The isopod *Asellus aquaticus*: A novel arthropod model organism to study evolution of segment identity and patterning[1]

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Abstract

Hox genes have been recognized as widely conserved metazoan genes relevant for patterning of embryonic axes. In insects, Hox genes display more or less strict tagmata-related expression patterns. For example, activity of *abdominal-A* (*abd-A*) correlates with the legless abdomen. In crustaceans, in contrast, expression domains are more divers. In the branchiopod *Artemia franciscana*, expression domains of posterior Hox genes display overlapping patterns. In the more modern malacostracan isopod *Porcellio scaber* however, *abd-A* is restricted to the developing pleon. Here we present the partial cloning and expression patterns of the Hox genes *abd-A* and *Antennapedia* from the freshwater crustacean *Asellus aquaticus*. In contrast to *Porcellio* this isopod shows a slightly different regulation of posterior segment patterning. The obtained *abd-A* signal points to a conserved early role in the pleon as well as to an anteriorly extended function in the pereon and pleon in later stages. In addition, we detected an as yet unknown endodermal expression domain of *abd-A* in the epithelium of the early developing digestive gland, which indicates a diverse role of this gene. The waterlouse *Asellus aquaticus* thus may provide a relevant and novel crustacean model organism for the study of evolutionary changes of segment identity and patterning.

Keywords: EvoDevo, *Asellus aquaticus*, Hox, *Antp*, *abd-A*, *dll*, evolution, segment identity, segment patterning.

Zusammenfassung

Hox-Gene sind stark konservierte Gene der Metazoa, die für die Musterbildung embryonaler Achsen von Bedeutung sind. In Insekten zeigen Hox-Gene mehr oder weniger streng an Tagmata gebundene Expressionsmuster. Die Aktivität von *abdominal-A* (*abd-A*) korreliert zum Beispiel mit dem beinlosen Abdomen. In den Krebstieren sind die Expressionsdomänen dagegen unterschiedlicher. In dem Kiemenfußkrebs *Artemia franciscana* zeigen die posterioren Hox-Expressionsdomänen überlappende Muster. In dem modernen Isopoden *Porcellio scaber* ist *abd-A* jedoch auf das entstehende Pleon beschränkt. Wir berichten hier von der partiellen Klonierung und den Expressionsmustern der Hox-Gene *abd-A* und *Antennapedia* des Süßwasserkrebses *Asellus aquaticus*. Im Gegensatz zu *Porcellio* zeigt dieser Isopode eine leicht unterschiedliche Regulierung der posterioren Segmente. Das erhaltene *abd-A*-Signal deutet sowohl auf eine konservierte frühe Rolle im Pleon als auch auf eine in späteren Stadien nach anterior hin erweiterte Funktion in Pereon und Pleon hin. Zusätzlich entdeckten wir eine bislang unbekannte endodermale Expression von *abd-A* im entstehenden Mitteldarmdrüsenepithel, was auf eine vielfältige Rolle für dieses Gen hindeutet. Die Wasserassel *Asellus aquaticus* könnte damit einen neuen und relevanten Modellorganismus zur Studie von evolutionären Änderungen von Segmentidentität und -musterbildung darstellen.

Contents

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1. Introduction

The Hox genes have been recognized as widely conserved metazoan genes relevant for patterning of the embryonic axes (Tabin 1992; Krumlauf 1993; Akam et al. 1994; Ruddle et al. 1994; Akam 1995; Holland & Garcia-Fernandez 1996; Averof 1997; Wolpert 2000; Popodi & Raff 2001; Vervoort 2002; Deutsch & Mouchel-Vielh

