Evolutionary variation in neural gene expression in the developing sense organs of the crustacean *Daphnia magna*

Marleen Klann[1], Angelika Stollewerk*

School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK

[1]Current address:

Whitney Laboratory for Marine Bioscience, University of Florida, 9505 Ocean Shore Blvd, St. Augustine, Florida 32080, USA

*Corresponding author: a.stollewerk@qmul.ac.uk

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Abstract

Arthropods have numerous sense organs, which are adapted to their habitat. While some sense organs are similar in structure and function in all arthropod groups, structural differences in functionally related sense organs have been described, as well as the absence of particular sense organ subtypes in individual arthropod groups. Here we address the question of how the diverse structures of arthropod sense organs have evolved by analysing the underlying molecular developmental processes in a crustacean, an arthropod group that has been neglected so far. We have investigated the development of four types of chemo- and mechanosensory sense organs in the branchiopod *Daphnia magna* (Cladocera) that either cannot be found in arthropods other than crustaceans or represent adaptations to an aquatic environment. The formation of the sensory organ precursors shows greater similarity to the arthropod taxa Chelicerata and Myriapoda than to the more closely related insects. All analysed sense organ types co-express the proneural genes *ASH* and *atonal* regardless of their structure and function. In contrast, in *Drosophila melangoaster, ASH* and *atonal* expression does not overlap and the genes confer different sense organ subtype identities. We performed experimental co-expression studies in *D. melanogaster* and found that the combinatorial expression of *ato* and *ASH* can change the external structure of sense organs. Our results indicate a central role for ASH and Atonal family members in the emergence of structural variations in arthropod sense organs.

Introduction

Arthropods have diverse, small internal and external sense organs, which can receive and process a wide range of mechanical (e.g. touch, vibration) and chemical (olfactory, gustatory) (Hartenstein, 2005) stimuli. These sense organs mediate essential behaviour such as mating, foraging and reproduction and are therefore directly involved in the communication with the environment and likely subject to ecological adaptation.

Here we address the question whether different developmental genes are expressed in different types of mechano- and chemosensory organs in insects and crustaceans and whether differential gene expression can be correlated with evolutionary changes in sense organ structure. A uniform system to classify mechano-and chemosensory organs in arthropods has not yet been developed and various terms are used for sense organs with similar structures in the different groups. Generally the literature distinguishes between external and internal sense organs in all arthropods. External sense organs show various shapes, ranging from hair-like structures to cones and perforated plates and can be mechano- and/or chemosensitive (Jarman and Ahmed, 1998; McIver, 1975). In many cases the structure of a sense organ can be directly related to its function. For example, in contrast to external mechanosensory organs, chemosensory organs usually have a pore or another opening in the bristle (McIver, 1975). Chemosensory organs are densely packed in the head appendages of arthropods and have been extensively investigated in insects and decapod crustaceans (Hallberg et al., 1997; Schmidt and Gnatzy, 1984). In crustaceans, the specialised olfactory organs in the first antennae are called aesthetascs. They are arranged in groups and their thin cuticles are permeable to large molecules (Hallberg and Hansson, 1999).

Chordotonal organs, also known as scolopidial organs, are primarily internal stretch receptors in insects but on the antennae they form the so-called Johnston's organ which acts as a hearing organ (Eberl, 1999; McIver, 1975). The main feature is an elongated spindle-shaped sheath cell, the scolopale cell, which contains densely packed, rod-shaped tubulin and actin filaments (the so-called scolopale) and envelops the dendrite of the sensory neuron (Eberl, 1999; Jarman and Ahmed, 1998). Scolopidial organs are absent in chelicerates and myriapods, but in aquatic crustaceans, all types of external mechanosensory organs contain scolopidalial structures (Hallberg and Hanson, 1999).

Despite structural differences, the large majority of mechano- and chemosensory sense organs in arthropods show similar cellular compositions. Each sense organ consists of only 4 to 5 different cell types and is innervated by one or several neurons, which respond to specific stimuli (Hartenstein, 2005). The stimuli are received by modified (sub-) epidermal cells (e.g. hairs) and transferred to the sensory neurons resulting in an action potential that is transmitted via the axons towards the central nervous system. All cells that contribute to the internal and external structure of the sense organ are initially clustered together in and underneath the epidermis (Hartenstein, 2005).

The molecular processes of mechano- and chemosensory organ development have been studied in great detail in insects, particularly in dipterans, but only few publications are available in other arthropod groups, namely in chelicerates and myriapods (Gold et al., 2009; Pioro and Stollewerk, 2006; Stollewerk and Seyfarth, 2008), and none in crustaceans. In *Drosophila melanogaster* the different cell types within a sense organ are generated in many cases from a single sensory organ precursor (SOP) cell in four consecutive divisions (Lai and Orgogozo, 2004). The five bHLH transcription factors *achaete*, *scute*, *lethal of scute*, *atonal* and *amos* determine the three different classes of sense organs (external mechanosensory, chemosensory and chordotonal organs) that

develop from the SOPs (Hartenstein, 2005). Several other transcription factors are switched on slightly later; among others, *cut*, which is exclusively expressed in external mechanosensory organs, and *pox-neuro*, which can be detected in all precursors of chemo-, thermo- and hygroreceptors (Awasaki and Kimura, 1997; Blochlinger et al., 1990; Blochlinger et al., 1991; Dambly-Chaudière et al., 1992). The importance of these SOP identity genes is seen in loss of function experiments where *cut* and *pox-neuro* mutant sense organs are transformed into chemo- and mechanosensory organs, respectively (Hartenstein, 2005). A cascade of genes is expressed in the developing sense organ that determines the identity of the individual cell types (i.e., neural/accessory) within the SOP lineage. *Drosophila asense*, *prospero* and *snail* establish the neural part of the SOP lineage in all types of sensory organs (Doe et al., 1991; Ip et al., 1994; Jarman et al., 1993a).

Here we analyse for the first time the gene expression patterns in four different types of developing external sense organs in a crustacean, the waterflea (Cladocera) *Daphnia magna*. Insects and crustaceans are closely related and together form the Pancrustacea (also called Tetraconata). The internal relationships of pancrustaceans are controversially discussed and different groups of the paraphyletic crustaceans have been suggested as sister group to the monophyletic hexapods (which include insects) (e.g., Andrew, 2011; Regier et al., 2010). Our data suggest that evolutionary changes in the gene expression patterns correlate with differences in sense organ structure in insects and crustaceans.

Material and Methods

Cloning, sequences and probe preparation

PCR primers for *Dam ato* were designed (*Dam ato* fwd 5'-TACAACACT-CCCAGCCCAAT-3'; *Dam ato* rev 5'-CCACAATGCCGTGATGTAAC-3') and PCR amplified using an oligo-cDNA template generated from mixed *D. magna* stages. The gene fragment was cloned using the pGEM®-T Easy Vector System II (Promega) and sent for sequencing (either Eurofins, MWG Operon or The Genome Centre, Barts and London School of Medicine and Dentistry). The *D. magna atonal* sequence has been identified independently by Gilbert, D.G., Choi, J.-H., Mockaitis, K., Colbourne, J. and Pfrender, M. and published in GenBank (Accession number: KZS01707.1). DIG and/or fluorescin labelled RNA probes were prepared according to standard protocols (Roche). The following primers were used to amplify and clone the *Dam ato* fragment that was used as template for the probes: TACAACACTCCCAGCCCAAT (forward), CCACAATGCCGTGATGTAAC (reverse). The fragment includes the open reading frame except for 12 nucleotides at the 3' end. *Dam ASH*, *Dam snail* and *Dam pros* were previously cloned and described (Ungerer at al., 2011).

Collection and staining of *Daphnia magna* embryos

A culture of *Daphnia magna* was kept in the laboratory and eggs were collected after previously described methods (Ungerer et al., 2011). For *in situ* hybridization and antibody staining *D. magna* embryos were fixed with 25% formaldehyde in fixation buffer for 30 min at room temperature, subsequently manually dechorionated/devitellinized and stored in 100% methanol at -20 °C. The colorimetric *in situ* protocol published in Ungerer et al. (Ungerer et al., 2011) was followed with

RNA probe hybridization over night at 60 °C. The antibody staining protocol, we used to visualize acetylated α-tubulin and Phalloidin, was also described by Ungerer et al. (2011). We used the fluorescent *in situ* hybridization protocol previously described by Biffar and Stollewerk, (2014). Embryos were counterstained using Hoechst 33258, Sytox green or SYBR® Green and transferred into 70% glycerol/PBS.

Scanning electron microscopy

For scanning electron microscopy, *D. magna* embryos, larvae and adults as well as *Drosophila* adults were fixed with Bouin for two hours. The specimens were washed with distilled water several times, and embryos were manually dechorionated and devitellinized (see above). Samples were then gradually dehydrated in hexamethyldisilazane (HMDS, Sigma-Aldrich). The samples were incubated for 1 hour in 50% HMDS in ethanol then left over night in 100% HMDS. The dried specimens were carefully mounted on Aluminium Specimen Stubs using Carbon sticky Tab and sputtered with an Agar Auto Sputter Coater. Scanning electron microscope pictures were taken with a SEM FEI Inspect F (10kV, spot 3.5).

Documentation and Analysis

Colorimetric *in situ* hybridizations together with SYBR Green counterstaining were documented with a Leica DM IL FLUO inverse microscope with a Leica DFC420C camera. Fluorescent *in situ* hybridizations, acetylated α-tubulin and Phalloidin staining were documented with a Leica SP5 confocal microscope. The software Helicon Focus (d-Studio Ltd.) was used to combine the image stacks of individual embryos taken with

the Leica DM IL FLUO microscope. The 3D-reconstruction software IMARIS (Bitplane AG) was used to analyse the confocal image stacks. The obtained pictures were further processed in Adobe Photoshop CS3. Picture plates and schematic representations were composed with Adobe Illustrator CS3.

D. melanogaster misexpression experiments

All *D. melanogaster* experiments were performed under standard conditions and on standard fly food. For misexpression experiments the *Gal4*/UAS system was used (Brand and Perrimon, 1993; Phelps and Brand, 1998). For stable germ line transformation the ΦC31 system was used (Bischof et al., 2007; Thorpe et al., 2000). The entire open reading frame of *D. magna atonal* was cloned and subsequently inserted into the pUASTattB vector (kindly provided by Prof. Ralf Stanewky's lab) and injected into ΦX-51C fly embryos. Each possible transformant (13 in total) was balanced in single crosses with yw; CyO/BL flies and the eye colour of the offspring was determined one to two days after eclosion. Offspring with light orange eyes (successful integration of the gene) and curly wings (Balancer) were selected as virgins and crossed with each other to establish a stock. In total four independent yw; UAS*ato*^{Dam} lines were generated. The external phenotype of these UAS*ato*^{Dam} flies is the same as for wild-type *D. melanogaster* except for an occasional (up to 7%; 3 of 46 flies analysed) duplication of the anterior scutellar macrochaetae, which is also seen in the *D. melanogaster* control line UAS*ato*^{8} (up to 17%; 7 of 42 flies analysed). The UAS*ato*^{Dam} flies were crossed to 3 different *Gal4* lines: *sca*$^{109\text{-}68}$*-Gal4*, *sca-Gal4* (kindly provided by Prof. Ralf Stanewsky's lab), *c784-Gal4*. The functionality of the UAS*ato*^{Dam} construct was confirmed by in situ hybridisations with the *Dam-ato* probe

on *D. melanogaster* embryos expressing the construct (data not shown). For control experiments the line UAS*ato*^{8}/TM3,Sb^{1} was used to repeat all misexpression experiments. Bloomington *Drosophila* Stock Center numbers: ΦX-51C*,* #24482; *sca*$^{109\text{-}68}$*-Gal4, #*6479*; c784-Gal4, #*6985*;* UAS*ato*^{8}/TM3,Sb^{1,} #39679

Results

Identification of different types of external sense organs in Daphnia magna

In contrast to insects, the hair- or bristle-like cuticular protrusions (setae) of many external sense organs in crustaceans exhibit secondary outgrowths called setules, which are articulated and vary in lengths. Although a standardized classification is missing, the following seven types of sense organs have been distinguished in various crustaceans based on the structure of their setae: serrulate, serrate, papposerrate, pappose, plumose, simple and cuspidate (Garm, 2004; Watling, 1989). In the following we will focus on the latter four sense organ types because they are located in prominent positions, which can be correlated with distinct areas of gene expression (Fig. 1A; Table 1). *D. magna* is mainly parthenogenetic under normal conditions; thus the description of setae primarily relates to females. Pappose setae have a long shaft with long serrated, articulated setules, which are randomly distributed. Plumose setae also show a long shaft but their long setules are arranged in rows (normally two opposite rows) giving them a feather-like appearance (Garm, 2004; Watling, 1989). Simple setae lack any cuticular outgrowth on their shaft; some have a terminal pore, indicating a chemosensory function. Finally, cuspidate setae resemble simple setae in that they do not have secondary outgrowths; however, in contrast to the latter, they have a stout appearance. Like simple setae, they can exhibit a pore (Garm, 2004; Watling, 1989).

