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Axial variation in the threespine stickleback: relationship to *Hox* gene expression

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Abstract Despite mounting evidence that key developmental regulator genes are involved in significant macroevolutionary changes, there have been few studies demonstrating the functional significance of variation in such genes for the generation of population-level variation. In this study we examined and compared the expression domains of three *Hox* gene homeobox sequences in embryos derived from two morphologically distinct populations of the threespine stickleback, *Gasterosteus aculeatus*. We found within-population variation in the location of anterior limits, particularly in more 5' *Hox* genes whose anterior expression domains showed graded distributions of transcripts over several somites. However, despite considerable and statistically significant differences in the anteroposterior pattern of the axial and median skeletons between the two stickleback populations, this phenotypic variation was not found to be correlated with any of the variation in *Hox* gene expression. The possible functional significance of the combinatorial *Hox* code in fish species is discussed with respect to the buffering of development in fluctuating environments, and it is argued that population and quantitative genetic perspectives should also be taken into account in considering the function and evolution of *Hox* genes.

Key words Hox *genes* · Hox *code* · *Variation* · Gasterosteus aculeatus · *Axial skeleton*

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Introduction

According to the modern synthesis of evolutionary theory with genetics, morphological divergence at higher taxonomic levels is ultimately a consequence of the segregation of variation within species (Dobzhansky 1937). Yet there remain few demonstrations of the functional significance of polymorphism in specific genes that diverge in function or expression between species or genera. Despite the mounting evidence that key regulatory genes are involved in significant macroevolutionary changes, there is little indication that these genes contribute to population-level variation (Gibson and van Helden 1997; Palopoli and Patel 1996). On the contrary, some of these genes are involved in the generation of highly stable phenotypes such as the general form of the body plan. Until it is shown whether and how developmental control genes contribute to morphological variation, it will not be possible to understand fully how ecological and historical factors impinge upon the evolution of genetic pathways.

One promising character for studying links between micro and macroevolutionary changes is the structure of the vertebral column and associated skeletal elements. Variation in the number of the different types of vertebrae is prevalent in both tetrapods (Danforth 1930; Green 1941; Jockush 1997) and teleosts (Ahn 1998; Ford 1937). Shifts in the axial location of morphological landmarks in the vertebral column of rabbits and chickens can be explained by the relatively simple segregation of a few mendelian loci (Green 1939; Promptoff 1928), and meristic and homeotic changes in vertebral structures have been related to evolutionary trends affecting traits such as posture and cranial size (see Davis' classic monograph on the giant panda: Davis 1964). In fish, axial variation is more complex due to the presence of other bony elements derived from somites and the neural crest, and is also heavily influenced by environmental variables (Lindsey 1988).

The threespine stickleback, *Gasterosteus aculeatus*, exemplifies many of these features (Ahn 1998) and has

been a favored organism for ecologists interested in the proximate causes of morphological variation (Bell and Foster 1994; Schluter 1994). Variation in the number and arrangement of elements of the axial and median skeletons (the vertebral column and associated bones), collectively referred to as axial morphology, is strongly correlated with trophic specialization (Ahn 1998). At least one aspect of such differences, the ratio of abdominal to caudal vertebrae, is known to be under balancing selection in the wild, as it affects burst swimming speed and thus susceptibility to predation, and the optimal ratio changes during larval growth (Swain 1992).

It is now well established that vertebral morphology is heavily influenced by transcription factors encoded by the *Hox* genes. There are at least four *Hox* complexes with a total of approaching 40 genes in most higher vertebrates that have been surveyed to date (Holland and Garcia-Fernàndez 1996). These genes are thought to act in combination to specify positional information in various tissues (Hunt and Krumlauf 1992), including the paraxial mesoderm from which the axial skeleton is derived. Differences between mouse and chicken in the relative location of transitions between cervical, thoracic, lumbar, and sacral vertebrae have been shown to correlate well with shifts in the relative anterior limits of expression of particular *Hox* genes (Burke et al. 1995). Staggering of the anterior limits of *Hox* gene expression is also observed in zebrafish (Prince et al. 1998) and in the threespine stickleback (Ahn and Gibson 1999a) but occurs within the somites that give rise to the trunk region of the fish, which does not generally show discrete differences between vertebrae. The pattern of overlapping expression of *Hox* genes consequently appears to have arisen prior to the separation between ray-finned and lobe-finned fishes, and hence to have provided a precode that was used in the structural differentiation of the vertebral column at least in the lineage leading to the tetrapods (Ahn and Gibson 1999a).

The questions thus arise: what is the function of the *Hox* code in fish, and is variation in the expression of specific *Hox* genes causally associated with aspects of axial variation at the population level as well? As an attempt to address these issues we examined and compared expression domains of a subset of *Hox* genes among individuals of two threespine stickleback populations that differ in the organization of skeletal elements along the body axis. We show that there is subtle variation in expression domains among individuals within each population, but that such variation is not obviously correlated with differences in the axial patterns seen between the two populations. The implications for function of the combinatorial *Hox* code in fish species are discussed, and it is argued that in fish variation in *Hox* gene expression may contribute to the buffering of morphological variation against the influence of environmental fluctuation.