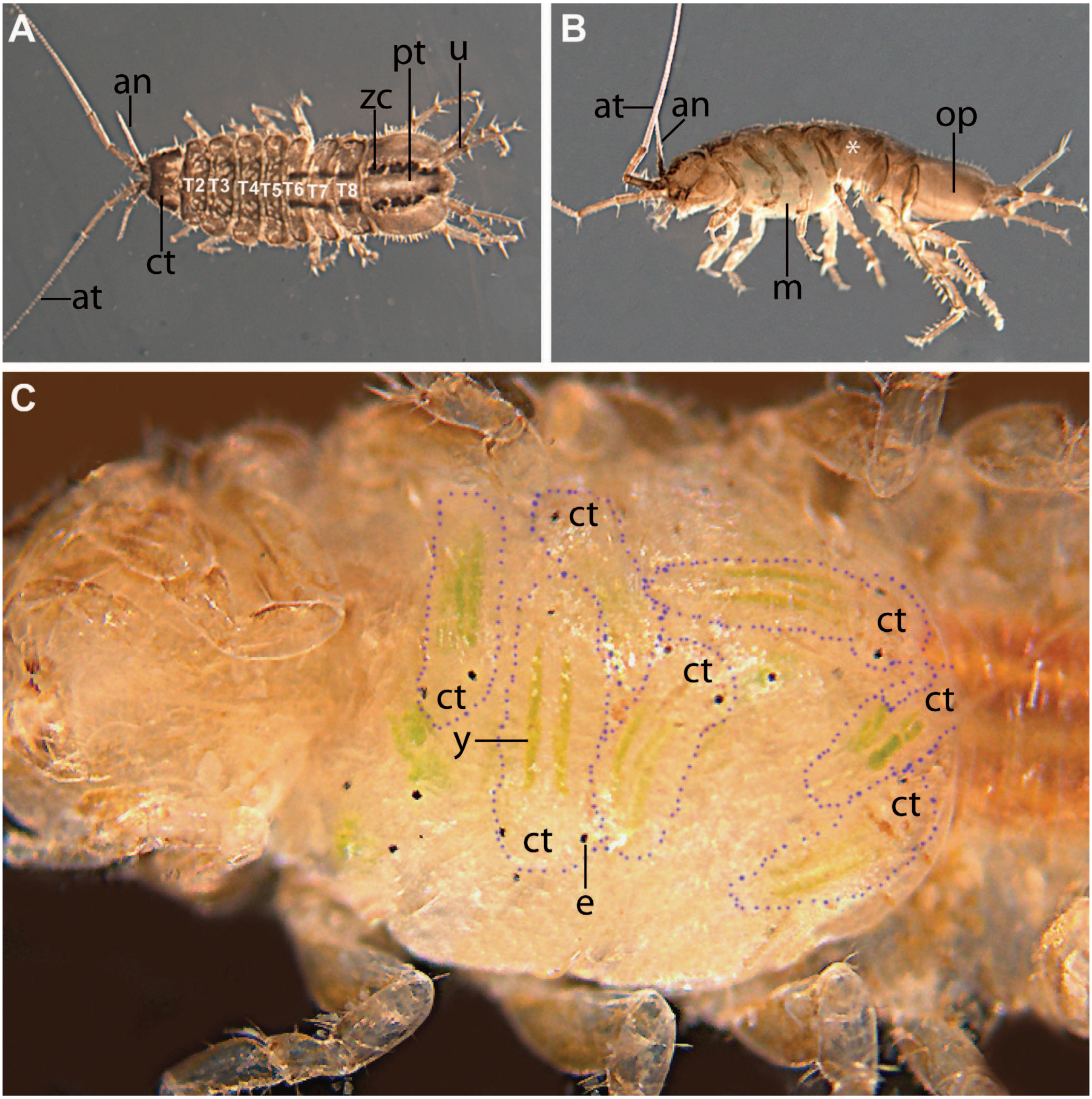

Fig. 1. *Asellus aquaticus*: adult female morphology. – **A**. Dorsal view of an adult female displaying the cephalothorax (ct) with a pair of antennules (an) and antennae (at), seven free thoracic segments (T2–T8), two free pleonic segments and the pleotelson (pt) bearing the uropods (u). **B**. Lateral view of same specimen displaying the seven pairs of pereopods (walking legs), marsupium and operculum. Please note the absence of the 5th pereopod (marked by an asterisk). **C**. Ventral view of a female with the marsupium in high magnification. The breeding room is filled with developing embryos. For some embryos the body shape is outlined by dotted lines. Eyes have developed and are visible as pigmented dots (e), and the green yolk inside the paired digestive glands is clearly discernable. – Anterior to the left. m, marsupium; op, operculum; T2–T8, thoracomer 2–8; y, yolk; zc, Zenker cells (Zenker 1854; Dohrn 1867).

2003; Garcia-Fernandez 2005; Hoegg & Meyer 2005; Pearson et al. 2005; Duboule 2007; Hueber & Lohmann 2008; Wellik 2009). In particular, they act as selector genes for segment identity throughout the animal kingdom. In insects, Hox genes display more or less strict tagmata-related expression patterns. For example, activity of *abdominal-A* (*abd-A*) correlates with the legless abdomen. In crustaceans, in contrast, expression domains are more divers. In the branchiopod brine shrimp *Artemia franciscana*, for example, expression domains of the posterior Hox genes follow the insect-type overlapping pattern (Averof & Akam 1995). In the more modern malacostracan isopod *Porcellio scaber* however, *abd-A* is restricted to the developing pleon (Abzhanov & Kaufman 2000a). Here we present the partial cloning and expression patterns of the Hox genes *abd-A* and *Antennapedia* (*Antp*), and the homeobox gene *distal-less* (*dll*) from the freshwater crustacean *Asellus aquaticus*. In contrast to *Porcellio* this isopod shows a similar but slightly different regulation of posterior segment patterning. The *abd-A* signal obtained by whole-mount in situ hybridization points to a more anterior extended function in the posterior pereon as well as to a conserved role in the pleon. In addition, we detected an as yet unknown endodermal expression domain of *abd-A* in the epithelium of the early developing digestive gland, which indicates a diverse role of this gene. Furthermore, we analyzed the expression of *Asellus Antp*, a Hox gene known to be expressed in the crustacean trunk, and of *distal-less* (*dll*), a gene expressed specifically in body appendages. Both expression domains are conserved in *Asellus*, underlining the significance of the derived expression patterns of *abd-A*. The waterlouse

