

Paranotal Lobes Are Appendicular in Origin: Elucidation by Micro-CT Analysis of the Thoracic Muscular System in the Larvae of *Carabus insulicola* (Insecta, Coleoptera)

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Abstract

In the embryo of *Carabus insulicola*, Kobayashi et al. (2013: J. Morphol., **274**, 1323–1352) revealed that the subcoxa can be divided into subcoxae-1 and 2 by the paracoxal suture (PCXS), and these subcoxae form the larval pleuron. Mashimo and Machida (2017: Sci. Rep., **7**, 12597), however, rejected the appendicular nature of subcoxa-1 and interpreted the PCXS as the boundary between the tergum and appendage (BTA), and thus they regarded subcoxa-1 as a tergal structure. To resolve the disagreement over the PCXS, we examined the larval thoracic muscular system of *C. insulicola* using an X-ray micro-CT. We conclude that subcoxae-1 and 2 are independent podomeres having intrinsic muscles in each region and that the paranotal lobe of the *C. insulicola* larvae is derived from the dorsal half of subcoxa-1. Thus, the paranotal lobe is appendicular in origin, contradictory to traditional interpretations of tergal origin. By comparing the muscle arrangement with the expression of leg patterning genes in *Tribolium castaneum* embryos, we suggest that subcoxa-1 corresponds to the crustacean precoxa, and the molecular BTA, or the boundary between the tergum and precoxa, is located more dorsally than the morphological BTA identified in *C. insulicola*. Combining these morphological and molecular data, we propose the “precoxal theory” for the origin of insect wings.

Embryological evidence for the subcoxal theory

The subcoxal theory of insects postulates that the pleuron is derived from the proximal region of the thoracic appendage, i.e., the subcoxa. The theory was pioneered by the embryological observations of Heymons (1899) in the hemipteran *Naucoris cimicoides*. Although the theory was then advocated and developed by such authorities in insect morphology as Snodgrass (1927), Weber (1928, 1952), and Matsuda (1970), there were few embryological studies (Roonwal, 1937). In recent years, however, embryological evidence for the theory has accumulated in *Galloisiana yuasai* (Grylloblattodea) (Uchifune and Machida, 2005), *Dineutus mellyi* (Coleoptera) (Komatsu and Kobayashi,

2012), *Carabus insulicola* (Coleoptera) (Kobayashi et al., 2013), *Zorotypus caudelli* (Zoraptera) (Mashimo et al., 2014), and *Gryllus bimaculatus* (Orthoptera) (Mashimo and Machida, 2017). Kobayashi et al. (2013) notably demonstrated that the subcoxa of *C. insulicola* at about 35% developmental time (DT) becomes divided into a proximal subcoxa-1 and a distal subcoxa-2, and as development proceeds, a semi-circular furrow, which corresponds to the future paracoxal suture (PCXS), appears between the two subcoxal regions. By comparing these two regions with the spatial arrangement of larval thoracic pleurites, Kobayashi et al. (2013) pointed out that the embryonic subcoxa-1 and subcoxa-2 correspond to the larval

anapleuron and katapleuron, respectively. Kobayashi (2017) continued this work by showing the presence of subcoxae-1 and 2 in the embryos of *D. mellyi*, *Protohermes grandis* (Megaloptera), and *Nemotaulius admorsus* (Trichoptera). However, the subdivision of the embryonic subcoxa has not yet been established in non-holometabolous orders.

An alternative interpretation of the insect subcoxa is offered by Mashimo and Machida (2017).

According to these authors, the thoracic pleuron of *Gr. bimaculatus* completes by the rapid dorsal extension of a single subcoxal region in late embryonic stages. They interpreted the subcoxa of *Ga. yuasai* and the subcoxa-1 of *C. insulicola* as part of the tergum, and thus the PCXS of the latter is not an internal structure of the subcoxa (i.e., a subdivision within the appendage) but the boundary between the tergum and the appendage (BTA).

