The ParaHox gene Gsx patterns the apical organ and central nervous system but not the foregut in scaphopod and cephalopod mollusks

Tim Wollesen¹, Sonia Victoria Rodríguez Monje¹, Carmel McDougall², Bernard M. Degnan² and Andreas Wanninger¹

Abstract

Background: It has been hypothesized that the ParaHox gene Gsx patterned the foregut of the last common bilaterian ancestor. This notion was corroborated by Gsx expression in three out of four lophotrochozoan species, several ecdysozoans, and some deuterostomes. Remarkably, Gsx is also expressed in the bilaterian anterior-most central nervous system (CNS) and the gastropod and annelid apical organ. To infer whether these findings are consistent with other mollusks or even lophotrochozoans, we investigated Gsx expression in developmental stages of representatives of two other molluscan classes, the scaphopod Antalis entalis and the cephalopod Idiosepius notoides.

Results: Gsx is not expressed in the developing digestive tract of Antalis entalis and Idiosepius notoides. Instead, it is expressed in cells of the apical organ in the scaphopod trochophore and in two cells adjacent to this organ. Late-stage trochophores express Aen-Gsx in cells of the developing cerebral and pedal ganglia and in cells close to the pavilion, mantle, and foot. In postmetamorphic specimens, Aen-Gsx is expressed in the cerebral and pedal ganglia, the foot, and the nascent caputacula. In early squid embryos, Ino-Gsx is expressed in the cerebral, palliovisceral, and optic ganglia. In late-stage embryos, Ino-Gsx is additionally expressed close to the eyes and in the supraesophageal and posterior subesophageal masses and optic lobes. Developmental stages close to hatching express Ino-Gsx only close to the eyes.

Conclusions: Our results suggest that Gsx expression in the foregut might not be a plesiomorphic trait of the Lophotrochozoa as insinuated previously. Since neither ecdysozoans nor deuterostomes express Gsx in their gut, a role in gut formation in the last common bilaterian ancestor appears unlikely. Gsx is consistently expressed in the bilaterian anterior-most CNS and the apical organ of lophotrochozoan larvae, suggesting a recruitment of Gsx into the formation of this organ in the Lophotrochozoa. The cephalopod posterior subesophageal mass and optic ganglia and the scaphopod pedal ganglia also express Gsx. In summary, Gsx expression only appears to be conserved in the anterior-most brain region during evolution. Accordingly, Gsx appears to have been recruited into the formation of other expression domains, e.g., the apical organ or the foregut, in some lophotrochozoans.

Keywords: Brain, Cephalopoda, Evolution, Development, Hox, Homeobox genes, Invertebrate, Lophotrochozoa, Mollusca, Ontogeny, Scaphopoda, Lophotrochozoa
Background

The *Hox* and *ParaHox* gene clusters are considered to be derived from a hypothetical *ProtoHox* cluster by duplication [1]. Both belong to the homeobox gene family and exhibit highly conserved amino acid sequences in phylogenetically distantly related animals [1, 2]. In the majority of bilaterians investigated, it has been shown that *Hox* genes are expressed in tempo-spatial collinearity during development, in particular in neuroectodermal domains [2, 3]. Cephalopod and gastropod mollusks were among the first examples among bilaterians that apparently do not exhibit such a collinear mode of *Hox* gene expression [4, 5]. Tempo-spatial collinear expression of the three *ParaHox* genes has also been proposed for the last common bilaterian ancestor [1]. It has been hypothesized that *Gsx* was expressed in the foregut, *Xlox* in the midgut, and *Cdx* in the hindgut in the last common bilaterian ancestor [1, 6]. While *Xlox* expression in the midgut and *Cdx* expression in the hindgut was found in various bilaterians, no *Gsx* expression has been reported in the foregut of any deuterostome representative to date [1, 6]. This was explained by the fact that the blastopore does not develop into the prospective mouth in deuterostomes. Deuterostomes instead evolved a new mouth and hence *Gsx* might have lost its role in patterning the anterior-most region of the digestive tract. Interestingly, the deuterostome hemichordate *Ptychodera flava*, for example, does express *Gsx* around the blastopore, however, apparently not in the digestive tract of subsequent developmental stages [7]. Holland anticipated that protostome invertebrates may show *Gsx* expression in the foregut since their blastopore usually does become the future mouth [1, 6].

Data on the ecdysozoan and lophotrochozoan condition show, however, an ambiguous picture. While all ecdysozoans investigated so far do not appear to express *Gsx* in their digestive tract, the situation in lophotrochozoans is less clear (Table 1). The annelids *Platynereis dumerilii* and *Nereis virens* and the gastropod *Gibbula varia* express *Gsx* in their foregut [12–14], while the annelid *Capitella teleta* does not [15]. Comparisons with the condition in the Cnidaria, the putative bilaterian sister group, do not appear to contribute to inferring the ancestral state of *Gsx* expression in the Bilateria since the different germ layers cannot be homologized convincingly among the Cnidaria and the Bilateria. In addition, *Gsx* expression patterns are not consistent among cnidarians. In the planula larvae of *Nematostella vectensis*, *Clynthia hemisphaerica*, and *Podocoryne carnea* [9–11], *Gsx* is expressed in the endoderm, while it is expressed in the ectoderm of the planula of *Acropora millepora* [8].

