The ups and downs of amphioxus biology: a history

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ABSTRACT Humans (at least a select few) have long known about the cephalochordate amphi-oxus, first as something to eat and later as a subject for scientific study. The rate of publication on these animals has waxed and waned several times. The first big surge, in the late nineteenth century, was stimulated by Darwin's evolutionary ideas and by Kowalevsky's embryologic findings suggesting that an amphioxus-like creature might have bridged the gap between the invertebrates and the vertebrates. Interest declined sharply in the early twentieth century and remained low for the next 50 years. An important contributing factor (in addition to inhibition by two world wars and the Great Depression) was the indifference of the new evolutionary synthesis toward broad phylogenetic problems like the origin of the vertebrates. Then, during the 1960s and 1970s, interest in amphioxus resurged, driven especially by increased government support for basic science as well as opportunities presented by electron microscopy. After faltering briefly in the 1980s (electron microscopists were running out of amphioxus tissues to study), a third and still-continuing period of intensive amphioxus research began in the early 1990s, stimulated by the advent of evolutionary developmental biology (evo-devo) and genomics. The volume of studies peaked in 2008 with the publication of the genome of the Florida amphioxus. Since then, although the number of papers per year has dropped somewhat, sequencing of additional genomes and transcriptomes of several species of amphioxus (both in the genus *Branchiostoma* and in a second genus, *Asymmetron*) is providing the raw material for addressing the major unanswered question of the relationship between genotype and phenotype.

KEY WORDS: *Cephalochordata, amphioxus, lancelet, history of science, evodevo*

Preliminary: cephalochordate names (west and east)

It is useful to begin with a few words about common names for cephalochordates. When referring informally to these animals in English, contemporary biologists use *lancelet* (plural: *lancelets*) or, more commonly, *amphioxus.* The plural of the latter is awkward because: *amphioxi* looks pretentious and *amphioxuses* is almost unpronounceable; the common solution is to use *amphioxus* regardless of whether the singular or the plural is intended. Occidental languages other than English have recognizable cognates for *lancelet* and *amphioxus*, but oriental languages do not. The Japanese name is *slug fish* (*namekuji-uo*), which traces back to an eighteenth century classification of amphioxus as a mollusc (Nishikawa, 1995). The Chinese name is *fish of the god of literature* for somewhat involved reasons that will be explained in the next paragraph.

Amphioxus — the early years (A in Fig. 1)

Amphioxus is quite suitable for human consumption. In fact, Gibbons (1964), author of *Stalking the blue-eyed scallop*, ate some and reported that he had "seldom dined better." In the rare locations where large numbers of amphioxus live shallowly, humans could conceivably have been preying on amphioxus for thousands of years. However, only one old record of such human predation has survived—as oral tradition recorded by Light (1923), who interviewed Chinese fishermen during his visit to the city of Xiamen (formerly Amoy). When he was there in the early 1920s, small boats equipped with primitive dredges were harvesting hundreds of tons of amphioxus each year from the nearby shallows. Light could not discover how long the fishery had existed, but estimated it was at least hundreds of years old. He also recounts how the Chinese name for amphioxus is derived from a mixture of religion, mythology, and fishermen's tales. The Chinese co-gods of literature are Wen Chang and Kui Xing, although the latter is often considered a lesser deity or even a mere attendant of the former. The essential part of the tale is that Kui Xing was elevated above the sea surface on the head of a crocodile (or, in variants of the story, a turtle, a fish, or a dragon). For reasons that are never explained, the crocodile died and was transformed into Crocodile Island a short distance from Xiamen. The rotting corpse produced maggots that were

really amphioxus. Although Kui Xing was the main actor in this story, the Chinese named amphioxus after the higher ranking deity, as *Wen Chang yu*, which can be translated literally as *fish of Wen Chang* or more loosely as *fish of the god of literature*. Parenthetically, by the turn of the present century, the amphioxus harvest in Xiamen had fallen to about 1 ton per year due to construction of a causeway, land fill, toxic runoff, intensive aquaculture in floating cages, and electrofishing (Chua and Gorre, 2000). By now, collecting the animals is illegal there, although they are still available on the black market (Lanna Cheng, personal communication).

The formal debut of amphioxus into the world of science took place in 1774 and was a truly international affair. Two pickled specimens were sent from England to Russia where they were described in Latin by a German named Peter Pallas. The short species description, translated into English, reads as follows: "Planar, lanceolate body, tapering to a point at either end. The ventral side has two longitudinal ridges flanking a narrow slug-like foot. There are no tentacles. Either side of the body is decorated with a series of v-shaped lines, each pointing anteriorly. These make the animal look like a little fish." In an explanatory addition, Pallas (1774) likened the ventral side of amphioxus to the narrow foot of a sea slug (*Scyllaea sp.*) that is adapted for crawling on sargasso weed. This incorrect homology convinced him that amphioxus was a molluscan slug, so he assigned his new species to the genus *Limax*. Progress in science was leisurely in those days, and it was 1817 before Stewart referred to the description of Pallas and brusquely concluded that the animal was "Hardly a *Limax*."