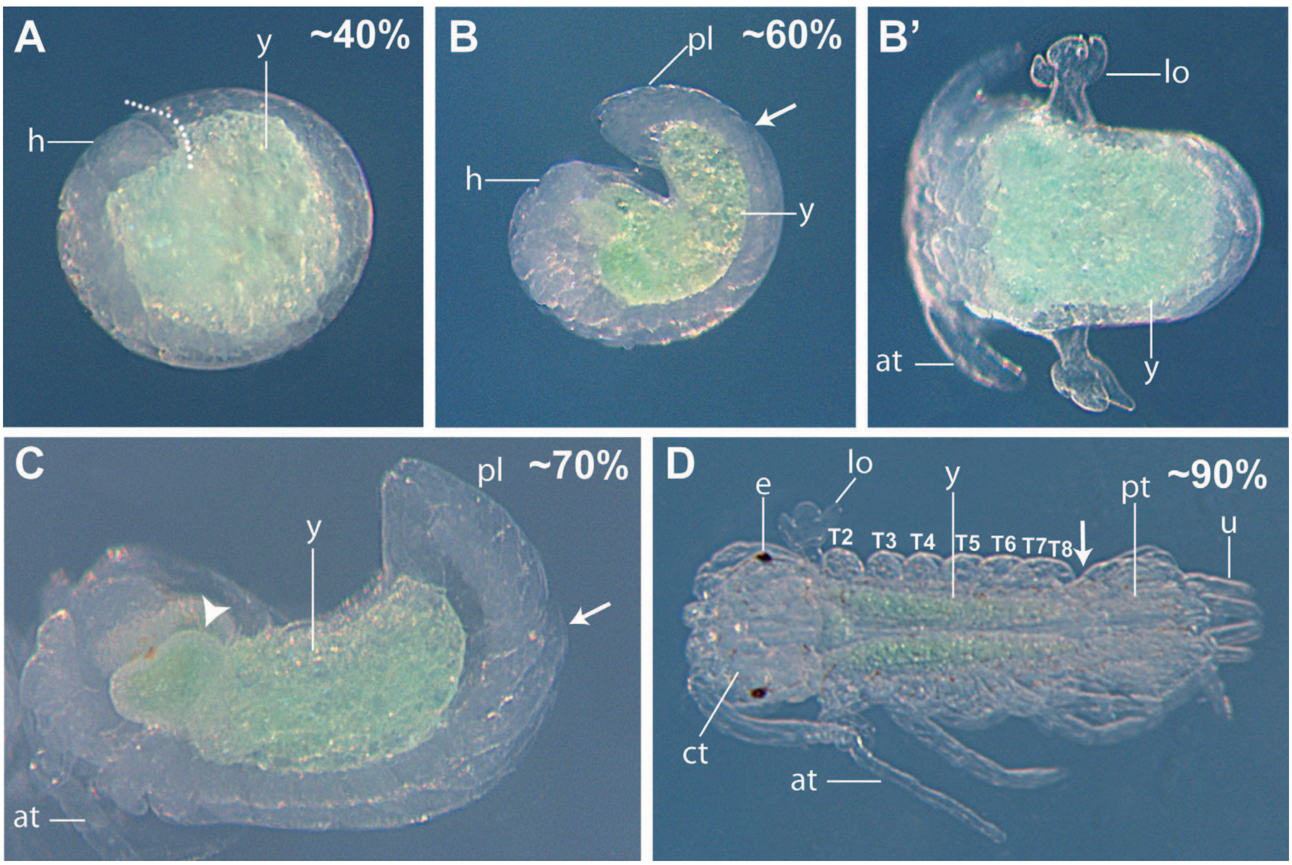

Fig. 2. Embryonic development of *Asellus aquaticus*. – **A**. Lateral view of an embryo at about 40 % of development, still inside the chorion. The starting point of the dorsal curvature between head and posterior end of the pleon is indicated by a dotted line. **B**. Lateral view of an embryo at about 60 % of development. **B'**. Ventral view of the embryo shown in B. Lateral organs can be seen at the level of the first thoracomer. **C**. Lateral view of an embryo at about 70 % of development. Anteriorly, the yolk begins to be incorporated into the paired digestive glands (arrowhead). Appendages are well developed and dorsal bending is nearly fully retracted. **D**. Dorsal view of an embryo just before the end of embryonic development (first molt). The yolk now is fully incorporated into the glands and thoracomers are clearly distinguishable. Please note the greenish color of the yolk. – Staging of embryos according to Whitington et al. (1993). Anterior to the left. White arrows mark approximate border between pereon/thorax and pleon. at, antenna; ct, cephalothorax; e, eye; h, head; lo, lateral organ; pl, pleon; pt, pleotelson; T2–T8, thoracomer 2–8; u, uropod; y, yolk.

Asellus aquaticus thus may provide a relevant and novel crustacean model organism for the study of evolutionary changes of segment identity and patterning.

2. Results and discussion

2.1. Adult morphology of *Asellus aquaticus*

Fig. 1 shows the adult morphology of *Asellus aquaticus*, both as a reference point and to correlate embryonic expression patterns shown below. In the genus *Asellus* the head and the first trunk segment are fused to form a cephalothorax, followed by seven free thoracic segments (the so-called pereomers), two small free pleonic segments and a pleotelson, in which four pleon segments are fused with the telson (Fig. 1A, B). Females temporarily develop a marsupium on the ventral side, representing an isopod-characteristic breeding room, in which the whole embryonic development takes place (Fig. 1C). The marsupium is bordered by the anterior pereonic sternites and the specialized, flattened epipodites of the same segments. Embryos are visible through the marsupium and may easily be obtained (Gruner 1965; Vick et al. 2009).