*Gsx* is also involved in the development of the CNS in bilaterians, and it is expressed in distinct cells of the apical organ in the gastropod mollusk *G. varia* and the annelid *P. dumerilii* (Table 1; [12, 13]). In addition, *Gsx* expression was also found in the radula sac, a molluscan evolutionary novelty [12]. Recent phylogenomic analyses on mollusks have revived a classical hypothesis placing the Aculifera, i.e., the worm-shaped and spicule-bearing aplacophorans and the eight-shelled polyplacophorans, as a sister group to the Conchifera [30–32]. The Conchifera is an anatomically diverse clade comprising scaphopods, gastropods, bivalves, monoplacophorans, and cephalopods. Until now, conchiferan interrelationships are unsettled, and attempts to infer the evolution of their body plans are scarce (c.f. [31, 32]; but see [33, 34]).

The present study deals with two conchifers, the scaphopod *Antalis entalis* Jeffreys 1869 and the cephalopod squid *Idiosepius notoides* Berry, 1921 (Fig. 1). Adult scaphopods and cephalopods exhibit a pronounced dorso-ventral body axis as opposed to the majority of bilaterians that exhibit a pronounced antero-posterior body axis (Fig. 1). In adult scaphopods, the mouth and foot are located ventrally, while the mantle (i.e., the mantle cavity opening on the opposite side) marks the dorsal pole (Fig. 1b). In adult cephalopods, the funnel and (parts of) the circumoral brachial crown are considered to be homologous to the foot of other mollusks [35] (Fig. 1c). The brachial crown and the funnel define the ventral side, while the mantle apex is located dorsally (Fig. 1c). Thus, the dorso-ventral axis constitutes the major body axis in these animals. In both clades, the cerebral ganglia are located anteriorly (labeled blue in Fig. 1), while the statocysts are located at the posterior pole (dashed circles in Fig. 1b, c).

### Ontogeny of the scaphopod *Antalis entalis* and the cephalopod *Idiosepius notoides*

In the scaphopod, *A. entalis* gastrulation occurs at 12 h after fertilization (hpf) at 21–23 °C (Fig. 2a). At 14 hpf, a trochophore larva develops that exhibits an episphere with an apical organ and tuft (red dashed circles in Fig. 2). The episphere is divided from the hyposphere by a prototroch (Fig. 2b; see also [36–38]). The gastropod trochophore resembles the latter, but while the apical region develops into the prospective anterior region in gastropods, it develops into the prospective ventral region in scaphopods (see scaphopod condition in Fig. 2; [12, 36–38]). The blastopore of the gastrula develops into the mouth in *A. entalis* and lecithotrophic early-stage trochophore larvae already possess a through-gut with mouth and anus (Fig. 2a, b). The apical organ exhibits two serotonin-like immunoreactive cell somata (labeled red in Fig. 2b), and the nascent shell field is located in the anterior region of the hyposphere (Fig. 2b; [39]). The apical organ of mid-stage trochophore larvae (21 hpf)
Table 1  Gsx gene expression domains in metazoan developmental stages as revealed by in situ hybridization experiments