Materials and methods

Stickleback husbandry

Two threespine stickleback populations were established in the laboratory with specimens derived from two sympatric populations native to Paxton Lake on Texada Island (British Columbia, Canada). These two populations are referred to as benthic and limnetic species in the literature in order to emphasize their adaptation to benthic (lake bottom) and limnetic (water column) lifestyles as well as their reproductive isolation (McPhail 1992). They are known to exhibit statistically significant and heritable differences in their axial morphology (Ahn 1998), which appears to be due to the activities of several genes with moderate phenotypic effects (Ahn and Gibson, in press). The populations were founded from six specimens caught in their native habitat and were maintained in the laboratory for two generations before the experiments described here, through random inbreeding using artificial fertilization (for details see Ahn 1998). Embryos and larval fish were raised in 10% Hank's saline and 5% sea water, respectively, at 18°C with constant aeration to provide uniform growth conditions. All specimens examined in this study were drawn from third-generation laboratory populations of the benthic and limnetic species.

Whole-mount in situ hybridization

Embryos of selected stages were collected and fixed overnight in MEMFA (Harland 1991) and stored at –20°C in 100% methanol until processing for whole mount in situ hybridization. Digoxygenin-labeled riboprobes were generated from cloned PCR-generated homeobox fragments that likely correspond to the stickleback *Hoxb-5*, *Hoxa-9*, and *Hoxa-10* homologs (see Ahn and Gibson (1999) for procedures used for cloning, as well as a complete description of transcription profiles throughout embryogenesis of these and other stickleback *Hox* genes), using a Genius-4 nonradioactive RNA-labeling kit (Boehringer-Mannheim, Germany). In situ hybridization was performed essentially as in Harland (1991) with the modifications described in Ahn and Gibson (1999a). For the comparison of anterior expression limits, embryos of 20- to 24-somite stages were chosen, based on consideration of the ease of handling and identification of anterior limits. Since maintenance of the anterior limits appeared to be quite stable for all three genes during gastrulation and somitogenesis (see "Results"), our choice of stages should not influence the conclusions drawn in this study. For documentation, images were captured using a video camera (Optronics Engineering) attached to a Zeiss Axiovert microscope, and processed using Adobe Photoshop 3.0 on a Power-Macintosh computer.

Staining was allowed to develop for up to 24 h to ensure detection of low levels of transcript at the anterior limits of expression in the paraxial mesoderm. Embryos were subsequently separated from the yolk and observed under Nomarski differential interference optics (Zeiss) in a flat mount. For each of the genes examined in this study, expression was found to be restricted to a group of ventrolateral cells in each somite in its anterior domain (Fig. 1). The anterior limit of expression was recorded for each embryo using the serial numbers assigned to each individual somite in an anteroposterior sequence. There was often a gradual decrease in the staining intensity from posterior to anterior, particularly for more posterior *Hox* genes, and therefore each embryo was visually scanned under the microscope from the posterior to anterior until the staining intensity (or number of expressing cells) went below detectable levels, and this point was assigned as the anterior limit of visible expression.

Since the probes were complementary to only 120 (*Hoxb-5* and *Hoxa-9*) or 159 (*Hoxa-10*) nucleotides of the highly conserved portion of the homeobox, some cross-hybridization to paralogs may have occurred. This does not appear to have been a major problem since under the stringency conditions that were used (60°C in 50% formamide) expression of *Hoxb-5* was distinguished from that of its paralog *Hoxa-5* (see Ahn and Gibson 1999a) as

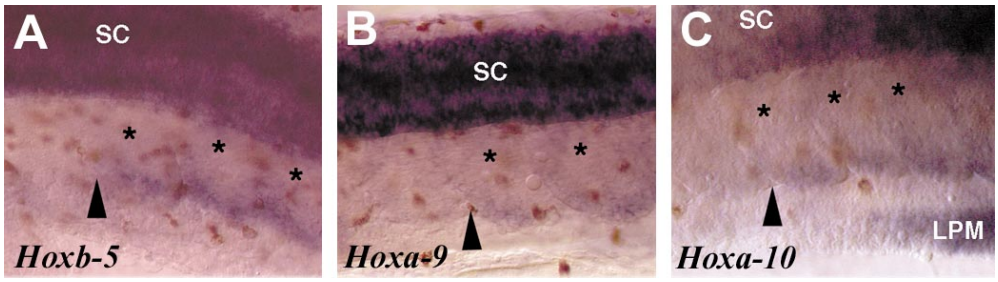

Fig. 1A–C Determination of the anterior limits of expression of *Hox* genes in the paraxial mesoderm of threespine stickleback embryos. **A** *Hoxb-5*. **B** *Hoxa-9*. **C** *Hoxa-10*. *Asterisks* Positive somites; *arrowheads* anterior limits; *SC* spinal cord; *LPM* lateral plate mesoderm. Anterior *to the left* in all images. *Arrowheads* s3/s4 border (**A**), s6/s7 border (**B**), and s8/s9 border (**C**)

