



Lissamphibian limbs and the origins of tetrapod *hox* domains

Joost M. Woltering^{*}, Michaela Holzem^{1,2}, Axel Meyer

Chair in Zoology and Evolutionary Biology, Department of Biology, Universität Konstanz, Universitätsstraße 10, 78464, Konstanz, Germany

ABSTRACT

The expression and function of *hox* genes have played a key role in the debate on the evolution of limbs from fins. As an early branching tetrapod lineage, lissamphibians may provide information on the origin of the limb's *hox* domains and particularly how the plesiomorphic tetrapod pattern compares to the *hox* pattern present in fish fins. Here, we comparatively investigated the expression of *hox* genes in the developing limbs of axolotl and *Xenopus laevis* as well as in the fins of the direct developing cichlid *Astatotilapia burtoni*. In contrast to axolotl, which has only very low digital expression of *hoxd11*, *Xenopus* limbs recapitulate the reverse collinear *hoxd* expression pattern known from amniotes with clearly defined proximal and distal *hoxd11* expression domains. For *hoxa* genes, we observe that in *Xenopus* limbs, as in axolotl, a clear distal domain of *hoxa11* expression is present, although in the presence of a *hoxa11* antisense transcript. Investigation of fins reveals the presence of *hoxa11* antisense transcription in the developing fin rays in a domain similar to that of *hoxa13* and overlapping with *hoxa11* sense transcription. Our results indicate that full exclusion of *hoxa11* from the autopod only became firmly established in amniotes. The distal antisense transcription of *hoxa11*, however, appears to predate the evolution of the limb, but likely originated without the concurrent implementation of the transcriptional suppression mechanism that causes mutually exclusive *hoxa11* and *hoxa13* domains in amniotes.

1. Introduction

The universally conserved anatomical architecture of the tetrapod limb, as first highlighted in 1849 by Richard Owen (1849), is an often and widely repeated textbook example of homology. This *Bauplan* consists of the proximal longbones of the stylopod and zeugopod, followed by the nodular bones of the wrist and ankles (mesopodium), which articulate distally with the long bones of the metacarpals and digits. This “cross-articular” pattern is highly constrained throughout the radiation of tetrapods (land animals) and is believed to have originated at the fin-to-limb transition around 380MYA. The bimodal anatomy of tetrapod limbs is mirrored in the expression and function of the *hox11* and *hox13* paralogs from the *Hoxa* and *Hoxd* clusters, which are instructive for the formation of the zeugopod and the autopod respectively (Woltering and Duboule, 2010). In the amniote limb, expression of *hoxa13* and *hoxd13* is restricted to the autopod and digits (Nelson et al., 1996; Fromental-Ramain et al., 1996), while *hoxa11* is transcribed in the zeugopod only (Nelson et al., 1996). Recent work has suggested that the mutually exclusive expression of *hoxa11* and *hoxa13* is mediated through antisense transcription of *hoxa11* in the autopod (Kherdjemil et al., 2016; Kherdjemil and Kmita, 2017). *Hoxd11* is expressed in both proximal and distal limb domains as a result of the two temporally and spatially separate phases that activate *hoxd* gene expression (Kmita et al., 2002; Beccari

et al., 2016).

The consensus is that a tetrapod-like cross-articular organisation is absent from the endochondral skeleton of fish fins (Woltering and Duboule, 2010; Wagner and Chiu, 2001). Comparative expression profiling in ray-finned (actinopterygian) fish has indeed shown that expression of *hoxa11* and *hoxa13* overlap (Tulenko et al., 2017; Metscher et al., 2005; Ahn and Ho, 2008), and allegedly no distally confined *hoxa11* antisense transcription occurs (Kherdjemil et al., 2016; Kherdjemil and Kmita, 2017). The presence of separated proximal and distal *hoxd* phases in fish fins remains debated, but the most recent data on basal lineages such as paddlefish describe a single continuous proximo-distal domain with a lower level of expression in the developing fin rays (Tulenko et al., 2016). Similarities exist between the patterning of the digits and the dermal fin rays and both are dependent on the function of *hox13* genes (Ahn and Ho, 2008; Nakamura et al., 2016; Wood and Nakamura, 2018; Gehrke et al., 2015). Therefore, it appears that if any “bimodal” *hox* gene signature exists in fish fins, this is between the articulating parts of endochondral radials and the dermal fin rays. How the fish fin *hox* patterns evolved into the bimodal *hox* domains of the tetrapod limb during the fin-to-limb transition remains however highly debated (Woltering and Duboule, 2010; Nakamura et al., 2016; Wood and Nakamura, 2018; Woltering et al., 2014; Schneider and Shubin, 2013; Stewart et al., 2017).