In *D. magna*, the first setae become visible in stage 7 embryos (staging system according to Mittmann et al., 2014) as buds on the second antennae and posterior to the proctodeum (Mittmann et al., 2014). By stage 9 the buds have developed into simple setae (Suppl. Fig. 1A and B). During further embryonic development, all appendages show initially unbranched setae (Fig. 1A). Most of the setae develop secondary outgrowths, called setules, and appear feathered or branched in larval stages (Fig. 1G-K; Suppl. Fig. 1C-E,H). In the following, we describe cuspidate, simple, plumose and pappose setae, which develop in prominent positions in *D. magna* embryos.

Cuspidate setae

Crustaceans possess a pair of unique olfactory sense organs on the first antennae (Hallberg et al., 1992) (Fig. 1A,B; Suppl. Fig. 1A,C,D,F). They are composed of groups of cuspidate setae, called aesthetascs, which are covered with a thin, permeable cuticle. We found that in *D. magna* the first aesthetascs appear at the tip of the first antennae in stage 9 embryos (Suppl. Fig. 1A). In the first larval stage, the final number of 9 aesthetascs per antenna is visible. They are arranged in a group, giving the olfactory sense organ a tuft-like appearance (Fig. 1B; Suppl. Fig. 1D,F). Each aesthetasc has a porous plate at its tip (Fig. 1C). The first antennae of male *D. magna* are elongated (Suppl. Fig. 1D) and not fused as in females, but nevertheless the number and arrangement of aesthetascs is the same (Suppl. Fig. 1D).

Simple setae

Short hair-like (i.e. bendable) simple setae are located on the coxae of the first and second antennae. On the first antenna, one short hair-like simple seta can be detected, which is partially covered by the head shield in larval stages (Fig. 1A,B). The setae have a pore at the tip indicating a chemosensory function (Fig. 1D). We named it

‘female antennal coxal seta’. In males, there is a plumose seta at the same position, which is considerably longer than the female simple seta and exhibits a feathered tip (Suppl. Fig. 1D). We named it ‘male antennal coxal seta’. On the second antennae two short hair-like simple setae are located next to each other on the proximal-lateral side of the coxae (Fig. 1E). Furthermore, the basal segment of the second antenna shows a short bristle-like (i.e. stiff) simple seta close to the branching point of the endo- and exopodite (Fig. 1A,E; Suppl. Fig. 1E,F). These setae were named ‘antennal coxal’ and ‘antennal basal setae’, respectively.

Plumose setae

Plumose setae, with their long hair-like appearance and rows of evenly distributed setules are found on the second antennae of daphnids and on their flat leaf-like thoracic legs. The second antennae are used for swimming assisted by the plumose swimming setae that are spread out like fingers during the swimming motion (Agar, 1950). We found that *D. magna* has 9 swimming setae each on the second antennae (Fig. 1F; Suppl. Fig. 1F). The number and arrangement fits the description for other *Daphnia* species (Agar, 1950; Kotov and Boikova, 2001). The setae become first visible as tiny buds in stage 7.5 embryos (Mittmann et al., 2014). The exopodite (outer branch of the second antenna) has three segments and bears four swimming setae, one at the distal end of the second segment and three at the tip of the third (distal-most) segment (Fig. 1F; Suppl. Fig. 1F). The endopodite (inner branch) shows five swimming setae, one each on the distal part of the first and second segment and three at the tip of the third (distal-most) segment (Fig. 1F; Suppl. Fig 1F). The swimming setae can be classified with the plumose setae. They grow very long, have a hair-like appearance and exhibit rows of evenly distributed setules (Fig. 1G).

Daphnia are filter feeder, which produce a current with their flat leaf-like thoracic legs to strain small organisms out of the water. *D. magna* has five pairs of thoracic legs (Suppl. Fig. 1G). The first and second pair of legs shows a similar arrangement of several plumose setae on their endo- and exopodites. The setae have a bristle-like morphology and are covered with two opposite rows of long setules (Suppl. Fig. 1H). The water current is mainly generated by the third and fourth thoracic legs and thus they are the main filter apparatus (Watts and Petri, 1981). This function is reflected by their enlarged gnathobases, which are covered with dense rows of very long hair-like plumose setae called filtering setae (Fig. 1H,I). Each seta develops two opposite rows of short setules that terminate in small hooks so that the space between the setae can be closed like a zipper (Fig. 1I).

Pappose setae

The pair of long setae posterior to the proctodeum of most Cladocera (Flossner, 2000), called postabdominal bristles, are pappose setae (Fig. 1A,J; Suppl. Fig. B,C,I). They are thought to act as gravity sensors (Laforsch, personal communication). In *D. magna* they can first be detected in stage 7.5 embryos, where they emerge as small buds (Mittmann et al., 2014). We found that the postabdominal bristles grow out during further development (Suppl. Fig. 1B) and extend long irregularly distributed setules on their distal parts, which become more elaborate in larval stages (Fig. 1J,K; Suppl. Fig. I). The setules (Fig. 1K) are serrated which, together with the remaining morphological features, classifies them with the pappose setae. Since the expression studies below also show unique spots of neural gene expression above the proctodeum, we would like to mention the two short claw-like structures that develop in this area (Suppl. Fig. 1J). They are thought to be used for cleaning and removing accumulated food from the food grove (Watts and Petri, 1981).

All External sense organs analysed seem to contain scolopales

The main distinguishing feature of chordotonal organs is the array of actin and tubulin-rich rods that are produced by the scolopale cell and surround the dendrides of the sensory neurons. We show F-actin staining and strong α-tubulin staining in the dendrite segments of the swimming and filtering setae, the postabdominal setae and most setae of the head and trunk (Suppl. Fig. 2). The staining suggests that all external sense organs analysed contain scolpales, which is in line with previous publications (Hallberg and Hanssen, 1999).

Gene expression patterns in the developing sense organs

Daphnia magna atonal and Achaete-Scute Homologue

In *D. melanogaster*, the proneural bHLH proteins Atonal (Ato) and Achaete-Scute are essential for the formation of specific sense organs and are expressed in non-overlapping patterns (Powell et al., 2004). A single *Achaete-Scute* homologue (*ASH*) has been identified in *D. magna* previously and is expressed in neuroblasts in the central nervous system (CNS) (Ungerer et al., 2011). Here we have identified and cloned a *D. magna ato* gene. The deduced amino acid sequence of the *Dam* Ato bHLH domain is 78% identical to the same sequence in *Dm* Ato but only 36% identical to the *Dm* Achaete-Scute bHLH domains (Suppl. Fig. 3). The gene was independently identified as an *ato* homologue by Gilbert, D.G., Choi, J.-H., Mockaitis, K., Colbourne, J. and Pfrender, M. published in GenBank with the accession number KZS01707.1.

In the main text, we mainly limit the description of the gene expression to the domains that can be correlated with the sense organs we identified. A detailed presentation of the

complete gene expression patterns in all relevant developmental stages is presented in Suppl. Figs. 4 and 5, respectively. An example of a negative control is shown in Suppl. Fig. 6.

We observed that the proneural gene *Dam ato* is expressed first in the three naupliar segments of stage 5 embryos: the first antennal, the second antennal and the mandibular segment (Fig. 2A,A'). Furthermore, we detected *Dam ato* transcripts anterior to the Scheitelplatten, a half-moon-shaped area that contributes to the formation of the brain and the eye (Kuehnemund, 1929; Mittmann et al., 2014), and in the area of the future proctodeum (Fig. 2A,A'; Suppl. Fig. 4A,A',B,B'). We found additional expression domains in the limb anlagen of the thoracic segments during stages 6 and 7, when the maxillary and thoracic segments develop (Fig. 2C,D,D',E; Suppl. 4A-G'). We could not perform *in situ* hybridization assays with *D. magna* embryos older than stage 7 due to the formation of the embryonic cuticle.

Similar to *Dam ato*, *Dam ASH* expression starts in stage 5 embryos. *Dam ASH* positive domains are located in the segments of the first antennal and the second antennal anlagen as well as in the area of the future proctodeum (Fig. 2B,B'). Besides the expression in the peripheral nervous system (PNS) *Dam ASH* is expressed in the developing CNS (Fig. 2D"; Suppl. Fig. 4H'-N'), which has been described previously (Ungerer et al., 2011). Although several additional segments have formed by stage 7.2 the expression domains of *Dam ASH* in the PNS remain the same (Suppl. Fig. 4H-K, H'-K'). However, in stages 7.4 and 7.5 embryos exhibit a more pronounced and refined expression pattern of *Dam ASH* (Fig. 2E; Suppl. Fig. 4L-N,L'-N'). Additional expression domains are located in the first and second antennae and in the developing thoracic appendages, among others (Fig. 2E; Suppl. Fig. 4L-N,L'-N'). By stage 7.5 the overall *Dam ASH* expression pattern in the PNS consists of many small cell clusters,

especially in the head region (Fig. 2E; Suppl. Fig. 4N,N'). The thoracic appendages show broad domains in the proximal region as well as small cell clusters (Fig. 2E; Suppl. Fig. 4N,N').

Both *Dam ato* and *Dam ASH* show prolonged expression and most importantly, their expression overlaps in most areas of SOP formation (Fig. 3A-D). The few areas showing expression of single proneural genes include the region anterior to the proctodeum, which expresses *Dam ASH* and gives rise to the claw-like structure (Fig. 2C,D,E; Suppl. Fig. 1J), and scattered areas in the developing thoracic appendages, which either express *Dam ato* or *Dam ASH* (Fig. 2E). Furthermore, *Dam ato* is expressed in the area of the Scheitelplatten, which gives rise to the eye (Fig. 2C, magenta asterisk). This expression conforms to the highly conserved role of *ato* in eye development, which has been documented across the animal kingdom (Ben-Arie et al., 2011).

A comparison of the overlapping expression domains of *Dam ato* and *Dam ASH* with the outgrowing setae of stage 11 embryos revealed that several SOPs can be mapped to the identified sense organs (Table 1; Fig. 2F,G; Fig. 3A-D). On the first antenna, the *ato-ASH* positive expression domains prefigure the two regions from which the female/male antennal coxal setae and the aesthetascs, respectively, emerge (Fig. 2F,G; Fig. 3D,D',D"). The second antenna shows four regions of *ato-ASH* positive SOPs from which setae arise: the two short hair-like simple antennal coxal setae, which are located on the proximal-lateral side of the coxa and the short bristle-like simple antennal basal seta which is positioned close to the branching site proximal to the exopodite (Fig. 2F,G; compare to Fig. 2C,D,E; Fig. 3D,D',D"). In addition, the *ato-ASH* co-expression domains at the tip of both the endo- and exopodite of the second antennae correspond to the area where the three long distal swimming setae arise (Fig. 2F,G; compare to Fig.

2C,D,E; Fig. 3A,B,B',B"). The broad co-expression domains of *Dam ato* and *Dam ASH* in the gnathobases of the third and fourth thoracic appendage give rise to hundreds of filtering setae which are arranged in rows (Fig. 2F,G; compare to Fig. 2E; Fig. 3C,D,D',D"). Finally the postabdominal bristles can be traced back to two SOP clusters expressing *Dam ato* and *Dam ASH* posterior to the proctodeum (Fig. 2E-G).

Dam asense, prospero and snail

We examined the expression of *Dam asense (ase), prospero (pros)* and *snail* because these genes are expressed in the developing sense organs of *D. melanogaster*. In *D. melanogaster, ase* is expressed shortly after the proneural genes in neuroblasts as well as SOPs and the same sequence of expression has been shown in the CNS of *D. magna* (Ungerer et al. 2011). However, we found that *Dam ase* is not expressed in the developing sense organs of *D. magna* during the embryonic stages analysed here.

In *D. melanogaster*, both *prospero* (*pros*) and *snail* are expressed in the neural part of the sensory organ lineage and both genes are expressed in the developing CNS in *D. melanogaster* and in *D. magna* (Fichelson and Gho, 2003; Ip et al., 1994; Ungerer et al. 2011; Ungerer et al. 2012). In the PNS, we first detected *Dam pros* expression in small clusters of cells in the appendages of the developing head (Suppl. Fig. 5A,A'). During further development, we observed *Dam pros* transcripts in small groups of cells in the emerging thoracic appendages and in two clusters posterior to the proctodeum (Fig. 4A,B; Suppl. Fig. 5B,B'). Additional small cell groups express *Dam pros* in the head and thoracic appendages in subsequent stages, which seem to cover most areas of peripheral neurogenesis (Fig. 4C-E,H: Suppl. Fig. 5C,C',D,D').

The expression of *Dam snail* in the developing sense organs of the thoracic legs is first obscured due to an additional role of *Dam snail* in segmentation (Eriksson et al., 2013). From stage 5 onward, the gene is expressed in transverse stripes in the areas where the segmental borders form (Fig. 4F). In stage 7.4, the stripes start to resolve and we found that *Dam snail* expression appears in large clusters of cells in the proximal parts of the thoracic limb anlagen at stage 7.5, while smaller clusters are seen in the distal parts, which overlap or are in close proximity to the *Dam pros* positive cell groups (Fig. 4E,G,H). We found that *Dam snail* expression is similar to *Dam pros* in the head appendages and also extends into areas from which the filtering setae emerge (Fig. 4E,G). We found further that *Dam snail* and *pros* expression coincides in the areas which will form aesthetascs, the female/male first antennal coxal setae, the second antennal coxal and basal setae, the swimming and filtering setae, and the postabdominal bristles (Fig 4A,E; Suppl. Fig. 5).