The next advance was Costa's (1834) report on amphioxus living abundantly in Naples. He was unaware of the work of Pallas and considered the new animals to be a kind of fish that should be classified somewhere between sea horses and sharks. Costa misidentified the cirri around the mouth as gills and accordingly coined the genus name *Branchiostoma* (literally, gill mouth). A few years later, Yarrell (1836), who had read Pallas but not Costa, independently decided that amphioxus was a fish and added that it was the simplest kind of vertebrate. Yarrell also introduced *lancelet* as a common name and changed the generic name from *Limax* to *Amphioxus.* Ultimately, however, Costa's *Branchiostoma* was given precedence as the formal genus, and *amphioxus* was demoted to a common name. The work of Yarrell attracted the attention of anatomists, who commenced detailed studies on adults of amphioxus (Müller, 1839; Rathke, 1841; Goodsir, 1844; de Quatrefages, 1845). In addition, taxonomists began finding cephalochordate species in addition to the European one initially discovered: Gray described *B. belcheri* from Borneo in 1847, and Sundevall described *B. elongatum* from Peru in 1852 and *B. caribaeum* from the Caribbean and Brazil in 1853. Soon after that, the first contributions to amphioxus developmental biology were made by Schulze (1851) and Leuckart and Pagenstecher (1858)
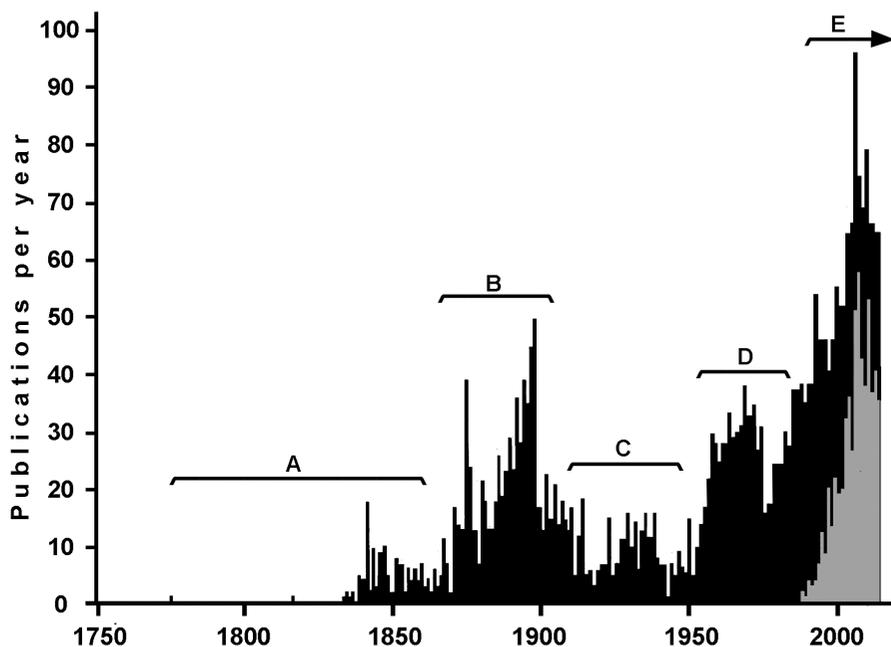

Fig. 1. Number of publications on amphioxus from 1774 through 2015. *This graph builds on the analysis of Gans (1996), who analyzed the annual frequency of amphioxus research up through 1995. We have extended the graph from 1996 through 2015. From 1774 through 2015, the history of amphioxus research is divided into five eras:* **(A)** *the early years;* **(B)** *the Golden Age of zoology;* **(C)** *the era of disillusionment with Haeckel's law of recapitulation followed by world wars and the great depression;* **(D)** *the post-World War II decades of prosperity, including electron microscopy; and* **(E)** *the current era of evolutionary development and genomics (the gray portion of each bar from 1992 to 2015 indicates the number of publications on those subjects per year).*

who worked on late larvae captured from the plankton. Almost none of these early students of amphioxus mentioned evolution in their publications—the lone exception was Goodsir (1844), who proposed, somewhat vaguely, that amphioxus might be halfway between an invertebrate and a vertebrate.

Amphioxus during the Golden Age of zoology (B in Fig. 1)

The years between the 1859 publication of Darwin's *Origin of species* and the end of the nineteenth century have been regarded as the Golden Age of zoology (Goldschmidt, 1966). In that era, interest in amphioxus was stimulated in general by the new evolutionary viewpoint, and in particular by Alexander Kowalevsky's seminally important embryological work. The circumstances leading up to that work were as follows: during his undergraduate years Kowalevsky studied at several German universities from 1859 to 1861 (Fokin, 2012). At Heidelberg, he came under the influence of Heinrich Pagenstecher (already mentioned for his study of the late larvae of amphioxus), who evidently encouraged his young guest to describe the early embryology of amphioxus (Vucinich, 1970). It is not clear why such a study would be considered so important at that time—amphioxus was thought to be a fish, and the broad outlines of fish embryology were already known from the work of Rusconi and Weber (1836). Perhaps Kowalevsky thought amphioxus embryos would *not* be fish-like and would, therefore, be especially interesting. We will probably never know the answer.