^{*} Corresponding author.

E-mail addresses: joost.woltering@uni-konstanz.de, jmwoltering@gmail.com (J.M. Woltering).

¹ Current address: Division of Signaling and Functional Genomics, German Cancer Research Center (DKFZ), Heidelberg, Germany.

² Current address: Department of Cell and Molecular Biology, Medical Faculty Mannheim, Heidelberg University, Heidelberg, Germany.

The question of the fin-to-limb transition has been mostly addressed with reference to the well-known *hox* patterns of amniotes (mouse and chicken), which may not be the best suited for inferring the ancestral tetrapodial condition. Lissamphibians (i.e. frogs, salamanders and caecilians) were the earliest extant lineage to branch from the tetrapod stem (Parsons and Williams, 1963; Jurgens, 1971; Irisarri et al., 2017) and given this phylogenetic position, their *hox* domains could be informative for a reconstruction of the ancestral patterns present at the time of fin-to-limb transition. In spite of the overall highly constrained nature of the tetrapod limb, notable differences in limb structure and ontogeny exist in amphibians. Whereas frog limbs mostly resemble the amniote condition, urodeles (newts and salamanders) show departures in limb structure and ontogeny that are difficult to reconcile with their phylogenetic position. These for instance concern a pre-axial dominance during digit formation and an unusual ontogenetic sequence of mesopodial development (Jarvik, 1965, 1980; Frobisch and Shubin, 2011; Holmgren, 1933, 1949; Johanson et al., 2007). Although the similarity between frogs and amniotes suggests that the urodele condition is derived, recent paleontological analysis (Frobisch et al., 2015) does not necessarily support this notion and scenarios of convergence should perhaps not be excluded with respect to the closer resemblance of frog and amniote limbs. Therefore a detailed anatomical and molecular comparison between urodele and frog limbs, set side by side with fish fins, appears justified for the purpose of reconstructing the ancestral tetrapod limb condition.

In this study, we revisit and provide a further comparison of the *hox11* and *hox13* expression patterns in urodele and frog limbs within the context of their differing modes of limb development and their phylogenetic position as an early branching tetrapod lineage. In addition, we provide data for the expression of these genes in a direct developing ray finned fish, the cichlid *Astatotilapia burtoni* (Woltering et al., 2018). This species shows a rapid developmental progression from fin budding to the

formation of dermal lepidotrichia, and therefore provides easy access to the full continuum of developmental events involved in fin formation.

2. Results

2.1. The non-canonical expression domains of axolotl limbs

In the urodele axolotl (*Ambystoma mexicanum*), non-canonical expression domains of *hox* genes have been reported (Carlson et al., 2001; Bickelmann et al., 2018; Gardiner et al., 1995; Torok et al., 1998; Wagner et al., 1999) whereby the expression domains of the *hoxa11*, *hoxa11*-antisense and *hoxd11* genes are of particular interest. *Hoxa13* and *hoxd13* are expressed in distal autopodial and digital arch territories, with as previously reported absence of *hoxd13* expression from the anterior-most digit (Bickelmann et al., 2018) (Fig. 1, red arrowhead). We further investigated the expression of *hoxa11* in the autopod using two separate non-overlapping probes to provide additional validation of the unusual expression domains reported before. To detect sense transcription through the locus we used one probe corresponding to exon 1 and an additional probe corresponding to the 3' UTR region. Using either of these probes we detect a near homogenous expression domain including the autopod and the zeugopod, without any indications of the autopodial repression implemented in amniotes (Wagner et al., 1999; Bickelmann et al., 2018; Kherdjemil and Kmita, 2017) (Fig. 1, blue arrowhead). To detect potential *hoxa11* antisense transcription we used a “sense” probe derived from the *hoxa11* exon 1 construct, which does not result in noticeable staining. For *hoxd11* (Torok et al., 1998; Bickelmann et al., 2018), we also detect a strong zeugopodial domain and much lower levels of expression distally in the autopod as well as more proximally in the region of the stylopod. A separation of discrete proximal and distal domains by a “mesopodial gap” appears to be non-existent.