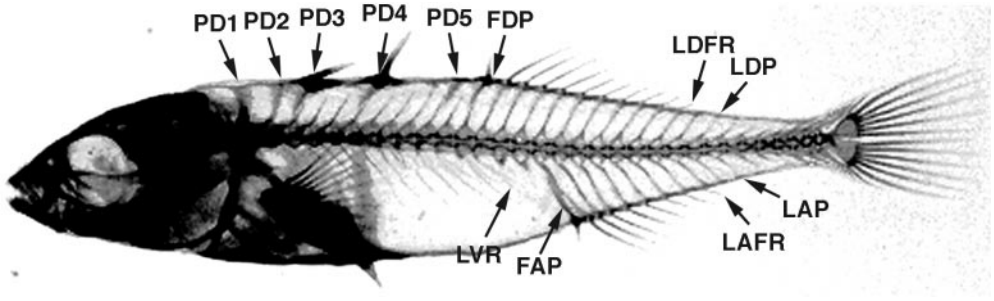

Fig. 2 Elements of the axial and median skeleton of the threespine stickleback. *PD1* first predorsal; *PD2* second predorsal; *PD3* third predorsal; *PD4* fourth predorsal; *PD5* fifth predorsal; *FDP* first dorsal fin pterygiophore; *FAP* first anal fin pterygiophore; *LVR* last ventral rib; *LDFR* last dorsal fin ray; *LAFR* last anal fin ray; *LDP* last dorsal fin pterygiophore; *LAP* last anal fin pterygiophore. The axial location of each element was designated as the ordinal number assigned to the vertebra with which the element made the most intimate association through their bony processes. For example, in this specimen the location of PD5 is 10. This image is taken from a juvenile specimen stained for bones that has a total length of approximately 3.5 cm

well as from *Hoxc-6*, which were almost as similar to *Hoxb-5* in nucleotide sequence as *Hoxa-5* (data not shown). Furthermore, since we were primarily interested in identifying variation in expression domains per se rather than tracing the variation to particular *Hox* genes, any cross-hybridization that occurred would not affect the interpretation of our results in this context.

Experimental design and statistical analysis

For each gene 12 independent pairs of second-generation parents (6 limnetic and 6 benthic) were used to produce embryos of the benthic and limnetic species, which were subsequently used in the whole mount in situ hybridization. Experiments were performed in three rounds, using 20 randomly selected embryos (10 embryos/pair) from each species in each round. This resulted in a total of approximately 60 embryos examined for each gene in each of the two species. In situ hybridization was performed on 20- to 24-somite stage embryos, and anterior limits of expression were determined using the criteria illustrated in Fig. 1. Data from the six crosses were pooled before statistical analysis to ensure minimization of bias due to genetic differences between parents within each species or variation in the staining reactions of each round.

Differences in axial morphology between the benthic and limnetic laboratory populations were characterized by scoring the ordinal positions of median skeletal elements measured in relation to the vertebral column (Fig. 2). For this purpose randomly selected juvenile specimens of the third-generation laboratory populations were prepared for whole-mount staining of bone and cartilage (Taylor and Van Dyke 1985) and examined under a dissection microscope. Statistical significance of differences in the mean axial locations of individual elements as well as differences in the anterior limits of expression of *Hox* genes were determined by two-tailed *t* tests (α=0.05).

Results

Differences in axial morphology between benthic and limnetic sticklebacks

The two laboratory populations of sticklebacks that were compared in this study are both derived from a single lake in British Columbia. They differ significantly in the axial arrangement of median skeletal elements, which are fairly representative of differences between populations from different types of habitats (Ahn 1998). Such differences are highly heritable and resemble those of wild specimens (a detailed description of the genetic and environmental contributions to variation in axial morphology in these populations will be published elsewhere; Ahn and Gibson, in press). Each of the two populations derive from only three wild fish, and thus are genetically inbred, although third-generation families within each population do show variation in the location of median skeletal elements relative to the vertebral axis (Fig. 3). However, the posterior shift of the second and third predorsals and the first anal pterygiophores in benthic relative to limnetic sticklebacks is consistent, as is the anterior shift of the fifth predorsals and most caudal fin rays and pterygiophores (Fig. 3). Benthic sticklebacks also tend to have a smaller total number of vertebrae than limnetic sticklebacks.