What we do know is that he returned to Russia, finished his undergraduate degree, and set out for Naples, Italy, arriving there late in 1863. Because the Zoological Station of Anton Dohrn did not yet exist, Kowalevsky rented an apartment on the waterfront to serve as a laboratory as well as living quarters.

After setting up a sea-water aquarium in his apartment, Kowalevsky began collecting amphioxus regularly in hopes that they would spawn. Although the animals had conspicuous gonads from December 1963 through May 1864, no gametes were forthcoming. During those six months, Kowalevsky was far from idle: he studied the embryos of phoronids (the beginnings of his PhD thesis), entoprocts, holothurians, ctenophores, and ascidians. Finally, on 18 May 1864, his amphioxus spawned and he described development through the early larval stage, at which point all of his animals starved to death (fortunately he was able to net more advanced larvae from the plankton). The overall result of the study was that the early development resembled that of a sea urchin, but the larval stages had much in common with vertebrate embryos. This research formed the basis of his master's thesis in 1865.

When Kowalevsky publicly defended his thesis at St. Petersburg University, the aged and anti-Darwinian professor von Baer was pleased with the technical excellence of the work, but was irritated by the evolutionary implications. Metchnikoff (1866) went right to the heart of the matter when he wrote: "It will not escape anyone how much the major features of amphioxus development are intermediate between the development of vertebrates and that of lower animals." When Kowalevsky published his amphioxus embryology *in extenso* as a journal article in 1867, he attracted international attention and controversy (thoroughly reviewed by Beeson in 1978). Amphioxus was variously interpreted as (1) a member of some phylum (for instance, molluscs) with little to do with vertebrates, (2) a degenerate vertebrate, (3) a bridge connecting vertebrates with various invertebrates, or (4) a proxy for the ancestor of the vertebrates, but with yet-undiscovered antecedents. By now, the first two of these possibilities can be ruled out, but discussion of the last two continues (Holland *et al.*, 2015).

Amphioxus research in the doldrums: first half of the twentieth century (C in Fig. 1)

By the early twentieth century, the number of biologists studying amphioxus dropped precipitously. This nadir had several causes. In part, there was growing disillusionment with evolutionary speculations based on Haeckel's law of recapitulation (Rasmussen, 1991). Rejection of that law had a chilling effect on proposals for long-range transitions between distantly related phyla (the new evolutionary synthesis largely ignored and side-stepped this problem by focusing on evolutionary mechanisms over relatively narrow scales of diversity and time). Another reason for the near eclipse of amphioxus research was that embryologists had lost interest in the evolutionary implications of their work and focused on experimental approaches to elucidate mechanisms of development (Nyhart, 1995). Amphioxus was not one of their narrow selection of favored animals because it inconveniently spawned unpredictably on days of its own choosing and had a relatively short breeding season each year. Moreover, some populations, particularly in Italy, had disappeared. Last, and hardly least, academic science was severely inhibited during the first half of the twentieth century by two world wars and the intervening Great Depression.

Amphioxus studies in post-World War II decades (D in Fig. 1)

The 1960s and 1970s were years of resurgence in amphioxus research. Government funding for basic sciences was increasing in many countries. Support was especially generous in the United States after the shock of *Sputnik* loosened the government's purse strings. Due to the favorable intellectual climate, more people were attracted to careers in academe. Scientific faculties at existing universities were expanding, and new institutions were creating biology departments *de novo*, all of which translated into more biologists to study amphioxus. In addition, amphioxus research was no longer dominated by morphology and light-microscopic histology, but now included ecology, physiology, biochemistry, and especially electron microscopy. Electron microscopists described tissue after tissue so avidly that, by the early 1980s, few body parts remained that they had not already examined. The number of fine structural studies therefore dwindled—so much that the total number of amphioxus publications declined briefly (Fig. 1) before an intensifying of research on amphioxus biochemistry and endocrinology began to reverse the trend.

Amphioxus in the era of evolutionary developmental biology (E in Fig. 1)

The discovery of *Hox* genes in 1983 (Lawrence, 1992) led to comparative studies of developmental genetics. Initially, relatively few major groups (for instance, insects and vertebrates) were compared, and studies limited to such drastically pruned trees hindered consideration of broader phylogenetic questions (Jenner, 2000). It is fair to say that the field of evolutionary developmental biology was only getting started at the beginning of the 1990s as relevant data began accumulating for a wider spectrum of taxa.

The first developmental genetic publication on amphioxus (Holland *et al.*, 1992) focused on *Hox* genes. In mammalian genomes, due to two whole-genome duplications early in vertebrate evolution, there are four *Hox* clusters, each with a subset of the 13 ancestral genes. The genes in each cluster are arranged collinearly on their chromosomes. For the most part, they are also expressed collinearly in the CNS, with *Hox1* through *Hox4* expressed in the hindbrain and the remainder in the spinal cord (Krumlauf *et al.*, 1993; Tschopp *et al.*, 2012). Amphioxus, not having undergone genome duplications, has one cluster of 15 Hox genes, arranged collinearly on a single chromosome (Garcia-Fernandez and Holland, 1994; Holland *et al.*, 2008a). Because *Hox* genes are collinearly expressed in the vertebrate CNS, we and our colleagues reasoned that they would be similarly expressed in the amphioxus CNS and consequently that their expression patterns could be used as characters to address the question of whether the amphioxus brain was quite small (Steida, 1873), relatively large (Huxley, 1874) or completely absent (Schmidt, 1897). The first amphioxus gene examined was amphioxus *Hox3* (Holland *et al.*, 1992). This study showed that the anterior limit of *Hox3* expression in the amphioxus nerve cord is adjacent to the boundary between muscular somites 4 and 5, similar to the anterior limit of *Hoxb3* at the boundary between rhombomeres 4 and 5 in the vertebrate hindbrain. This indicated that amphioxus likely has an equivalent of the vertebrate hindbrain and suggested that the more anterior regions of the amphioxus CNS might correspond to a forebrain and/or midbrain (Holland