2.2. Embryonic development of *Asellus aquaticus*

For a comparative analysis of embryonic development of *Asellus aquaticus* in relation to other crustacean species, staging of embryos was adapted to Whitington et al. (1993). *Asellus* develops no ventral groove and is thus characterized by a dorsal curvature, which separates head and pleon early during development (Fig. 2A, B). This represents a typical feature of isopods and a few other crustacean orders, which all lack development of a ventral groove (Gruner 1965). Another typical characteris-

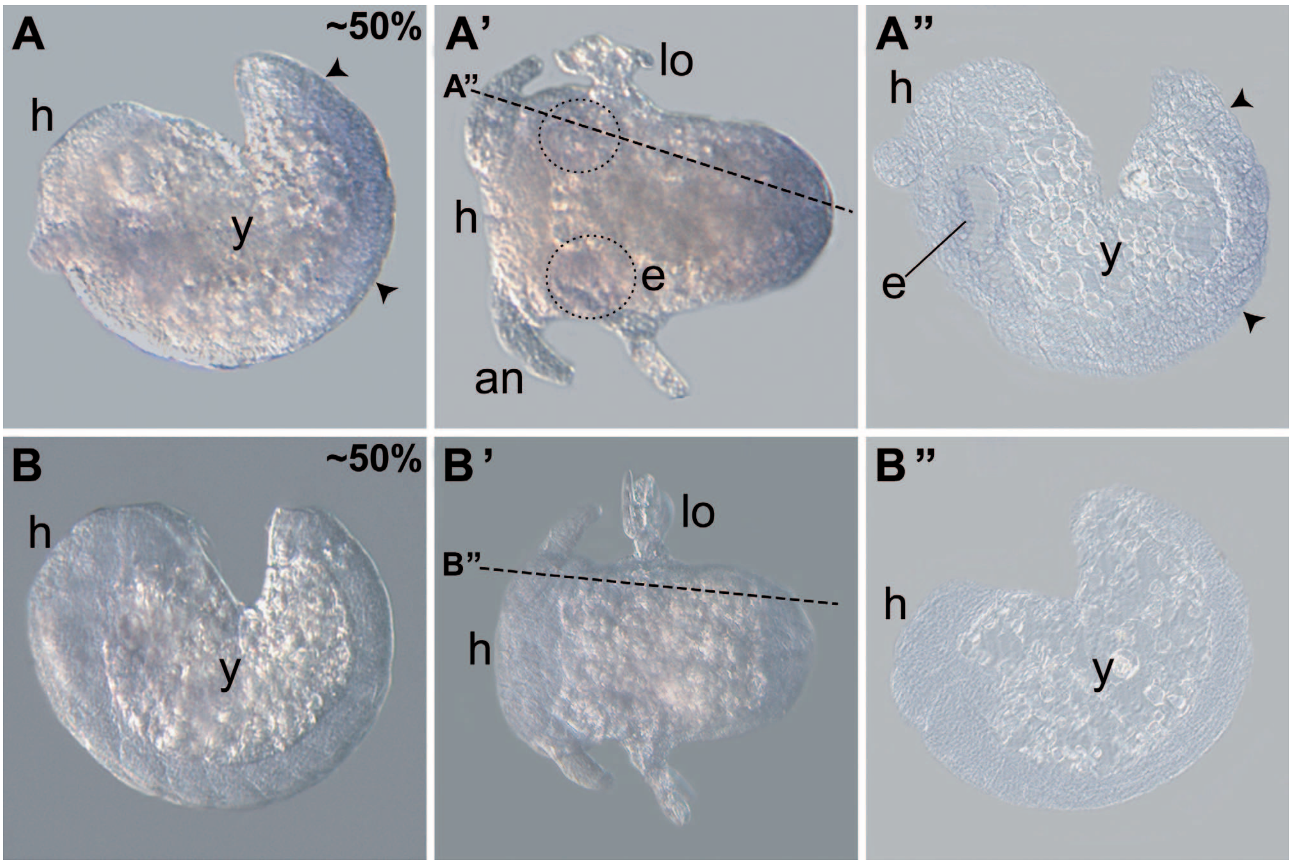

Fig. 3. Early embryonic expression patterns of *abd-A* in *Asellus aquaticus* embryos. – **A–A''**. Whole-mount in situ hybridization (WMISH) of an embryo at ~50 % of development showing *abd-A* mRNA expression mainly in the pleon and in the paired endodermal anlagen of the digestive glands (e). **A**. Only the posterior expression domain can bee seen. **A'**. Ventral view displaying lateral organs (lo) and endodermal primordia, which are visible through the translucent ectoderm (dotted rings). **A''**. A sagittal section (plane indicated in A') highlights expression domains. **B–B''**. Control embryo at ~50 % of development hybridized with a sense probe showing no expression in any tissue. – A, A'', B, B'' lateral views, anterior to the left, dorsal to the top; A', B' ventral views, anterior to the left. an, antennule; h, head; y, yolk. Black arrowheads indicate approximate anterior and posterior expression boundaries.

tic of this species are the so-called lateral organs (WEYGOLDT 1959). These arise very early in development and persist until the first ecdysis, i. e. they mark embryonic development (Fig. 2B', D). During development, appendages arise from all segments formed. Parallel to the stretching of the anterior-posterior axis, through which the dorsal curvature disappears, the yolk is consumed or incorporated into the digestive glands (Fig. 2B–D). Before the first molt, which transforms the embryo into a juvenile isopod, the embryo is fully stretched, bears developed appendages, eye spots, and a continuous gut, and thus roughly resembles an adult specimen (Fig. 2D). However, this late embryo as well as the following juvenile (or manca) stage still lack the eighth thoracic appendages, i. e. the last pair of walking legs.

