Hox genes and the diversification of insect and crustacean body plans

Michalis Averof & Michael Akam

Wellcome/CRC Institute, Tennis Court Road, Cambridge CB2 1QR, and Department of Genetics, University of Cambridge, Cambridge, UK

CRUSTACEANS and insects share a common origin of segmentation[1,2], but the specialization of trunk segments appears to have arisen independently in insects and various crustacean subgroups[3,4]. Such macroevolutionary changes in body architecture may be investigated by comparative studies of conserved genetic markers[5–7]. The *Hox* genes are well suited for this purpose, as they determine positional identity along the body axis in a wide range of animals[8–10]. Here we examine the expression of four *Hox* genes in the branchiopod crustacean *Artemia franciscana*, and compare this with *Hox* expression patterns from insects. In *Artemia* the three 'trunk' genes *Antp*, *Ubx* and *abdA* are expressed in largely overlapping domains in the uniform thoracic region, whereas in insects they specify distinct segment types within the thorax and abdomen. Our comparisons suggest a multistep process for the diversification of these *Hox* gene functions, involving early differences in tissue specificity and the later acquisition of a role in defining segmental differences within the trunk. We propose that the branchiopod thorax may be homologous to the entire pregenital (thoracic and abdominal) region of the insect trunk.

We have previously shown that gene duplication and diversification in the arthropod *Hox* clusters pre-date the divergence of crustaceans and insects[5,11]. Thus, in spite of striking differences in body organization, crustaceans and insects share similar sets of homeotic genes. Here, we use *in situ* hybridization and immunochemical techniques to study expression of several of these genes during the larval (postnaupliar) stages of *Artemia* development, while trunk segments are forming[12,13] (Fig. 1).

The monoclonal antibody FP6.87 recognizes a conserved epitope in the Ubx and AbdA proteins of diverse insects[14,15]. This epitope has been mapped to a region which is also conserved in the Ubx and AbdA proteins of *Artemia* (Fig. 2*a*); we therefore use this antibody to examine *Ubx* and *abdA* expression during *Artemia* development (Fig. 2*d–h*). We observe staining with nuclear localization restricted to the thoracic region of the body. Staining first appears when the thoracic region is forming, before segments are externally distinct (Fig. 2*d*). The expression has a discrete anterior boundary that persists throughout development and co-incides with the morphological boundary between the gnathal and postgnathal regions of the body. Ventrally, this boundary appears to be parasegmental[1,2], starting in the posterior part of the last gnathal segment (Mx2; Fig. 2*f*). In the body wall, staining starts from the first thoracic segment (T1) and extends backwards during development, as new segments are formed from the posterior growth zone (Fig. 2*g*, *h*). Expression extends transiently into the prospective genital and abdominal segments, but later retracts and is confined to the thorax (as shown for *abdA* in Fig. 2*k*). As thoracic segments develop (in antero-posterior progression), staining becomes strongest in the developing neuromeres of the central nervous system and then gradually fades. In mature segments levels of expression appear to be low.

To explore the expression of *abdA* and *Ubx* further, we have raised antibodies against a peptide which is diagnostic for the AbdA protein and is conserved between *Artemia* and insects (Fig. 2*a*). In insects this antibody can specifically detect the AbdA protein distribution (Fig. 2*b*, *c*). We can therefore use this antibody to examine *abdA* expression in *Artemia* independently from *Ubx*. Unfortunately, owing to the low sensitivity of the antibody, we cannot detect expression during early stages, but we obtain clear nuclear staining during middle to late stages of postnaupliar development (Fig. 2*i–k*). This confirms that the AbdA protein is expressed in the *Artemia* thorax but is absent in the abdomen. Expression appears strongest in the neuromeres and weaker in the body wall and limbs. The anterior boundary of expression during early stages is unclear, but at later stages we detect weak expression in a few cells of the T2 neuromere and in many cells of the T3 neuromere, and stronger widespread expression in the more posterior neuromeres of the thorax (Fig. 2*i*, *j*). A few cells stain in the genital segments (Fig. 2*k*), but not in genital neuromeres.