et al., 1992). Subsequently, expression of other amphioxus Hox genes showed that, as in vertebrates, they are generally expressed collinearly in the amphioxus CNS (Pascual-Anaya *et al.*, 2012; Schubert *et al.*, 2006). These studies established amphioxus as a model for understanding how the vertebrate CNS evolved from the simpler CNS in an invertebrate ancestor.

After the initial study on *Hox3*, comparisons of expression of many more developmental genes between amphioxus and vertebrates indicated that the anteriormost part of the amphioxus CNS was probably equivalent to a diencephalic forebrain and perhaps a small midbrain; there is no telencephalon (diagrammed by Shimeld and Holland, 2005). Thus, expression of many genes is similar in the brains of the two chordates. For example, *BF1* (*FoxG1*), *Pax6*, and *Otx* are expressed in the anteriormost part of the amphioxus CNS indicating that amphioxus has a forebrain, while the domain of *Fezf* abuts that of *Irx*, suggesting that within the forebrain there is an equivalent of the vertebrate zona limitans intrathalamica (ZLI). In addition, the domain of *Otx* abuts that of *Gbx* where the enlarged anterior part of the CNS, the cerebral vesicle, narrows just anterior to the anteriormost Hox domains, suggesting the presence of a homolog of the vertebrate midbrain/hindbrain boundary (MHB). In vertebrates, the ZLI and MHB function as organizers as shown by transplantation studies; when transplanted either anterior or posterior to their normal position in the brain, they change the fate of the host tissue. It is unlikely that the corresponding regions in amphioxus have organizer properties since some of the critical genes that confer such properties in vertebrates are not comparably expressed in amphioxus. Even so, comparative data from gene expression strongly suggest that the vertebrate brain evolved from something like the simpler brain of a modern amphioxus.

Inferences of homologies are always strengthened when data from more than one approach concur. For the brain, the conclusions from mapping domains of gene expression in amphioxus correspond well with results from 3D reconstructions of neurons and their connections made from serial electron microscopic sections (Lacalli *et al.*, 1994; Wicht and Lacalli, 2005). As in gene expression studies, the neural wiring diagram of a late larva indicated that amphioxus lacks a telencephalon, but does have a diencephalon, a small midbrain, and hindbrain. At the anterior end of the CNS is a medial photoreceptor, which has been homologized with the vertebrate paired eyes, and posterior to that a balance organ, an infundibular organ, which secretes a fiber similar to Reissner's fiber in the vertebrate brain, and the lamellar body (a homolog of the vertebrate pineal) followed by a motor neuron center (reviewed in Wicht and Lacalli, 2005). Together with these studies, mapping of neurons expressing specific neuropeptides and neurotransmitters, and proteins expressed in the anterior photoreceptor have demonstrated marked similarities of many cell types in the amphioxus and vertebrate CNSs (Candiani *et al.*, 2012; Holland and Holland, 1993; Vopalensky *et al.*, 2012). These studies strongly support the idea that the common ancestor of amphioxus and vertebrates had a brain

that was regionalized into forebrain, midbrain and hindbrain, and indicate that the telencephalon was likely a vertebrate innovation.

For amphioxus, the mapping of major brain regions by domains of gene expression was soon followed by a spate of research on genes and development aimed at finding additional homologies between amphioxus and vertebrates. As these studies are far too numerous to review here, we will focus on three examples showing how expression of only one or a few developmental genes can illuminate quite broad evolutionary issues. First, Holland *et al.*, (1996) showed that amphioxus *Distal-less* is expressed in ectoderm adjacent to the neural plate, which detaches from the neural plate and walks over it by means of lamellipodia (Fig. 2). Expression of *Distal-less* in this cell population is similar to that of vertebrate *Dll 5* in the neural plate border region where it functions in defining the border between neural plate and the pan placocdal ectoderm (McLarren *et al.*, 2003; Woda *et al.*, 2003). These results led us to make the first suggestion that the ancestral chordate had a cell population at the edges of the neural plate that set the stage and provided the raw material for the subsequent evolution of vertebrate neural crest.