2.3. Early expression patterns of *abdominal-A* in embryos of *Asellus aquaticus*

Following partial cloning of the *Asellus aquaticus abd-A* gene (VICK et al. 2009; accession number ACG63499), an expression analysis was performed by whole-mount in situ hybridization using an antisense-specific riboprobe. At about 50 % of development, mRNA transcription could be detected in the developing pleon, excluding the very posterior part, i. e. the telson (Fig. 3A–A''). Most remarkably, an additional expression domain was detected in the epithelial lining of the two endodermal anlagen of the digestive glands in the anterior thorax (Fig. 3A', A''). A sense control probe revealed no staining, underscoring the specificity of the obtained mRNA signals (Fig. 3B–B'').

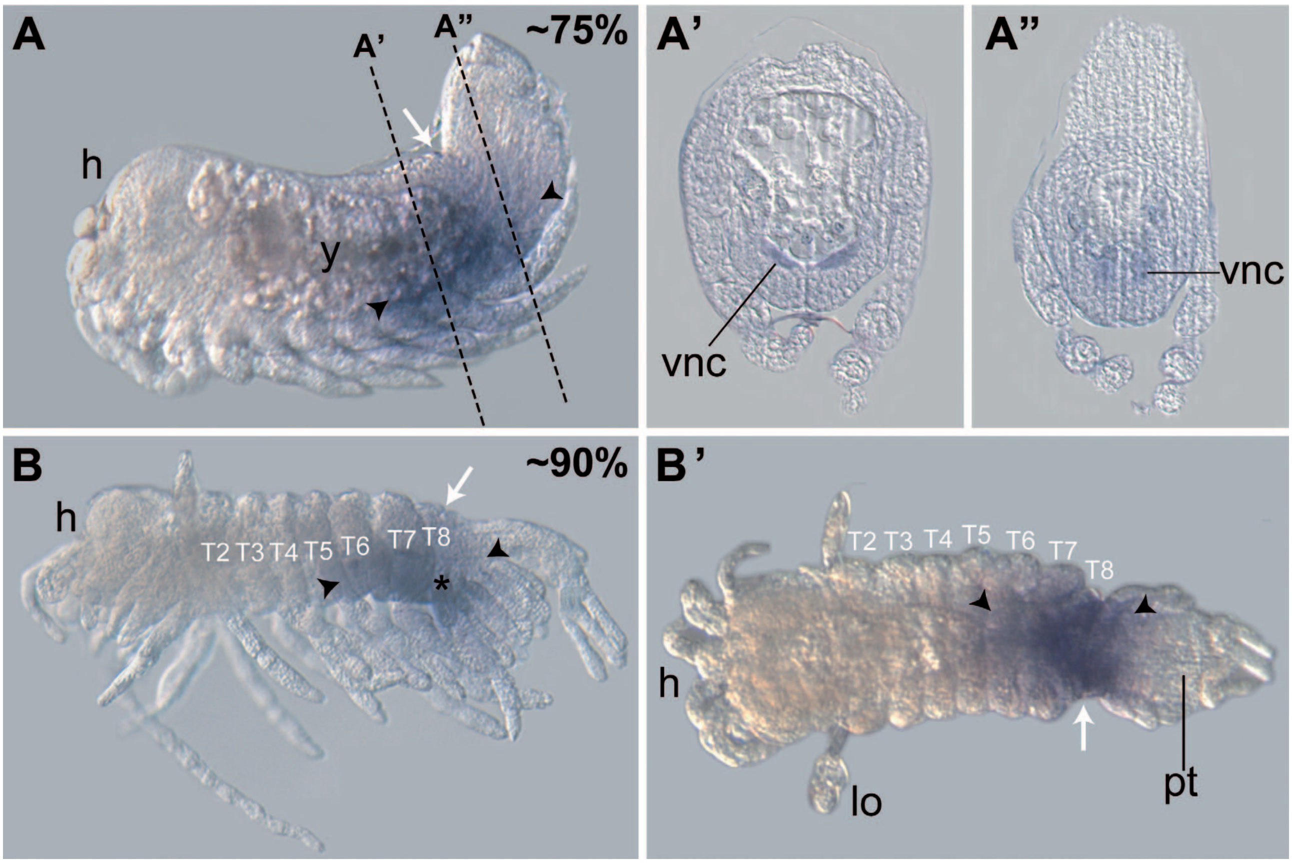

Fig. 4. Late expression patterns of *abd-A* in embryos of *Asellus aquaticus*. – **A–A''**. *abd-A* mRNA expression in a ~75 % embryo. Expression was seen between the 5th pereonic segment and the anterior pleon. Transversal sections (A', A'') showed strong expression in the ventral nerve cord (vnc) and weaker signals in the proximal limbs and the remainder of the mesodermal and ectodermal trunk tissue, which gradually faded towards the dorsal side. **B, B'**. Expression of *abd-A* in an embryo at ~90 % of development. The signal was clearly restricted to a region between T5 and pleonic segment 3, with strongest expression in T7 and T8 (B'), the developing 7th pereopod (* in B), and the proximal limbs of the anterior pleonic segments (B). – A and B, lateral views, anterior to the left, dorsal to the top; B', dorsal view, anterior to the left; A' and A'', transversal sections, dorsal to the top. White arrows mark tagmata boundary between pereon and pleon. Black arrowheads indicate approximate anterior and posterior expression boundaries. Dashed lines indicate approximate planes of section. h, head; lo, lateral organ; pt pleotelson; T2–T8, thoracomers 2–8; y, yolk.