In situ hybridization was used to examine the expression of *Antp* and *AbdB*. *Antp* is expressed throughout the *Artemia* thorax and extends anteriorly into the last gnathal segments (posterior Mx1 and entire Mx2; Fig. 2*l*). Expression appears strongest in the limbs, but is weak or absent in the neuromeres, where *abdA* and perhaps *Ubx* are expressed at similar developmental stages. *AbdB* is expressed in the genital segments of *Artemia*, immediately posterior to the domain where *Antp*, *Ubx* and *abdA* are coexpressed (Fig. 2*m*).

The expression patterns described are consistent with a role of these genes in the positional specification of segmental fate; they are limited to well-defined regions of the trunk, and appear before visible segment differentiation. Our results suggest that *Antp*, *Ubx* and *abdA* are collectively involved in defining the segmental identity of the thoracic region. Although these genes may have different spatio-temporal or cell-specific functions in the thorax (such as late function of *abdA* predominantly in the neuromeres), they appear not to be used to define distinct segment identities within the postgnathal trunk; all thoracic segments have identical morphology, except for small differences in size (Fig. 1). *Antp* may also be involved in the specification of the posterior gnathal region, although we would predict on the basis of conventional homologies[16] that *Scr* would be more important in defining segmental identity in this region[17].

The expression of *Antp*, *Ubx* and *abdA* in *Artemia* contrasts with what has been described in insects, where these genes are expressed in discrete (although still partly overlapping) domains along the trunk and are responsible for specifying the differential fates of thoracic and abdominal segments[8,18,19]. Most striking is the expression of *abdA* in the *Artemia* thorax; in insects it is expressed exclusively in the abdomen, and its function is thought to define the identity of that region[15,19–21]. We consider the contrasting expression patterns of these genes in *Artemia* and insects under a general model for the evolution of *Hox* gene functions (Fig. 3).

The regulation of eukaryotic genes depends on distinct regulatory elements, each responsible for different spatio-temporal or tissue-specific aspects of a complex pattern. Thus we expect that when ancestral *Hox* genes duplicate, different subsets of these regulatory elements may become partitioned into separate genes. If so, these duplications would produce functionally identical homeoproteins expressed in different tissues, regions or developmental stages. Each of the newly duplicated genes would then be subject to independent selection on the basis of its unique features of expression. Similar cases of regulatory diversification have been documented for duplicated genes with functionally identical protein products[22,23]. We speculate that such a process occurred in some common ancestor of crustaceans and insects, giving rise to the closely related *Antp*, *Ubx* and *abdA* genes; initially these were involved in specifying a common regional identity, the 'trunk', with no distinct segment-specific functions. Furthermore, we suggest that this ancestral state may be reflected in the overlapping expression domains of *Antp*, *Ubx* and *abdA* in *Artemia*, and to a large extent of *Ubx* and *abdA* in *Drosophila*[20]. Even today, AbdA and Ubx proteins have identical effects in many genetic assays[24,25]. An analogous situation has also been documented in vertebrates, where duplications of

entire *Hox* gene clusters gave rise to paralogous sets of genes with partly redundant functions in the hindbrain[10].

In the lineage that gave rise to the insects it appears that the expression of these genes resolved to more discrete anteroposterior domains, through changes in gene regulation and competing cross-regulatory interactions[10]. Ultimately, the diversification of *Hox* gene functions to regulate different sets of downstream genes within each of these domains led to the morphological diversification of segments in the thorax and abdomen of insects. One example would be the repression of *Distal-less* expression by *Ubx* and *abdA*, and the consequent development of a limbless abdomen in insects[15,25].

Our observations provide a framework for direct morphological comparisons between arthropod body plans, where positional homologies can be defined on the basis of underlying genetic information (Fig. 4*a*). We suggest that the thoracic region of branchiopods is homologous to the entire pregenital trunk region of the insects (thorax and most of the abdomen), a region that we designate as 'trunk'. It is intriguing that the number of segments in this region is conserved between most branchiopods and insects, with only limited variations in each group. This scheme identifies the genital segments as homologous in these two groups, defined by the activity of *AbdB*[26], and occupying conserved positions at the end of the trunk. It also implies that the *Artemia* abdomen is a terminal structure, perhaps comparable to the highly modified and reduced postgenital segments of the insect abdomen.