Further studies showed that expression of the genes specifying the neural plate and the edges of the neural plate is highly conserved between amphioxus and vertebrates; however, homologs of many the genes that specify neural crest are not expressed at the edges of the amphioxus neural plate (Yu *et al.*, 2008). Subsequent work indicated how the additional genes deriving from two whole genome duplications at the base of the vertebrates facilitated
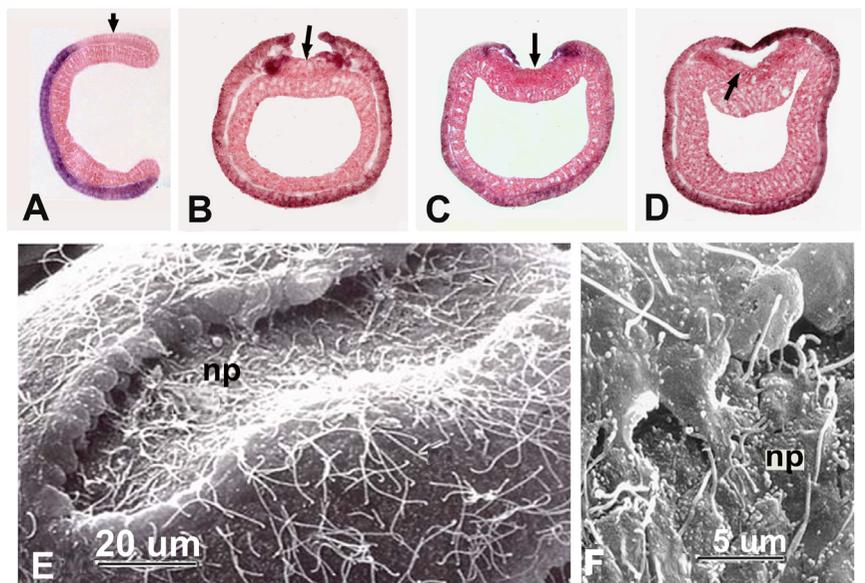

Fig. 2. Ectoderm at the neural plate border in amphioxus and vertebrates expresses Distal-less. (A) *Longitudinal section of a mid-gastrula of amphioxus (Branchiostoma floridae) showing Distal-less expressed in the non-neural ectoderm.* **(B-D)** *Cross-sections through successively later stages of amphioxus neurulae.* **(B)** *Early neurula with Distal-less expressed in the non-neural ectoderm, which has begun to migrate over the neural plate (arrow) and in the edges of the anterior neural plate.* **(C)** *The Distal-less expressing non-neural ectoderm has migrated about half-way across the neural plate (arrow).* **(D)** *The Distal-less expressing ectoderm has completed migration over the neural plate (arrow) and fused in the dorsal midline.* **(E)** *A scanning electron micrograph of the dorsal side of a neurula at the same stage as in (C) showing the migrating ectoderm moving over the neural plate. All the ectodermal cells are ciliated.* **(F)** *Higher power magnification of the leading edge of the migrating ectoderm showing the lamellipodia. From Holland* et al., *1996.*

evolution of neural crest. The best example is *FoxD3*. Amphioxus has a single *FoxD* gene, which is not expressed at the edges of the neural plate, whereas vertebrates have five duplicates, only one of which, *FoxD3*, is expressed at the edges of the neural plate and can induce expression of other neural crest specifiers such as *Sox10*. Yu (2010) demonstrated that the acquisition of new *cis*-regulatory elements allowed FoxD3 to be expressed at the edges of the neural plate, while Ono *et al.,* (2014) showed that the vertebrate FoxD3 protein acquired new sequences near the amino terminal allowing it to induce expression of other neural crest genes. Although this is just one gene, it exemplifies how both new regulatory elements and new amino acid sequences in gene duplicates can evolve to create new gene functions.

In the light of the initial work on amphioxus, the subsequent discovery of some cells in ascidian tunicates that migrate from the neural tube or its vicinity (Jeffery *et al.*, 2004; Stolfi *et al.,* 2015) together with the finding that *Twist*, if ectopically expressed in the ascidian neural tube, can induce cells to migrate (Abitua *et al.*, 2012) fit with phylogenetic trees based on concatenated nuclear gene sequences that place tunicates as the closest extant relatives of the vertebrates (Delsuc *et al.*, 2006). However, determining the complexity of the brain of the common ancestor of tunicates and vertebrates remains problematic as tunicates are quite derived. For example, the nervous system of an ascidian has only about 330 cells and even fewer neurons compared to an estimated 20,000 neurons in amphioxus and millions or even billions in vertebrates (Meinertzhagen and Okamura, 2001; Nicol and Meinertzhagen, 1991, Olkowicz *et al.*, 2016). Moreover, ascidian and appendicularian tunicates have both lost several genes expressed in amphioxus and vertebrate brains including *Otx, Gbx* and several *Hox* genes, but the *Hox* genes lost differ between the two tunicates (Edvardsen *et al.*, 2005; Ikuta and Saiga, 2005; Wada *et al.*, 2003). Therefore, if a feature is missing in both amphioxus and tunicates, but present in vertebrates, it is not clear whether it is a vertebrate invention or evolved at the base of the tunicate-vertebrate clade and was lost in the tunicate lineage.