To summarise, *Dam pros* and *Dam snail* are expressed in most areas of peripheral neurogenesis either in an overlapping pattern or in cells close to each other, which presumably belong to the same developing sense organs.

Does co-expression of achaete-scute and atonal lead to changes in sense organ structure in Drosophila melanogaster?

In order to analyse if misexpression of the *Drosophila* and *Daphnia ato* genes results in morphological changes in sense organs that are specified by *achaete-scute*, we generated a line of *D. melanogaster* that expresses *UAS D. magna ato* (*UASato*^{Dam}) and, in addition, we used the available *UAS D. melanogaster ato* line *UASato*^{8} for misexpression experiments. We crossed the *UAS* lines to three different *Gal4* lines

which were either expressed in the whole imaginal discs giving rise to the wings and legs of the fly (*c784-Gal4*) or in the ectodermal cells of the CNS and the imaginal discs giving rise to neural progenitor cells (*sca(109-68)-Gal4*; *sca-Gal4*). We analysed the external stretch receptor organs in the *D. melanogaster* wing, the campaniform sensilla, which show a fixed pattern along the wing veins (Fig. 5A).

The wild-type *D. melanogaster* wing has seven mechanosensory campaniform sensilla (Huang et al., 1991). They are located on the third wing vein, the anterior cross vein and close to the wing hinge (Fig. 5A). Campaniform sensilla show a dome-like structure with a surrounding collar (Fig. 5B). When either $UASato^{Dam}$ or $UASato^{8}$ are misexpressed in the wing, using the three different *Gal4* lines (*sca(109-68)-Gal4*, *sca-Gal4, c784-Gal4*), two types of phenotypic changes occur in the campaniform sensilla: more than one sensillum develops at a specific position (Fig. 5C,D) or the campaniform sensillum is transformed into a seta-type sense organ (Fig. 5E,F), sometimes incompletely (Fig. 5F). Furthermore, combinations of both types of changes could be observed where one or more campaniform sensilla and a seta-type sense organ arise at the same position.

Between 4% and 13% of campaniform sensilla showed the transformation phenotype, depending on the *Gal4* line (Table 2). When using the *c784-Gal4* line, which drives expression in the larval wing and leg disc amongst others, a phenotype was only observed with the $UASato^{Dam}$ line (Table2).

To summarise, both the *D. melanogaster* and *D. magna UAS* lines show the same phenotype and co-expression of endogenous *achaete-scute* and exogenous *ato* can result in structural changes of sense organs.

Discussion

Co-expression of ASH and atonal might have contributed evolutionary variations in sense organ structure

We have analysed here the early development of four sense organs in *D. magna* that either cannot be found in arthropods other than crustaceans such as the aesthetascs, or represent adaptations to an aquatic environment, like the swimming, filtering and postabdominal setae. Our data show that all analysed sense organ types in *D. magna* express the same early neural genes regardless of structure and function (i.e., mechano-, chemosensory). Most importantly, *ASH* and *ato* are co-expressed in all types of SOPs and there are only few locations in the developing PNS where these genes are expressed separately. This is in contrast to *D. melanogaster* where Achaete-Scute and Ato specify different sense organ types and their expression does not overlap, except for one case: *scute* and *ato* are co-expressed in the SOP (P cell) that gives rise to the metameric lateral chordotonal organ 5 in the fly embryo (Jarman et al., 1993b; Vaessin et al., 1994). In all other cases *achaete* and *scute* are co-expressed and required for the generation of external mechanosensory organs (Cubas et al., 1991; Dambly-Chaudiere and Ghysen, 1987; Ghysen and Dambly-Chaudiere, 1988; Romani et al., 1989; Ruiz-Gomez and Ghysen, 1993), while *ato* specifies internal chordotonal (scolopidial) organs, a subset of olfactory organs (sensilla coeloconica) on the antennae and maxillary palps as well as a few multidendritic neurons in the PNS (Goulding et al., 2000; Grillenzoni et al., 2007; Gupta and Rodrigues, 1997; Jarman et al., 1993b; Jarman et al., 1994; Jarman et al., 1995; Jhaveri et al., 2000).

In the remaining arthropod groups, the chelicerates and myriapods, *ato* expression has only been analysed in eye development in a spider (chelicerate)(Samadi et al., 2015) but in the millipede *Glomeris marginata* (myriapod) expression of *ato* and *ASH* was shown in areas of sense organ development in the appendages where it overlaps in a limited number of sense organs (see also below) (Dove and Stollewerk, 2003). The widespread co-expression of *Dam ASH* and *Dam ato* in diverse SOPs in the *D. magna* embryo suggests that the structure of crustacean sense organs is fundamentally different.

Structural variations have indeed been described in the external mechanosensory organs of terrestrial arthropods and aquatic crustaceans. While terrestrial arthropods possess sensory neurons with a tubular body, external mechanosensory organs of crustaceans are built according to the scolopale type (Crouau, 1995), which is confirmed by the present data in *D. magna.* These structural modifications result in dissimilarities in the mechanical conduction of the sensory stimulus in terrestrial and aquatic arthropods, respectively. The tubular body is a small electron dense structure that is situated at the distal tip of the dendrite (Crouau, 1995). The mechanosensory stimulus is received by deformation of the seta, which results in compression of the tubular body (Crouau, 1995). In contrast, in the scolopidial sense organs, the scolopale cells produce a circular array of microtubules surrounding the dendrite, and stretch, rather than compression, is the main detected force (Hallberg and Hansson, 1999; Hartenstein, 2005; Keil, 1997). It can be speculated that this mode of mechanical stimulus transduction for external mechanosensory organs is only feasible in an aquatic environment since it involves thinning of the outer cuticle, which in turn would lead to water evaporation in terrestrial habitats due to the difference in density between the internal (haemolymph) and external (air) environment. In an aquatic environment, however, the density of the outer medium (water) is similar to the inner medium (haemolymph) so that the thinning of the

cuticle does not pose a problem (Crouau, 1995). Thus, the differences in the ultrastructure of aquatic crustaceans and terrestrial arthropods might be adaptations to their respective environment. This hypothesis is supported by the presence of tubular bodies in the mechanosensory organs of a terrestrial crustacean, the isopod *Titanethes alba* (Crouau, 1995).

The presented misexpression data demonstrate that *Dam ato* acts as a proneural gene in *D. melanogaster* and that the flies exhibit the same phenotype as in *Drosophila ato* misexpression experiments. In previous misexpression studies it was already shown that *D. melanogaster ato* can transform external sensory organs specified by Achaete-Scute into chordotonal organs (Jarman and Ahmed, 1998). The transformation is not always complete, so that malformed external structures appear, consisting of small setae or sockets only associated with scolopidial structures.

Here we show for the first time that both *D. magna* and *D. melanogaster ato* can transform the external structure of the wing campaniform sensilla, which are normally specified by *achaete-scute*, into bristles. These data, together with the results published by Jarman and co-worker (Jarman and Ahmed, 1998), demonstrate that the combinatorial expression of *ato* and *ASH* can change the internal as well as external structure of sense organs in *Drosophila*.

We speculate that the evolution of arthropod sense organs has been facilitated on the one hand by the evolution of sense organ subtype specific functions of the proneural genes and on the other hand by their combinatorial expression. As in *D. melanogaster*, additional *ato* paralogues are present in the *D. magna* genome, named *amos* and *atonal-like* (published in GenBank by Gilbert, D.G., Choi, J.-H., Mockaitis, K., Colbourne, J. and Pfrender, M.). Future studies will show if these genes have evolved sense organ

subtype specific functions and thus have further supported the diversification of the sensory system in crustaceans.

Atonal expression is conserved in olfactory sense organs in insects and crustaceans

Ato seems to specify unimodal olfactory sense organs lacking scolopedial units, both in insects and crustaceans. Olfactory sense organs are located in the head appendages and are interspersed by taste sense organs integrating mechanosensory stimuli in both groups (Laissue and Vosshall, 2008; Tadesse et al., 2011). *Dam ato* is expressed early and continuously at the tip of the first antennae of *D. magna* throughout the stages analysed. This area gives rise to the specialised olfactory sense organs of crustaceans, the aesthetascs. The pattern of minor *Dam ASH-ato* co-expression in this region is in line with the presence of interspersed chemo-mechanosensory organs that might be specified through the combinatorial expression of both genes. Interestingly, one of the chemo-mechanosensory organs associated with the single compound olfactory organ (dorsal organ) of the *D. melanogaster* larva is affected in *ato-achaete/scute* double mutants. Co-expression of the proneural genes has not been demonstrated in this sense organ. Thus, the phenotype might result from indirect genetic interactions (Grillenzoni et al., 2007).

However, the fact that *Dam ASH* transcripts can be detected in smaller subsets of *Dam ato* expressing cells could also be interpreted as an expression of *Dam ASH* in immature olfactory receptor neurons. This would be in line with recent findings in the Caribbean spiny lobster *Panulirus argus* (Tadesse et al., 2011) and would therefore represent an evolutionary variation in insects and crustaceans.

Despite a potentially conserved role of *ato* in the formation of olfactory sense organs in

crustaceans and insects, there are significant differences in the structure of these organs in both groups. The olfactory sense organs specified by *ato* in *D. melanogaster* are peg-shaped, while they are hose-shaped in crustaceans. Furthermore, there are consistent differences in the internal structure of olfactory setae (Stensmyr, 2005). Both in insects and crustaceans the outer dendritic segment of the sensory neurons converges into a cilium surrounded by microtubles originating from the so-called basal body. The cilium and basal body have a subepithelial position surrounded by a lymph space in insects, while they are shifted into the external structure of the aesthetascs in aquatic crustaceans. This part of the arthropod olfactory sense organ is again subject to environmental adaptations since in the terrestrial giant robber crab the organisation of the cilium and basal body is similar to insects (Stensmyr et al., 2005). However, the internal projections of the olfactory neurons show mostly high conservation in terrestrial and aquatic crustaceans (Tuchina et al., 2015).

Proneural clusters are absent in D. magna

The temporal expression patterns of *Dam ASH* and *Dam ato* that we observed show that individual SOPs are not selected from larger domains of epithelial cells expressing these genes ('proneural clusters') as is the case in external mechanosensory organs in *D. melanogaster* (Cubas et al., 1991). Rather, small groups of *Dam ASH* and *Dam ato* expressing SOPs appear, which either cease expression, as for example in several areas of the second antennae, or increase in size during development as in the areas from which the filtering setae arise. Interestingly, the latter pattern corresponds to the mechanism of SOP selection seen in the olfactory sensilla and poly-scolopidial organs of *D. melanogaster* (Reddy et al., 1997; zur Lage and Jarman, 1999). In both cases groups of SOPs express *ato*, and additional cells are recruited during development of the sense organs. It seems that the selection of multiple, rather than single, SOPs reflects the ancestral mechanism since in members of two other arthropod groups, the

chelicerates and myriapods, this pattern is seen in the developing sense organs of the legs. In the millipede *Glomeris marginata*, for example, *Gm ato* is expressed in groups of SOPs at the tip of the appendages, an area from which chemosensory organs (cone sensilla) arise (Pioro and Stollewerk, 2006). Furthermore, two *ASHs* are expressed in groups of SOPs in the spider *Cupiennius salei*, from which neural and accessory cells develop, and are required for the formation of external mechanosensory hairs, among others (Stollewerk and Seyfarth, 2008). Thus, the selection of single SOPs, which give rise to all parts of the sense organ by clonal division, seems to be a derived feature of specific sense organ lineages in *D. melanogaster*.

Asense is not expressed in the developing sense organs of D. magna

A surprising variation of neural gene expression in *D. magna* is the complete absence of *Dam asense* transcripts in the PNS. In *D. melanogaster*, *asense* is a precursor-specific gene which is expressed downstream of the proneural *achaete-scute* and *ato* genes in the neuroblasts and sensory precursors, respectively (zur Lage and Jarman, 1999). In the PNS, Asense is required for the differentiation of the SOPs in most locations, which is shown by malformations of the external and internal parts of the sense organs in *asense* mutants (Domínguez and Campuzano, 1993). Two reasons for the lack of *asense* expression in *D. magna* are conceivable. Firstly, there might be a second *asense* gene in the *D. magna* genome. This is highly unlikely, however, since only a single copy of *asense* was identified in two other crustaceans, including *Daphnia pulex,* whose genome has been sequenced (Ayyar et al., 2010; Wheeler and Skeath, 2005). Furthermore, all insect genomes that have been sequenced bear single *asense* copies (Negre and Simpson, 2009), while the gene has not been identified in the remaining arthropod groups (Dove and Stollewerk, 2003; Stollewerk et al., 2001).