| Super-phylum/clade/species | Name of Gsx ortholog | Gsx expression domains                                                                                                      | References |
|---------------------------|----------------------|-----------------------------------------------------------------------------------------------------------------------------|------------|
| Cnidaria                  |                      | Planula larva                                                                                                               | [8]        |
| Acropora millepora        | Cnox-2Am             | Ectodermal cells along the oral/aboral body axis (rare in oral region)                                                    |            |
| Nematostella vectensis    | Anthox2              | Planula larva                                                                                                               | [9]        |
|                           |                      | Posterior endoderm, i.e., prospective oral end                                                                             |            |
|                           |                      | Developing mesenteries (ectoderm), Late planula larva                                                                       |            |
|                           |                      | Columnar ectodermal cells in tentacle buds                                                                                |            |
|                           |                      | Oral ectoderm                                                                                                               |            |
| Clytia hemisphaerica      | Gsx Ch               | Planula and embryos                                                                                                         | [10]       |
| Podocoryne carnea         | Gsx                  | Planula and embryos                                                                                                         | [11]       |
| Lophotrochozoa            |                      | Planula and embryos                                                                                                         |            |
| Gastropoda                | Gva-Gsx              | Vicel larva                                                                                                                 | [12]       |
| Gibbula varia             |                      | Endodermal cells in oral and aboral region                                                                             |            |
| Lophotrochozoa            |                      | Endodermal cells in oral and aboral region                                                                             |            |
| Scaphopoda                | Aen-Gsx              | Early-stage trophophore                                                                                                      | Present study |
| Antalis entalis           |                      | 2 cells each in the lateral episphere on both sides                                                                        |            |
|                           |                      | 1 cell each lateral to the anus on both sides                                                                              |            |
|                           |                      | Mid-stage trophophore                                                                                                        |            |
|                           |                      | 1 pair of cells in the apical organ and another pair lateral to latter                                                    |            |
|                           |                      | 1 cell each lateral to the anus on both sides                                                                              |            |
|                           |                      | 1 cell each in postero-lateral mantle on both sides                                                                          |            |
|                           |                      | Late-stage trophophore                                                                                                       |            |
|                           |                      | Several cells in the region of the cerebral and pedal ganglia and ventral foot                                              |            |
|                           |                      | Metamorphic competent trophophore                                                                                             |            |
|                           |                      | Several cells in the region of the cerebral and pedal ganglia, the ventral foot, and the capitula                          |            |
|                           |                      | Postmetamorphic individual                                                                                                   |            |
|                           |                      | Several cells in the region of the cerebral and pedal ganglia, the ventral foot, and the capitula                          |            |
| Cephalopoda               | Ino-Gsx              | Stage 19–20 Cerebral, optic, and palliovisceral ganglia                                                                      | Present study |
| Idiosepius notoides       |                      | Stage 23 Cerebral, optic, and palliovisceral ganglia                                                                        |            |
|                           |                      | Stage 25 Inferior frontal lobes, precommissural lobes, anterior and posterior basal lobes, inferior buccal lobes,       |            |
|                           |                      | Stage 26 Inferior frontal lobes, precommissural lobes, anterior and posterior basal lobes, inferior buccal lobes, peduncle lobes, and optic lobes |            |
|                           |                      | Stages 27–30 Region around eyes                                                                                              |            |
possesses four serotonin-like immunoreactive cells that are located next to two lateral cells that do not belong to this sensory organ (Fig. 2c). The episphere including the apical organ migrates in direction of the dorsal side and the cerebral ganglia develop below the latter and ventrally to the esophagus (Fig. 2c) [40]. In mid-stage trochophore larvae, the statocysts become visible in the foot (black dashed circles in Fig. 2c), and the dorsal-most region of the mantle, the pavilion, serves as second opening of the mantle cavity. In late-stage trochophore larvae (63 hpf)
and advanced developmental stages, the dorso-ventral body axis elongates considerably and the foot grows out into ventral direction (Fig. 2d). The apical organ migrates in dorsal direction and most probably disappears with all serotonin-like immunoreactive cells in metamorphic competent trochophore larvae (Fig. 2e). The cerebral ganglia are located anteriorly (blue domain in Fig. 2e) and connect to the pedal ganglia that are located ventrally to the statocysts (green domain in Fig. 2e). During metamorphosis, trochophores settle and are able to retract their prototroch and foot into the shell. Postmetamorphic individuals do not exhibit a prototroch and possess two captacula anlagen. These are the forerunners of the multiple cephalic tentacles that are used to collect food (Fig. 2f). Settled individuals show a well-differentiated midgut gland, a pronounced trilobed foot, and a buccal cone with a mouth (Fig. 2f). Notably, adult scaphopods generally lack eyes and a distinct head.

In the cephalopod I. notoides, cleavage only occurs on the cytoplasmic cap of the yolk-rich embryo (stages 2–13 according to [41]; reviewed in [42]). During the gastrulation process at stage 13, the outermost blastomere rows migrate below the inner blastomeres and a two-layered epithelium is formed on the yolk syncytium. Stage 18–19 individuals are roundish in shape and various organ systems are formed as placodes, among others the CNS, the arms, the funnel, the eyes, the mantle, and the arms (Fig. 3a). The brachial ganglia are located in the anlagen of the arms, the stellate ganglia are situated in the anterior portion of the mantle, and the optic ganglia are connected to both eyes (Fig. 3a). The cerebral ganglia develop dorsally to the mouth opening, the palliovisceral ganglia lie between the mantle and the statocysts, and the pedal ganglia are located ventrally to the statocysts. The dorso-ventral axis of stage 23 individuals is elongated compared to earlier stages (Fig. 3b). The esophagus is situated adjacent to the inner yolk duct and the individual ganglia connected to each other. Stage 25 embryos exhibit a more centralized brain and all individual central ganglia are termed brain masses herein in accordance with the classical literature (Fig. 3c). The cerebral ganglia give rise to the supraesophageal mass, the pedal ganglia develop into the anterior and middle subesophageal masses, and the palliovisceral ganglia are then termed posterior subesophageal mass [43]. Contrary, the peripheral stellate and brachial ganglia are still termed ganglia. The dorso-ventral body axis of stage 30 hatchlings is more elongated, and the CNS is more centralized than in earlier stages (Fig. 3d).

In this study, we describe hitherto unknown Gsx orthologs and their expression domains in the scaphopod Antalis entalis and the cephalopod squid Idiosepius notoides (Fig. 1). Our results question the widely assumed role of Gsx in patterning the foregut of the last common bilaterian ancestor and highlight similarities as well as differences among mollusks, lophotrochozoans, and bilaterians.