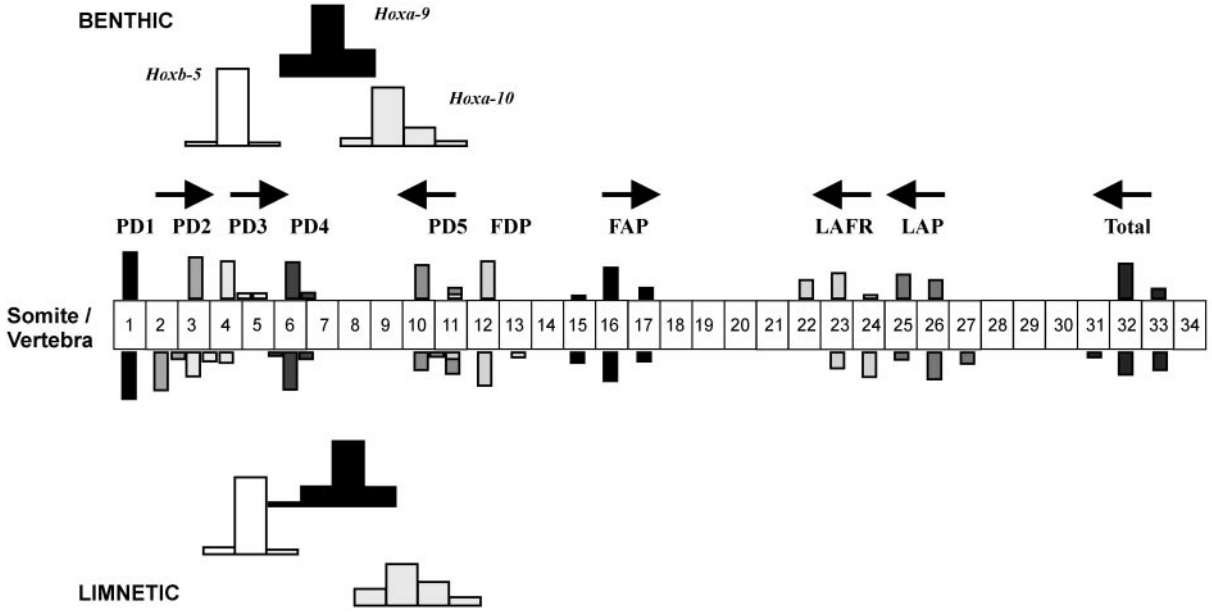

Fig. 3 Summary of variation in *Hox* expression and the axial location of skeletal elements in benthic and limnetic sticklebacks. *Boxes in the center* Somites (or vertebrae), numbered from anterior to posterior (*left to right*). Histograms adjacent to the boxes summarize relative frequencies of the location of the indicated skeletal elements in the third-generation laboratory populations of benthic (*n*=290, *above*) and limnetic (*n*=300, *below*) sticklebacks. *Arrows* direction of statistically significant shifts in the mean location of elements in the benthic relative to limnetic samples. For clarity of presentation, only frequencies greater than 5% are shown in the histogram, and data on the variation in the axial locations of LVR, LDP, LDFR are omitted. *Histograms at top and bottom* locations of anterior expression limits of *Hoxb-5*, *Hoxa-9*, and *Hoxa-10* in the paraxial mesoderm (Table 1). There were no significant differences in the mean location of anterior limits of any of the three genes (two-tailed *t* test: *Hoxb-5*, *P*=0.71; *Hoxa-9*, *P*=0.40; *Hoxa-10*, *P*=0.91)

Expression of *Hox* genes in the paraxial mesoderm of stickleback embryos

Threespine stickleback *Hox* genes are known to be expressed in a variety of tissues during embryogenesis, including the central nervous system, paraxial mesoderm, tail bud, and pectoral find buds (Ahn and Gibson 1999a). Transcription of the three *Hox* genes examined in this study – *Hoxb-5*, *Hoxa-9*, and *Hoxa-10* – was first observed in the embryos during late gastrulation before the completion of epiboly, as diffuse expression in the area of the spinal cord and paraxial mesoderm that corresponds to the future anterior expression domains. Initial expression in the paraxial mesoderm, which at this stage had not yet undergone segmentation, was usually low and uniform in intensity but with a clear anterior limit, indicating that the anteroposterior pattern is already specified during gastrulation and is maintained stably (Fig. 4A). As somites start to form, expression becomes upregulated at the anterior and ventrolateral portion of each of the forming somites but it remains low in other parts of the somite (Fig. 4B).

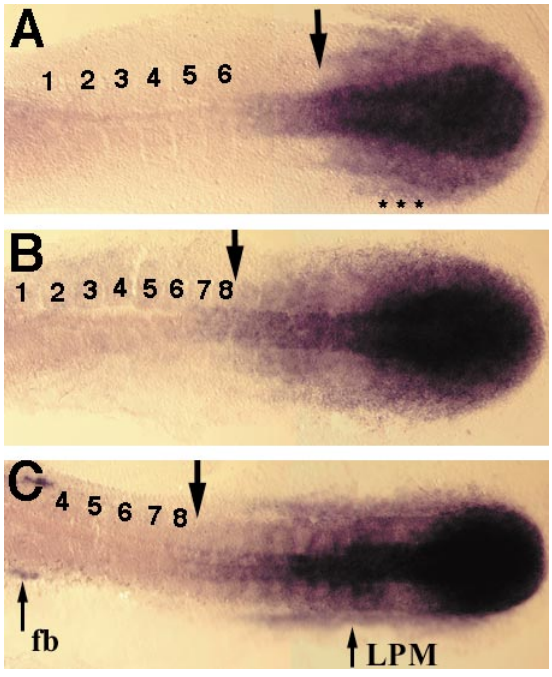

Fig. 4A–C Early expression of *Hoxa-10* in threespine stickleback embryos. **A** The 6-somite stage. Anterior limit of expression within the somitic paraxial mesoderm (*arrow*) is already recognizable. **B** The 10-somite stage. Anterior expression limit is located at the boundary between 8th and 9th somites. **C** The 15-somite stage. Anterior expression limit is again at the level of the 9th somite. Expression domains extend posteriorly through terminal addition of expressing cells as the tail bud extends in a caudal direction. *fb* Pectoral fin bud; *LPM* lateral plate mesoderm. All images are dorsal views with anterior *to the left*. *Asterisks* (**A**) represent prospective lateral plate mesoderm. *Numbers in each figure* serial numbers assigned to individual somites. Note that in addition to the paraxial mesoderm, spinal cord and lateral plate mesoderm also show the expression with stable anterior limits in the stages shown in this figure