The proposed regional homologies will have to be reconciled with the phylogenetic status and body plans of other arthropod groups. At present, the proposed scheme may be extended to

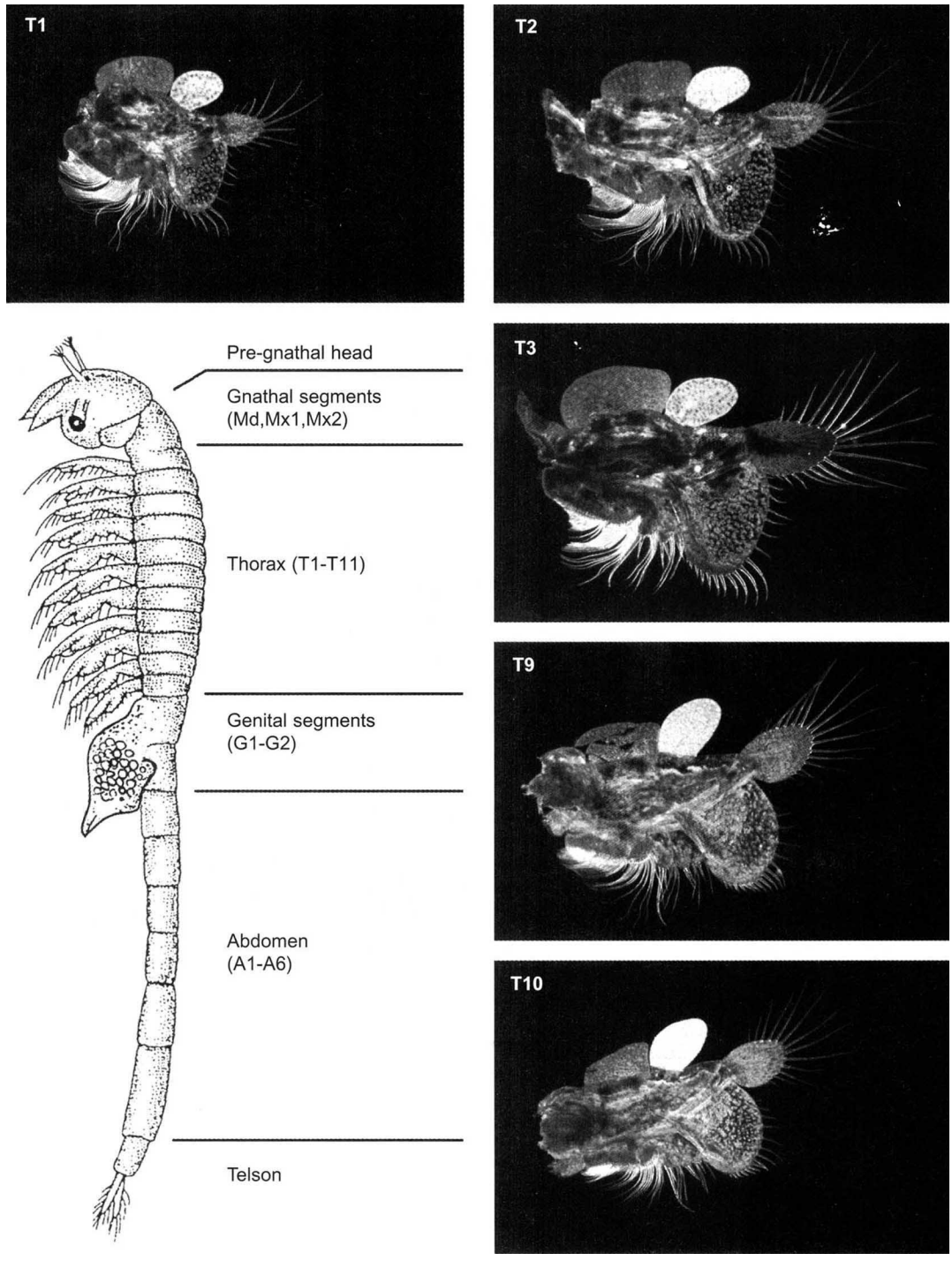

FIG. 1 A diagrammatic representation of the *Artemia* body plan, consisting of a head (including 3 gnathal segments; Md, Mx1 and Mx2), thorax (11 segments: T1–T11), genital region (2 segments: G1 and G2), abdomen (6 segments: A1–A6) and telson. Apart from differences in size, all thoracic segments have similar morphology. We illustrate this by showing the structure of the limbs on thoracic segments T1, T2, T3, T9 and T10.