**Methods**

**Collection and culture of animals**

Adults of the scaphopod Antalis entalis were collected from approximately 30 m depth by the staff of the research vessel Neomys off the coast of Roscoff (France). Individuals were immediately transferred into dishes filled with seawater (see also [39]). Spawning occurred spontaneously or was induced by heat shocks, i.e., individuals were exposed to alternating water temperatures. Unfertilized eggs were rinsed several times and fertilized with sperm. Early- and mid-stage trochophore larvae were cultured in Millipore-filtered seawater (MFSW) with 50 mg streptomycin sulfate and 60 mg penicillin G per liter MFSW. Early cleavage stages, metamorphic competent larvae, and settled individuals were cultured in MFSW without antibiotics. Water was changed every other day. Metamorphosis occurred spontaneously or was induced by adding shell-gravel from the collection site.

Adults of the pygmy squid Idiosepius notoides were dip-netted in the sea grass beds of Moreton Bay, Queensland, Australia. Embryos were cultured and staged as described previously [43]. Development from freshly
laid fertilized eggs (stage 1) to hatchlings (stage 30) takes 9–10 days at 25 °C.

RNA extraction and fixation of animals
For *Antalis entalis*, a total of several hundred individuals of mixed developmental stages including early cleavage stages, trochophore larvae, metamorphic competent individuals, and early juveniles were collected and stored at −20 to −80 °C in RNAlater (Life Technologies, Vienna, Austria). RNA was extracted with a RNA extraction kit (Qiagen, Roermond, Netherlands) and stored at −80 °C.

For *Idiosepius notoides*, the egg jelly and chorion were removed from approximately 300 specimens covering freshly laid zygotes (stage 1) to hatchlings (stage 30). RNA was extracted using TriReagent according to the manufacturer’s instructions (Astral Scientific Pty. Ltd., Caringbah, Australia, see also [44]). Individuals of all the above-described developmental stages were fixed for in situ hybridization experiments as previously described [44].

RNAseq and transcriptome assembly
Total RNA from pooled developmental stages of *Antalis entalis* was sequenced by Illumina technology (Eurofins, Ebersberg, Germany). Paired-end reads of an average read length of 100 bp were obtained and subsequently filtered (rRNA removal). Adapter and low-quality sequences were trimmed, normalized, and assembled de novo into contigs with the assembler Trinity [45].

RNA from developmental stages of *Idiosepius notoides* was sequenced by 454 and Illumina technology (both Eurofins) as described previously [44]. After filtering, the adapter and low-quality reads were trimmed, normalized, and assembled de novo by Eurofins (454 transcriptome) or using Trinity (Illumina transcriptome).

Alignment and phylogenetic analysis
Known amino acid sequences of bilaterian Gsx orthologs were retrieved from the National Center for Biotechnology Information (NCBI) and used in BLAST searches against both assembled transcriptomes. Amino acid sequences were aligned using ClustalX v.2.0 [46].
trimmed by hand with the program AliView [47], and only conserved regions were retained (Fig. 4; untrimmed alignments are available upon request). This alignment was used to construct the neighbor-joining tree shown in Fig. 5 using the JTT matrix with 1000 bootstrap replicates within the Phylip v.3.695 [48] suite of programs.

**Molecular isolation of RNA transcripts**

First-strand cDNA synthesis of the RNA pooled from different developmental stages of *Antalis entalis* and *Idiosepius notoides*, respectively, was carried out by reverse transcription using the First-strand cDNA Synthesis Kit for rt-PCR (Roche Diagnostics GmbH, Mannheim, Germany). Identified Gsx orthologs of *A. entalis* and *I. notoides* were used to design gene-specific primers, and PCR products were size-fractioned by gel electrophoresis. Gel bands of the expected length were excised and cleaned up using a QIAquick Gel Extraction Kit (QIAgen, Hilden, Germany). By insertion into pGEM-T Easy Vectors (Promega, Mannheim, Germany), cleaned-up products were cloned. Plasmid minipreps were grown overnight, cleaned up with the QIAprep Spin Miniprep Kit (QIAgen), and sent for sequencing. The sequenced minipreps matched both transcripts identified as *Aen-Gsx* and *Ino-Gsx* in the phylogenetic analysis (Figs. 4, 5).