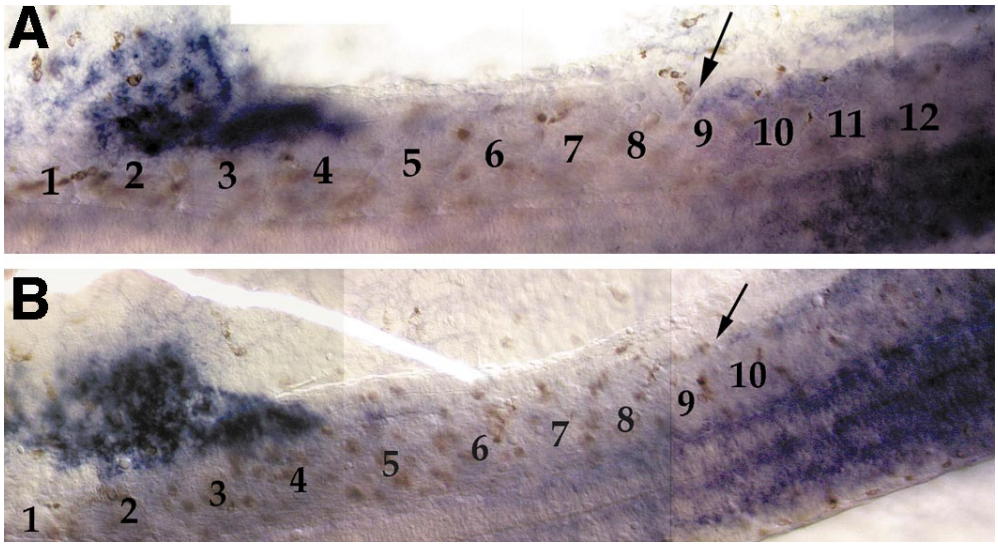

Fig. 5A, B Variation in the anterior limit of expression within the paraxial mesoderm. High magnification composite images of specimens with anterior limits of expression of *Hoxa-10* at the 20-somite stage. *Arrows* boundaries between the 8th and 9th (**A**) and 9th and 10th (**B**) somites. Dark staining adjacent to somites 2, 3, and 4 is expression in the pectoral fin bud

Posteriorly, as new somites are added through tail morphogenesis, expression domains become extended toward the posterior via de novo activation of expression within the cells of the tailbud (Fig. 4C; also see Ahn and Gibson 1999a). With the progression of embryogenesis, expression within each somite gradually becomes restricted to a small number of cells lying on the ventrolateral surface, which remains relatively strong throughout the segmentation period, allowing unambiguous identification of visible anterior limits at later stages. Interestingly, unlike some *Hox* genes of mouse and chicken (e.g., Deschamps and Wijgrede 1993), no substantial changes in anterior limits of expression were observed during gastrulation or segmentation for any of the stickleback *Hox* genes examined in this study (Fig. 4).

Expression of *Hox* genes became weaker after the completion of somitogenesis (approx. 34-somite stage), although faint staining was still visible on the ventrolateral surface of somites for a considerable time. Curiously, no expression was ever detected in the cells surrounding the notochord during later stages of development, including the stages in which the morphogenesis of vertebral anlage is clearly visible (data not shown). This is in stark contrast to the conditions seen in the mouse where prevertebral condensations exhibit combinatorial expression of *Hox* genes, which has greatly facilitated the inference of correlation between *Hox* expression domains and vertebral morphology (e.g., Kessel and Gruss 1991). The reason for this lack of late expression of *Hox* genes in the prevertebral condensations of stickleback embryos is not clear, but the relatively small number of sclerotome cells present in fish compared to tetrapod somites (Morin-Kensicki and Eisen 1997) might make it very difficult to detect such expression if it is present.

Variation in anterior limits of expression within the paraxial mesoderm

All three *Hox* genes showed some variation in the location of the somite in which the most anterior limit of expression was detected (e.g., Fig. 5), as documented in Table 1. It is clear that (a) the anterior limit of expression can vary over up to four somites, although the distribution of limits is reasonably normal in all cases, and (b) the mean and modal locations of the anterior limits are the same in benthic and limnetic sticklebacks. The only possible difference is in the shapes of the distribution of locations of anterior expression limits of *Hoxa-10*, which has a greater variance in the limnetic sticklebacks, although this difference was not significant as judged by the nonparametric Kolmogorov-Smirnov test.