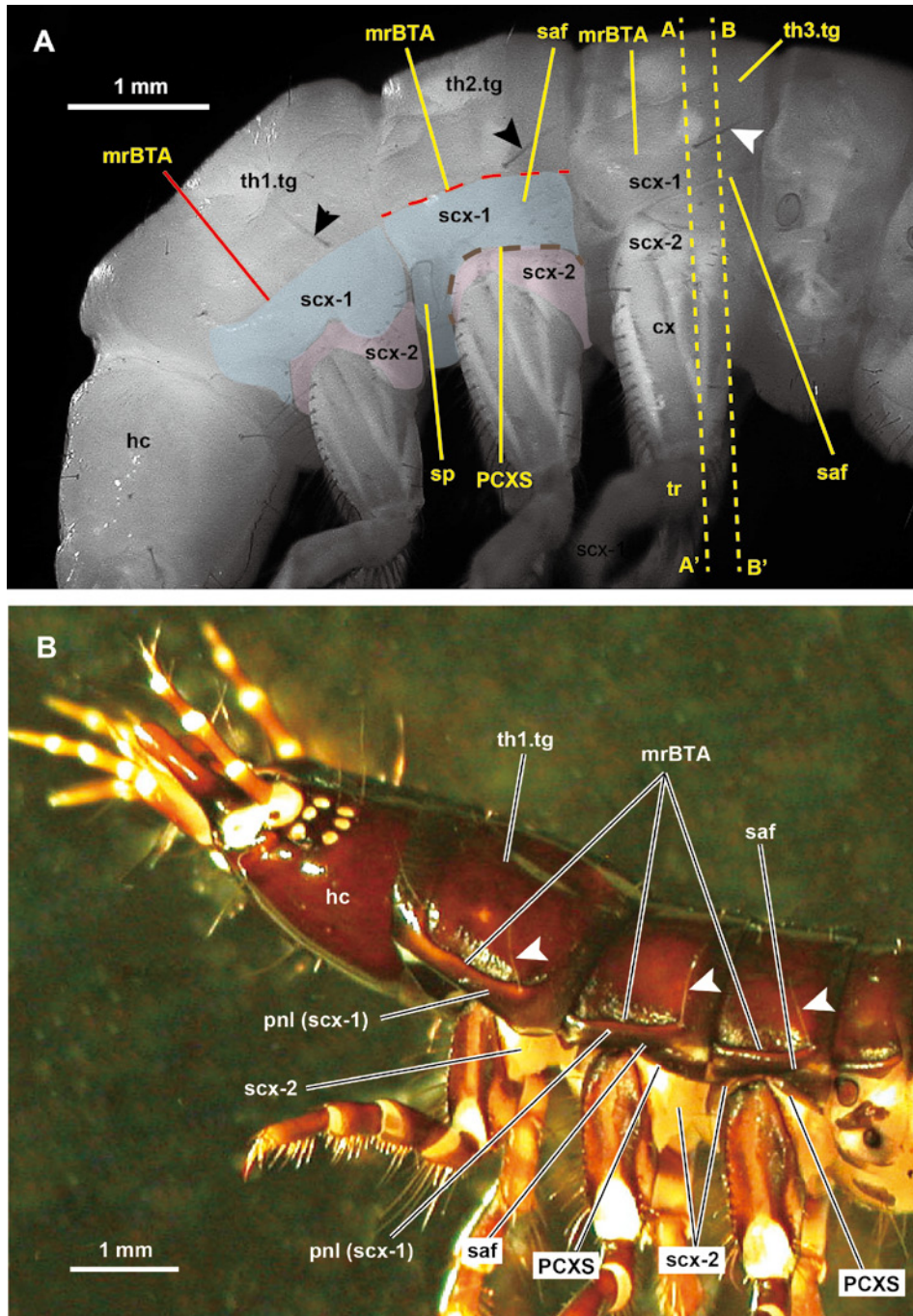


Fig. 1 Lateral views of the larva of *Carabus insulicola*, fixed with alcoholic Bouin's fluid. A. Newly hatched larva. The cuticle is not yet sclerotized, so some muscles can be observed through the semi-transparent cuticle. The regions derived from the embryonic subcoxae-1 and 2 are highlighted in blue and red, respectively. B. About two days after hatching. A-A', B-B': levels of cross sections in Fig. 3A, B, respectively. cx: coxa, hc: head capsule, mrBTA: morphological boundary between tergum and appendage, PCXS: paracoxal suture, pnl: paranotal lobe, saf: subalar fold, scx-1, 2: subcoxa-1, 2, sp: spiracle, th1-3.tg: pro-, meso-, and metathoracic tergum, tr: trochanter, arrowhead: long seta near mrBTA.

Micro-CT analysis of the muscular system in the *Carabus insulicola* larva

To resolve the disagreement over the PCXS, we started with the fact that podomeres (leg segments) are defined as having joints on which muscles either originate or insert. We reasoned that, if subcoxae-1 and 2 are indeed derived from ancestral podomeres, then the ancestral joint between them (our PXCS) should have muscles originating or inserting on it. To determine whether the PCXS has muscles originating or inserting on it, we examined the thoracic muscular system of newly hatched *C. insulicola* larvae using an X-ray micro-CT scanner (XRadia Micro XCT-400 system, ZEISS). Here, we identify all meso- and metathoracic muscles according to Matsuda's (1970) system. Prothoracic muscles were excluded from the examination because the deformation of the segment would deter identification of musculature.