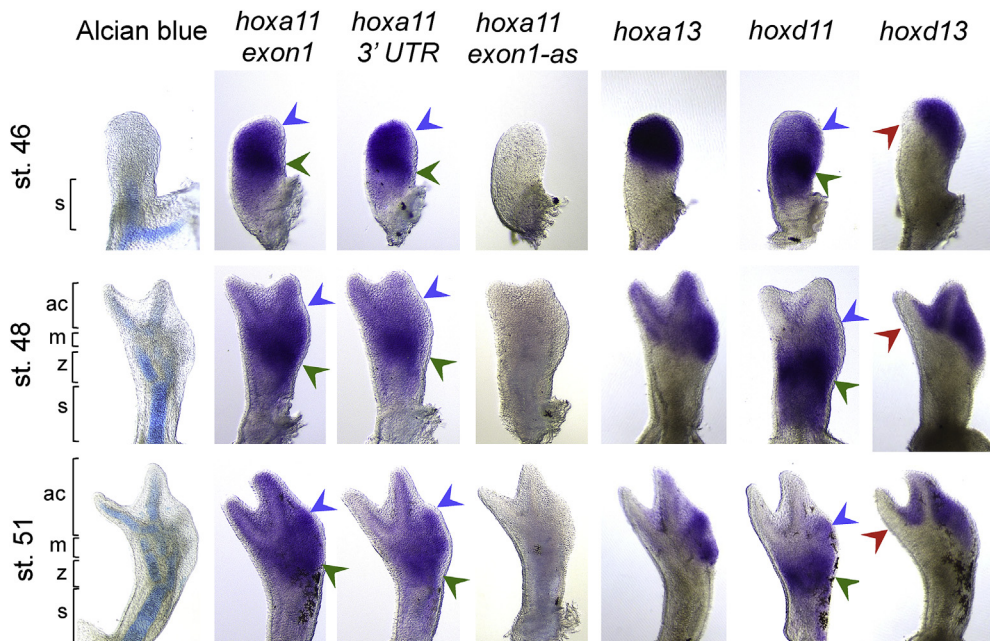


Fig. 1. Expression of *hox* genes during development of the forelimbs in axolotl. Expression domains of a panel of *hox* genes analysed during the formation of the axolotl limb covering developmental stages during which the zeugopodial and the autopodial domains are specified (see Alcian blue cartilage stains in the left most column). *Hoxa11* is expressed in a near continuous domain covering the zeugopod (green arrowhead) and the autopod (blue arrowhead). The expression of this gene was assayed using two separate non-overlapping probes corresponding to exon1 and the 3'UTR in order to control for staining specificity. A probe designed to detect a potential antisense transcript of *hoxa11* corresponding to exon1 fails to detect any specific staining, suggesting the absence of such transcript. *Hoxd11* is expressed in a strong zeugopodial domain (green arrowhead) but in a much weaker autopodial domain (blue arrowhead). A gap in expression between these domains corresponding to the mesopodium appears absent. *Hoxa13* and *hoxd13* show expected distal expression domains in the entire autopod (*hoxa13*) and the acropod (*hoxd13*) respectively, whereby *hoxd13* is absent from the *Anlage* of the first digit at all three stages investigated (red arrowhead). Developmental stages investigated are stage 46 to stage 51 (defined after Nye et al., 2003.). Abbreviations; as: antisense, ac: acropod, m: mesopod, z: zeugopod, s: stylopod, st.: stage. Anterior is to the left.