Variation in *Hox* expression limits and variation in axial morphology

For each of the three *Hox* genes we examined in this study, the anterior limits of expression were determined in approximately 60 embryos of each of the inbred limnetic and benthic stickleback lines, and compared with the variation in axial organization of skeletal elements found in juvenile specimens of the same generation. The visible anterior limit of transcription of *Hoxb-5* was determined as the fourth somite in over 90% of specimens of both the benthic and limnetic sticklebacks, which was not correlated with significant differences in the location of the predorsals found at this axial level (Fig. 3). The anterior limits of expression of *Hoxa-9* and *Hoxa-10* were determined to be within the seventh and ninth somites, respectively, in the majority of embryos examined (Table 1), neither of which lie at the same axial levels as any of the median skeletal elements examined (Fig. 3). Interestingly, these two genes showed

Table 1 Location and variation in anterior *Hox* expression limits

Somite	Benthic			Limnetic		
	b-5 (*n*=56)	*a-9* (*n*=59)	*a-10* (*n*=55)	*b-5* (*n*=57)	*a-9* (*n*=64)	*a-10* (*n*=54)
3	1			3		
4	54			52		
5	1			2	1	
6		10			12	
7		36			40	
8		13	3		11	10
9			39			27
10			12			14
11			1			3
Mean±SD	4.0±0.2	7.1±0.6	9.2±0.6	4.0±0.6	7.0±0.7	9.2±0.8

much greater variation in their anterior expression limits than *Hoxb-5*, suggesting that there is increasing variance in anterior limits of expression in more posteriorly expressed genes (Table 1). Expression of these two genes in the adjacent lateral plate mesoderm also showed some intrapopulational variation, although no significant interpopulational differences in expression limits were detected (data not shown).

Discussion

The data presented above can be summarized in three major conclusions: (a) there is variation within *G. aculeatus* in the anterior limits of expression of *Hox* genes expressed in the paraxial mesoderm; (b) anterior limits of expression tend to be more variable in more posteriorly expressed *Hox* genes; and (c) heritable differences in organization of the body axis are not obviously correlates with differences in *Hox* expression limits. The first two results are not unexpected, given that there is also variation in the location of mesodermal derivatives in the stickleback's body that increases in more posterior parts of the axis (Fig. 3). Nevertheless, it establishes the existence of intraspecific variation in the domains of regulatory gene expression for a vertebrate. The third result might simply be explained if there are other *Hox* genes that we have not examined, and that directly modulate development of the predorsals, pterygiophores, and fin rays. However, as discussed below, we think this explanation obscures a better understanding of the function and evolution of *Hox* codes in vertebrates that may be obtained when population and quantitative genetic perspectives are also taken into consideration.

Sources of variation in *Hox* expression

Interpretation of the significance of intraspecific variation in *Hox* gene expression depends to some extent on how much of the variation is real, and how much is artifactual. There are at least three potential sources of error in scoring anterior limits of expression of *Hox* genes in sticklebacks. First, the low level of transcription in the most anterior somite that expresses group 9 or 10 genes can lead to some observational error, although we found that there were no significant differences in overall expression profiles when the same embryos were scored on two different occasions. Second, variation in the intensity of staining within each somite may result in failure to accurately identify anterior limits. For example, slightly different results were obtained when we used the stepped increase in the overall tone of staining in the somites rather than the presence of staining in the ventrolateral portion of somites (data not shown). Third, although we observed that the anterior limits are stable once established, it is possible that there were cases in which the most anterior limits of expression had undergone gradual decay as somitogenesis proceeded, especially in older embryos. However, this is unlikely to account for much of the variation in our data from somite stages 20–24, as there was no significant correlation between the total number of somites observed in each specimen and the location of the anterior limits (data not shown). Thus, at least part of the variation in anterior limits observed in this study is likely to be a genuine reflection of the variation in the expression domains of stickleback *Hox* genes.

Increasing variation in anterior limits of expression in more posterior body parts

Somite formation and the initiation of *Hox* expression do not seem to be tightly coupled processes in vertebrates (Ahn and Gibson 1999a; Richardson et al. 1998). This is also indicated here by the observation that expression of *Hox* genes was initiated in the paraxial mesoderm before the formation of somite boundaries (Fig. 4A). In other words, at least in threespine stickleback embryos the newly generated segmental plate is already patterned by differential expression of *Hox* genes before its subdivision into individual somites. One potential consequence of such uncoupling is the possibility that spurious homeotic variation results from meristic mechanisms as explained below.

If the anterior limits of expression of genes that define the positional identity of certain skeletal elements are normally set at a certain body length (or at a certain