Subcoxae-1 and 2 are independent podomeres

In insects, the PCXS (Fig. 1A) forms an internal paracoxal ridge PCXR (Figs. 2, 6, PCXR) on which several transverse muscles attach (e.g., Matsuda, 1970; Vilhelmsen, 2000; Willkommen, 2008; Wipfler et al., 2015). We compared our data to previous studies of the muscular system of other insects, including representatives from Ephemeroptera, Plecoptera,

Orthoptera, and Odonata (e.g., Snodgrass, 1935, Matsuda, 1970, Willkommen, 2008). However, the most detailed and precise description of muscles attaching to the PCXR has been provided only by Willkommen (2008) in Ephemeroptera. In her paper, muscles t-p 3 and p-cx 5 sensu Matsuda are attached to the dorsal side and ventral side of the anterior PCXR, respectively (cf. Willkommen, 2008; Figs. 16, 17, muscle AN.Pm = t-p 3, P.Cm = p-cx 5). These two muscles specifically attached to the PCXR are observed in the exact same positions in *C. insulicola* (Fig. 2). Therefore, the PCXS/PCXR is not the BTA, hence the division of the subcoxa into subcoxae-1 and 2 is valid (Figs. 1A, 3A, PCXS).

As postulated by Weber (1952), the furca should be regarded as a specialized apodeme formed in the medial part of the PCXS (Fig. 4, fup). In fact, in *Carabus* larvae, a furcal apodeme is located in the sternum slightly distant from the medio-posterior margin of the coxal base of each thoracic leg (Zaitsev, 2008). Many muscles are observed to be attached to the furca in the larvae of *C. insulicola* (Fig. 3A, B, circles indicated by white dotted lines), providing further evidence that the PCXS is the remnant of the ancestral joint between subcoxae-1 and 2.

To determine whether subcoxa-1 (anapleuron) and subcoxa-2 (katapleuron) are derived from two independent podomeres, we analyzed the intrinsic

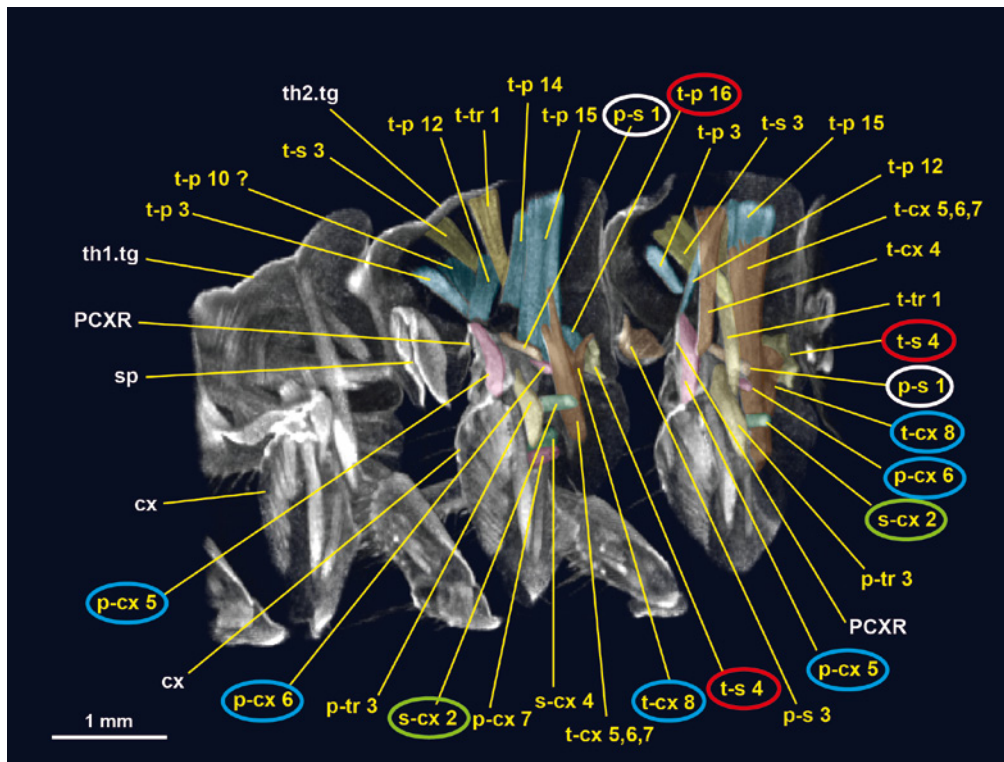


Fig. 2 Parasagittal-sectioned micro-CT image of the newly hatched larva of *Carabus insulicola*, mesal view of the right half of pterothoraxes. The larva in this image is the same as shown in Fig.1A. The image of the alimentary canal was digitally removed from the original image. Abbreviations for muscles follow the Matsuda's system and are highlighted in yellow letters. Red circles indicate intrinsic muscles of subcoxa-1. Blue, green, and white circles indicate pleuro-coxal muscles, "furca-coxal" muscles, and a "pleuro-furcal" muscle of subcoxa-2, respectively. cx: coxa, PCXR: paracoxal ridge, sp: spiracle, th1, 2.tg: pro- and mesothoracic tergum.