2.2. Expression of posterior *hoxa* and *hoxd* genes in *A. burtoni*

In certain aspects the *hox* patterns observed in axolotl could be interpreted as being “fish-like” given the overlapping expression of *hoxa11* and *hoxa13* and the absence of *hoxa11* antisense transcription. Such similarities between axolotl and fish are well illustrated by the *hox* expression territories in a fin staging series of the direct developing cichlid *A. burtoni* (Woltering et al., 2018) (Fig. 2). Due to the teleost specific genome duplication, *A. burtoni* possesses two *Hoxa* clusters and two *Hoxd* clusters, although the 5' end of the *Hoxd* cluster is only preserved in the *Hoxda* cluster (Hoegg et al., 2007). *Hoxa11b* clearly shows expression in a continuous territory along the proximo-distal fin axis, and distal expression of *hoxa11b* overlaps with the expression of *hoxa13a* and *hoxa13b*. The *hoxa11a* copy is expressed proximally only, likely due to the subfunctionalization within the acanthomorph fish lineage, which has resulted in muscle specific expression for the *hoxa11a* duplicate gene, as has been described for Medaka (Takamatsu et al., 2007). In contrast to previous reports investigating the existence of antisense transcription through the *hoxa11* loci (Kherdjemil et al., 2016), we do detect expression of the antisense transcripts derived from exon 1 of *hoxa11b* and of *hoxa11a* (albeit the latter is weaker and starts later during development) in the distal fin overlaying the fin rays in a domain similar but not identical to that of the *hoxa13* genes (Fig. 2, blue arrowhead). This indicates that the antisense transcription of *hoxa11* loci in the distal paired appendages predates the evolution of tetrapods, but without apparent consequences for the expression of the sense strand given that the *hoxa11b* sense transcript is strongly expressed distally in an overlapping territory. As *A. burtoni* has lost the *hoxd13a* gene (Hoegg et al., 2007), we investigated the expression of *hoxd11a* and *hoxd12a*, which are the most 5' genes present in the *A. burtoni* *Hoxda* cluster (Hoegg et al., 2007). Throughout development, expression of *hoxd11a* and *hoxd12a* is confined to the posterior half of the fin, which extends distally into the dermal region of the skeleton, without the appearance of a clear intermediate zone of low expression as present in the tetrapod limb at the position of the mesopodium.

2.3. *Hox* expression domains in *Xenopus laevis*

Frogs have a more amniote like pattern of limb development than urodeles, showing post-axial dominance during the formation of the digital arch. As not all aspects of their *hox* expression domains have been extensively investigated it remains open to what extent these recapitulate the canonical amniote pattern. The *hoxa* expression domains in *X. laevis* were previously characterised in whole mounts (Blanco et al., 1998; Satoh et al., 2006) and on sectioned limb buds, and *hoxd* expression was analysed on sections (Satoh et al., 2006). Given that a direct comparison of expression domains between sections and whole mounts can be complicated, and because past *in situ* hybridizations experiments were likely performed without the anticipation of a potential distal *hoxa11* domain, (which therefore might have been disregarded as background due to technical issues), we decided to reinvestigate the expression domains of *hoxd11*, *hoxd13* as well as of *hoxa11*, *hoxa11*-antisense and *hoxa13* in a staging series of *Xenopus* limb development (~st. 52–54 (Nieuwkoop and Faber, 1994)) (Fig. 3). *Hoxd13* is detected in a canonical domain in the digital arch spanning all four digits. In the proximal limb, expression of *hoxd11* is detected in two separated domains, one corresponding to the zeugopod and one domain more proximally, probably associated with the condensing humerus (Fig. 3, orange arrowhead). In the autopod, *hoxd11* is expressed along the distal margin in the forming digits, more distally than *hoxd13*. At the position of the most anterior digit (Fig. 3, red arrowhead) there is a conspicuous domain of lower *hoxd11* expression showing reverse collinear expression, similar to that reported for amniote limbs (Woltering and Duboule, 2010; Nelson et al., 1996; Vargas et al., 2008; Montavon et al., 2008). *Hoxa13* is expressed in a distal domain covering the entire autopod including the mesopodium. Expression of *hoxa11* is detected in a strong zeugopodial domain (Fig. 3, green arrowhead), which however, clearly extends into the autopod (Fig. 3, blue arrowhead), whereas proximal expression at the base of the limb is entirely absent, providing a control for the general background staining in the experiments. The autopodial domain of *hoxa11* expression is particularly obvious in the two younger stages investigated, but also clearly detectable at stage 54 when the condensations in the digital arch form. At all stages investigated however, the expression in the distal