2.4. Late expression pattern of *abd-A*

A careful expression analysis of the final stages of embryonic development of *Asellus aquaticus* revealed a striking change of *abd-A* transcript distribution. From 75 % of embryonic development onwards, *abd-A* was active more anteriorly in the embryo. *abd-A* was found to be expressed in the anterior-most pleon (pleomeres 1 and 2) and most strongly in the posterior thoracic segments (T5–T8; Fig. 4A, B). Transversal histological sections revealed strong staining of the ventral nerve cord and of the surrounding ventral ectodermal and mesodermal tissues (Fig. 4A', A''). The appendages of these segments showed positive *abd-A* signals only in the very proximal parts (asterisk in Fig. 4B). Interestingly, this positive staining of the pleonic ventral nerve cord was not found in *Porcellio scaber* (Abzhanov & Kaufman 2000a).

2.5. Late expression pattern of *Antp* in *Asellus aquaticus*

To learn more about the significance of this derived expression pattern of *abd-A*, *Antennapedia* (*Antp*), another trunk Hox gene, was cloned (accession number

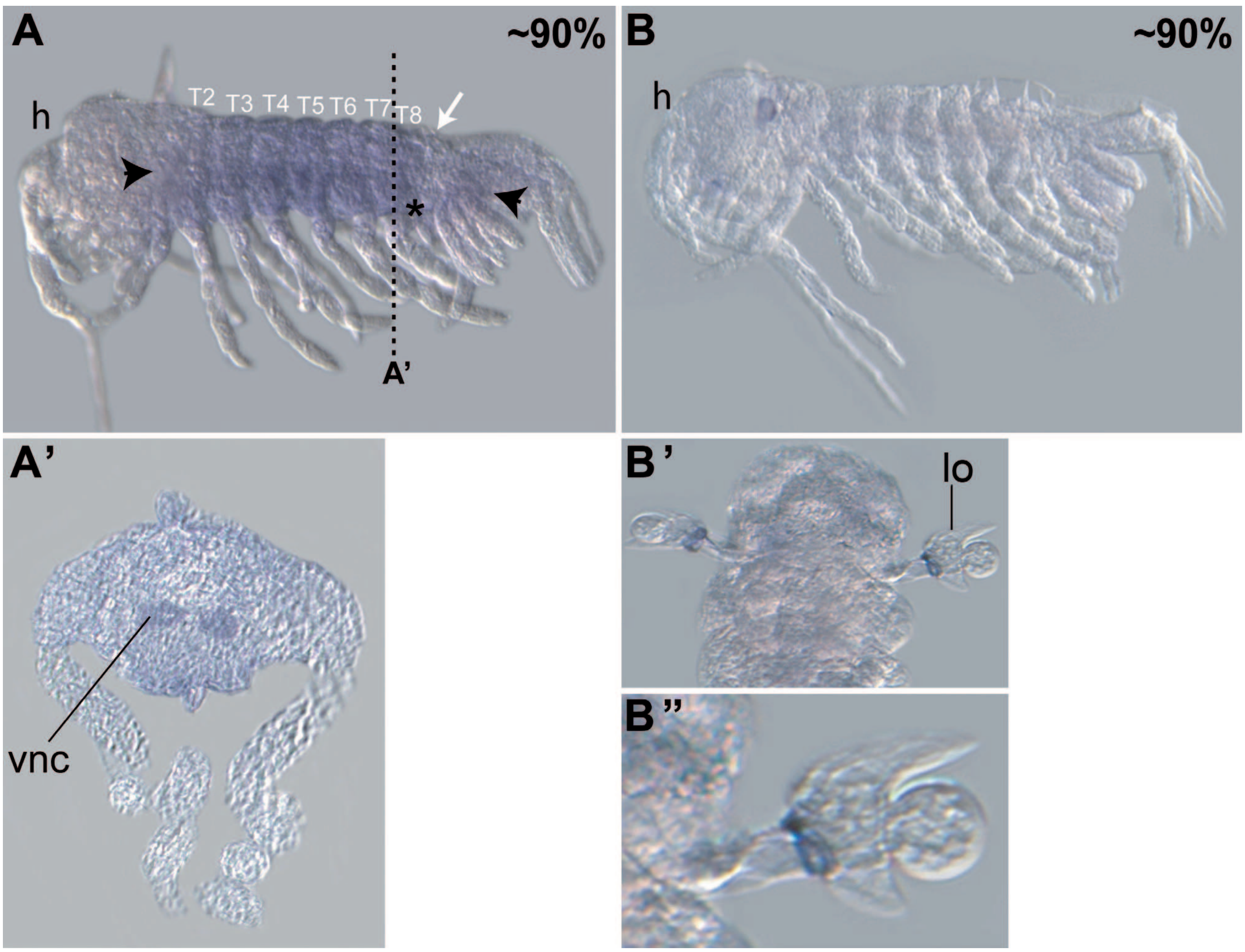

Fig. 5. Late embryonic expression patterns of *Antp* in embryos of *Asellus aquaticus*. – **A**. Lateral view of an embryo at about 90 % of development after WMISH. A strong signal was detectable in T2–T8 and in the proximal appendages. Weak expression was seen in T1 and the ventral pleonic segments. **A'**. Transverse histological section through the pereon. Besides weak ventral and lateral expression patterns, a strong signal was detected in the ventral nerve cord (vnc). **B**. Lateral view of a sense control embryo of the same stage, in which no staining could be detected besides an artificial signal at the base of the lateral organ. **B', B''**. Higher magnification of the lateral organ region to visualize artificial staining at its basis. – Dorsal to the top in A, A', B. Anterior to the top in B', B''. Black arrowheads indicate approximate anterior and posterior expression boundaries. White arrow marks tagmata boundary between pereon/thorax and pleon. Dashed line in (A) indicates approximate plane of section. h, head; lo, lateral organ; T2–T8, thoracomers 2–8; y, yolk.