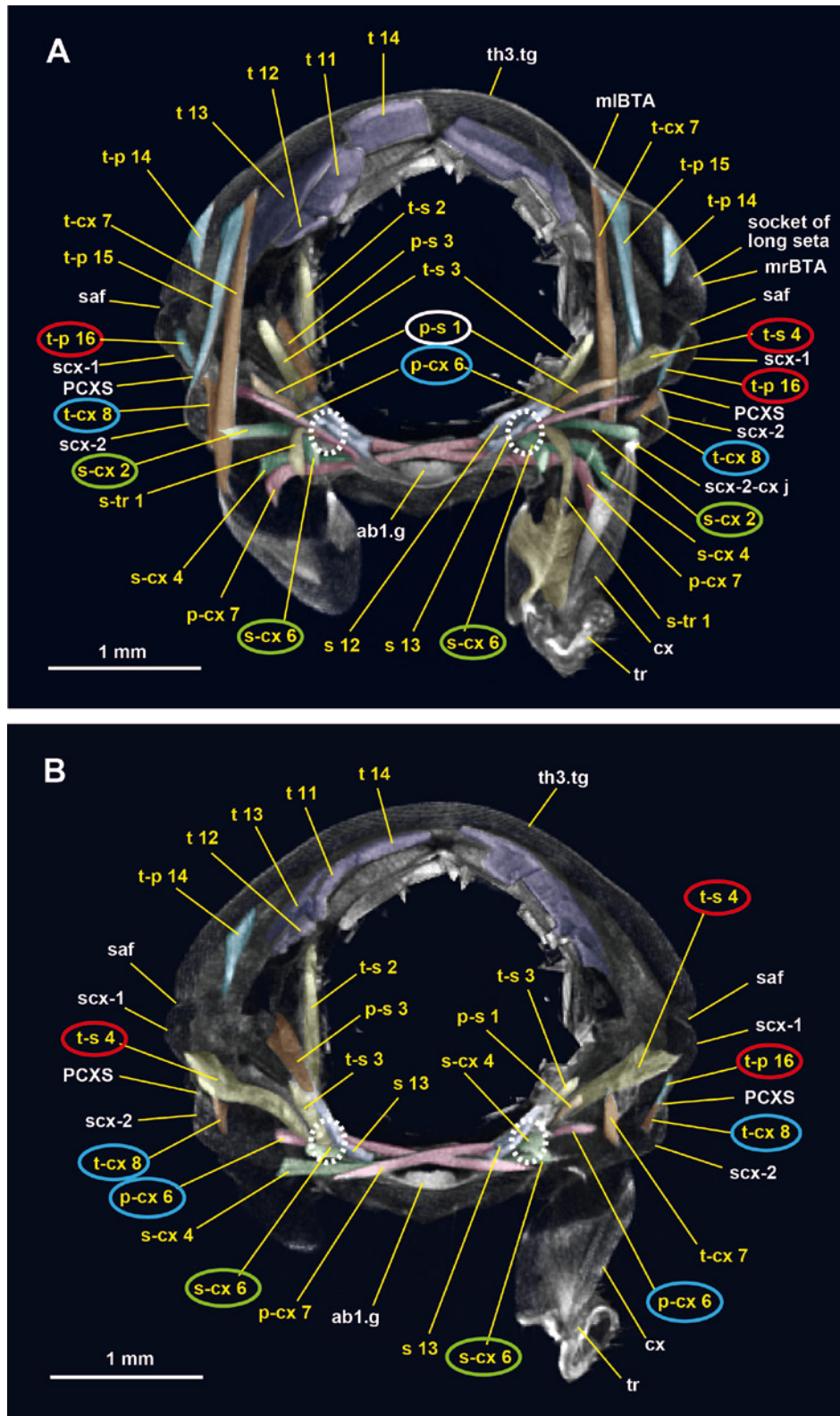


Fig. 3 Cross-sectioned micro-CT images of the posterior part of the metathorax of *Carabus insulicola* at the levels of A-A' (A) and B-B' (B) in Fig. 1A. The larva in this image is the same as in Fig. 1A. The image of the alimentary canal was digitally removed from the original image. Abbreviations for muscles follow the Matsuda's system and are highlighted in yellow letters. Red circles indicate intrinsic muscles of subcoxa-1 (precoxa). Blue, green, and white circles indicate pleuro-coxal muscles, "furca-coxal" muscles, and a "pleuro-furcal" muscle, respectively, of subcoxa 2. Circles indicated by white dotted lines show the places where a furcal invagination is formed in the larva with sclerotization. ab1.g: first abdominal ganglion, cx: coxa, mIBTA: molecular boundary between tergum and appendage inferred from gene expression data of *Tribolium castaneum* embryos, mrBTA: morphological boundary between tergum and appendage, PCXS: paracoxal suture, saf: subalar fold, scx-1, 2: subcoxae-1 and 2, scx-2-cx j: subcoxa-2-coxa joint, th3.tg: metathoracic tergum, tr: trochanter.

muscles of each region. In subcoxa-2, six muscles were found to have both their origin and insertion within this region (Figs. 2, 3A, B, 4); that is, three of them were pleuro-coxal muscles (p-cx 5, p-cx 6, and t-cx 8 sensu Matsuda), two were newly defined “furca-coxal” muscles (s-cx 2 and s-cx 6 sensu Matsuda), and the other one was the newly defined “pleuro-furcal” muscle (p-s 1 sensu Matsuda). Given that muscles originate or attach on both the proximal and distal “joints” of subcoxa-2, this region (katapleuron) should be regarded as an independent podomere.