a

FP6.87	putative epitope	WFQNRRMK**L**KKExx**A**x**K**Ex**NEQ**
	Artemia AbdA	WFQNRRMKLKKELRAVKEINEQ
	Artemia Ubx	WFQNRRMKLKKEIQAIKELNEQ
	Artemia Antp	WFQNRRMKWKKENKSKVDSGCL
		* * ** ***
Anti-AbdA	putative epitope	LRAVKEINEQAR
	Artemia AbdA	LRAVKEINEQAR
	fly AbdA	LRAVKEINEQAR
	locust AbdA	LRAVKEINEQAR
	Artemia Ubx	IQAIKELNEQEK
		** * * **
	Artemia Antp	NKSKVDSGCLDG
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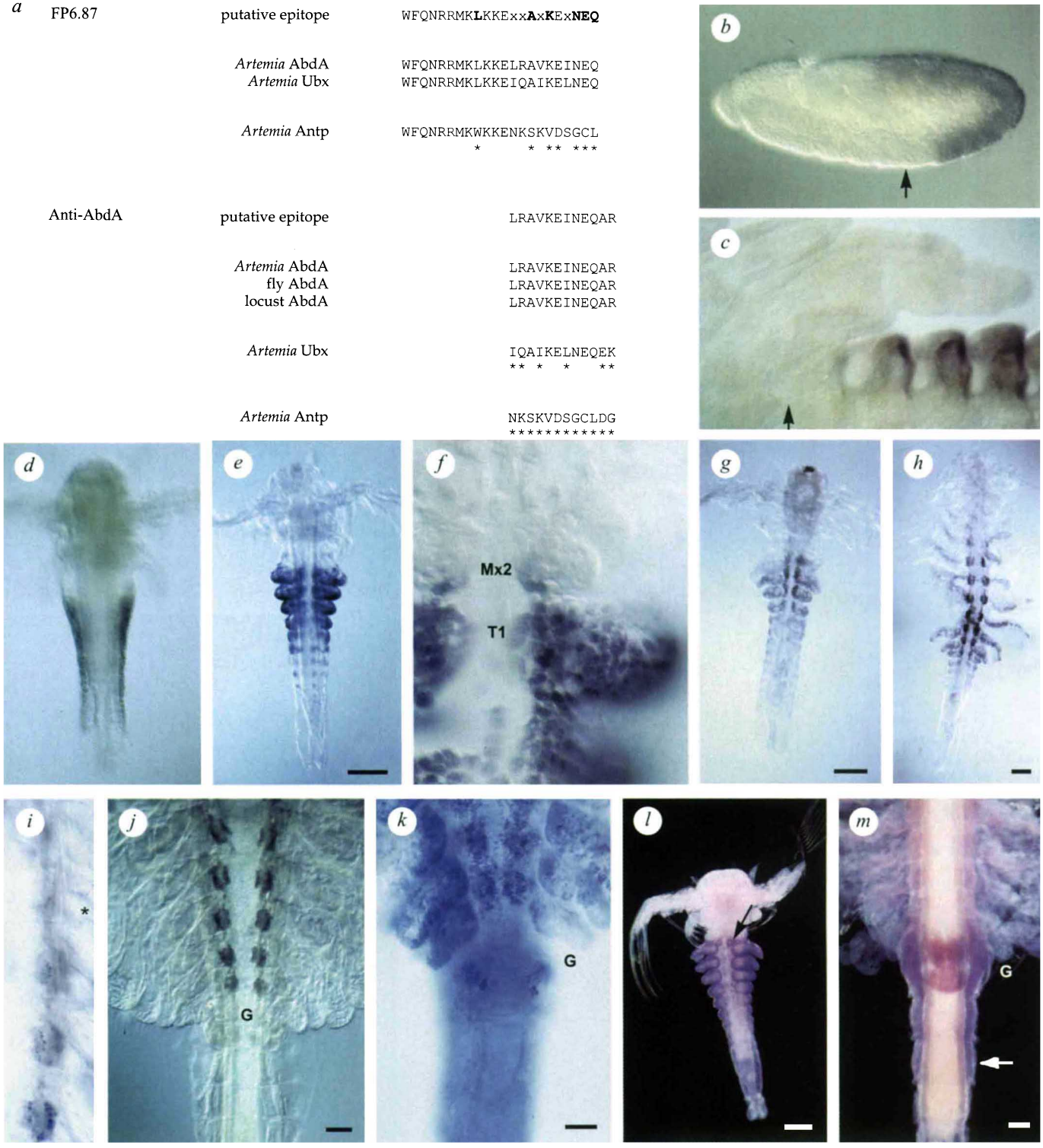