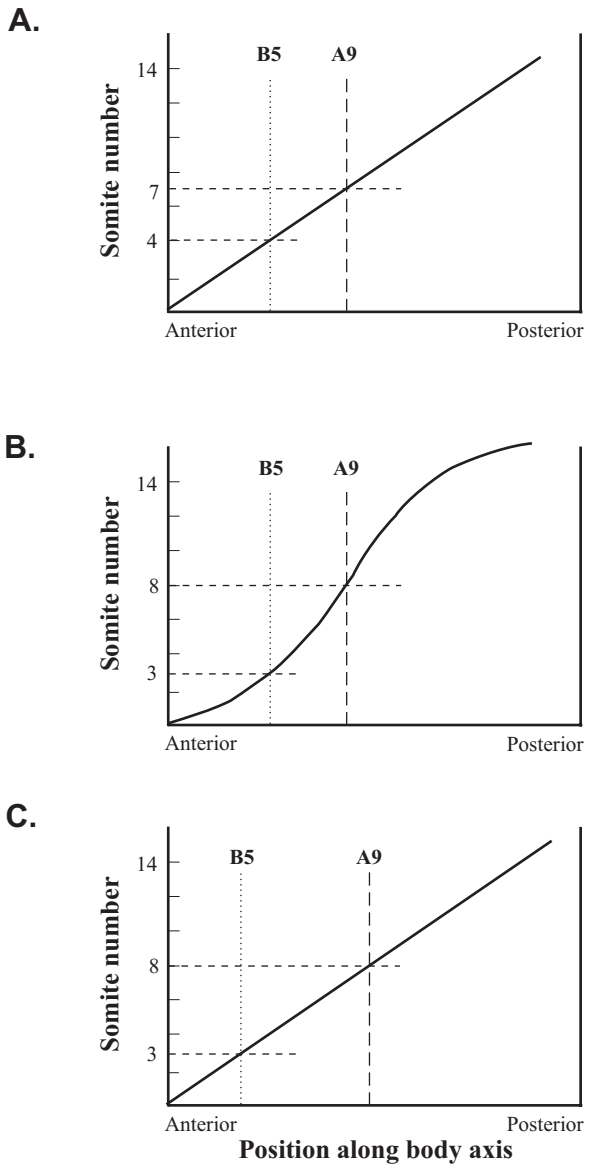

Fig. 6A–C Relationship between meristic and homeotic mechanisms for generation of axial variation. **A** Anterior limits of expression of *Hox* genes (or alternatively, locations of skeletal elements) are distributed along the body axis from anterior to posterior (*x-axis*). The somites (or vertebrae) at which the limits occur are determined by the relationship between the number of somites (*y-axis*) and distance along the body axis (*solid diagonal line*). For example, the anterior limit of *Hoxb-5* may normally lie in the 4th somite, and that of *Hoxa-9* in the 7th somite. **B** A meristic change occurs when the number of somites lying between any two points along the body axis changes. This results in a change in the relationship between somite number and distance along the body axis (*curved solid line*). If the positioning of the *Hox* expression limits is held constant relative to the body axis, the observed limits with respect to the number of somites changes, in this case to the 3rd and 8th somites for *Hoxb-5* and *Hoxa-9*, respectively. **C** An apparently identical transformation can be produced by shifting the location of the gene expression boundaries relative to the body axis, while holding the number of somites per portion of the body axis constant. Both mechanisms probably contribute to the variation documented in this study, and the figure is not meant to imply that the two mechanisms are mutually exclusive

time point of development), their location relative to the number of somites is set by the number of somites that develop anterior to that region of the body axis, as diagrammed in Fig. 6A. A meristic mechanism that results in fusion or deletion of somites, or changes a paramater such as the number of cells that give rise to each somite or the local rate of cell division, leads to a change in the relationship between limits of gene expression (or the position of morphological landmarks), and the number of segments (Fig. 6B). An identical change could alternatively be produced by a true homeotic mechanism if the limits of gene expression shift relative to the location along the body axis, while the relationship between number of somites formed and distance along the body axis remains constant (Fig. 6C).

The meristic model predicts that variance in location of gene expression limits (or morphological boundaries) increases in more posterior regions of the body, since there is an increasing likelihood of asynchrony between *Hox* expression domains and somite boundaries as somitogenesis proceeds. This is in fact observed with respect to the location of elements of the axial and median skeletons (Fig. 3). Consequently, the morphological variance at a particular axial level can be used as a benchmark for comparison with the amount of variance in the anterior limits of *Hox* expression. When this is done, the variance in gene expression is significantly greater than expected. For example, the standard deviation in location of predorsals 4 and 5 in the specimens used in this study is up to 0.3 segments, whereas that of the anterior expression limit of *Hoxa-9* and *Hoxa-10* is twice as great (Table 1; see Fig. 3). This implies that meristic effects can account for only half of the observed variance in *Hox* gene expression. The remainder would be due to scoring errors as well as to the homeotic effects that cause the anterior limits of visible *Hox* transcription to shift along the body axis. A careful analysis involving both the measurement of distances and examination of the counts of somites between anterior limits of *Hox* gene transcription through double labeling might be used to investigate the relative contribution of meristic and homeotic mechanisms, although the low levels of expression in the relevant somites make this a technically demanding experiment.

Comparison of *Hox* expression between morphologically divergent lines

The expression profiles in benthic and limnetic inbred populations of three stickleback *Hox* genes were similar, despite significant heritable differences in their axial morphology (Fig. 3). With respect to *Hoxb-5*, it is simple to conclude that the anterior limit of expression of this gene is not involved in regulating the location(s) of skeletal elements that arise at a similar axial level. Both predorsals 2 and 3 are typically displaced by one segment in a caudal direction in benthic sticklebacks relative to limnetic ones (Fig. 3), but *Hoxb-5* has a sharp anterior

limit that is almost always found at the boundary between the third and fourth somites in both forms of sticklebacks (Fig. 1A). The morphological difference in this region must be due to genetic variation that acts downstream, or independent, of the *Hox* gene.

Interpretation of the results with *Hoxa-9* and *Hoxa-10* is less straightforward. The anterior expression limit of *Hoxa-9* in the paraxial mesoderm is at the axial level where predorsal 4 forms, and this element is one of the few that does not significantly differ in location between benthic and limnetic sticklebacks. The anterior limit of *Hoxa-10* lies between the locations of predorsals 4 and 5, and is not associated with any morphological landmarks. If the level of expression required for function is higher than the detectable level of transcription, or if translation occurs in only part of the expression domains, the functional anterior limit would shift caudally, possibly to the level of predorsal 5. It is thus intriguing that the distribution of anterior limits of *Hoxa-10* is more spread out in limnetic than benthic sticklebacks as is the distribution of the location of predorsal 5.