Secondly, *Dam ASH* and *Dam ato* might fulfill the role of Asense in sense organ

formation. This hypothesis is based on previous observations in arthropods that do not have *asense* genes. In the spider *Cupiennius salei*, for example, one of the two ASHs, *CsASH2*, has taken on a precursor-specific (Asense-like) function. *CsASH2* is neither expressed in proneural clusters in the CNS nor in the PNS but transcripts are exclusively upregulated in neural precursors/SOPs (Stollewerk et al., 2001; Stollewerk and Seyfarth, 2008). There is evidence that gene duplications, originating from a common *ASH*/*asense* precursor gene, occurred independently in the different arthropod groups, separating proneural from precursor-specific function (Ayyar et al., 2010). This might explain the partial overlap and/or variations in the roles of the *ASH* and *asense* genes in the different arthropod lineages.

Dam snail and Dam pros expression in all sense organs is conserved in D. magna and D. melangaster

Similar to *D. melanogaster*, *Dam snail* and *Dam pros* are expressed in all different types of sense organs. The genes are not always expressed at the exact same location; however, the close proximity of expression, together with the position of the sense organs arising in the respective domains, suggests that the genes are expressed in the same developing sense organs. Both genes seem to be expressed in a subset of SOPs in the developing sense organs as judged by the smaller expression domains relative to *Dam ASH* and *Dam ato*. Although the identity of these precursors cannot be resolved without additional markers, the conserved expression of both *snail* and *pros* in the neural precursors of all arthropod groups, including the neural precursors in the CNS of *D. magna*, strongly suggests a conserved function in the neural lineage of the *D. magna* sense organs. In turn, the presence of a neural component in all sense organs analysed is supported by previous publications (Hallberg et al., 1997; Tuchina et al., 2015; Weiss et

al., 2012) and our morphological data, which show an array of microtubules and F-actin filaments associated with the internal part of the sense organs indicating the presence of scolopidial structures and/or dendrites.

Conclusion

Our results show variations in the expression patterns of neural developmental genes that might correspond to differences in sense organ structure in insects and crustaceans. Experimental co-expression studies in *D. melanogaster* support a central role of the Achaete-Scute and Atonal family members in the evolutionary process. Future studies in additional crustacean and insect groups, including sense organ subtype specific genes, will give a detailed understanding on how the structural variations have evolved in arthropods.

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Figure legends

Fig. 1 Mechano- and chemosensory organs in late embryonic and larval stages of *D. magna*. Scanning electron micrographs of *Daphnia magna* embryonic and larval stages. (A) Stage 11 embryo showing most of the identified sense organs. (B) Aesthetascs and female antennal coxal setae on the first antennae of a larva. (C) Aesthetasc with porous plate at the tip (arrow). (D) Larval female antennal coxal seta on the first antenna with pore at the tip (arrow). (E) Antennal coxal and basal setae on the second antenna of a stage-11 embryo. (F) Distal and lateral swimming setae on the exo- and endopodite of the second antenna of a stage-11 embryo. (G) Larval swimming setae (arrows) showing the regular arrangement of setules (arrowheads). (H) Larval filtering setae on the third thoracopod. (I) Larval filtering setae (arrows) on the third thoracopod. The arrowheads point to the setules, which terminate in a hook-like structure. (J) Larval postabdominal bristles. (K) Larval postabdominal bristles (arrows) with irregular setules (arrowheads). a1 to 2, antenna 1 to 2; ab, abdomen; endo, endopodite; exo, exopodite; lb, labrum. Scale bars: 100 μm in A, F, H, J; 50 μm in B, E; 10 μm in G, I, K; 5 μm in C; 1 μm in D.

Fig. 2 Overlapping expression domains of *Dam ASH* and *Dam ato* correlate with the position of identified mechano- and chemosensory organs. Whole mount *in situ* hybridisations of DIG labelled RNA probes (dark blue) for *Dam ASH* and *Dam ato,* respectively, and of nuclei staining (light blue)(A',B'D',D"), schematic illustrations (A,C,D,E,F) and scanning electron micrograph (G). (A,A') Stage 5 embryo; *Dam ato* expression is visible in the anlagen of the head appendages, the first antenna (asterisks), the second antenna (arrowheads), and the mandible (open arrowheads) as well as

anterior to the Scheitelplatten (arrows) and the region of the future proctodeum (double arrowhead). (B,B') Stage 5 embryo; *Dam ASH* expression is visible in the same regions in the developing first (asterisk) and second antennae (arrowheads) and the proctodeum (double arrowhead). However, expression is absent in the Scheitelplatten area and the emerging mandibular appendages. (C,D) Schematic illustrations of *Dam ASH* and *Dam ato* expression in stage 6.2 and 7.2, respectively. Magenta asterisk in C indicates *Dam ato* expression in the Scheitelplatten; the blue asterisk in C, D and E indicates *Dam ASH* expression in the bilateral clusters that presumably give rise to the abdominal claws. (D') At stage 7.2, *Dam ato* expression shows additional domains predominantly in the thoracopod anlagen (arrow). (D") An additional *Dam ASH* domain appears proximally in the second antennae in stage stage 7.2 embryos. The arrow points to *Dam ASH* expression in the CNS. (E) Schematic illustration showing the elaborate pattern of *Dam ASH* and *Dam ato* expression in the PNS. The arrows point to the broad areas of co-expression in the third and fourth thoracopod where the filtering setae form. (F) Schematic drawing of a stage 11 embryo showing the identified sensory organs which presumably arise from the overlapping expression domains of *Dam ASH* and *Dam ato* in orange. The shorter setae are encircled (orange) for clarity. (G) Artificial staining of sense organs (orange) presumably arising from *Dam ASH* and *Dam ato* domains in a stage 11 embryo. a1 to 2, antenna 1 to 2; md, mandible; mx1 to 2, maxilla 1 to 2; pro, proctodeum; sp, Scheitelplatten; t1 to t3, thoracic segment 1 to 3; tp1 to tp4, thoracopod 1 to 4. Scale bars: 100 μm.

Fig. 3 Expression patterns of *Dam ASH* and *Dam ato.* Fluorescence double *in situ* hybridisation of *Dam ato* (red) and *Dam ASH* (green), co-expression (yellow) (B,B',B",D,D',D") (20 embryos analysed). Schematic illustration of *Dam ASH* and

Dam ato co-expression; *ato* (magenta), *ASH* (cyan), co-expression (purple) (A,C). (A,B,B',B") Stage 6.2 embryo; the endopodite of the second antenna exhibits one co-expression domain. The arrows in B and B" point to the CNS in the developing head ('head V'). The arrowheads in B', B" indicate the expression anterior to the Scheitelplatten. (C,D,D',D") Stage 7.5 embryo, flat preparation showing one half of the germband. The domains, from which the aesthetascs, the second antennal coxal setae and the filtering setae develop, show co-expression of *Dam ASH* and *Dam ato*. White asterisks in D,D',D" indicate unspecific staining in the developing gut. a1 to 2, antenna 1 to 2; endo, endopodite; exo, exopodite; md, mandible; mx1 to 2, maxilla 1 to 2; tp1 to tp3, thoracopod 1 to 4. Scale bars: 100 μm.

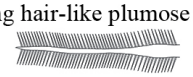
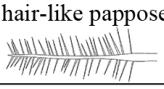
Fig. 4 Expression patterns of *Dam pros* and *Dam snail*. Whole mount *in situ* hybridisations of DIG labelled RNA probes (dark blue) for *Dam pros* (67 embryos analysed) and *Dam snail* (50 embryos analysed), respectively, and of nuclei staining (light blue)(B-D, F-H), schematic illustrations (A, E). The arrowheads point to *Dam pros* and *Dam snail* staining in the CNS. (A) Overlapping expression pattern of *Dam snail* and *Dam pros* at stage 7.3. (B) At stage 7.3 *Dam pros* PNS staining is mainly restricted to the developing head appendages. The arrow indicates a small lateral expression domain in the lateral thoracic segments. *Dam pros* is expressed in the bilateral domains posterior to the proctodeum. (C) Stage 7.4 embryo, anterior view; *Dam pros* expression is visible in the first and second antennae in the areas from which the aesthetascs, first antennal coxal setae and the distal swimming setae arise. (D) Stage 7.4 embryo; posterior view showing the *Dam pros* expression domains in the area where the postabdominal bristles develop. (E) Many additional expression domains appear in the head appendages and in the thoracopod anlagen at stage 7.5. (F)

Expression pattern of *Dam snail* at stage 7.3. Similar to *Dam pros*, *Dam snail* is mainly expressed in the head appendages at this stage. Expression can be seen in the domains of the first and second antennae that give rise to the aesthetascs as well as the coxal and basal setae, and the distal swimming setae. Expression is also visible posterior to the proctodeum. The arrows point to *Dam snail* expression in the intersegmental furrows (Eriksson et al., 2013). (G) At late stage 7.5 *Dam snail* is expressed in the areas from which the filtering setae develop. (H) At late stage 7.5 *Dam pros* expression can be detected in many additional expression domains, in particular in the developing thoracopods. a1 to 2, antenna 1 to 2; endo, endopodite; md, mandible; mx1 to 2, maxilla 1 to 2; t1 to 3. thoracic segment 1 to 3; tp1 to tp3, thoracopod 1 to 4. Scale bars: 100 μm

Fig. 5 Misexpression of *atonal* results in transformation and duplication of campaniform sensilla. Scanning electron micrographs of *D. melanogaster* wings. (A) Pattern of campaniform sensilla in the wing. The circles indicate the position of the distribution of campaniform sensilla: twin sensilla (red circle), anterior cross-vein sensillum (yellow circle), third wing vein sensilla (white circles). (B) External structure of a campaniform sensilla. The sensillum has a dome-like structure. The center is surrounded by a rim of small white bristles (arrow). (C,D) Duplication of the anterior cross-vein sensillum (encircled in yellow). (E,F) Misexpression of the *D. magna* and *D. melanogaster UAS-ato* construct results in transformation of the campaniform twin sensilla (encircled in red). Scale bars: 500 μm in A; 10 μm in B – F.

Tables

Table 1 Identified mechano- and chemosensory sense organs in *Daphnia magna*

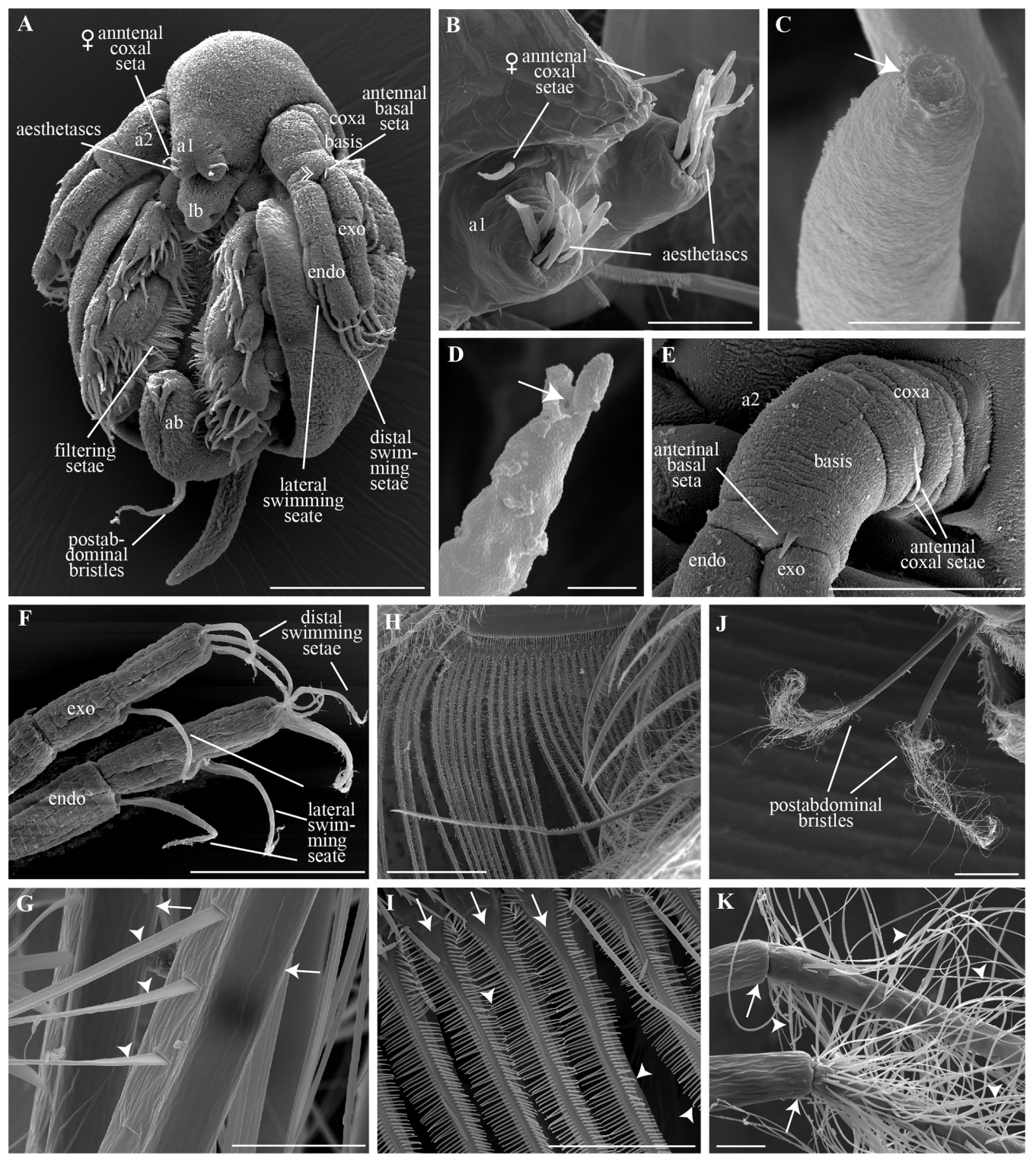
Name	No.	Sense organ type	Location	Function	Genes expressed
Aesthetascs	18	Compound cuspidate 	1st antenna	Olfactory	*ASH, atonal, prospero, snail*
Female 1st antennal coxal setae	2	short hair-like simple 	1st antenna, coxa	Chemosensory	*ASH, atonal, prospero, snail*
Male 1st antennal coxal setae	2	long hair-like plumose 	1st antenna, coxa	Chemosensory?	*n/a*
2nd Antennal coxal setae	4	short hair-like simple 	2nd antenna, coxa	Chemosensory?	*ASH, atonal, prospero, snail*
2nd Antennal basal setae	2	short bristle-like simple	2nd antenna, basis	Mechanosensory?	*ASH, atonal, prospero, snail*
Distal swimming setae	12	long hair-like plumose 	2nd antenna, exo- and endopodite	Mechanosensory	*ASH, atonal, prospero, snail*
Filtering setae	~200	long hair-like plumose 	Gnathobases of 3rd & 4th thoracic limb	Mechanosensory	*ASH, atonal, prospero, snail*
Postabdominal bristles	2	long hair-like pappose 	Posterior to proctodeum	Gravity receptor	*ASH, atonal, prospero, snail*

The table summarises the number, structure, location, possible function and gene expression of the sense organs identified. The numbers correspond to the overall number of the sense organs in both appendages of a pair. For example, there are 9 aesthetascs on each of the 1st antennae, thus the overall number is 18. 17 embryos and 24 larvae were analysed for determining the number and position of the listed sense organs. We did not detect any deviations in the positions and numbers of sense organs in embryos and larvae of corresponding stages.

