Lissamphibian limbs and the origins of tetrapod hox domains

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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 pleiomorphic 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 hox 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 a hoxa11 antisense transcription in the developing fin rays in a domain similar to that of hoxd13 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 hoxd13 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 hoxd13 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).

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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 tetrapod condition. Lissamphibians (i.e. frogs, salamanders and cęcilians) 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 mesodermal 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 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 mesodermal 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.

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; Tokor 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 autopodal 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 hoxa11 (Tokor 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 autopodal 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 zeugopodal domain (green arrowhead) but in a much weaker autopodal 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 S’ 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 hoxa13a gene (Hoegg et al., 2007), we investigated the expression of hoxd11a and hoxd12a, which are the most S’ 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 hox 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 zeugopodal 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 Astatotlaplia burtoni.](image-url)
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 *hoxa11* 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 Knörr, 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
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

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References