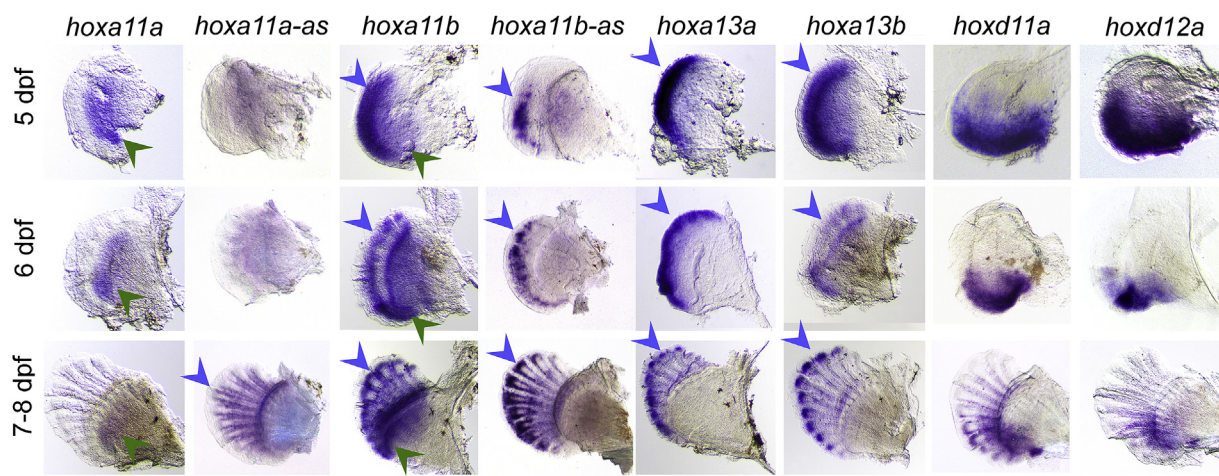


Fig. 2. Expression of *hox* genes during development of the pectoral fin in *Astatotilapia burtoni*.

Analysis of the expression of *hoxa11*, *hoxa13* paralogs as well as *hoxd11a* and *hoxd12a* in the pectoral fins of *A. burtoni* covering 5–8 days after fertilization (Woltering et al., 2018). *Hoxa11b* displays the expected expression in both proximal and distal fin as reported for instance *Polyodon* (Tulenkov et al., 2017) (red and blue arrowheads). Note that as a result of subfunctionalization, as has been reported for Medaka (Takamatsu et al., 2007), *hoxa11a* is only expressed in the myotome overlying the pectoral fin while *hoxa11b* is expressed in the mesenchyme. *Hoxa13a* and *hoxa13b* are expressed in the distal fin, at later stages corresponding to the developing fin rays (blue arrowheads). Contrary to previous reports in zebrafish, we do detect expression of the antisense transcripts of *hoxa11b* and *hoxa11a* (the latter at a distinctly lower level) in the distal fin. *Hoxa11b* expression is observed in cells migrating into the fin fold (5 dpf, blue arrowhead) and in the forming fin rays (7–8 dpf, blue arrowhead). *Hoxd11a* and *hoxd12a* are expressed in the posterior half of the fins, with at a later stages expression at the distal margin of the endoskeleton (7–8 dpf, green arrowhead) and weaker expression more distally in the developing fin rays (7–8 dpf, blue arrowhead). Abbreviations; as: antisense, dpf: days post fertilization. Anterior is up, distal is to the left.