Another example of a gene expression study relevant to a broad biological question is a study of the *engrailed* gene in developing amphioxus. In amphioxus, the somites extend to the anterior tip of the animal. As they form, *engrailed* is expressed in the posterior part of each of the 8-12 anteriormost ones (Holland *et al.*, 1997). This pattern is reminiscent of the expression of *engrailed* during segmentation in *Drosophila* and raised the possibility that a conserved developmental mechanism involved in segmentation was a legacy from a segmented bilaterian ancestor. However, the situation is complicated by data for onycophorans, which are placed either as sister group to chelicerates or as more closely related to insects and crustacea (Min *et al.,* 1998; Strausfeld *et al.,* 2006). In onycophorans, although *engrailed* and other segmentation genes are expressed in stripes in the embryo, they turn on in segmental patterns *after* the segmental furrows have formed (Franke and Mayer, 2014). Moreover, arthropod segmentation genes do not appear to be conserved in segmentation in an annelid (Seaver *et al.*, 2012). Thus, whether the ancestral bilaterian was segmented or not is still an open question.

A related question concerns the evolution of segmentation of the head mesoderm in chordates. It is segmented in amphioxus with *engrailed* expressed in stripes in the future posterior half of the somites as segmentation begins; in contrast, head mesoderm in gnathostomes is unsegmented. Since developing embryos of lampreys and sharks have head cavities, which form mesoderm and resemble the anterior somites of amphioxus, one hypothesis (segmentalist) is that the anterior somites of an ancestral chordate evolved into the head segments (head cavities) of lampreys and sharks and into some of the jaw and eye muscles of bony vertebrates. This idea of an ancestral chordate with a segmented head is supported by expression of *engrailed* in amphioxus, in the posterior wall of the mandibular head segment in lampreys, and in the mandibular mesoderm in the shark as well as in the jaw muscles of gnathostomes (reviewed in Holland *et al.,* 2008b). In contrast, anti-segmentalists (Olson *et al.,* 2005; Kuratani and Adachi, 2016) argue that the ancestors of the vertebrates lacked any such segmentation at the anterior end; thus, head segmentation arose independently in amphioxus, agnathans and sharks. Part of this controversy revolves around the presence of mesodermal condensations (somitomeres) in the heads of gnathostomes. While true somites in gnathostomes extend from adjacent the posterior hindbrain to the tailbud, the somitomeres appear to extend the series of somites anteriorly. Such anterior condensations of mesoderm are clearly present in shark embryos (Gilland, 1992; Holland *et al.*, 2008b). The most anterior one extends a ventral process into the mandibular arch that expresses *engrailed* (Adachi, 2012; Gilland, 1992). However, *engrailed* is but one gene. Expression of other relevant genes such as *Foxl2,* which is expressed in the mandibular head mesoderm and mandibular arch in the shark (Wotton *et al.*, 2007) as well as in the head mesoderm and head muscles in the mouse (Heude *et al.*, 2015) should also be examined in amphioxus and lampreys

The final example shows how studies on gene expression as well as on gene function can help evaluate homologies originally proposed from morphological and biochemical data. In aquatic chordates, a stream of water enters the pharynx through the mouth and exits via the pharyngeal gill slits. In invertebrate chordates and larvae of jawless vertebrates, a pharyngeal organ, the endostyle, secretes mucus that traps food particles. The mucus strand plus food particles are then transported deeper into the digestive tract. Studies on lampreys have shown that at metamorphosis, the endostyle becomes the thyroid (Müller, 1873; Schneider, 1879); reviewed in (Kluge *et al.*, 2005; Leach, 1939).

It has long been thought that the gill slits in hemichordates, amphioxus, tunicates and vertebrates are homologous, and thus that a pharynx perforated by gill slits existed before hemichordates branched from the chordates (Bateson, 1886); reviewed in (Tagawa, 2016). Molecular data have reinforced these homologies (Fig. 3). Our studies of amphioxus showed that as in vertebrates, retinoic acid specifies position along the anterior/posterior axis of the embryo; excess RA respecifies the pharynx as mid-gut and the forebrain as hindbrain/spinal cord (Holland and Holland, 1996; Escriva-Garcia *et al.*, 2002; Onai *et al.*, 2012; Schubert *et al.,* 2004, 2005, 2006). The effect of RA on pharyngeal development in hemichordates has not been studied; however, most genes in the RA pathway have been identified in hemichordates as well as in chordates (Cañestro *et al.*, 2006).

Expression of *Pax1/9* genes in the pharyngeal endoderm is conserved in hemichordates and throughout the chordates (Holland *et al.*, 1995; Müller *et al.*, 1996, Ogasawara *et al.*, 1999). In all groups, *Pax 1/9* becomes downregulated where the gill slits will penetrate. Expression of other pharyngeal markers is also

conserved throughout the chordates, but only partly conserved with hemichordates. For example, in chordates, Pax2/5/8, is expressed where the gill slits will penetrate as well as in the endostyle (gill slits have been lost in land vertebrates), while Tbx1/10, which mediates segmentation of the gill slit region in chordates, is expressed in the mesoderm of the branchial bars between the gill slits. Mutation of *Tbx/10* in the zebrafish inhibits partitioning of the pharynx into gill slits and pharyngeal arches but does not inhibit migration of neural crest into the pharynx (Piotrowski and Nüsslein-Volhard, 2000). This result confirmed what we had found in amphioxus, which lacks neural crest, that pharyngeal segmentation results chiefly from interactions between the endoderm and head mesoderm and is not caused by neural crest migrating into the pharyngeal arches. However, even though the gill bars in amphioxus, hemichordates and tunicates contain collagen, in hemichordates, *Tbx1/10* is not expressed in between gill slits (Gillis *et al.*, 2012.

