1 Title: Insect wings and body wall evolved from ancient leg segments

2 One Sentence Summary: CRISPR-Cas9 knockout of leg gap genes in a crustacean reveals

3 that insect wings are not novel structures, they evolved from crustacean leg segments

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8 Abstract: The origin of insect wings has long been debated. Central to this debate is 9 whether wings evolved from an epipod (outgrowth, e.g., a gill) on ancestral crustacean leg 10 segments, or represent a novel outgrowth from the dorsal body wall that co-opted some of 11 the genes used to pattern the epipods. To determine whether wings can be traced to 12 ancestral, pre-insect structures, or arose by co-option, comparisons are necessary between insects and arthropods more representative of the ancestral state, where the hypothesized 13 14 proximal leg region is not fused to the body wall. To do so, we examined the function of five 15 leg patterning genes in the crustacean Parhyale hawaiensis and compared this to previous 16 functional data from insects. By comparing gene knockout phenotypes of leg patterning 17 genes in a crustacean with those of insects, we show that two ancestral crustacean leg 18 segments were incorporated into the insect body, moving the leg's epipod dorsally, up onto 19 the back to form insect wings. Thus, our data shows that much of the body wall of insects, 20 including the entire wing, is derived from these two ancestral proximal leg segments. This 21 model explains all observations in favor of either the body wall origin or proximal leg 22 origin of insect wings. Thus, our results show that insect wings are not novel structures, but 23 instead evolved from existing, ancestral structures.

Main Text: The origin of insect wings has fascinated researchers for over 130 years. One theory proposes that the proximal portion of the ancestral crustacean leg became incorporated into the body (*1*), which moved the leg's epipod (lobe-shaped outgrowth, e.g. gill) dorsally, up onto the back to form insect wings (2). Another theory proposes that the wing is a novel outgrowth from the dorsal body wall that co-opted some of the genes used to pattern the epipods of leg segments (3). Alternatively, wings may be derived from a combination of leg and body wall (dual origin, (4)). To determine whether wings can be traced to ancestral, pre-insect structures, or arose by co-option, comparisons are necessary between insects and other arthropods more representative of the ancestral state, where the hypothesized proximal leg region is not fused to the body wall.

34 Towards this aim, we examined five leg gap genes, Distalless (Dll), Sp6-9, dachshund 35 (dac), extradenticle (exd), and homothorax (hth), in an amphipod crustacean, Parhyale 36 hawaiensis. While we have documented their expression at several developmental stages (Fig. 37 S1), our comparative analysis does not rely solely on these expression patterns, given that 38 expression is not always a reliable indication of function, and expression is often temporally 39 dynamic (5). Instead, we have systematically knocked out these genes in *Parhyale* using 40 CRISPR- Cas9 mutagenesis and compared this to our understanding of their function in 41 Drosophila and other insects (Figs. 2, S2).

42 Insects have six leg segments, while *Parhyale* has seven (Fig. 1). In insects, *Dll* is 43 required for the development of leg segments 2-6(6-9). In *Parhyale*, the canonical *Dll* gene, 44 Dll-e (10-12), is required for the development of leg segments 3-7 (Fig. 2b). In insects, Sp6-9 45 (13) is required for the development of leg segments 1 - 6 (14), and in addition in Drosophila, loss of Sp6-9 (i.e. D-Sp1, (13)) occasionally transforms the leg towards wing and lateral body 46 47 wall identity (14). In Parhyale, Sp6-9 (13, 15) is required for the development of leg segments 2 48 -7 (Fig. 2c), and in some legs, segment 2 is occasionally transformed towards a leg segment 1 49 identity (Fig S3). In *Drosophila*, dac is required in the trochanter through proximal tarsus (leg

segments 2-4, and first tarsus, (15, 16). Parhyale has two dac paralogs. dac1 does not seem to 50 51 be expressed in the legs or have a morphologically visible knockout phenotype. *dac2* is required 52 to pattern leg segments 3-5 (Fig. 2d). exd and hth are expressed in the body wall and proximal 53 leg segments of insects (17-20) and Parhyale (21) (Fig S1). They form heterodimers and 54 therefore have similar phenotypes (17-20). In insects, exd or hth knockout results in 55 deletions/fusions of the coxa through proximal tibia (leg segments 1-3, and proximal tibia, 17-56 20). In *Parhyale, exd* or *hth* knockout results in deletions/fusions of the coxa through proximal 57 carpus (leg segments 1 - 4, and proximal carpus; Figs. 2e, f). In both insects (17, 18, 22) and 58 *Parhyale*, the remaining distal leg segments are sometimes transformed towards a generalized 59 thoracic leg identity (compare Fig. 2 e, f and Fig S4). In both insects (17-20) and Parhyale (Fig. 60 S4), exd or hth knockout results in deletions/fusions of body segments. 61 In summary, the expression and function of *Dll*, *Sp6-9*, *dac*, *exd*, and *hth* in *Parhyale* are 62 shifted distally by one segment relative to insects. This shift is accounted for if insects fused an 63 ancestral proximal leg segment into the body wall (Fig. 2g). Thus, there is a one-to-one 64 homology between insect and *Parhyale* legs, displaced by one segment, such that the insect coxa 65 is homologous to the crustacean basis (23), the insect femur is the crustacean ischium, and so on 66 for all leg segments. This also means that part of the insect body wall is homologous to the 67 crustacean coxa.