Table 2 Atonal misexpression results in transformation and duplication of campaniform sensilla on the *D. melanogaster* wing

Genotype	$n_{campaniform\ sensilla}$ (n_{flies})	Duplication	Transformation
φ51C	210 (15)	0	0
UASato^{Dam}	392 (28)	0	0
UASato^{8}	336 (24)	0	0
sca$^{109\text{-}68}$*Gal4*/*UASato*^{Dam}	371 (27)	7 ± 0.34	25 ± 0.635
sca$^{109\text{-}68}$*Gal4*/*UASato*^{8}	434 (31)	6 ± 0.32	25 ± 0.645
sca-Gal4/*UASato*^{Dam}	329 (24)	14 ± 0.5	42 ± 1.45
sca-Gal4/*UASato*^{8}	*not viable*		
c-784-Gal4/*UASato*^{Dam}	203 (15)	12 ± 0.5	10 ± 0.73
c-784-Gal4/*UASato*^{8}	336 (24)	0	0

The *Daphnia magna UASato*^{Dam} and *Drosophila melanogaster UASato*^{8} lines show similar phenotypes in crosses with the four Gal4 driver lines, except for *c-784-Gal4*/*UASato*^{8}, which does not show an altered phenotype in the wing campaniform sensilla. The numbers in the 'Duplication' and 'Transformation' column refer to the total number of campaniform sensilla affected. The numbers vary depending on the *Gal4* lines used.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♀ anntenal coxal seta
aesthetascs
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antennal basal seta
coxa
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lb
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filtering setae
distal swim-ming setae
lateral swimming seate
postab-dominal bristles
B
♀ anntenal coxal setae
a1
aesthetascs
C
D
E
a2
coxa
antennal basal seta
basis
endo
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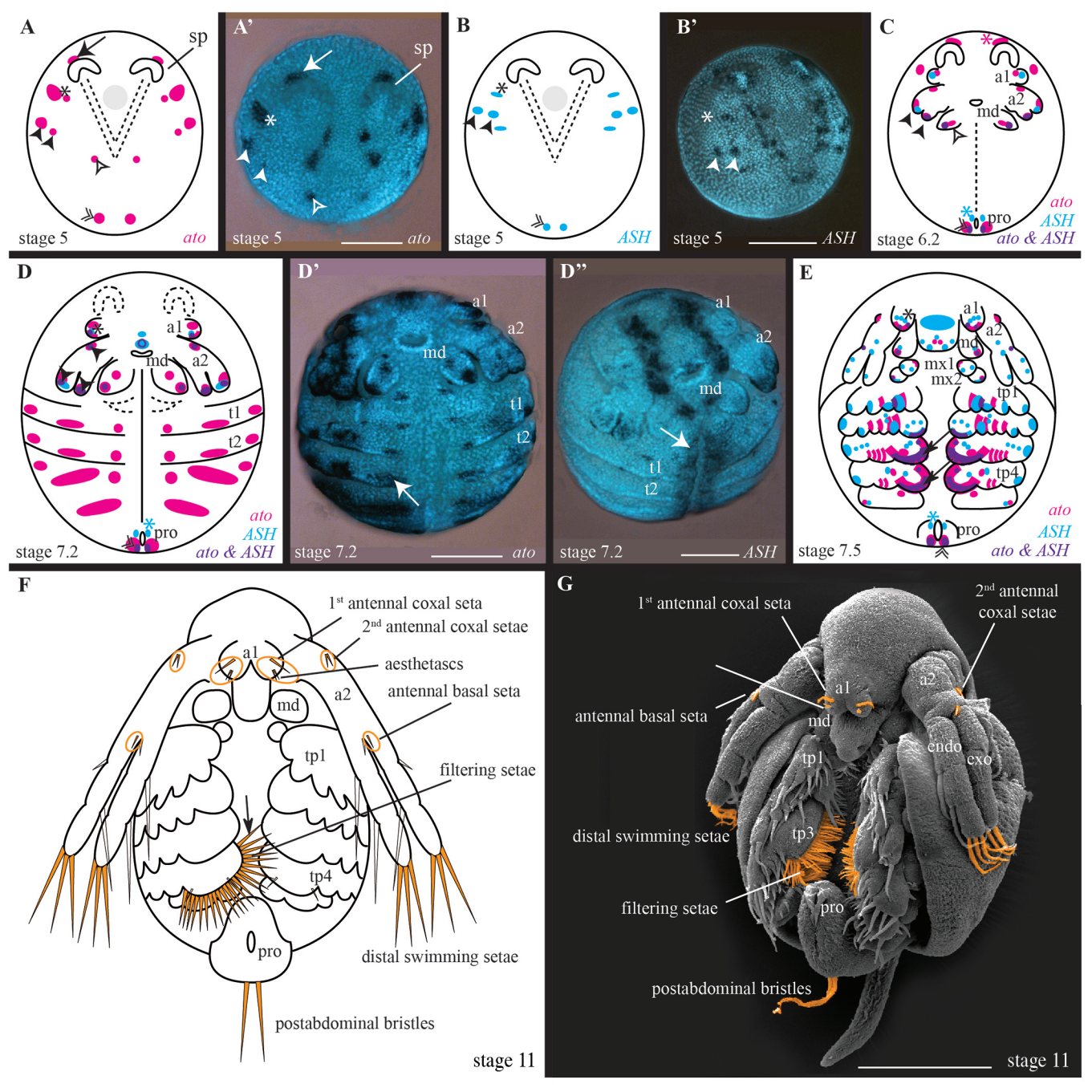
I
K