FIG. 2 *Hox* gene expression in *Artemia franciscana*. *a*, The putative epitopes recognized by the FP6.87 (ref. 14) and anti-AbdA antibodies are compared to the protein sequence of AbdA, Ubx and Antp from *Artemia franciscana*[5]. Both epitopes lie near the carboxy-terminal end of the homeodomain. Residues in bold are thought to define the specificity of the FP6.87 antibody; asterisks indicate differences between the putative epitope and a particular homeoprotein. *b*, *c*, Specificity of the anti-AbdA serum. Immunochemical staining on fly (*b*) and locust (*c*) embryos using anti-AbdA reveals a pattern identical to the previously characterized *AbdA* protein distribution[20,21] but with low sensitivity (arrowheads indicate the borders between abdomen and thorax). *d–h*, Staining with the FP6.87 antibody during *Artemia* development. Staining appears very early, before external segmentation of the thorax (*d*), and persists throughout development of the thoracic region (*e*, *g*, *h*) in a domain that starts at the posterior end of the Mx2 limb bud (*f*) and spans the entire thorax. *i–k*, Staining with the anti-AbdA antibody in *Artemia*. At relatively late stages, strong staining can be seen in the thoracic neuromeres, starting weakly in T2 (marked by asterisk in *i*) and extending posteriorly throughout the thorax. At late stages, staining appears in a few cells within the genital region (G) (*k*), but is completely absent from the genital neuromeres and abdomen (*j*, *k*). *l*, *Antp* expression in *Artemia*. *In situ* hybridization shows expression in the developing thorax, in the limb buds of Mx2 (indicated by the arrowhead) and the posterior of Mx1. *m*, *AbdB* expression in *Artemia*. *In situ* hybridization with a short probe shows expression in the two genital segments (G) and the gonads. The gonads develop in the genital region and extend laterally into the abdomen (arrowhead). Scale bars, 0.1 mm.

METHODS. The anti-AbdA antiserum was raised against a synthetic AbdA-specific peptide (LRAVKEINEQAR). This was coupled to ovalbumin through an amino-terminal cysteine and used to immunize rabbits. The serum was affinity-purified using a locust AbdA fusion protein[21]. Immunochemical stainings were performed by standard methods[14], after sonication[13]. *In situ* hybridizations were performed with digoxygenin-labelled antisense RNA probes for *AfAntp*[5] and *AfAbdB* (method adapted from ref. 13). The *Artemia AbdB* fragment was isolated by using degenerate-primer PCR (F. Casares, unpublished data; sequence, EMBL accession number X87250).