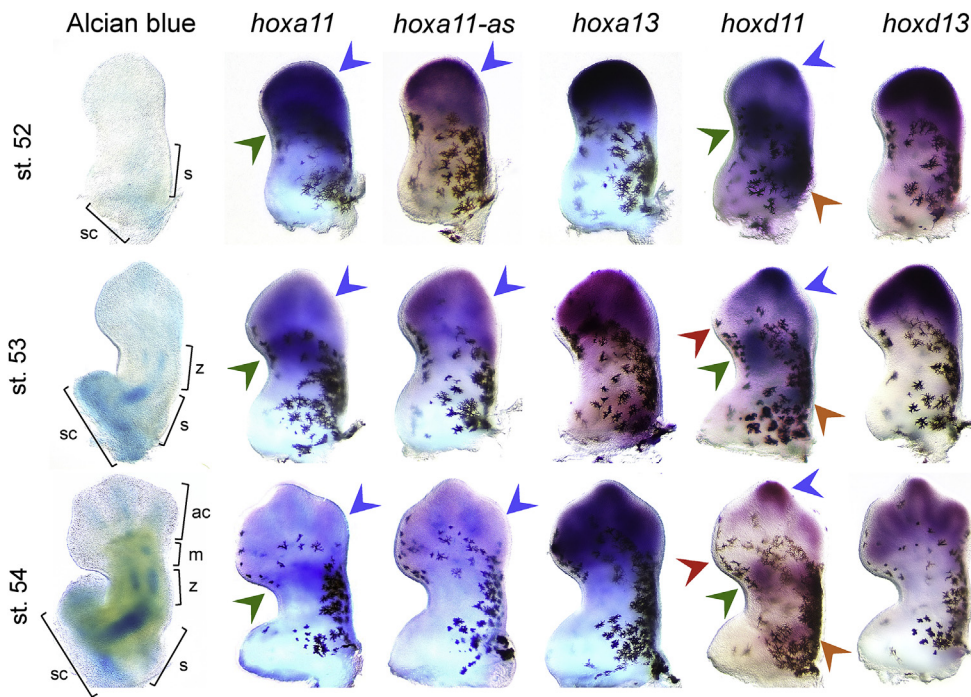


Fig. 3. Expression of *hox* genes during development of the forelimbs in *Xenopus laevis*.

Expression domains of a panel of *hox* genes analysed during the formation of the forelimb of *Xenopus laevis* covering the developmental stages during which zeugopodial and autopodial domains are specified (see Alcian blue cartilage stains in left-most column). *Hoxa11* is expressed in the expected zeugopodial domain (green arrowhead) but also in a weaker autopodial domain (blue arrowhead, and compare to the complete absence of staining at the base of the limb). A *hoxa11*-antisense transcript is detected in the autopod, in a domain similar to that of *hoxa13* using a probe derived from the exon1 sequence. *Hoxd13* is expressed in the expected distal domain covering the entire digital arch. *Hoxd11* is expressed in a zeugopodial (green arrowhead) and autopodial (blue arrowhead) separated by a mesopodial gap in expression. Consistent with the expression of *hoxd11* in amniotes this gene is absent, or expressed at a distinctly lower level, from the most anterior digit (red arrowhead) (Woltering and Duboule, 2010; Vargas et al., 2008; Nelson et al., 1996). Additional expression of *hoxd11* is detected more proximally in a putative stylopodial domain (orange arrowhead). Developmental stages investigated are stage 52 to stage 54 (defined after Nieuwkoop and Faber, 1994). Abbreviations; as: antisense, ac: acropod, m: mesopod, z: zeugopod, s: stylopod, sc: scapulo-coracoid, st.: stage. Anterior is to the left.

domain of *hoxa11* is less intense than in the zeugopodial domain, and a near continuous domain as seen in axolotl limbs or fins is never observed in *Xenopus*. To investigate the presence of a potential *hoxa11* antisense transcript, we used a “sense” probe derived from the *hoxa11* exon 1 plasmid. Using this probe we detect expression in the autopodial part of the limb (Fig. 3, blue arrowhead) in a pattern similar to that of *hoxa13* and mutually exclusive with the strong zeugopodial domain of *hoxa11*.

3. Conclusions and discussion

3.1. Lissamphibian *hox* gene expression

When compared to the amniote *hox* domains known from mouse and chicken several departures are observed in *Xenopus* and axolotl. In axolotl, the expression of *hoxd11*, and as further reported *hoxd10* (Torok et al., 1998), are characterized by the lack of discrete proximal and distal territories separated by a mesopodial gap and an overall lower level of distal gene expression. The close resemblance between the frog and the amniote patterns, whereby *hoxd11* is expressed in discrete proximal and distal phases and also recapitulates the absence of *hoxd11* from the anterior-most digit, strongly suggests that this non-canonical *hoxd* pattern in urodeles is best interpreted as being derived. Considering the *hoxa* genes, in both *Xenopus* and axolotl *hoxa11* is expressed in a distal domain overlapping with *hoxa13*. The overlap of *hoxa11* and *hoxa13* thus at least suggests that the mutually exclusive pattern of *hoxa11* and *hoxa13* expression only became canalised in amniotes (Fig. 4). In this sense, the overlapping domains of 5' *hoxa* domains can be seen as reflecting the ancestral expression domains in the distal appendages as present in fish fins.