A
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stage 5
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A'
sp
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ato
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stage 5
ASH
B'
stage 5
ASH
C
a1
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md
pro
ato
ASH
ato & ASH
stage 6.2
D
a1
md
a2
t1
t2
pro
ato
ASH
ato & ASH
stage 7.2
D'
a1
a2
md
t1
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stage 7.2
ato
D''
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a2
md
t1
t2
stage 7.2
ASH
E
a1
a2
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mx1
mx2
tp1
tp4
pro
ato
ASH
ato & ASH
stage 7.5
F
1st antennal coxal seta
2nd antennal coxal setae
aesthetascs
antennal basal seta
filtering setae
distal swimming setae
postabdominal bristles
a1
a2
md
tp1
tp4
pro
stage 11
G
1st antennal coxal seta
2nd antennal coxal setae
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md
tp1
tp3
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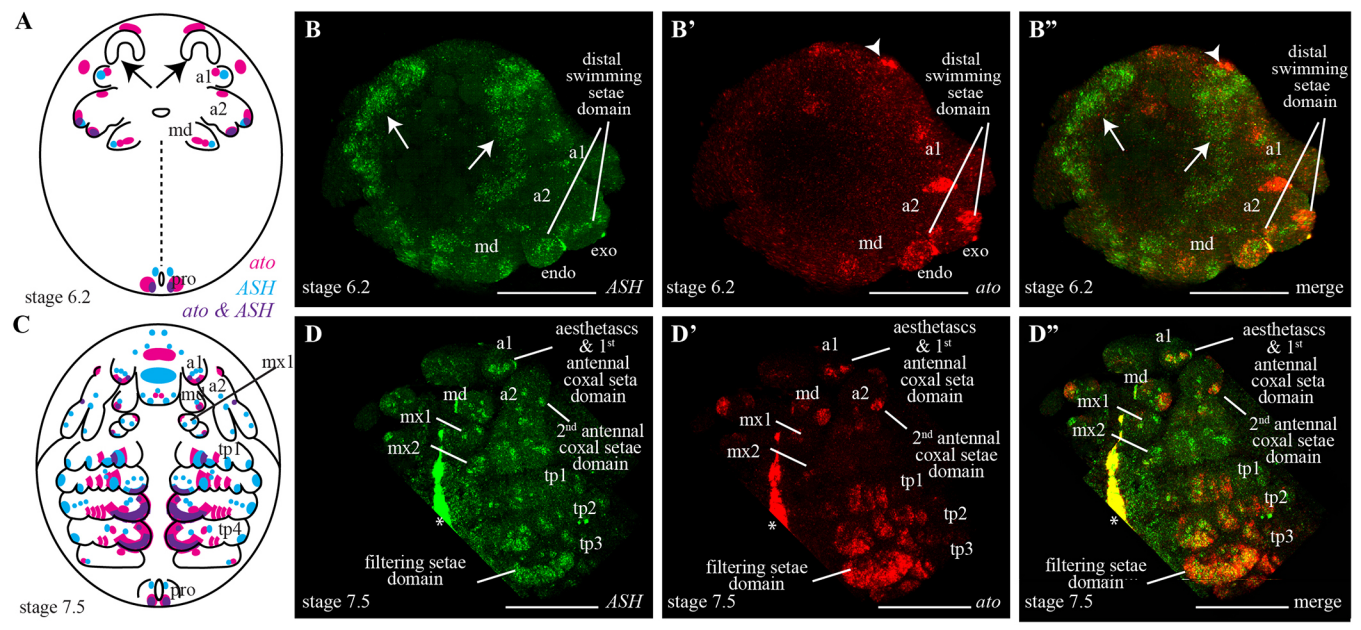
endo
exo
stage 11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a2
md
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ASH
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stage 6.2
B
distal swimming setae domain
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endo
stage 6.2
ASH
B'
distal swimming setae domain
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a2
md
exo
endo
stage 6.2
ato
B"
distal swimming setae domain
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stage 6.2
merge
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mx1
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md
tp1
tp4
pro
stage 7.5
D
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a2
mx1
mx2
2nd antennal coxal setae domain
tp1
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tp3
*
filtering setae domain
stage 7.5
ASH
D'
a1
aesthetascs & 1st antennal coxal seta domain
md
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mx1
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2nd antennal coxal setae domain
tp1
tp2
tp3
*
filtering setae domain
stage 7.5
ato
D"
a1
aesthetascs & 1st antennal coxal seta domain
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tp2
tp3
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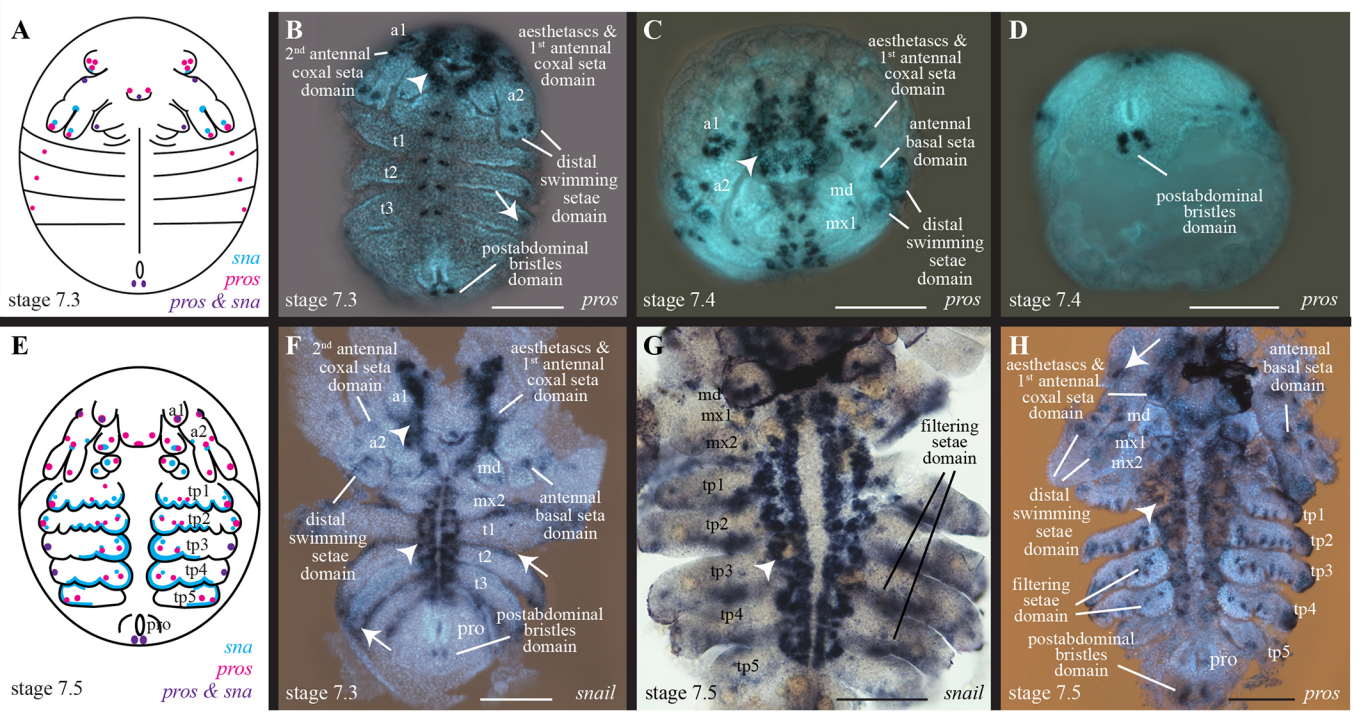
filtering setae domain
stage 7.5
merge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pros
pros & sna
stage 7.3
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a2
t1
t2
t3
distal swimming setae domain
postabdominal bristles domain
stage 7.3
pros
C
aesthetascs & 1st antennal coxal seta domain
a1
antennal basal seta domain
a2
md
mx1
distal swimming setae domain
stage 7.4
pros
D
postabdominal bristles domain
stage 7.4
pros
E
a1
a2
tp1
tp2
tp3
tp4
tp5
pro
sna
pros
pros & sna
stage 7.5
F
2nd antennal coxal seta domain
aesthetascs & 1st antennal coxal seta domain
a1
a2
md
mx2
t1
t2
t3
distal swimming setae domain
antennal basal seta domain
pro
postabdominal bristles domain
stage 7.3
snail
G
md
mx1
mx2
filtering setae domain
tp1
tp2
tp3
tp4
tp5
stage 7.5
snail
H
aesthetascs & 1st antennal coxal seta domain
antennal basal seta domain
md
mx1
mx2
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tp3
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pro
tp5
stage 7.5
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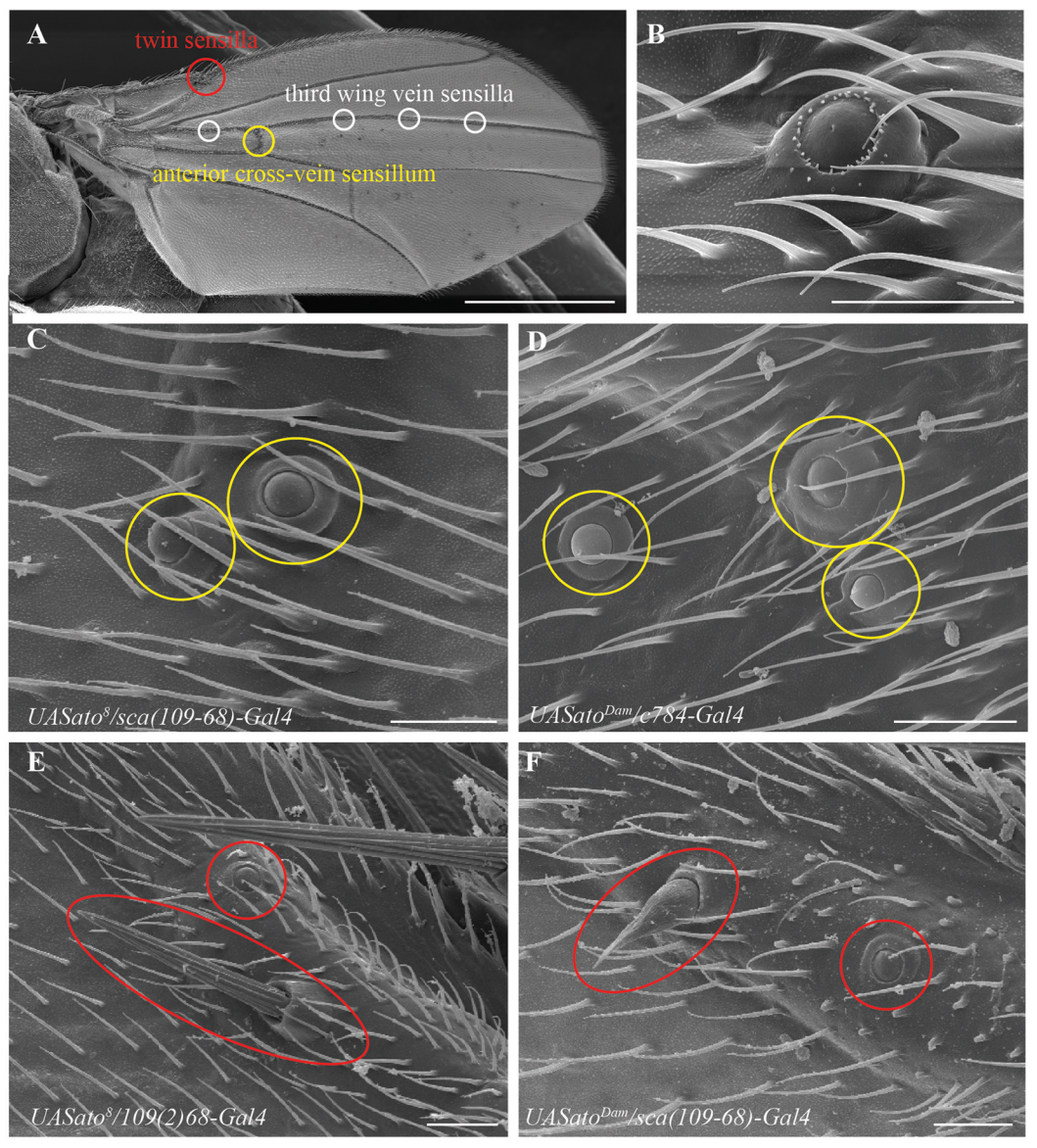
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third wing vein sensilla
anterior cross-vein sensillum
B
C
UASato8/sca(109-68)-Gal4
D
UASatoDam/e784-Gal4
E
UASato8/109(2)68-Gal4
F
UASatoDam/sca(109-68)-Gal4

Supplementary Figure legends

Suppl. Fig. 1 Sensory organs in embryonic and larval stages of *D. magna*. Scanning electron micrographs of *Daphnia magna* embryonic and larval stages. (A) The first external structures of the sense organs become visible in stage 9 embryos. (B) Post-abdominal setae in a stage 9 embryo. (C) Many setae develop secondary outgrowths (setulae, arrows) in larval stages. (D) In males the first antennae are elongated (arrow) and not fused. The male antennal coxal seta belongs to the long hair-like plumose sense organ type and has a feathered tip (arrowhead). The asthetascs show the same arrangement as in females. (E) Larval antennal basal seta on the second antenna. (F) In the stage 11 embryo all 9 swimming setae are visible. The arrowheads indicate the lateral and distal swimming setae on the exopodite, respectively. (G) Stage 11 embryo showing the five thoracic appendages (thoracopods) and the filtering setae. (H) Larval stage, first thoracopod. The arrows point to the setae and the arrowhead indicates the setules. (I) Postabdominal bristles in an early larval stage. (J) Larval stage, abdominal claws. a1 to 2, antenna 1 to 2; ab, abdomen; endo, endopodite; exo, exopodite; lb, labrum; md, mandible; tp1 to tp5, thoracopod 1 to 5. Scale bars: 100 μm in A, B, D, F, G, I, J; 500 μm in C; 50 μm in E, H.

Suppl. Fig. 2 Actin and α-tubulin staining show dendritic elements of sense organs in *D. magna*. Confocal micrographs of embryos and larva; red, acetylated α-tubulin in A,A',B (25 analysed); F-actin (Phalloidin) in C-E; green (23 embryos analysed), Sytox (A') or SYBR green (C,E) (nuclei staining). (A,A',B) Stage 8 embryos showing strong staining around the dendritic segments of the sensory neurons in swimming, filtering and postabdominal setae. Please note that at this stage

the external structure of the respective sense organs is only visible as small bud. The arrow in B points to the filtering setae. (C) Dendrites of the larval aesthetascs. The arrow points to the strong F-actin staining in the receptor lymph cavity. (D) Dendrites of the filtering setae in the third thoracopod of a stage 8 embyo. (E) Dendrites of the larval distal swimming setae in the second antenna. a1 to a2, antenna 1 to 2; lb, labrum; md, mandible; mx1 to 2, maxilla 1 to 2; tp1 to tp5, thoracopod 1 to 5. Scale bars: 50 μm in B-E; 100 μm in A, A'.

Suppl. Fig. 3 Alignment of the deduced amino acid sequences of the bHLH domains of the *Daphnia magna* and *Drosophila melanogaster* Achaete-Scute and Atonal genes. Amino acids in bold indicate identity in all bHLH domains. Asterisks indicate identity in the respective alignments separated by blank lines. The bHLH domain of *Dam* Ato shows 78% identity to the *Dm* Ato domain but only 36% identity to the *D. magna* and *D. melanogaster achaete-scute* bHLH domains. The *Dam ASH* and *Dam ase* bHLH domains are 67 % identical to the respective domains in the *D. melanogaster achaete-scute* and *ase* genes.

Suppl. Fig. 4 Expression patterns of *Dam atonal* and *Dam ASH* in the developing sense organs of *Daphnia magna*. Whole mount *in situ* hybridisations of DIG labelled RNA probes (dark blue) for *Dam ASH* (H – N') (100 embryos analysed) and *Dam ato* (A – G') (125 embryos analysed), respectively, and of nuclei staining (light blue)(A' – N') and schematic illustrations (A – N). (A,A') Stage 5 embryo; *Dam ato* expression is found in the anlagen of the head appendages, the first antenna (asterisks), the second antenna (arrowheads), and the mandible (open arrowheads) as well as anterior to the Scheitelplatten (arrows) and the region of the future

proctodeum (double arrowhead). (B,B') Stage 6.2 embryo; the head appendages with their respective expression domains of *Dam ato* are formed, the first antenna (asterisk), the second antenna (arrowheads), and the mandible (open arrowhead). (C,C') Stage 7.1 embryo; during further development additional expression domains arise in the future thoracic segment anlagen (arrows) and anterior to the stomodeum (double arrow). (D,D') Stage 7.2 embryo; additional expression domains are visible in the thoracic segment anlagen (arrows) as well as in the first and second maxilla (open arrows). (E,E') Stage 7.3 embryo; a new *Dam ato* positive cell cluster emerges in the second antenna, proximally of the branching point of exo- and endopodite (arrowheads). (F,F') Stage 7.4 embryo; the *Dam ato* expression pattern becomes refined in all appendages and shows a notably broad domain in the mid-proximal regions of the third and fourth thoracopods (arrows). (G,G') Stage 7.5 embryo; the expression of *Dam ato* begins to decrease in the head region.