One pharyngeal organ that may be absent in hemichordates but present in chordates is the endostyle, which is homologous to the vertebrate thyroid. Homology between the endostyle of invertebrate chordates and the thyroid gland of vertebrates was initially suggested by biochemical studies showing that endostyles, like the vertebrate thyroid gland, metabolise iodine to form iodo-thyronines and the synthesis of similar thyroglobulins and peroxi-dases (reviewed in Holland and Holland, 1999). Not so long ago, however, the available evidence left some biologists unconvinced; for example, Burrow (1989) stated, "evidence of thyroid evolution from prevertebrate ancestry is inconclusive." More recently, the homology has been strengthened by discoveries that correspond-ing genes were involved in the development of the amphioxus endostyle and vertebrate thryroid: namely *Pax2/5/9* (Kozmik *et al.*, 1999), and *Nk2-1*, also known as *thyroid transcription factor 1* (TTF1) (Venkatesh *et al.*, 1999). Thus an already respectable homology was considerably strengthened by the addition of gene expression data.

It has also been suggested that the stomochord of hemichordates, which is an extension of the pharynx may be homologous to the endostyle and thyroid gland of chordates since *FoxE* is expressed in the club-shaped gland in amphioxus (Yu *et al.,* 2002), which is a larval secretory organ adjacent to the larval endostyle, and in the endostyle and thyroid in other chordates (Satoh *et al.*, 2014). NKX2-1 is also expressed in the stomochord of hemichordates, but expression is fairly wide-spread in the endoderm leading to the idea that perhaps the gene started as a general endodermal gene that was coopted later in evolution for the development of the endostyle (Takacs *et al.*, 2002). Therefore, as the function of the stomochord is unknown, possible ho-mologies with the chordate endostyle remain uncertain.

Taken together, evidence from morphology, bio-chemistry and developmental genes indicates that a pharynx with gill slits and possibly an endostyle was present in the common ancestor of hemichordates and chordates. The gene network specifying the pharynx was modified at the base of the chordates. In the vertebrate lineage, the endostyle was modified into the thyroid gland, and subsequently, in land vertebrates, expres-sion of *Pax2/5/8* in the pharyngeal pouches of was lost together with the gill slits. When all lines of evidence agree, proposed homologies become more certain.
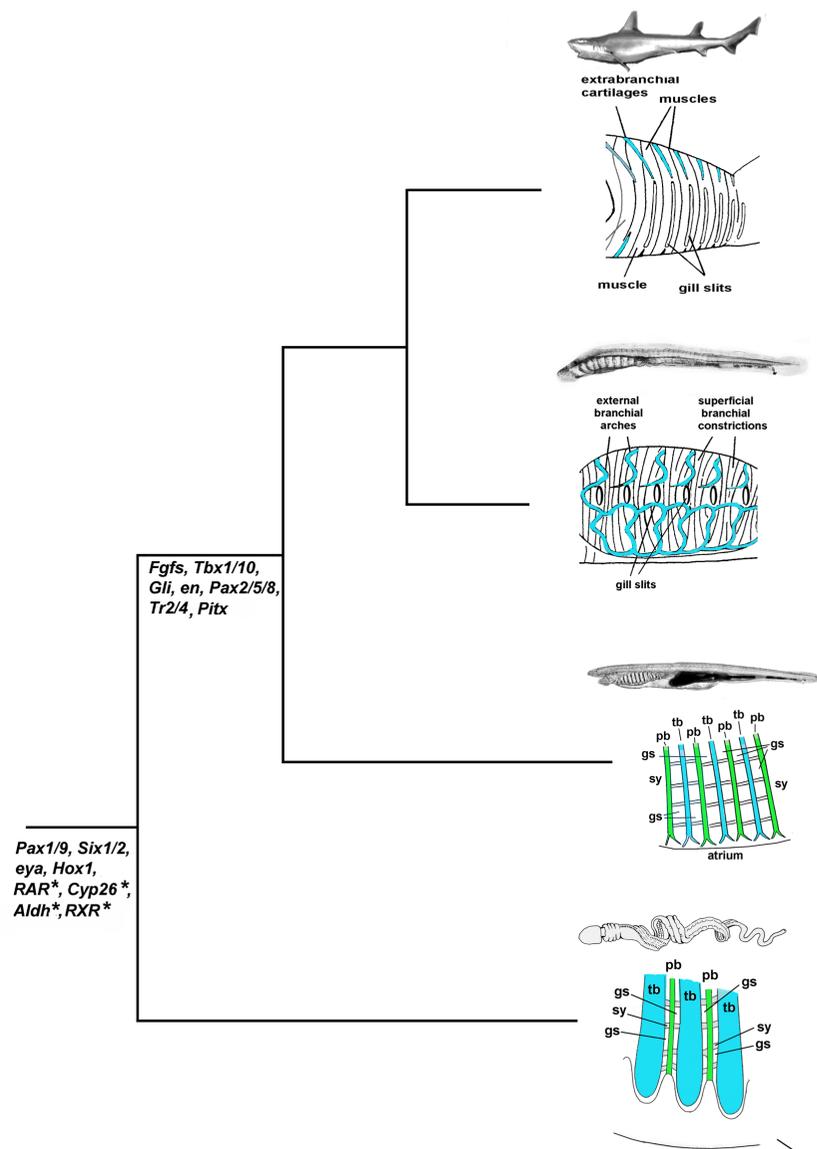

