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

Graphical abstract



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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.

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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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 work² 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 *Parhyale⁷* 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 Patel⁵ and Bruce⁶ 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 Patel⁵ 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* (*DI*), *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, and the two domains of *ara* will be located ventral to the carapace.

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segments²³ 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 *lroquois* 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 Patel⁵ 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-Peck²⁶ 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 Patel⁵⁴ 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 Patel⁵⁴ 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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SUPPLEMENTAL INFORMATION

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

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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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STAR***METHODS**

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER	
Chemicals, peptides, and recombinant prote	eins		
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 N/A Medical School		
Parhyale hawaiensis, strain Chicago-F	Nipam Patel, Marine Biological Laboratory	Nipam Patel, Marine N/A Biological Laboratory	
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: P		
Daphnia magna vestigial	GenBank: AB465512 Molecular Instruments Lot Number: PRG13		
Tribolium castaneum pannier	GenBank: XM_008202266.2	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: PR		
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.	Rambaut, A. FigTree v1.3.1.http://tree.bio.ed.ac.uk/software/figtree/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	Darriba et al. ⁶² https://github.com/ddarriba/prottest3	
Zen Black 2.3	Zeiss	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/	

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 medium⁶⁴ 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 Tomoyasu⁶⁵)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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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 2001⁵⁹). 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 GATA456⁶⁸ 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/).

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Supplemental Information

The *Daphnia* carapace and other novel structures evolved via the cryptic persistence of serial homologs Heather S. Bruce and Nipam H. Patel



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.



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}.



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 8^{th} 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}).



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^{84} 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^{85}). 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

Parhyale	Accession:	CCTCAACCTACCTCCAACaaTTGAATTCAACAAACTGGAACAAAC
hawaiensis	MG703506.1	AGCTTTAAACATTGCGGTCAGAGTTatTCTCACCATATTCGCTTC
		CCTCAACCTACCTCCAACaaTGGGAAGGGATTTTTATTTCATAAA
vestigiai	B4 initiator	GGTCAGTAATTTCGCTCTTTATTGCatTCTCACCATATTCGCTTC
	adapter	CCTCAACCTACCTCCAACaaTTCTTTGGTTTCGCGTTCCCTCAGA
	20 probe pairs	GTCCAAATTACAGGCAGAAATGCCAatTCTCACCATATTCGCTTC
	(40 sequences)	CCTCAACCTACCTCCAACaaGTTTTTATAAGTAAAACAAGTTAAT
		AGCTTTAAAACGTGCTTTCCCCAAGatTCTCACCATATTCGCTTC
		CCTCAACCTACCTCCAACaaATCAGAGATTGCGTGGAGCCGGGAA
		GACATCTGCCTATGATGCAAATTTAatTCTCACCATATTCGCTTC
		CCTCAACCTACCTCCAACaaAATATACGGCCGTGTGATGTGGTTT
		CTAGCTGCAGAATACGACATGACGTatTCTCACCATATTCGCTTC
		CCTCAACCTACCTCCAACaaCTGCCGACGTGAAGCAATTGATGAC
		GTATGCTGTAATATCCGTTAGTTGGatTCTCACCATATTCGCTTC
		CCTCAACCTACCTCCAACaaTTTACTGTCTTTAATCTATTTATC
		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).

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