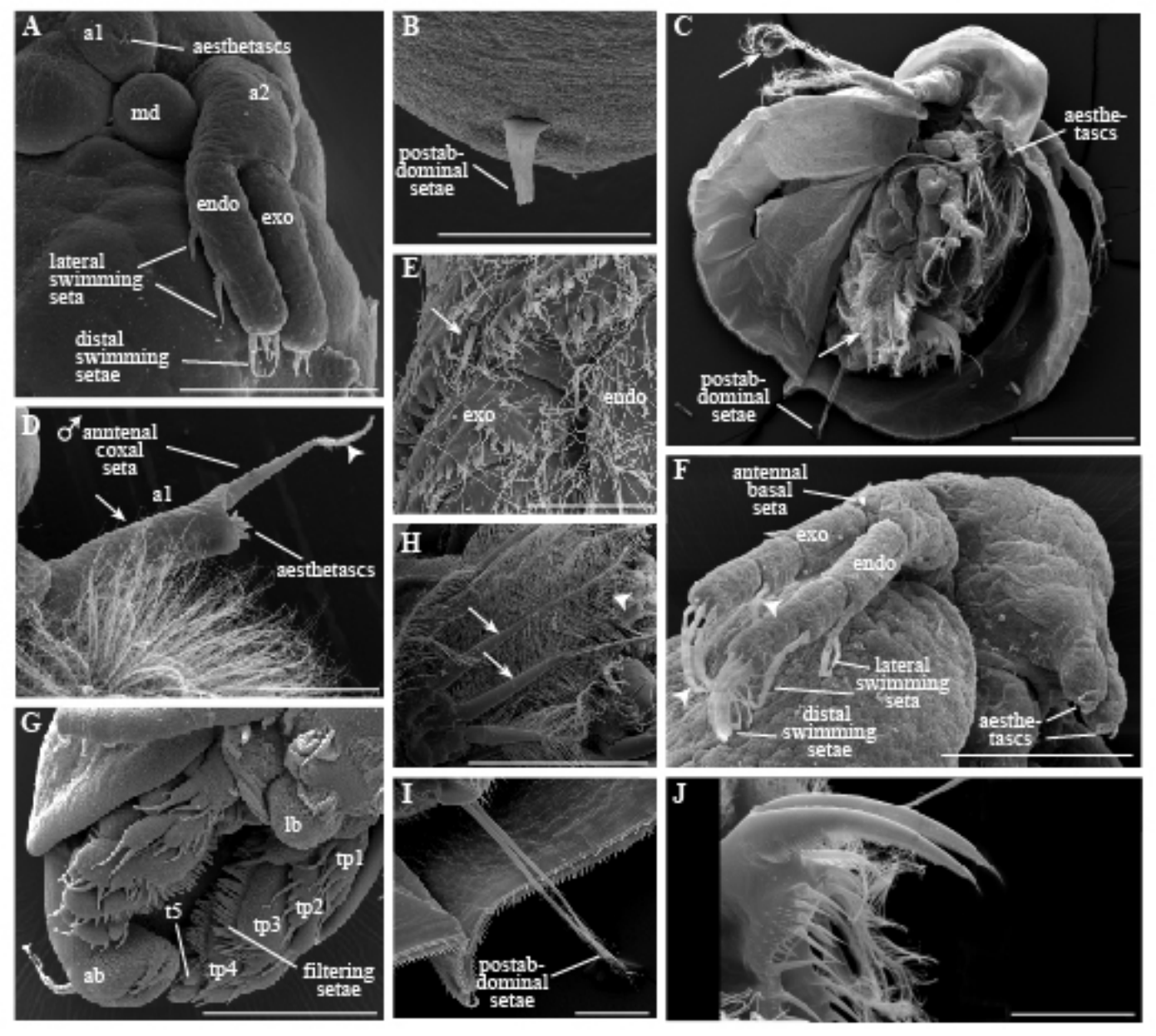
(H,H') Stage 5 embryo; *Dam ASH* expression is found in the anlagen of the first antenna (asterisks), the second antenna (arrowheads), and in the region of the future proctodeum (double arrowhead). (I,I') Stage 6.2 embryo; the head appendages with their respective expression domains of *Dam ASH* are formed: the first antenna (asterisks), the second antenna (arrowheads), and the mandible (open arrowheads). A second bilateral cluster appears in the region of the proctodeum, anterior to the first one (arrow). (J,J') Stage 7.1 embryo; during further development additional expression domains arise anterior to the stomodeum (double arrows). (K,K') Stage 7.2 embryo; an additional *Dam ASH* domain is located proximally in the second antenna (arrowheads). (L,L') Stage 7.3 embryo; new *Dam ASH* expression domains are situated in the first antenna (asterisks), the second antenna (arrowheads), the mandible (open arrowheads), both maxillae (open arrows), as well as in the emerging

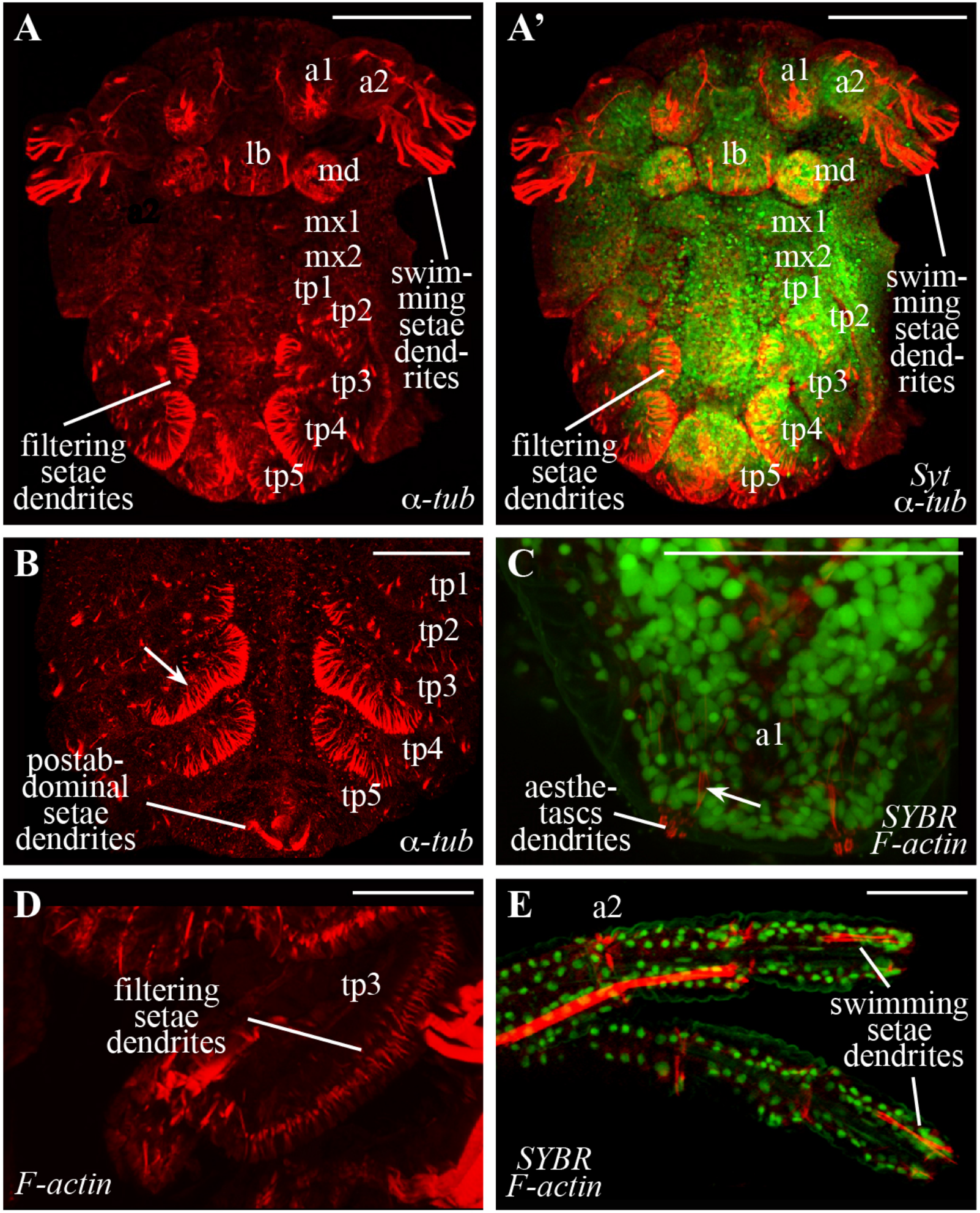
thoracic appendage anlagen (arrows). (M,M') Stage 7.4 embryo; further *Dam ASH* positive cell clusters arise in the thoracic appendages (arrows), while some domains in the head appendages start to cease. (N,N') Stage 7.5 embryo; the *Dam ASH* expression pattern becomes refined in all appendages and reveals a notably broad domain in the mid-proximal regions of the third and fourth thoracopods (arrows).
a1 to 2, antenna 1 to 2; md, mandible; mx1, maxilla 1; pro, proctodeum; sp, Scheitelplatten; t1 to t3, thoracic segment 1 to 3; tp1 to tp4, thoracopod 1 to 4. Scale bars: 100 μm.

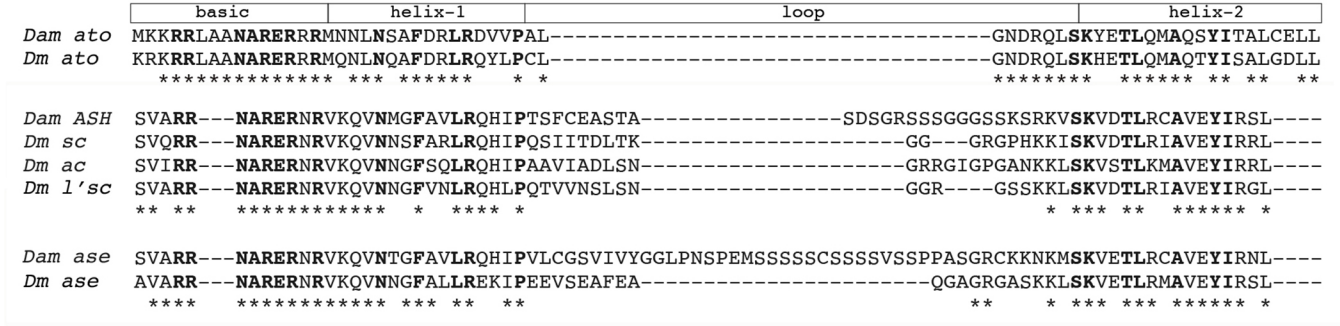
Suppl. Fig. 5 Expression pattern of *Dam pros* and *Dam snail* in the developing sense organs of *Daphia magna*. Whole mount *in situ* hybridisations of DIG labelled RNA probes (dark blue) for *Dam pros* (A'-D') and *Dam snail* (E'-H'), respectively, and of nuclei staining (light blue) and schematic illustrations (A-H).
(A,A') Stage 6.2 embryo; *Dam pros* is expressed anterior to Scheitelplatten (arrow), in the first antenna (asterisks), the second antenna (arrowheads) and in the region of the proctodeum (double arrowheads). (B,B') Stage 7.3 embryo; additional *Dam pros* expression can be found in the anlagen of the labrum (double arrows), in the first antenna (asterisks), in the mandible (open arrowheads), and distally in the first three thoracic segment anlagen (arrows). (C,C') Stage 7.4 embryo; all head appendages show an extensive *Dam pros* expression pattern, especially the second antenna. The thoracic appendages show distal and proximal expression domains (arrows). (D,D') Stage 7.5 embryo; additional *Dam pros* positive cell clusters arise in the thoracopods, while some domains in the head appendages start to cease. (E,E') Stage 6.2 embryo; *Dam snail* expression in the PNS can be found in the second antenna (arrowheads), the mandible (open arrowheads), and near the stomodeum (double arrows). (F,F')

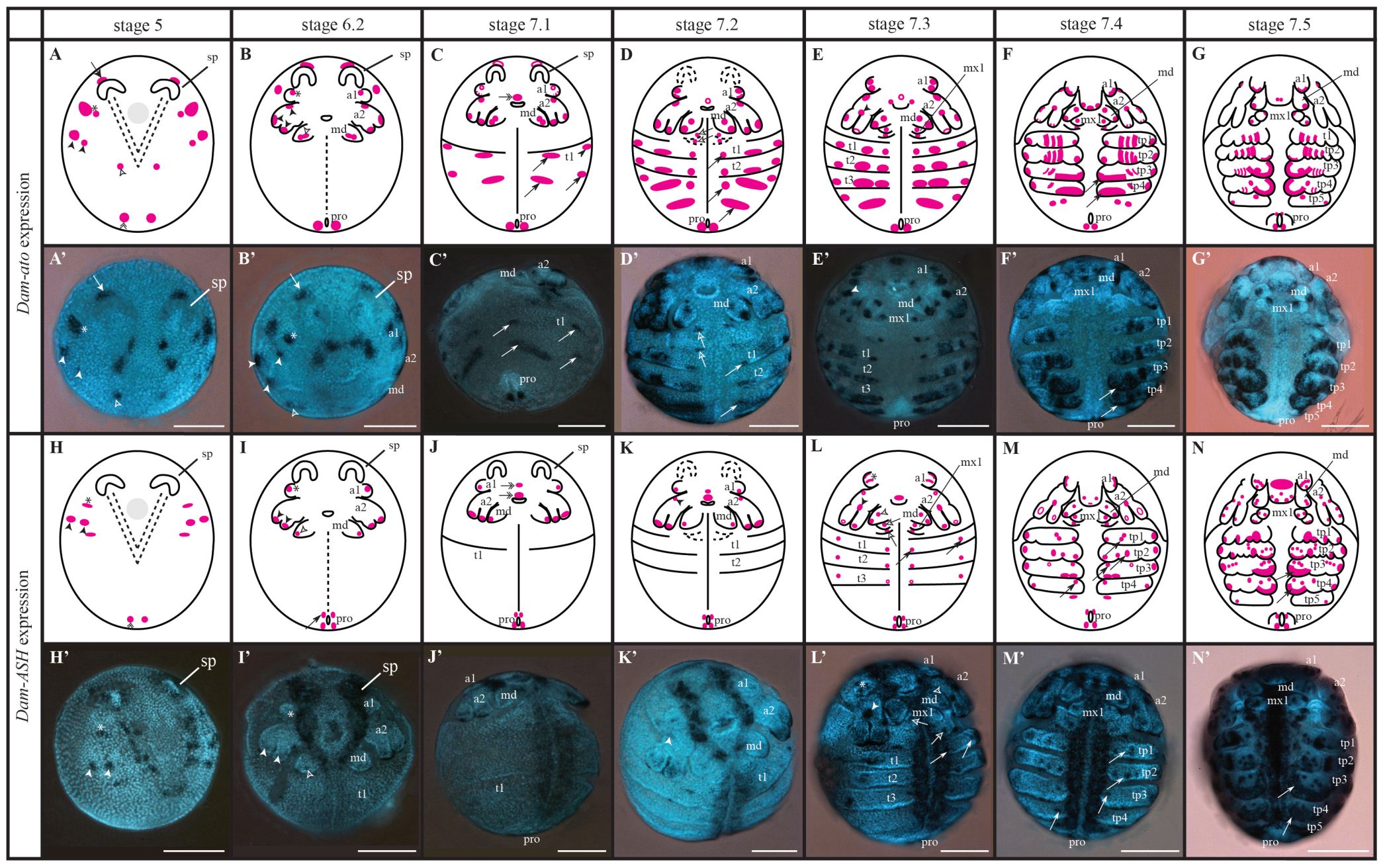
Stage 7.3 embryo; additional *Dam snail* expression emerge in the second antenna (arrowheads) and posterior to the proctodeum (double arrowheads). (G,G') Stage 7.4 embryo; additional *Dam snail* positive cell clusters arise in the first antenna (asterisks), the mandible (open arrowheads), and the thoracopods 1 to 4 (arrows). (H,H') Stage 7.5 embryo; thoracopods 1 to 4 exhibit broad *Dam snail* expression domains in their proximal margins (arrows). This is most prominent in the third and fourth thoracopod from which the filtering setae arise. Furthermore, *Dam snail* is visible in both maxillae (open arrows). a1 to 2, antenna 1 to 2; md, mandible; mx1 to 2, maxilla 1 to 2; pro, proctodeum; t1 to 3, thoracic segments 1 to 3; tp1 to 5, thoracopod 1 to 5. Scale bars: 100 μm.