However, in contrast to the very clear results of comparison of birds and mammals (Burke et al. 1995), we find no direct evidence in this study for "coincident transposition" of axial elements and *Hox* expression boundaries within sticklebacks. One possible explanation for this result is that *Hox* genes influence only the differentiation of vertebral elements while an unknown class of genes provide positional information along the body axis outside of the vertebral column. Alternatively, other *Hox* genes that we did not examine in this study might be responsible for the differences in axial morphology seen between the two populations. This could certainly be the case for the location of the trunk-tail boundary marked by the first anal fin pterygiophore (FAP: Fig. 2) where expression of one or more group 13 genes occurs in zebrafish (van der Hoeven et al. 1996). Variation in expression of group 13 genes may be predicted to be correlated with morphological variation within this region in sticklebacks, but judging from the expression patterns in zebrafish (Prince et al. 1998) and other vertebrates (Burke et al. 1995), the vast majority of *Hox* genes are likely to have anterior limits within the trunk region of the body and therefore cannot obviously explain the variation in the caudal half of the stickleback's body.

Expression domains and the origin of morphological structures

Two important issues in fish embryology that have not been completely addressed require particular caution in interpretation of our results. Currently it is not known whether the close serial correspondence between the median skeletal elements and individual vertebrae (Fig. 2) is due to shared embryonic origins or to secondary association between elements with separate embryonic origins. Careful lineage tracing of skeletogenic cells in several divergent species of fish will be necessary to completely resolve this issue, as well as to establish whether the possible incorporation of the anterior-most somites into the base of the skull may affect the serial correspondence of somites with median skeletal elements.

There is also the question of whether the anterior expression limits that we scored in stickleback embryos are appropriate measures of the anteroposterior identities of the axial and median skeletal elements that form at the same level. We were unable to find expression of *Hox* genes in prevertebral sclerotomal condensations surrounding the notochord, which made it difficult to determine the anterior limits within the skeletogenic cells. Nevertheless, evidence from study of the mouse indicates that the pattern of *Hox* gene expression within the prevertebral condensations closely mirrors the earlier expression within the parental somites (e.g., Püschel et al. 1990, 1991), which suggests that at least in mouse embryos sclerotome cells possess the same axial identities as the somites from which they originate. Therefore, if a similar somite patterning mechanism is utilized in fish embryos, the early expression of *Hox* genes might be sufficient to provide mesoderm cells with positional identities, which is then passively transmitted to the sclerotome cells as the somite undergoes differentiation. Since the initial expression of stickleback *Hox* genes within the cells of an entire somite was later maintained as a patch of expression restricted to a small subset of prospective dermomyotome cells in the same somite (Fig. 4; see Morin-Kensicki and Eisen), our criteria for determining the anterior limits of expression are likely to have provided an accurate prediction for the identity of sclerotome cells lying at the same axial level, even though these may no longer express *Hox* genes.

Hox genes and quantitative variation

Numerous studies of both vertebrates and invertebrates have emphasized that the roles of *Hox* genes in development can evolve either by changes in patterns of expression of the genes themselves or by modification of the function and target specificity of the encoded proteins (Gellon and McGinnis 1998). This dichotomization was necessary to explain qualitative morphological transitions, such as those between wings and halteres, and between thoracic and lumbar vertebrae (Warren and Carroll 1995). However, the data on *Hox* expression in teleosts suggest that quantitative contributions should also be considered. Although not discrete, there is variation in the trunk region, such as in the lengths of ribs and vertebral centra, and the volume of hypaxial muscles. If *Hox* genes regulate cell division and differentiation (Duboule 1995), they could well provide positional information that affects such variation, and this may have been the ancestral function of the genes that led to the establishment of a precode of staggered expression limits in the mesoderm. Thus, in a phylogenetic context, the possibility that quantitative function preceded the emergence of qualitative function should be considered.

There are two features of *Hox* usage in teleosts that lend themselves to this type of quantitative function. The first is that anterior boundaries of expression of the genes transcribed more caudally are graded over several somites (in terms of both number of cells and level of expression within cells; Fig. 1). Concentration gradients are intrinsically capable of giving quantitative activity. In theory, they can also provide plasticity of response in a fluctuating environment that for fish includes changes in physical variables such as temperature, oxygen tension, and ionic concentrations in the water, all of which are known to affect axial morphology to some extent (Lindsey 1988). The second feature is that the *Hox* complexes are duplicated, which could provide some level of buffering. Minor changes in expression of one gene can be compensated for by the redundant activity of a paralog. Whether this speculation is true, there is no doubt that variation in axial morphology in fish is prevalent, of great adaptive significance, and has a complex developmental basis involving both meristic and homeotic mechanisms. Although variation in *Hox* genes is perhaps unlikely to contribute to morphological variation in a Mendelian manner as seen in mouse gene knockout mutants, there is every possibility that it may make subtle contributions to the genetically complex trait of axial morphology.

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