In subcoxa-1, two muscles (t-p 16 and t-s 4 sensu Matsuda in Figs. 2 and 3A, B) are localized and both are attached to the subalar fold (Figs. 3A, 4, saf). The subalar fold is observed from the outside as a faint suture in subcoxa-1 running from its medio-ventral part to its dorsoposterior end (Fig. 1A, B, saf). This fold is first documented in the larvae of the megalopteran *Corydalus cornutus*, and muscle 161 (epimeral-subalar muscle) (t-p 16 sensu Matsuda) is attached to this fold (Kelsey, 1957). In the adult of this species, the subalare is formed just dorsal to this fold. Muscle t-s 4 has been known from only Ephemeroptera (in both adults and larvae) as the subalar-furcal muscle (SA. Fm sensu Willkommen, 2008). Our observation of this muscle in *C. insulicola* is therefore noteworthy. Although Matsuda (1970) regarded the subalare as a tergal structure, Snodgrass (1935) and Willkommen (2009) regarded it as belonging to the pleuron. Based on our analysis, muscle t-p 14 originates from near the

lateral edge of the tergum (Fig. 3A) and inserts in the antero-dorsal part of the subalar fold (not pictured in Fig. 3A). In Ephemeroptera, the superior axillar-pleural muscle (Ax.PmS sensu Willkommen, 2008) runs from the third wing base sclerite to the anterior part of the subalar. In the adult of *Carabus*, t-p 14 also runs from the third axillary sclerite to the pleuron (epimeron) (Larsén, 1966), although, in *Carabus* as in other Neoptera, the anterior subalar is absent (only posterior one is present). Willkommen (2008), thus, concluded t-p 14 in Neoptera and Ax.PmS in Ephemeroptera are homologous muscles. Therefore, the arrangement of t-p 14 also suggests that the subalar is indeed a pleural structure. This conclusion agrees with the recent findings from Ohde et. al. (2022), where ablation of the pleuron in 3rd instar nymphs of the cricket *Gr. bimaculatus* results in the loss of the epipleurites including the basalare and subalare. We therefore agree with the interpretation of Snodgrass and Willkommen. Subcoxa-1 (anapleuron), therefore, can be regarded as an independent podomere that would be homologous to the crustacean precoxa (the most proximal podomere). The existence of the precoxa in Crustacea has been demonstrated in the study of post-embryological development of decapods by Fukuda (1994) and also in the study of gene expression in amphipods by Bruce and Patel (2020) and Bruce (2021). The PCXS and PCXR, thus, should be newly interpreted as the segmental boundary between the precoxa and subcoxa.

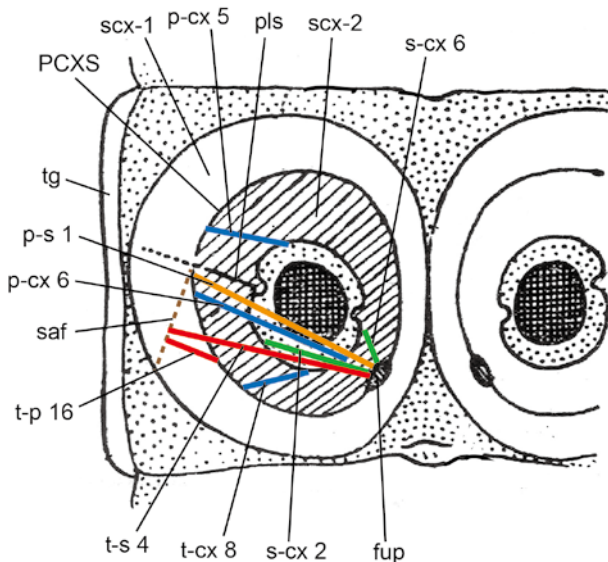


Fig. 4 Intrinsic muscles of subcoxae-1 and 2 mapped on the ventral view of a schematic thoracic segment (modified from Weber, 1952, Fig. 16c). Intrinsic muscles of subcoxa-1 (precoxa) are highlighted in red. Intrinsic muscles of subcoxa-2, i.e., pleuro-coxal muscles, are highlighted in blue, furca-coxal muscles in green, and pleuro-furcal muscle in orange. Abbreviations for muscles follow Matsuda (1970). fup: furcal pit, pls: pleural suture, PCXS: paracoxal suture, saf: subalar fold, scx-1, 2: subcoxae-1 and 2, tg: tergum.