68 Clark-Hachtel (accompanying manuscript) show that the plates on the *Parhyale* basis, 69 coxa, and lateral body wall are epipods. The body wall epipod is notable, because epipods are 70 characteristic of leg segments (*23*). This suggested to us that part of the *Parhyale* body wall 71 might actually be an additional leg segment. In fact, most groups of crustaceans have an 72 additional proximal leg segment, the precoxa (Fig. 3a). To determine whether *Parhyale* retains the precoxa, we examined dissected *Parhyale* using confocal and brightfield microscopy. We
identified a precoxal structure that meets the criteria for a true leg segment: it protrudes
conspicuously from the body wall; it forms a true, muscled joint; and it extends musculature to
another leg segment (Figs. 3 and S5, (23-25)). Importantly, the plate does not emerge from the
body wall, but from the precoxa (Fig. 3e), like the plates of the coxa and basis. Thus, much of
what appears to be lateral body wall in *Parhyale* is in fact proximal leg.

79 If the insect coxa is homologous to the crustacean basis, what happened to the leg 80 segments corresponding to the ancestral crustacean precoxa and crustacean coxa in insects? If 81 these two leg segments became incorporated into the body wall, then one would expect to find 82 two leg segments and two epipods dorsal to the insect coxa (Fig. 4a). As predicted, two leg-like 83 segments can be observed proximal to the coxa in basal hexapods (1) including collembolans 84 (26), as well as in the embryos of many insects (27-29), where these two leg-like segments 85 flatten out before hatching to form the lateral body wall (1, 26-31). Insects also appear to have 86 two epipods dorsal to the insect coxa, because when "wing" genes are depleted in insects via 87 RNAi, two distinct outgrowths are affected, the wing and the plate adjacent to the leg (Fig. 1c, 88 (32-35)).

Based on these data, insects incorporated two ancestral leg segments, the precoxa and crustacean coxa, into the body wall (Fig. 4a). Thus, like *Parhyale*, much of what appears to be lateral body wall in insects is in fact proximal leg. Clark-Hachtel's interpretation of the dual origin theory proposes that these two leg segments and their two epipods fused to form the wing. While we agree that both leg segments may contribute wing muscle, we propose that only the more dorsal precoxa epipod formed the insect wing, while the more ventral crustacean coxa epipod formed the insect plate (Fig. 4b).

96 Our results may settle the long-standing debate regarding the origin of insect wings as 97 derived from either an epipod of the leg or from body wall. Our model accounts for all 98 observations in favor of either of these hypotheses, including the dorsal position of insect wings 99 relative to their legs, the loss of ancestral leg segments in insects, the two-segmented 100 morphology of the insect subcoxa in both embryos and adults, the complex musculature for 101 flight, and the shared gene expression between wings and epipods. Our model also explains the 102 apparent "dual origin" of insect wings from both body wall and leg epipod: much of what 103 appears to be insect body wall is in fact the remnant of two ancestral leg segments and their 104 epipods.

105 In fact, a number leg-associated outgrowths in arthropods could be explained by this 106 model, in addition to insect wings. The Daphnia carapace (36) is the epipod of the precoxa(37); 107 the *Oncopeltus* small plate outgrowth (Fig. 1c) is the epipod of the crustacean coxa; and the 108 thoracic stylus of jumping bristletails (Fig. 4, st) is the epipod of the crustacean basis(38, 39). 109 This also explains many insect abdominal appendages, like gills (40), gin traps (33), prolegs (41), 110 and sepsid fly appendages (42), which are often proposed as de novo structures (43-45). However, 111 most insects form abdominal appendages as embryos(40, 46), some even with an epipod nub, but 112 these fuse to the body wall before hatching to form the sternites (28, 39). The existence of insect 113 abdominal appendages is supported by a re-analysis of the expression of Sp6-9 and its paralog, 114 buttonhead, in insect embryos in a previous study (13). According to the leg segment homology 115 model presented here (Fig. 4), the paired dots of *btd* expression on each abdominal segment of 116 insect embryos demonstrates that these appendages are comprised of three leg segments: the 117 precoxa (pink), crustacean coxa (red), and insect coxa (orange). These abdominal appendages are 118 truncated, lacking all distal appendages from the trochanter (vellow) down, because *Dll* and *dac*,