**Probe synthesis and whole-mount in situ hybridization**

Riboprobe templates were amplified via standard PCR from miniprepped plasmids using M13 forward and reverse primers. *In vitro* transcription reactions were performed with these templates, digoxigenin-UTP (DIG RNA Labeling Kit, Roche Diagnostics) and SP6/T7 polymerase (Roche Diagnostics GmbH) for the syntheses of antisense riboprobes according to the manufacturer’s instructions. For whole-mount in situ hybridization experiments, specimens were rehydrated into PBT (PBS + 0.1 % Tween-20) and treated with Proteinase-K (25 µg/ml for *Idiosepius notoides* and 45 µg/ml for *Antalis entalis*) in PBT at 37 °C for 10 min. Specimens were prehybridized in hybridization buffer for 4 h at 50 °C (*A. entalis*) or 65 °C (*I. notoides*), and hybridization with a probe concentration of 0.5 µg/ml (*I. notoides*) to 1 µg/ml (*A. entalis*) was carried out overnight at 50 °C (*A. entalis*) or 65 °C (*I. notoides*). For *A. entalis* as well as *I. notoides*, a minimum of 20 individuals per stage were investigated, and negative controls were carried out with sense probes for all genes and developmental stages. The majority of whole-mount preparations were cleared in a solution of benzyl benzoate/benzyl alcohol (2:1), mounted on objective slides, and analyzed. Preparations were documented with an Olympus BX53 Microscope.
In addition, scaphopod developmental stages were scanned with a Leica confocal SP5 II microscope (Leica Microsystems, Wetzlar, Germany) using bright-field, autofluorescence, and reflection mode scans [49]. If necessary, images were processed with Adobe Photoshop 9.0.2 software (San Jose, CA, USA) to adjust contrast and brightness.

**Histology**

After in situ hybridization experiments, developmental stages of *Antalis entalis* were post-fixed in 100 % EtOH and embedded in agar low viscosity resin (Agar Scientific, Essex, United Kingdom). Specimens were semithin sectioned with a diamond knife (Histo Jumbo Diatome) at a thickness of 0.5 µm with an ultramicrotome (Leica EM UC6, Wetzlar, Germany). Sections were mounted on objective slides, stained with Eosin using standard histological protocols, and covered with cover slips. Alternatively, after in situ hybridization, specimens were embedded in O.C.T. medium (VWR, Vienna, Austria) and cut into 15–30 µm cryosections with a cryotome (Leica CM 3050S). Sections were stained with Dapi (Sigma-Aldrich, St. Louis, MO, USA) and Cellmask Green plasma membrane stain (ThermoFisher, Waltham, MA, USA) in order to stain cell nuclei and cell membranes. Sections were mounted in Fluoromount G (Southern Biotech, Birmingham, Alabama, USA) and covered with cover slips. Semithin as well as cryotome sections was documented with an Olympus BX53 Microscope (Olympus).

**Statement of ethical approval**

Developmental stages and adults of the pygmy squid *Idiosepius notoides* were collected, anesthetized, and fixed according to internationally recognized standards (University of Queensland Animal Welfare Permit No. 158/09 “The cultivation of *Idiosepius* (pygmy squid) for studies in developmental biology” to BMD).

**Results**

*Aen-Gsx* expression in developmental stages of the scaphopod *Antalis entalis*

The alignment of multiple amino acid sequences shows that *Aen-Gsx* and *Ino-Gsx* exhibit high sequence similarity with their bilaterian orthologs (Fig. 4). *Aen-Gsx* as well as *Ino-Gsx* clusters with their bilaterian orthologs in the phylogenetic analysis (Fig. 5).
Aen-Gsx is first expressed in two cells in the episphere of early-stage trochophore larvae (14 hpf) (arrowheads in Fig. 6a, b, f–h). In addition, each one Aen-Gsx-expressing cell is located laterally to the anus (“4” in Fig. 6c–e, g). In mid-stage trochophore larvae (19 hpf), Aen-Gsx is expressed in two flask-shaped cells of the apical organ (“1” in Figs. 7a, b, 8a, b) and two lateral cells (“2” in Figs. 7a, b, 8a, b). Mid-stage trochophore larvae at 21 hpf also exhibit both aforementioned groups of cells (Fig. 7c, d). While two Aen-Gsx-expressing cells are located in the apical organ (“1” in Figs. 7d, g, 8a, b), both lateral Aen-Gsx-expressing cells do not appear to belong to the latter (“2” in Figs. 7d, f, 8a, b). Another pair of Aen-Gsx-expressing cells is present on the posterolateral side of the mantle (“3” in Figs. 7d, 8a, b) and below the mantle laterally to the anus (“4” in Figs. 7e, 8a, b). In late-stage trochophore larvae, two clusters of Aen-Gsx-expressing cells are present at the base of both captacula, in a region where the future cerebral ganglia develop (Fig. 8c, d; black dashed circle in Fig. 9a). Two additional clusters of Aen-Gsx-expressing cells are located ventro-laterally to the statocysts (Fig. 8c, d; red dashed circle in Fig. 9b). Two flask-shaped Aen-Gsx-expressing cells are located in the region of the pavilion (Fig. 8c, d; arrowheads in Fig. 9b). Another group of Aen-Gsx-expressing cells is located in the ventral portion of the foot (Fig. 8c; green dashed circle in Fig. 9c). In some individuals, one or two flask-shaped Aen-Gsx-expressing cells are visible in the region close to the cerebral ganglia (data not shown). In metamorphic competent trochophore larvae, each one Aen-Gsx-expressing cell cluster is situated ventro-laterally to the statocysts in the region of the pedal ganglia (green dashed circles in Fig. 10b, c) and in the region of the cerebral ganglia (red dashed circles in Fig. 10b, c). Other Aen-Gsx-expressing cell clusters are located in the region of the nascent captacula (black dashed circles in Fig. 10b, c). Postmetamorphic specimens exhibit a similar distribution of Aen-Gsx-expressing cells in the regions of the cerebral and pedal ganglia (Figs. 8e, f, 11a–c). Aen-Gsx-expressing cells are also present in the region of the nascent captacula (arrowheads in Fig. 11a, b) and in the ventral foot region (Figs. 8e, f, 11a–c).