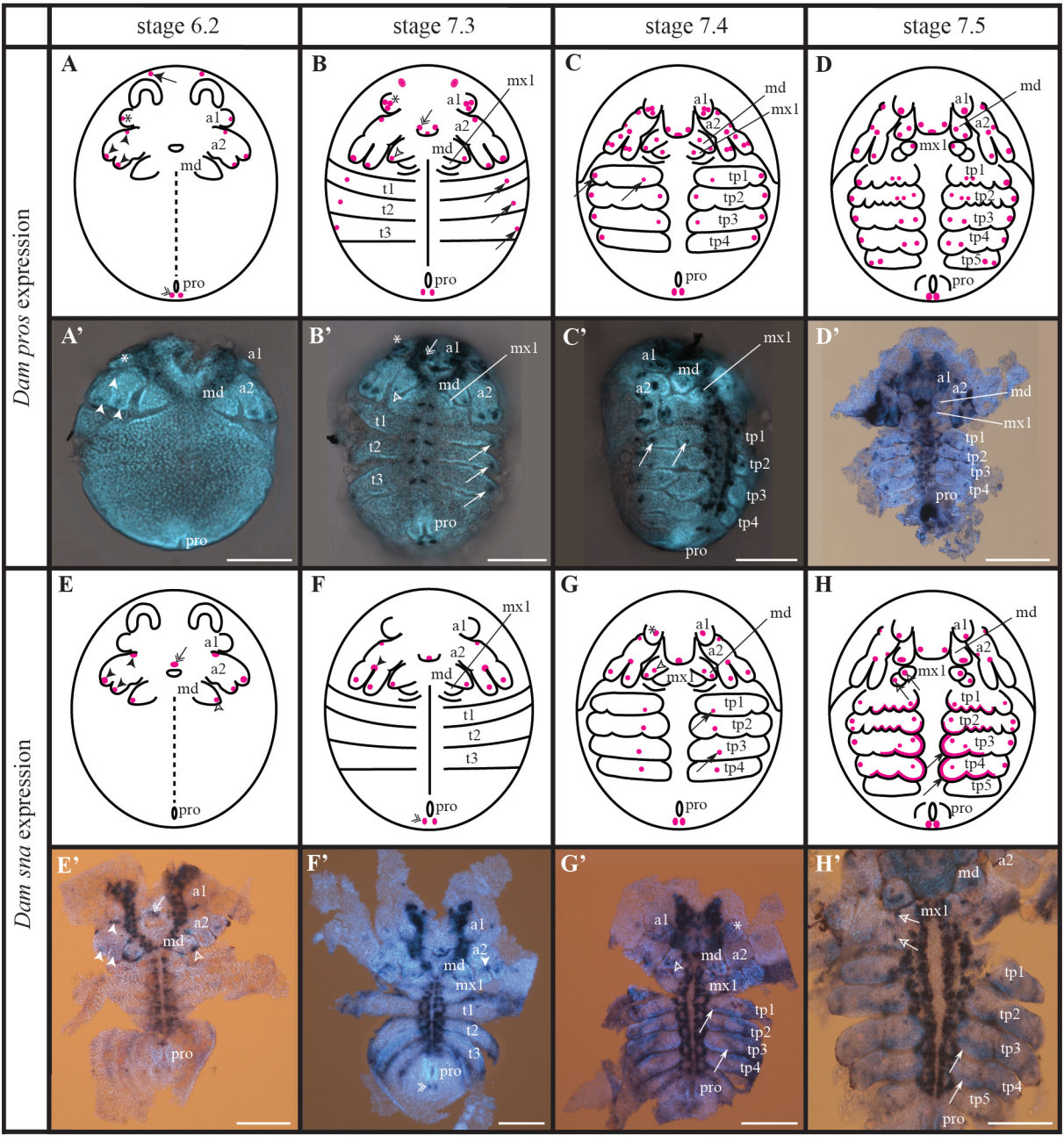
Suppl. Fig. 6 Example of a negative control for the in situ hybridisations. The DIG labelled RNA sense probes of all genes analysed show a similar unspecific staining in later stages (stages 7.4 and 7.5), which is mainly due to staining solution trapped underneath the outgrowing head appendages (arrows). Scale bar: 100 μm.

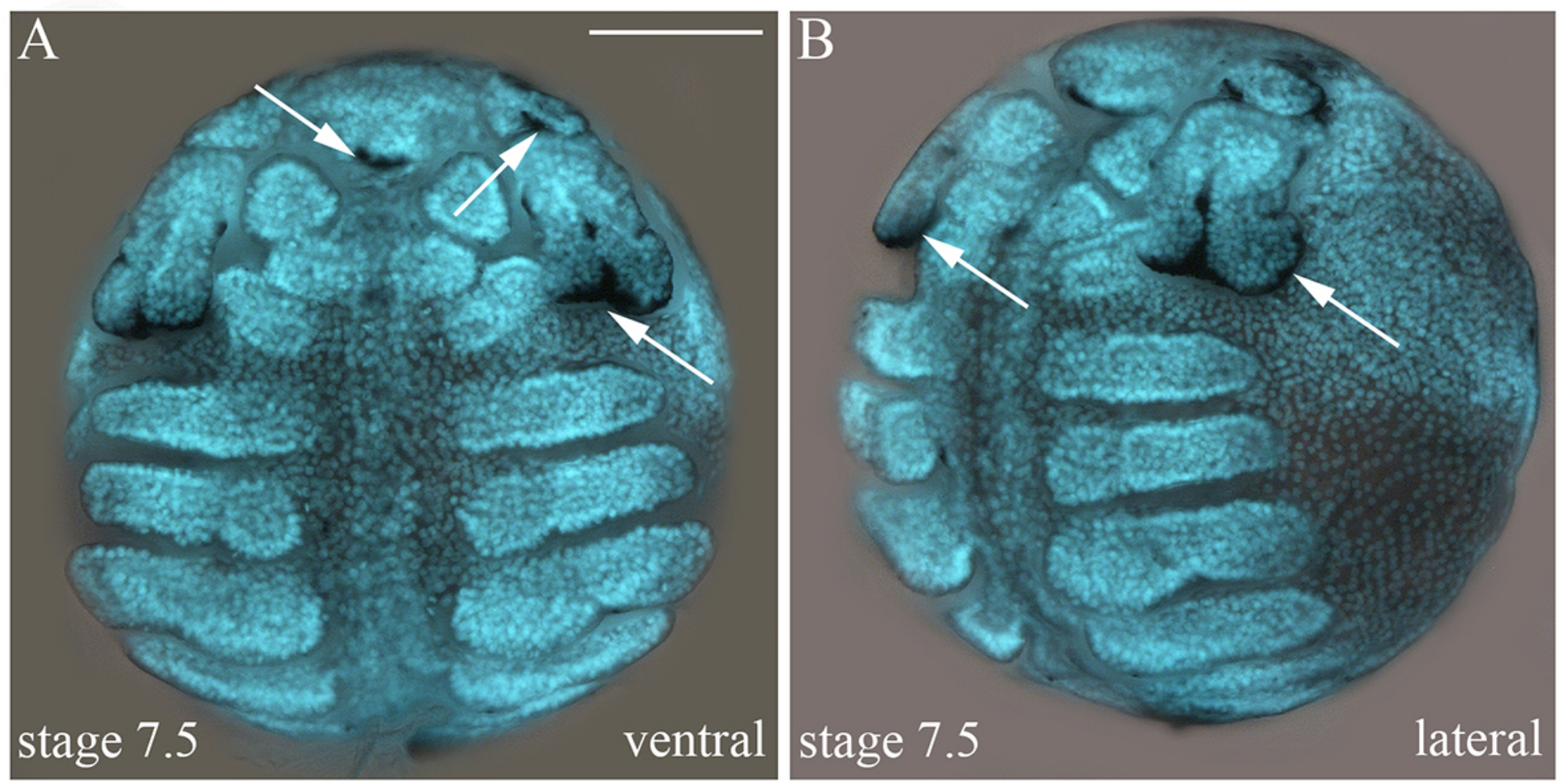

A
a1
aesthetascs
a2
md
endo
exo
lateral swimming seta
distal swimming setae
B
postab-dominal setae
C
aesthe-tascs
postab-dominal setae
D
♂ anntenal coxal seta
a1
aesthetascs
E
exo
endo
F
antennal basal seta
exo
endo
lateral swimming seta
distal swimming setae
aesthe-tascs
G
lb
tp1
tp2
tp3
t5
tp4
ab
filtering setae
H
I
postab-dominal setae
J


A
a1
a2
lb
md
a2
mx1
mx2
tp1
tp2
swim-
ming
setae
dend-
rites
tp3
tp4
filtering
setae
dendrites
tp5
α-tub
A'
a1
a2
lb
md
mx1
mx2
tp1
tp2
swim-
ming
setae
dend-
rites
tp3
tp4
filtering
setae
dendrites
tp5
Syt
α-tub
B
tp1
tp2
tp3
tp4
postab-
dominal
setae
dendrites
tp5
α-tub
C
a1
aesthe-
tascs
dendrites
SYBR
F-actin
D
filtering
setae
dendrites
tp3
F-actin
E
a2
swimming
setae
dendrites
SYBR
F-actin


basic
helix-1
loop
helix-2
Dam ato MKKRRLAANARERRRMNNLNSAFDRLRDVVPAL----------GNDRQLSKYETLQMAQSYITALCELL
Dm ato KRKRRLAANARERRRMQNLNQAFDRLRQYLPCL----------GNDRQLSKHETLQMAQTYISALGDLL
Dam ASH SVARR---NARERNRVKQVNMGFAVLRQHIPTSFCEASTA----------SDSGRSSSGGGSSKSRKVSKVDTLRCAVEYIRSL----
Dm sc SVQRR---NARERNRVKQVNNSFARLRQHIPQSIITDLTK----------GG---GRGPHKKISKVDTLRIAVEYIRRL----
Dm ac SVIRR---NARERNRVKQVNNGFSQLRQHIPAAVIADLSN----------GRRGIGPGANKKLSKVSTLKMAVEYIRRL----
Dm l'sc SVARR---NARERNRVKQVNNGFVNLRQHLPQTVVNSLSN----------GGR----GSSKKLSKVDTLRIAVEYIRGL----
Dam ase SVARR---NARERNRVKQVNTGFAVLRQHIPVLCGSVIVYGGLPNSPEMSSSSSCSSSSVSSPPASGRCKKNKMSKVETLRCAVEYIRNL----
Dm ase AVARR---NARERNRVKQVNNGFALLREKIPEEVSEAFEA----------QGAGRGASKKLSKVETLRMAVEYIRSL----


stage 5
stage 6.2
stage 7.1
stage 7.2
stage 7.3
stage 7.4
stage 7.5
Dam-ato expression
Dam-ASH expression
A
B
C
D
E
F
G
A'
B'
C'
D'
E'
F'
G'
H
I
J
K
L
M
N
H'
I'
J'
K'
L'
M'
N'
sp
a1
a2
md
mx1
pro
t1
t2
t3
tp1
tp2
tp3
tp4
tp5


stage 6.2
stage 7.3
stage 7.4
stage 7.5
Dam pros expression
Dam sna expression
A
B
C
D
A'
B'
C'
D'
E
F
G
H
E'
F'
G'
H'
a1
a2
md
mx1
pro
t1
t2
t3
tp1
tp2
tp3
tp4
tp5


A
stage 7.5
ventral
B
stage 7.5
lateral