Fig. 3. Evolution of gill slits in deuterostomes. *The pharyn-geal endoderm of the ancestral deuterostome was patterned by Pax1/9, Six1/2 and Eya, with Hox1, possibly regulated by retinoic acid (RA) signaling, establishing the posterior bound-ary of the pharynx. RA binds to heterodimers of the retinoic acid receptor (RAR) and retinoid X receptor (RXR). Cyp26 is an enzyme that degrades RA. Tr2/4 is a competitive inhibitor of RA, that is co-expressed with Pax2/5/8 where endoderm and ectoderm are fusing to form gill slits. In the ancestral chordate, Engrailed (En) and Tbx1/10 are expressed in the anteriormost somites that send Tbx1/10-expressing extensions into the pharynx between the forming gill slits. Fibroblast growth factors (FGFs) and Pitx are also vital in pharyngeal patterning. In vertebrates, neural crest cells contribute to the developing pharynx. Asterisk = pharyngeal expression unknown in hemichordates; expression of Tr2/4 in the verte-brate pharynx has not been determined. Abbreviations: gs, gill slits; sy, synapticles; tb, tongue bar (secondary pharyngeal bar); pb, primary pharyngeal bar.*

The genomics era

The genomics era (grey part of curve in Fig. 1) has provided new tools for understanding how chordates evolved. For amphioxus, such studies began in the early 2000s with the construction and end-sequencing of gridded cDNA libraries [EST (expressed sequence tags) analysis] of the Florida amphioxus, *Branchiostoma floridae*, and BAC libraries of its genome (Yu *et al.*, 2008c). Subsequently, the *B. floridae* genome was sequenced (Holland *et al.*, 2008a; Putnam *et al.*, 2008). The genome sequence of a second species of *Branchiostoma*, *B. belcheri*, was published in 2014 (Huang *et al.*, 2014). The genome of the European amphioxus, *B. lanceolatum*, is close to publication and analysis of its transcriptome during development has been done (Oulion *et al.*, 2012). These studies have shown that even though amphioxus and vertebrates split over 500 mya, the 520 mb *B. floridae* genome has a great deal of synteny with the much larger vertebrate genomes (3 gb for humans) and confirmed that vertebrate genomes underwent 2 rounds of whole genome duplication. Many of the duplicates were lost, but duplicate genes for signaling pathways and developmental genes were disproportionately retained.

Recently, transcriptomics and genomics of *Asymmetron lucayanum*, the sister genus of the two other genera (*Branchiostoma* and *Epigonichthys*) have been published (Yue *et al.*, 2014, 2016). Comparisons between *Asymmetron* and *Branchiostoma* genomes showed that the two genera, even though they split about 128-160 mya, are about the right distance apart for comparisons of the non-coding regions to reveal functional elements such regulatory elements (Yue *et al.*, 2016). This demonstrates remarkably slow evolution, which was confirmed by comparisons of about 430 gene groups, showing that amphioxus are evolving more slowly than the slowest-evolving vertebrate known, the elephant shark (Yue *et al.*, 2014). In light of this slow evolution, the report that protein evolution is especially rapid in amphioxus (Huang *et al.*, 2014) was quite surprising. However, this result has been questioned as likely being due to high rates of errors in gene prediction (Bányai and Patthy, 2016).

In addition, the availability of genome and transcriptome sequences has led to a great deal of "genome mining" and numerous papers on phylogeny and evolution of various gene families. From 2008 to present, the average rate of publications on amphioxus doubled, compared to the period from 1992 when the first paper on genes and development appeared (Holland *et al.*, 1992), Among other things, these papers have focused on identification of microRNAs (Candiani *et al.*, 2011; Chen *et al.*, 2009); understanding their function, the genes they regulate and the role of miRNA function in evolution will likely receive considerable attention in the future.

Conclusions

Since the second half of the 19th century, amphioxus has played a prominent role in discussions of how the vertebrates evolved from their invertebrate ancestors. Initially, the focus was on comparative anatomy. That was followed in the mid-20th century by comparative biochemistry. A major breakthrough was the discovery in the late 20th century that developmental genes such as the *Hox* family were widely conserved across phyla and in the early 21st century that comparative genomics could give insights into how genomes have evolved to create new structures such as vertebrate neural crest. When several lines of evidence point to the same conclusion concerning homologies among several organisms, the inferences are most likely correct. However, when there are discrepancies, it can be very difficult, for example, to decide whether or not there has been convergent evolution or secondary simplification. In the coming years, comparative genomics, biochemistry and evo/devo of amphioxus will continue playing a major part in gaining insights into the evolution of gene networks and the relation between genotype and phenotype.

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