HM044765). *Antp* is known to be expressed in the crustacean thorax (AVEROF & AKAM 1995; ABZHANOV & KAUFMAN 2000a) and therefore served as a probe to compare conservation of expression patterns among different crustacean species. In contrast to *abd-A*, expression of *Antp* was found to be conserved as compared to other crustacean and isopod species. Expression was detected in all thorax segments (including T1) as well as in the ventral parts of the anterior pleon and some proximal pleonic appendages (Fig. 5A). The domain in the pleonic appendages represented the sole difference to *Porcellio Antp* (ABZHANOV & KAUFMAN, 2000a). Histological sections revealed intense signals in the ventral nerve cords (Fig. 5A'). Sense control riboprobes showed no specific staining (Fig. 5B).

2.6. *Distal-less* (*dll*) expression in developing appendages of *Asellus aquaticus* embryos

To verify conservation of expression patterns of major developmental genes in *Asellus aquaticus*, we analyzed *dll* as a further example (accession number HM044766). In all arthropod species analyzed so far, *dll* is expressed in developing appendages (PANGANIBAN et al. 1994, 1995, 1997; ABZHANOV & KAUFMAN 2000b; PANGANIBAN & RUBENSTEIN 2002). Expression of *Asellus aquaticus dll* was found in most developing appendages in embryos developed to 60 % and 70 % of embryogenesis (Fig. 6A, B), while sense control riboprobes were negative in all cases (cf. inset in Fig. 6A). In accordance with their very early occurrence and origin, the lateral organs showed no expression (WEYGOLDT 1959). Histological sections verified specific staining of the appendages (Fig. 6A'', B', B''').
Therefore, *Asellus aquaticus* represents another example of conserved *dll* expression in developing appendages.

2.7. Summary and outlook: comparison of Hox expression patterns in *Asellus* and *Porcellio*

In summary, the expression patterns of the developmental genes were largely conserved, particularly with