Ino-Gsx expression in Idiosepius notoides

In stage 19–20 individuals, Ino-Gsx is expressed in the region of the optic and palliovisceral ganglia (Figs. 8g, h, 12a–c). The cerebral ganglia, which are located dorsally to the mouth and expand anteroventrally in direction of the eyes, also express Ino-Gsx (Fig. 8h; arrowheads in Fig. 12b). In subsequent developmental stages, the expression domains remain the same and stage 23 individuals express Ino-Gsx in the optic and palliovisceral ganglia.
(Fig. 12d). The expression domain in the cerebral ganglia is relatively smaller compared to the domain reported for previous stages, and it is restricted to two patches ventrolaterally to the eye and close to the forming buccal mass (double arrowheads in Fig. 12d). In subsequent developmental stages, individual lobes of the supraesophageal mass as well as the posterior subesophageal mass and the optic lobes express \textit{Ino-Gsx} (Fig. 13a–e). In the supraesophageal mass of stage 25 individuals, \textit{Ino-Gsx} expression occurs in the inferior frontal and precommissural lobes as well as in the anterior basal and posterior basal lobes including the dorsal basal and dorsolateral lobes (Figs. 8i, j, 13a–c). In addition, \textit{Ino-Gsx} is still expressed around the eyes and laterally of the buccal mass. This area might correspond to the region where the inferior buccal lobes develop (Figs. 8i, j, 13a, b). No expression was observed in the vertical, subvertical, and the superior frontal lobes or the anterior or middle subesophageal masses (Figs. 8i, j, 13a–c). Compared to stage 25 individuals, stage 26 individuals strongly express \textit{Ino-Gsx} in their optic lobes (Figs. 8i, j, 13a–c). In addition, lobes of the supraesophageal mass such as the peduncle lobes or the buccal lobes express \textit{Ino-Gsx} (Figs. 8i, j, 13a–c). Stronger \textit{Ino-Gsx} expression is also observed laterally of the buccal mass, most likely corresponding to the inferior buccal lobes (Fig. 13e). Subsequent developmental stages until hatching only express \textit{Ino-Gsx} around the eyes but not in the CNS (Figs. 8k, l, 13f, g).
Discussion

Gsx does not pattern the digestive tract of scaphopods and cephalopods

To date, it is commonly hypothesized that the digestive tract of the last common bilaterian ancestor expressed Gsx in a collinear fashion together with the two other ParaHox genes, Cdx and Xlox [1, 6, 12, 20, 50]. This hypothesis is seemingly corroborated by the fact that among the Lophotrochozoa, the annelids Platynereis dumerilii and Nereis virens, as well as the gastropod Gibbula varia, express Gsx in their anterior digestive tract (Table 1; [12–14]). Our results for the scaphopod Antalis entalis and the cephalopod Idiosepius notoides, however, show that this is not the case for all mollusks, and therefore, neither for all lophotrochozoans, a scenario that was already suggested by data on the annelid Capitella teleta ([15]; Table 1). Moreover, all ecdysozoan representatives investigated lack Gsx expression in their digestive tract, and among the deuterostomes investigated, only the hemichordate Ptychodera flava expresses Gsx around the blastopore [7]. The lack of Gsx expression in the foregut of the other deuterostomes has been explained by the fate of the blastopore that does not transform into the definite mouth in deuterostomes as it does in protostomes, but, instead, into the anus [6]. Accordingly, the latter hypothesis would argue for Gsx expression in the deuterostome hindgut which, however, appears to be absent ([50]; Table 1). It is important to mention that Gsx orthologs have either not been found or are indeed absent in representatives of the Acoelomorpha, which are characterized by having a single mouth/anus opening in their digestive tract and may form the sister taxon to all remaining Bilateria (the so-called Nephrozoa; [51, 52]; but see [53] for a controversial view). In cnidarians, Gsx is endodermally expressed in the planula larva of Nematostella vectensis, Clytia hemisphaerica, and Podocoryne...
Fig. 8 Summary of Gsx expression (blue) during the development of the scaphopod Antalis entalis and the cephalopod Idiosepius notoides. Dorsal (d)–ventral (v), anterior (a)–posterior (p), and left (l)–right (r) axes indicate the orientation and are the same in each of the six columns. Shown: Gsx-expressing cell somata do not represent absolute numbers. a–f Sketch drawing depicting a mid-stage trochophore (a, b), a late-stage trochophore (c, d), and a postmetamorphic (settled) individual (e, f) of Antalis entalis. Gsx-expressing cell somata are labeled with red numbers 1–4 (c.f. Figures 6, 7). g–l Sketch drawing depicting developmental of stage 19 (g, h), stage 25 (i, j), and stage 28 (k, l, yolk sac removed) of the pygmy squid Idiosepius notoides. ab anterior basal lobe, ao apical organ, ar arm, bc buccal cone, cg cerebral ganglion, cp captacula, ey eye, f foot, fn funnel, ib inferior buccal lobe, if inferior frontal lobe, m mantle, mf mantle fold, mo mouth, o optic ganglion/lobe, pb posterior basal lobe, pg pedal ganglion, pt prototroch, pv pavilion, pvg palliovisceral ganglion, ps posterior subesophageal mass and y yolk. Scale bars a–f 50 µm, g–l 150 µm