Paranotal lobe is derived from the dorsal half of subcoxa-1 (precoxa)

Snodgrass (1935) defined a morphological boundary between the “dorsum” and “appendage” (subcoxa), which appears to correspond to the BTA of Mashimo and Machida (2017). Snodgrass’ “dorsum”, however, includes laterotergites on both sides of the median “principal tergum” (Snodgrass, 1935, Fig. 139). Based on the muscular system described here, we conclude that his laterotergite corresponds to the ventral half of our subcoxa-1 (precoxa) and his “subcoxa” to our subcoxa-2. Snodgrass’ definition thus cannot be applied to our morphological boundary between the tergum and appendage (mrBTA) described below.

In the newly hatched larvae of *C. insulicola*, the mrBTA can be seen as a faint horizontal suture (Fig. 1A). In the larvae after sclerotization, a highly sclerotized ridge is observed in the same place where the suture was present (Fig. 1B). A long seta is present near the posterior end of this suture or sclerotized ridge as a landmark of the mrBTA in each thoracic segment (Fig. 1A, B, arrowhead). Within a day after hatching the dorsal half of the larval precoxa (subcoxa-1) slightly

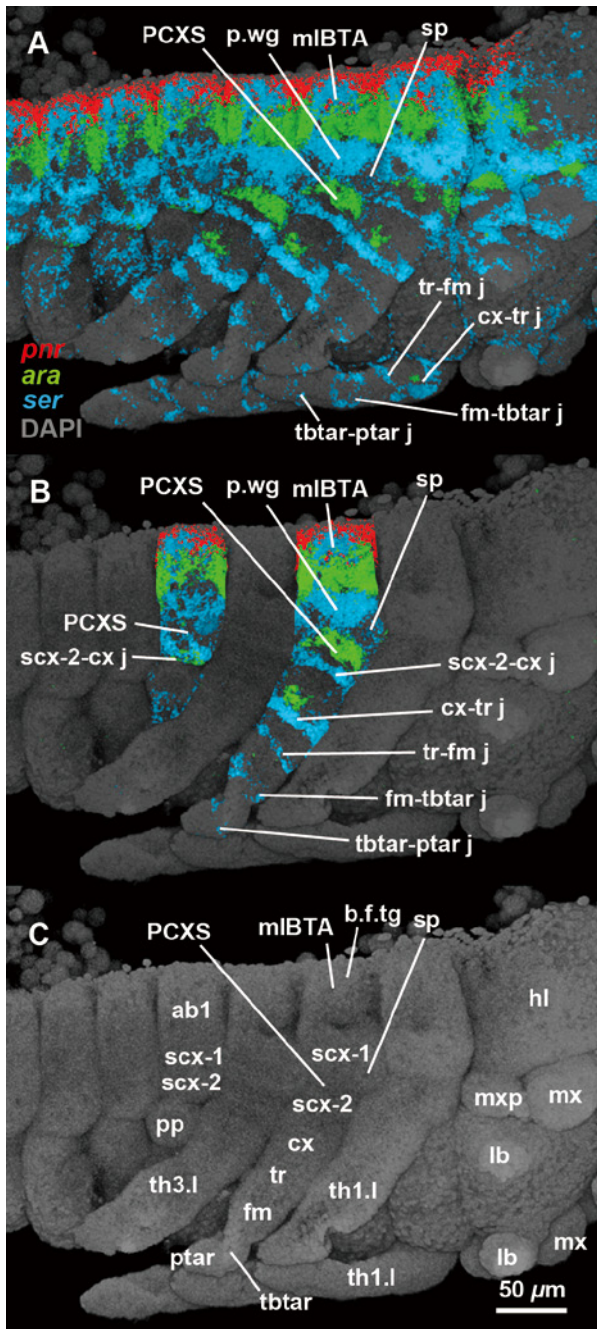


Fig. 5 Expression of *pannier*, *auracan*, and *serrate* in *Tribolium castaneum* embryo at the stage of leg segmentation, ventrolateral view. A. *pannier* (*pnr*) in red marks the dorsal-most tissue, or the *bona fide* tergum. *auracan* (*ara*) in green brackets subcoxa-1 and is also expressed in a patch on the coxa. *serrate* (*ser*) in blue marks the location of joints, as well as the presumptive wing (Couso et al., 1995). B. Gene expression highlighted in the mesothoracic and the first abdominal segment. C. Embryo without gene expression. ab1: first abdominal segment, b.f.tg: *bona fide* tergum, cx: coxa, cx-tr j: coxa-trochanter joint, DAPI: DAPI staining, fm: femur, fm-tbtar j: femur-tibiotarsus joint, hl: head lobe, lb: labium, mlBTA: molecular boundary between tergum and appendage, mx: maxilla, mxp: maxillary palp, PCXS: paracoxal suture, pp: pleuropodium, ptar: pretarsus, p.wg: presumptive wing, scx-1, 2: subcoxae-1 and 2, scx-2-cx j: subcoxa-2-coxa joint, sp: spiracle, tbtar: tibiotarsus, tbtar-ptar j: tibiotarsus-pretarsus joint, th1, 3.l: prothoracic- and metathoracic leg, tr: trochanter, tr-fm j: trochanter-femur joint.