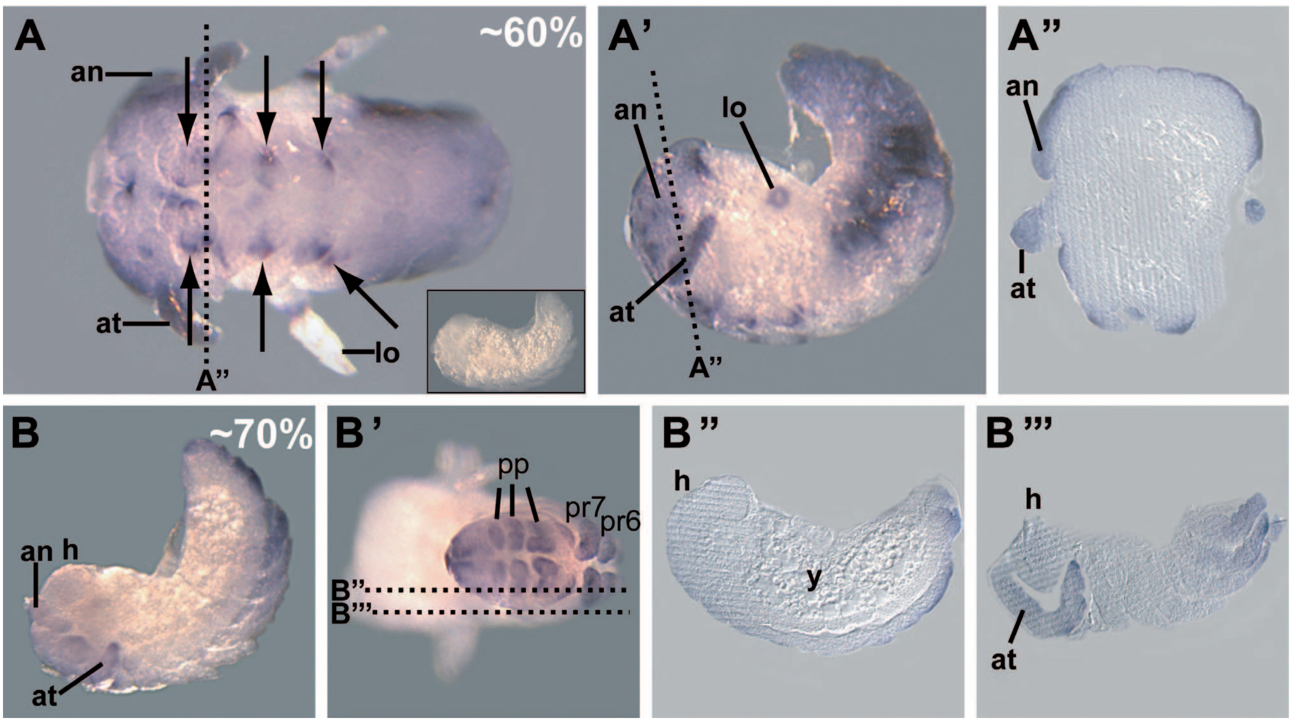

Fig. 6. Expression of *dll* in appendages but not in the lateral organs of *Asellus aquaticus* embryos. – **A**. Ventral view of an embryo at about 60 % of development showed expression specifically in developing appendages (arrows). Sense control (inset) was free of staining. **A'**. Lateral view of the same specimen showed strong expression in the antenna. Lateral staining at the posterior thorax and pleon represents an artifact. **A''**. Transversal section as indicated in (A) and (A') revealed staining in the appendages. **B**. Lateral view of an embryo at about 70 % exhibited expression in the mouth parts and appendages. **B'**. Ventral view of the same embryo showed distinct domains of *dll* expression in the pleonic and posterior thoracic appendages. **B'', B'''**. Sagittal sections of the specimen as depicted in (B') underlined appendage-specific *dll* mRNA transcription in the proximal and distal parts. – Anterior to the left in A, A' and B–B'''. In A'–B, B'' and B''' dorsal is to the top. an, antennula; at, antenna; h, head; lo, lateral organ; pp, pleopod; pr, pereopod; y, yolk.

respect to *Antp* and *dll*. In this light, the divergent patterns of *abd-A* deserve all the more attention (Fig. 7). *Asellus aquaticus* displayed strong expression patterns of *abd-A* in P6, P7 and the free pleonic segments 1 and 2, and weaker levels in P5 and pleonic segment 3. In contrast, strong expression in *Porcellio* was restricted to the pleonic segments. In P6 and P7 a weak expression was reported (ABZHANOV & KAUFMAN 2000a). Our *abd-A* expression analysis in *Asellus aquaticus* thus demonstrated a clear correlation of expression domains with distinct morphogenetic units in two cases: pleotelson fusion (absence of *abd-A* transcription; cf. Fig. 4B') and pereonic limb orientation (strong *abd-A* expression during the process of posterior bending in late stages; cf. Fig. 4A). Compared to *abd-A* expression in *Porcellio*, the adult morphology divergence is mimicked by a corresponding change in gene expression, i. e. *abd-A* activity throughout the pleonic segments (which do not fuse), and lack of activity in posterior pereopods (which do hardly orient posteriorly; VON HAFFNER 1937; GRUNER 1965). Thus, even between highly related crustaceans, shifts of Hox gene expression boundaries correlate with adult morphological variations. We thus hypothesize that temporal-spatial changes in *abd-A* gene expression are responsible for morphogenetic variations in adult morphologies of *Asellus aquaticus* and *Porcellio scaber*. Direct testing of this proposal might become possible in the future, provided that techniques for genetic manipulation of embryos become available.

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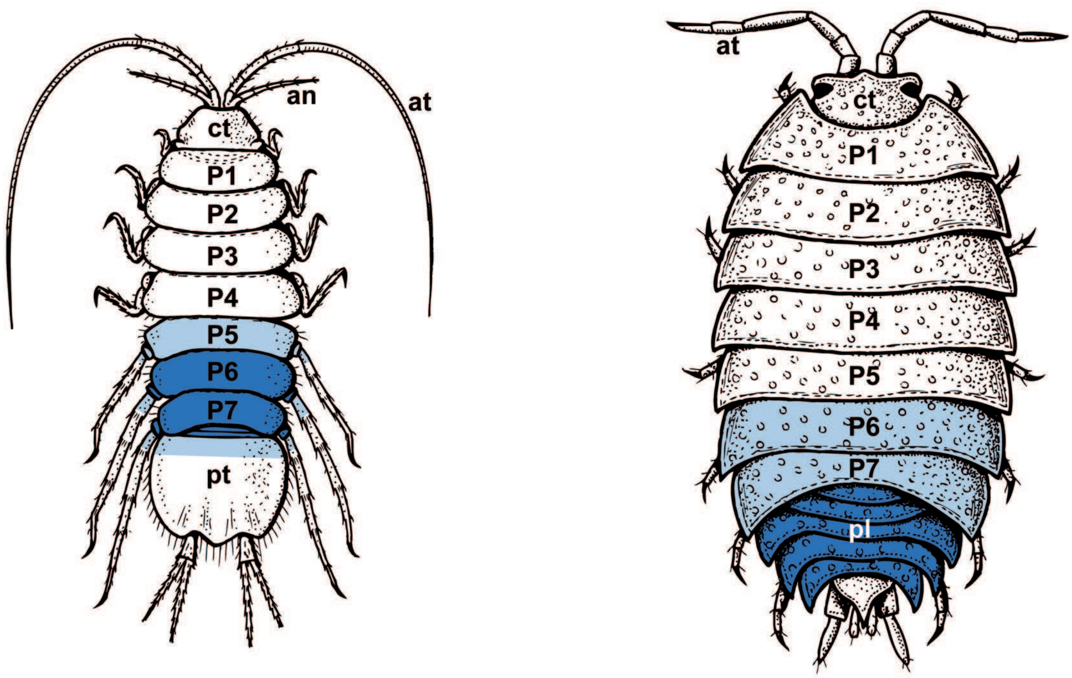

Fig. 7. Expression domains of *abd-A* mRNA in *Asellus aquaticus* (left) and *Porcellio scaber* (right). The experimentally derived embryonic patterns are shown in relation to the adult morphology. Dark blue coloring indicates strong gene activities, while a light blue color refers to weaker expression domains. – at, antennae; an, antennule; ct, cephalothorax; pl, pleon; pt, pleotelson.

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