119	which mark the trochanter and more distal leg segments, are not expressed in the insect
120	abdomen. Thus, rather than de novo co-options, abdominal appendages were always there,
121	persisting in a truncated, highly modified state, and de-repressed in various lineages to form
122	apparently novel structures. This provides a model for how insect wings can be both homologous
123	to the epipod of the crustacean precoxa, and yet not be continuously present in the fossil record:
124	epipod fields may persist in a truncated state, perhaps only visible as a nub in the embryo. We
125	therefore propose cryptic persistence via truncation as a general mechanism for the origin of
126	apparently novel structures that appear to be derived from serial homologs, rather than the
127	current model of extensive gene co-option.

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248 Supplementary Materials:

- 249 Materials and Methods
- 250 Figures S1-S5
- 251 Table S1
- 252 References 37-43

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Fig. 1. Crustacean and insect legs. (a) Adult *Parhyale*, with third thoracic leg (T3) outlined. (b)

- 256 Cartoon of *Parhyale* T3. The coxal plate extends over the leg. (c) Adult *Oncopeltus*, with T2
- 257 outlined. Inset shows magnified proximal leg, with body wall plate extending over the leg. (d)
- 258 Cartoon of *Oncopeltus* T2 leg.

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Fig. 2. Knockout phenotypes of leg gap genes. (a-f) *Parhyale* CRISPR-Cas9 phenotypes in dissected third thoracic legs (T3). Graded cyan in f indicates deletion/fusion of proximal leg segment 5. (g) Leg gap gene function in *Parhyale* and insects aligns only if insects incorporated the red leg segment into the body wall (0). Color bars correspond to remaining leg segments following knockout, transparent bars indicate deleted leg segments. Open bar in *dac* indicates slight extension of *dac* function into tarsus 1 of insects. Coxal plate (Cp), gill (G), tergal plate (Tp). Scale bar 50um.

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Fig. 3. Evidence for a precoxa in Parhyale. (a) Phylogeny based on Oakley 2012, precoxa 269 270 references in supplements. (b) Confocal image of Parhyale hatchling, autofluorescent cuticle in 271 blue. T5, T6 tergal plates (dotted outlines). (c) Confocal image of *Parhyale* hatchling, 272 autofluorescent cuticle in blue, muscle phalloidin stain in red. Compare blocks of simple, 273 anterior-posterior muscles of the body to orthogonal, complexly arranged muscles of the leg 274 segments. Note overlap between tergal plate (dotted lines) and orthogonal leg muscle. (d) 275 Brightfield image of right half of *Parhyale*, sagittal dissection, internal tissues removed, lateral 276 view. Wire used to position sample (w). The same orthogonal muscles in b are visible as 277 striations extending above the wire. The precoxa forms a joint with the coxa (47) (arrow). The 278 dorsal limit of the precoxa is unclear: a conservative estimate is to begin at the joint (arrow) and

279 follow the leg up to where it meets the adjacent leg, denoted by (<); however, the orthogonal 280 muscle striations continue farther up (pink outline). Either way, the precoxa protrudes quite a bit 281 from the body wall. (e) Posterior-lateral view of right T6, looking edge-on at tergal plate. The 282 tergal plate (dotted outline) emerges from the precoxa (contiguous pink between \leftarrow , >, and ---), 283 just as the coxal plate (dashed line) emerges from the coxa. In c, d, coxa is red (coxal plate not 284 shaded), gills (teal) partially cut for visibility, basis orange, precoxa pink. Note that all three 285 plates (tergal, coxal, and basal) form contiguous cuticle with their leg segment, i.e. there is no 286 distinguishing suture. Scale bar 100um.

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Fig. 4. Proposed leg segment homologies (colors) between insects, *Parhyale*, and an ancestral crustacean (a) based on gene function alignment (b). Ancestral precoxa epipod (pink ep),

- 290 *Parhyale* tergal plate (Tp), and insect wing are homologous (pink). Ancestral coxa epipod,
- 291 *Parhyale* coxal plate (Cp) and gill (G), and insect plate (see Fig. 1c) are homologous (red).
- 292 *Parhyale* basal plate (Bp). Insect numbering based on crustaceans.