Fig. 9 Expression of Aen-Gsx during late-stage trochophores of the scaphopod Antalis entalis. Dorsal (d)–ventral (v) and left (l)–right (r) axes indicate the orientation. The statocysts are labeled with asterisks. Optical sections from anterior (a) to posterior (b) in a 63 hpf old trochophore (all with same orientation and scale bar as indicated in a). a One cell cluster on each side expresses Aen-Gsx in the region of the cerebral ganglia (black dashed circles). b Two cell clusters (red dashed circles) express Aen-Gsx ventro-laterally to the statocysts, in the region of the prospective pedal ganglia. A pair of Aen-Gsx-expressing cells is located in the region of the pavilion (pv). c Aen-Gsx is expressed in the ventral portion of the foot (green dashed circle). m mantle, pt prototroch. Scale bar 50 µm
carnea [9–11]. In the coral Acropora millepora, Gsx is expressed in the ectoderm of the planula larva [8]. Comparisons of the cnidarian and nephrozoan expression domains are difficult since mouth and digestive system cannot be easily homologized. Hence, the data currently available argue for a last common nephrozoan and probably also bilaterian ancestor without Gsx expression in the digestive tract and for a recruitment of Gsx into foregut patterning in selected lineages. Accordingly, the gastropod G. varia and the polychaete annelids N. virens and P. dumerilli have acquired Gsx expression in the foregut secondarily during evolution (Table 1). In contrast, other genes such as Brachyury, Nkx2.1, or FoxA appear to be evolutionary highly conserved in the digestive system within the Lophotrochozoa [54–59].

Gsx is expressed in the anterior-most portion of the molluscan CNS

In contrast to the digestive tract, Gsx is consistently expressed in the anterior CNS of bilaterians and hence an ancestral role in CNS development was proposed (Table 1; [2]). Shared Gsx expression domains among mollusks are the cerebral ganglia that subsequently develop into the supraesophageal mass in cephalopods (present study; [12]). In scaphopod and gastropod larvae,
the apical organ is located in the anterior-most region. In the scaphopod *Antalis entalis*, *Gsx* is expressed in two flask-shaped cells of this organ and in two cells that are located laterally to it but do not constitute a part of the apical organ (Fig. 4d). With two apical tuft cells and further putative sensory cells, the larva of the gastropod *Gibbula varia* possesses more *Gsx*-expressing cells in the apical organ than the one of *A. entalis* (present study; [12]). The flask-shaped *Gsx*-expressing cells of *A. entalis* do not appear to be homologous to any of the *Gsx*-expressing cell types of *G. varia* judging by their morphology. However, detailed ultrastructural studies and molecular fingerprints on the various cell types occurring in lophotrochozoan apical organs are necessary to further assess homologies in this organ on the cellular level. Among all metazoans with an apical organ (Cnidaria, Ambulacraria, and Lophotrochozoa), only both above-mentioned mollusks and the annelid *Platynereis dumerilii* possess *Gsx*-expressing cells in the apical organ, suggesting that *Gsx* has been recruited into the patterning of this sensory organ in lophotrochozoans only (Table 1; present study; [12, 13]).

*Gsx* expression has also been reported for the polychaete annelids *Nereis virens* and *Capitella teleta* [14, 15]. As far as known, both species lack an apical organ as do cephalopods as direct developers (present study; [3, 15]). The vertical lobe as the anterior-most portion of the cephalopod CNS does not express *Gsx* (Figs. 9d, 10). This resembles the expression patterns of other homeobox genes such as *Otx* or the *POU* genes which are consistently expressed in the gastropod cerebral ganglia and large parts of the cephalopod cerebral ganglia/supræsophageal mass but not in the vertical lobe [44, 60, 61]. The vertical lobe is considered an evolutionary innovation of coleoid cephalopods, i.e., all cephalopods except the nautiluses as basal cephalopod offshoots [62]. As an evolutionary younger brain region confined to coleoid cephalopods, the vertical lobe also differentiates relatively late during ontogeny compared to other brain regions [63]. Hence, the vertical lobe probably evolved after *Otx*
expression domains had already been established in the supraesophageal mass of coleoid cephalopods.