extends laterally, accompanied by sclerotization, and forms an eave-like structure, or the paranotal lobe (Figs. 1B, 6, pnl), whereas the ventral half forms the pleuron. It is noteworthy that muscle t-p 14, or the axillary muscle, is attached to the tergum just dorsal to the mrBTA (Fig. 3A, t-p 14). As mentioned before, in the adult, this muscle is assumed to be inserted on the third axillary sclerite of the wing base (Larsén, 1966; Matsuda, 1970). Judging from the position of the mrBTA, therefore, axillary sclerites of the wing base are assumed to be in the tergum, but not in the paranotal lobe.

In comparison of our mrBTA to the BTA of Mashimo and Machida (2017), our mrBTA would correspond to their “paratergal groove” (e.g., their Figure 2F, G, white arrows), and our subcoxa-1 to their “paratergal bulge” from which a wing develops later. The BTA of Mashimo and Machida (2017) would thus correspond to the boundary between subcoxae-1 and 2, or our PCXS.

Incongruence between morphological and molecular boundary between tergum and appendage

According to Bruce and Patel (2020), the paranotal lobe is homologous to the insect wing and the crustacean tergal plate, both of which are the lateral expansions of the precoxa, the proximal-most

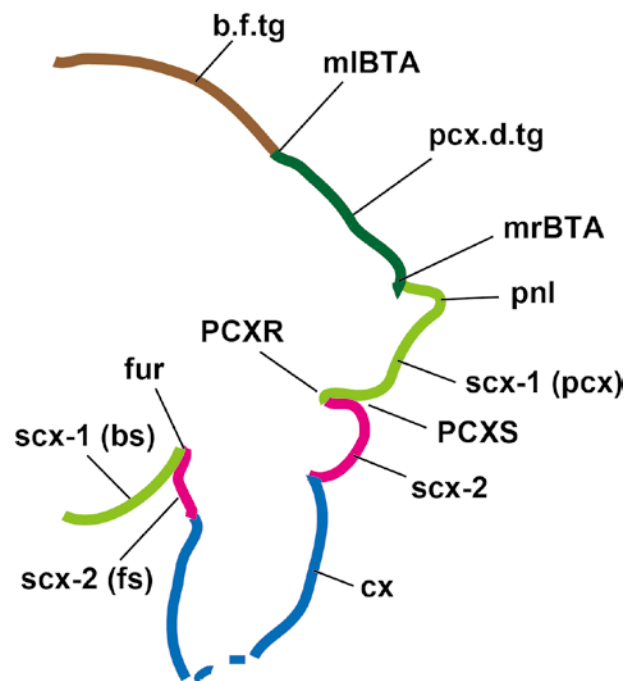


Fig. 6. Schematic diagram showing regionally categorized integuments of the pterothorax in the *Carabus insulicola* larva, cross sectioned. The regions of *bona fide* tergum (b.f.tg), precoxa-derived tergum (pcx.d.tg), and the location of the molecular BTA (mlBTA) are inferred from gene expression data of *Tribolium castaneum* embryos. bs: basisternum, cx: coxa, fs: furcasternum, fur: furca, mrBTA: morphological BTA, pcx: precoxa, PCXR: paracoxal ridge, PCXS: paracoxal suture, pnl: paranotal lobe, scx-1, 2: subcoxae-1 and 2.

podomere. If the paranotal lobe is the lateral expansion of a podomere, then the real BTA must be dorsal to the paranotal lobe. This hypothesis is supported by the following gene expression data.

Bruce and Patel (2020) and Bruce (2021) concluded that the *bona fide* tergum (true body wall) in all arthropods is marked by *pannier*, while *araucan* expression brackets the precoxa (subcoxa-1) dorsally – where the precoxa (subcoxa-1) meets the *bona fide* tergum – and ventrally – at the joint between subcoxae-1 and 2. Additionally, previous studies have found that *serrate* mediates the formation of leg joints (de Celis et al., 1998). Thus, the molecular BTA (mlBTA), or the boundary between the *bona fide* tergum and precoxa, should be located within the overlap between *pannier* and the dorsal domain of *serrate*. Furthermore, if subcoxae-1 and 2 are true podomeres, *serrate* should be expressed in each of their proximal and distal joints. Notably, the expression of these genes provides three separate indicators of the location of the mlBTA.