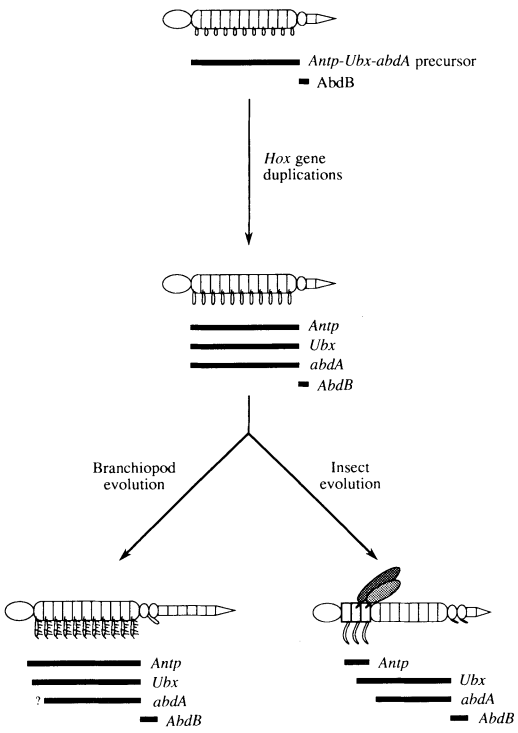

FIG. 3 A model for the evolution of *Hox* gene functions. We expect that the progenitor of *Antp*, *Ubx* and *abdA* was a *Hox* gene responsible for specification of the middle body region (the 'trunk') in a distant protostome ancestor. The gene duplications that gave rise to distinct *Antp*, *Ubx* and *abdA* genes must have occurred before the insect and crustacean lineages diverged[5,11]. These duplications, producing genes with functionally identical protein products, allowed the duplicated copies to acquire slightly different temporal, spatial, tissue- or cell-specific regulation. Further elaboration of this regulation (resolution of spatial domains of expression) and acquisition of differential downstream functions led to diversification of this region into the thorax and abdomen of insects. We expect that *AbdB* acquired a distinct function in the 'tail' region much earlier (before the divergence of major animal phyla), and that at some point during arthropod evolution it became associated with the genital segments.

include the body plans of phylogenetically interesting fossils such as *Rehbachiella*, *Lepidocaris* and the euthycarcinoids[4,27–29] (Fig. 4*b*). Interestingly, euthycarcinoids were originally identified as branchiopod crustaceans[28], and more recently were thought to be associated with the origin of the insects[29]. More problematic is the position of the myriapods and other crustacean classes, but plausible homologies which are consistent with our scheme have been suggested[4,30] (Fig. 4*b*). We expect that many of these issues will be resolved by extending our present study to diverse arthropod groups. □

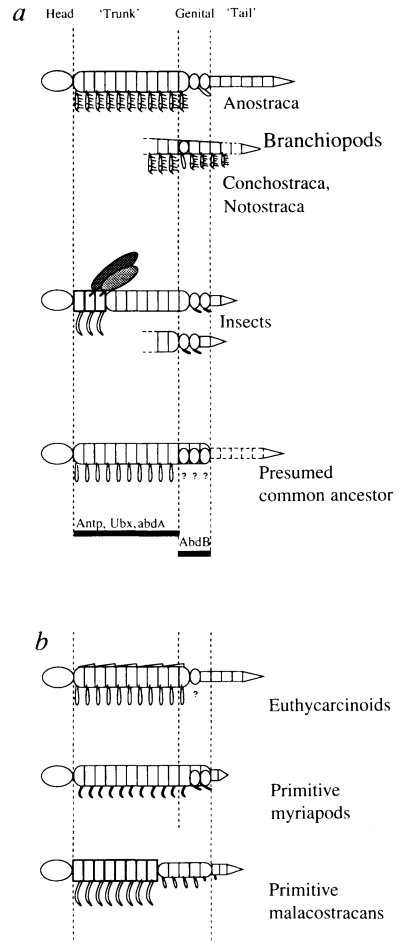

FIG. 4 *a*, A hypothesis for regional homologies between branchiopod and insect body plans. The homology of individual segments in the head region of crustaceans and insects (including the gnathal segments) is widely accepted[16]. We suggest that the thoracic region of branchiopod crustaceans (including Anostraca, Conchostraca and Notostraca) is homologous to the entire pregenital (thoracic and abdominal) region of the insects, and that genital segments hold homologous positions in both these groups. Genital segments are indicated by an oval shape. Our hypothesis suggests that the last common ancestor of crustaceans and insects was an animal with a distinct mandibulate head, a trunk defined by the activity of *Antp*, *Ubx* and *abdA*, genital segments defined by the activity of *AbdB*, and a postgenital 'tail'. *b*, Suggested homologies to other arthropod body plans. The body plans of euthycarcinoids[28,29] can be accomodated in our scheme. Homologies to myriapods and malacostracan crustaceans are more problematic, but the suggestions of Dohle[30], on the nature of primitive myriapods, and Walossek[4], on the homology between the branchiopod thorax and most of the malocostracan trunk (abdomen and thorax), provide some plausible solutions.

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