**Gsx is expressed in the posterior portion of the molluscan CNS**

*Idiosepius notoides* and *Antalis entalis* express Gsx in posterior portions of their CNS such as the scaphopod pedal ganglia and the cephalopod palliovisceral ganglia (the latter develop into the future posterior subesophageal mass). This is in contrast to the gastropod *Gibbula varia* and the annelid *Capitella teleta*, where Gsx expression is restricted to the anterior CNS [12, 15]. The scaphopod and cephalopod condition is, however, similar to the condition found in *Platyneris dumerilii* and certain vertebrates insofar that both mollusks and the polychaete express Gsx in more posterior regions of their nervous system. These domains comprise the scaphopod pedal ganglia, the cephalopod...
palliovisceral lobe/posterior subesophageal mass, the polychaeta nerve cord, and the hindbrain of vertebrates [13, 24–27]. Interestingly, Gsx is also expressed in portions of the developing visual system of few representatives of all three bilaterian superphyla. The mollusks *I. notoidea* and *Nereis virens*, the arthropods *Drosophila melanogaster*, as well as the teleost fish *Oryzias latipes*, express Gsx in portions of their visual system (Table 1; present study; [14, 17, 28]). Further studies on other bilaterian representatives are needed to assess if Gsx expression in the eyes and related brain regions may be an ancestral trait among nephrozoans or bilaterians.

**Conclusions**

This study suggests that Gsx expression in the foregut is not a molluscan plesiomorphy and together with already published data argues against Gsx expression in the foregut of the last common bilaterian ancestor. It is therefore most likely that Gsx has been independently recruited into the development of the foregut in some lophotrochozoan representatives. Gsx is consistently expressed in the developing anterior nervous system of bilaterians, which is probably an apomorphy of Bilateria. In contrast to other metazoan taxa, Gsx expression was only found in the larval apical organ in lophotrochozoans, indicating that Gsx expression in the apical organ may be a lophotrochozoan synapomorphy.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| a. Anterior | ab: Anterior basal lobe, Aen: Antalis entalis, A. entalis: Antalis entalis, an: Anus, at: Arm, at: Apical tuft, ao: Apical organ, BBA: Benzyl benzoate, bc: Buccal cone, Bf: Branchiostoma floridae, Bia: Branchiostoma lanceolatum, bm: Buccal muscle, bp: Blastopore, br: Brachial crown, c.f.: Confer, cg: Cerebral ganglia, cp: Captacula, CNG: Central nervous system, d: Dorsal, db: Dorsal basal lobe, Dme: Orosphila melanogaster, ep: Episphere, ey: Eye, f: Foot, fn: Funnel, Gsx: Genomic screened homeobox protein, G. varia: Gibbula varia, Gv: Gibbula varia, hpf: Hours after fertilization, hyposphere, ib: Inferior buccal lobe, i.e.: Id est, if: Inferior frontal lobe, ind: Intermediate neuroblasts defective, Ino: Idiosepius noides, I. noides: Idiosepius noides, I. Left, Lgi: Lottiaria gigantea, M: Mantle, ma: Mantle apex, mb: Median basal lobe, mc: Mantle cavity, mf: Mantle fold, mg: Midgut gland, MFSW: Millipore-Lottia gigantea, Lgi: Neomys, l: Left, L: Lophotrochozoan, L.): Gibbula varia, Nereis, Neomys, p: Posterior, P: dumerilii: Platynereis dumerilii, Pdu: Platynereis dumerilii, pb: Posterior basal lobe, pc: Precommissural lobe, ped: Peduncle lobe, pg: Pedal ganglion, Pmi: Pinnata miniata, ps: Posterior subesophageal mass, pt: Protostroch, pv: Pavilion, pv: Pallovisceral ganglia, r: Right, sb: Subesophageal mass, sh: Shell, shf: Shell field, sp: Supraesophageal mass, Tca: Trabulium castaneum, v: Ventral, y: Yolk.

**Authors' contributions**

TW designed the project together with AW. TW reared and fixed all developmental stages of *Idiosepius noides* and *Antalis entalis*, extracted the RNA, and assembled the transcriptomes. TW cloned all genes, and TW and SVRM carried out the in situ hybridization experiments. CMD performed the phylogenetic analysis. TW analyzed all data and drafted the manuscript. AW contributed to the in situ hybridization experiments. TW analyzed all data and drafted the manuscript. AW contributed to the in situ hybridization experiments. CMD performed the phylogenetic analysis. TW cloned all genes, and TW and SVRM carried out the in situ hybridization experiments. TW analyzed all data and drafted the manuscript. AW contributed to the in situ hybridization experiments. CMD performed the phylogenetic analysis.

**Author details**

1. Department of Integrative Zoology, Faculty of Life Sciences, University of Vienna, 1090 Vienna, Austria. 2. School of Biological Sciences, The University of Queensland, Brisbane, QLD 4072, Australia.

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**Competing interests**

The authors declare that they have no competing interests.

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