We examined the expression of the above three genes in *Tribolium castaneum* embryos. As predicted, *pannier* is expressed in the dorsal-most tissue, the *bona fide* tergum; *araucan* expression brackets subcoxa-1; and *serrate* is expressed in all joints, including the proximal and distal joints of both subcoxae-1 and 2 (Fig. 5A, B). Therefore, subcoxae-1 and 2 are true podomeres. Notably, the molecular location of the BTA (Fig. 5A, mlBTA) – the region of overlap between *pannier* and *serrate* – occurs dorsal to the thick horizontal domain of *serrate* expression. This *serrate* domain partially overlaps with the presumptive wing (Fig. 5A, B, p.wg), marked by *vestigial* expression (Bruce and Patel, 2020, Fig. 3l, extended data, Fig. 8b).

Another notable observation when considering the data in Bruce and Patel 2020 is that, like the BTA, the PCXS also has a molecular signature; that is, the ventral armband of *araucan* expression together with a *serrate* joint domain (Fig. 5A, B, PCXS). In insects, this marks the boundary between subcoxae-1 and 2, while in crustaceans it marks the boundary between the precoxa and the crustacean coxa. Thus, the BTA and PCXS have distinct molecular signatures, which can be applied to and investigated in other insect systems. Examination of *pannier*, *araucan*, and *serrate* expression in *Gr. bimaculatus* embryos, for example using the inexpensive and forgiving in situ HCR v3.0 (Choi et al., 2018; Bruce et al., 2021), may resolve the different interpretations of Kobayashi et al. (2013) and Mashimo and Machida (2017).

It is interesting to note the somewhat unexpected shape of *serrate* expression around the perimeter of subcoxa-1. This unexpected shape makes sense when one considers that a) *serrate* is expressed in a perimeter around each leg joint, and b) subcoxa-1 is a flattened

cylinder rather than an elongate cylinder like the distal leg segments. Thus, when the embryo is in ventrolateral view (Fig. 5C), the joint between subcoxa-1 and the *bona fide* tergum is viewed at an angle of about 60-degree, and appears more circular (Fig. 5A, B, mlBTA), while the distal leg joints are viewed edge-on, and appear like a line (Fig. 5B, scx-2-cx j, cx-tr j, tr-fm j, fm-tbtar j).

“Precoxal theory” for the origin of insect wings

Our musculature and gene expression data strongly suggest the positional incongruence between the morphological and molecular BTAs; that is, the latter is located more dorsally than the former (Fig. 6). We believe the mlBTA lies along the lateral edge of the budged area of the median tergum (Figs. 3A, 6, mlBTA). The incongruence is not necessarily unnatural, because sclerites do not always define anatomical areas (Snodgrass, 1935). However, if the molecular BTA but not the morphological BTA corresponds to a real BTA, the incongruence implies that the lateral part of the so-called tergum is really derived from the precoxa (precoxa-derived secondary tergum) (Fig. 6, pcx.d.tg) and only the medial part (*bona fide* tergum) (Fig. 6, b.f.tg) should be regarded as a primary tergum.

Based on the mlBTA, therefore, the tergo-sternal (furcal) (t-s 3) and tergo-pleural (t-p 14) muscles (Figs. 2, 3A), which are attached to the first and third axillary sclerites in the adult, must be included in the precoxal intrinsic muscles. Moreover, more medial muscles such as the tergo-pleural (t-p 15) and tergo-coxal (t-cx 3 to 7) muscles (Figs. 2, 3A) also must be categorized into a precoxal intrinsic muscle and precoxal-coxal muscles, respectively. Underneath the *bona fide* tergum, i.e., between the right and left mlBTAs, there exists only the broad tergal muscles consisting of t 11 to 14, and none of the dorso-ventral muscles are in this region (Fig. 3A). The detail of these changes in muscle category will be discussed in our subsequent paper.

The origin of insect wings has long been debated. One theory holds that wings derive from paranotal lobes (edges of the tergum) (Crampton, 1916), while another theory is that wings emerge from an ancestral proximal leg segment that now functions as the dorsal half of the pleuron (Kukalová-Peck, 1983, 2008). Recently, it has been proposed that both of these theories are correct, but in different ways. The “dual origin” theory of Clark-Hachtel and Tomoyasu (2020) proposes that wings represent a composite of both tergal and appendicular elements. In contrast, Bruce and Patel (2020) proposed that both the exite and paranotal theories are correct, but each pertains to different phylogenetic timepoints: the crustacean precoxal exite evolved into paranotal lobes, which later evolved into insect wings, and therefore wings

are derived only from the precoxa. In support of the latter interpretation, Ohde et. al. (2022) found that the pleuron does not contribute to the wing, but instead forms part of the epipleurites, such as the basalare and subalare.

Combining the positional information of the mrBTA and mlBTA obtained here, our data supports the growing consensus that paranotal lobes and insect wings evolved from the exite of the precoxa, and that the precoxa now forms a secondary tergum. We call this the precoxal theory for the origin of insect wings. We note that our precoxal theory is similar to Kukalová-Peck's epicoxal theory, with the exception that our precoxa is more expansive, encompassing all elements of wing articulation but also the lateral tergum and part of the sternum (basisternum). Our precoxal theory should be verified in many insect taxa in the future.

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