In *Xenopus*, we also detect the presence of autopodial *hoxa11* antisense transcription although this appears absent from axolotl. The strong signal we detect for the *hoxa11b* antisense transcripts in *A. burtoni* indicates that its expression in the distal appendages is likely an ancestral

trait, occurring in fish as well as in tetrapods, but secondarily lost from axolotl. How the lack of suppression of *hoxa11* sense transcription in fish and *Xenopus* can be reconciled with the presence of such antisense transcription remains to be further investigated, but it suggests that more is required in terms of molecular machinery than a mere transcript arising in opposite direction from the 5' end of *hoxa11* locus for transcriptional interference to occur.

3.2. The origins of tetrapod *hox* patterns

The cross-articular anatomy of the tetrapod limb became established during the conquest of land at the fin-to-limb transition. The paleontological record indicates significant subsequent modifications to the tetrapod limb during the course of its evolution, such as the establishment of pentadactyly (Galis, 2001; Woltering and Meyer, 2015) and changes in the structure of the mesopodium (Johanson et al., 2007). Axolotls and frogs undoubtedly have “modern” limbs exhibiting a full mesopodium and the Lissamphibia arose only after the evolutionary fixation of the pentadactyl *Bauplan*. Nevertheless, the departures from the amniote *hoxa* expression pattern do resemble those from ray-finned fish and likely represent an ancestral tetrapod state that was preserved in the lissamphibian lineage. Artificial overexpression of *hoxa11* results in polydactyly (Kherdjemil et al., 2016; Kherdjemil and Kmita, 2017) and our results support a scenario by which the early stem tetrapods still had overlapping domains of *hoxa11* and *hoxa13* expression contributing to their polydactylous phenotypes (Fig. 4). It is intriguing that urodeles and frogs often possess a so called “pre-pollux” or “pre-hallux” that can take the form of a sixth digit (Galis, 2001; Woltering and Meyer, 2015; Hayashi et al., 2015), and therefore could indeed result from the autopodial *hoxa11* expression in these species.

One emerging view concerning the origin of the autopod is that there might be regulatory convergence, co-option or shared ancestry between the autopod of tetrapods and the dermal rays of actinopterygian fish

