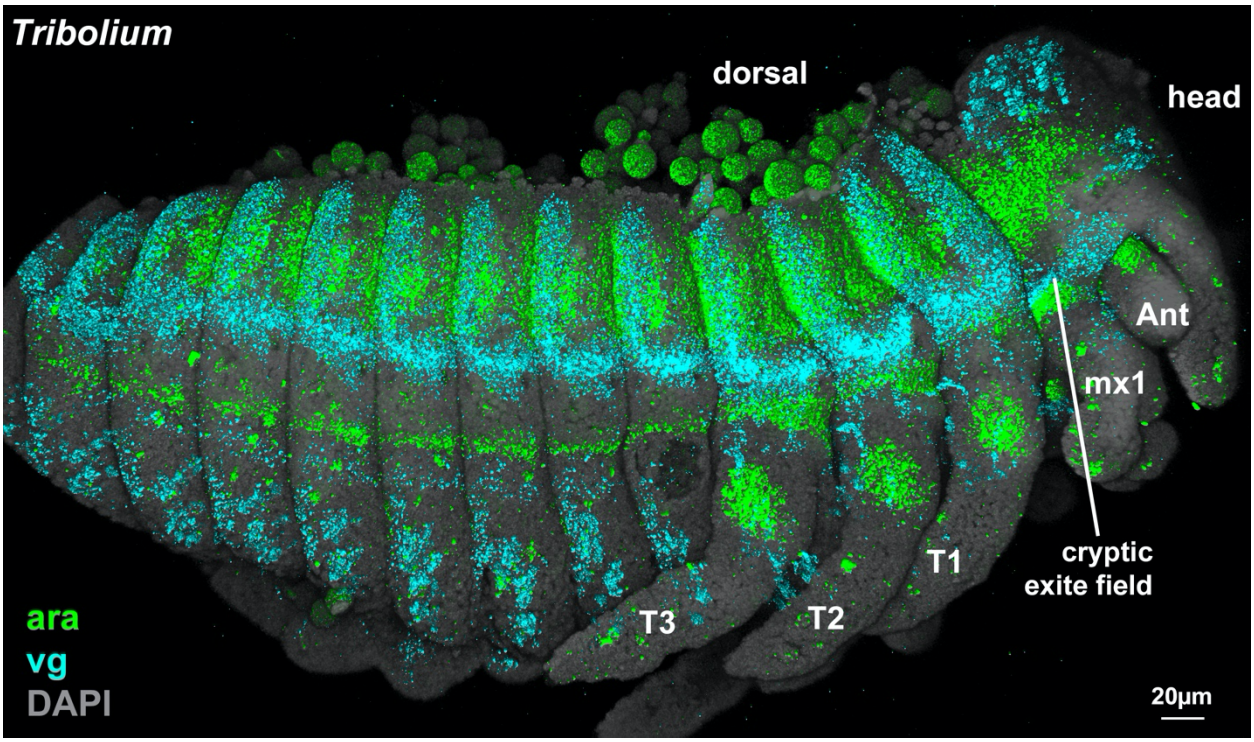
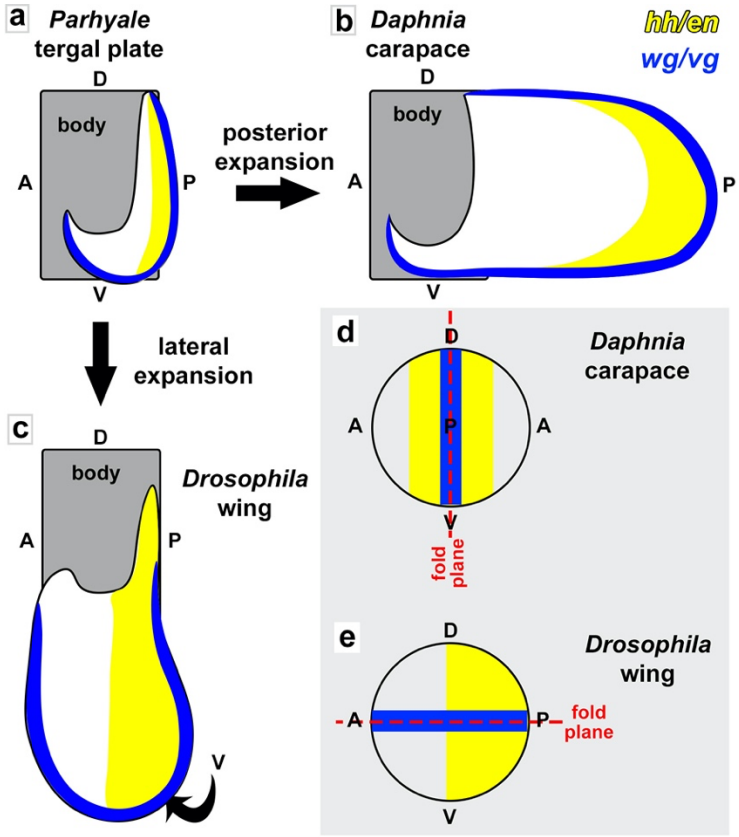

Tribolium
dorsal
head
Ant
mx1
cryptic
exite field
T1
T2
T3
ara
vg
DAPI
20µm

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

380

Fig. S4