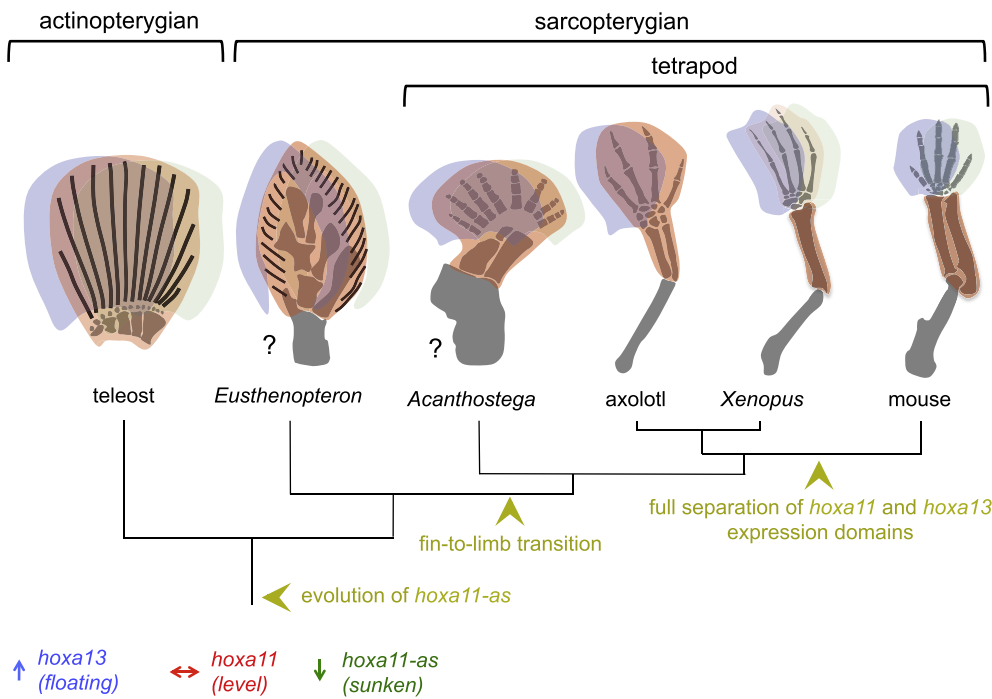


Fig. 4. Evolution of the *hoxa11/hoxa13* expression domains in fins and limbs.

In amniotes (mouse limb shown on the right), the patterns of *hoxa11* and *hoxa13*/*hoxa11*-antisense are mutually exclusive (Woltering and Duboule, 2010; Wagner and Chiu, 2001). In axolotl and *Xenopus*, autopodial expression of *hoxa11* occurs overlapping with the expression of *hoxa13* as such resembling the ancestral condition as present in fish fins. The autopodial expression of a *hoxa11*-antisense transcript (Kherdjemil et al., 2016; Woltering et al., 2014; Sheth et al., 2014) the generation of which is instrumental for the autopodial exclusion of the *hoxa11* sense transcript in the mouse (Kherdjemil et al., 2016), is detected in *Xenopus* but appears absent from axolotl (Bickelmann et al., 2018). Surprisingly we also have detected the presence of 5' antisense transcription of *hoxa11* in the distal, dermal fin skeleton of ray-finned fish. These observations suggest either lability or absence of the *hoxa11/hoxa13* exclusion mechanism in early branching tetrapods although it is clear that the distal anti sense transcription of *hoxa11* loci predates the evolution of the tetrapod limb. Loss of autopodial *hoxa11* expression could be related to the establishment of the canonical pentadactylous condition of tetrapods from the polydactylous condition as occurred in stem tetrapods (such as *Acanthostega*) (Kherdjemil and Kmita, 2017). It remains unclear how *hoxa13* is expressed in sarcopterygian fish (as well as in the direct ancestors of tetrapods, here illustrated using the fin of *Eusthenopteron*). These latter species also possess dermal fin ray and expression profiling in ray-finned fish (here illustrated using the fin of *A. burtoni*) indicates that expression of *hoxa13* occurs primarily in the dermal part of the fin skeleton (Tulenko et al., 2017; Nakamura et al., 2016). The *hox* domains are depicted as overlays to the skeleton, whereby *hoxa13* (blue) is floating, *hoxa11* (red) is level and *hoxa11-antisense* (green) is sunken. Anterior is to the left.

(Tulenko et al., 2016, 2017; Nakamura et al., 2016; Paco and Freitas, 2017). This in fact resembles Richard Owen's original notion of the similarity between fish fin rays and digits (Owen, 1849). Such scenario might find support in the very similar expression profiles of *hoxa11*, *hoxa11*-antisense and *hoxa13* in fish fins and amphibians limbs in each of which the proximal *hoxa13* boundary coincides with the main articulation (Fig. 4). However, whether the cross-articular architecture of the limb was indeed inherited from the articulation between the endochondral and dermal skeleton remains to be further investigated, preferably also involving sarcopterygian fish, which provide the closest living approximation to the direct ancestors of tetrapods.

4. Methods

In situ hybridization was performed according to Woltering et al. (2009) with modifications Woltering et al. (2014); Woltering and Duboule (2015). *In situ* hybridization probes were cloned in the pGEMT vector for RNA synthesis. Primer sequences used are listed in Supplementary Table I. For *Xenopus laevis* the probes cloned correspond to exon

1 of S or L paralogs. Sequence homology for the regions used is over 95% (BLASTN similarity) between L and S paralogs, therefore under the used stringency conditions (50% formamide/5x SSC/65 °C) probes are cross-reactive with both paralogous genes and will show the combined expression domain. Animal experiments were performed with permission of the responsible veterinarians (*Tierschutzbeauftragten*) under permit nr. T-15/05TFA, T-17/08TFA, T-17/16TFA, T18/04TFA, and T18/05TFA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ydbio.2019.08.014>.

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