Evolution of new appendage types by gradual changes in Hox gene expression – the case of crustacean maxillipeds[1]

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1. Hox genes and the evolution of segment specialization

One of the most striking features of arthropod evolution is the specialization of segments and associated appendages for particular functions in different parts of the body (Cisne 1974) – usually, sensory functions in the anterior part of the head (antennae), feeding in the area of the mouth (mandibles and maxillae), locomotion in the main thoracic/trunk region (swimming, walking and flight appendages) and reproduction in the posterior part (genital organs, ovipositors). This process of segment specialization is thought to have occurred several times independently, in each of the major arthropod lineages, from ancestors that had much more uniformly segmented ('homonomous') bodies, giving rise to different patterns of segment specialization in arachnids, myriapods, insects and various groups of crustaceans.

Studies in model organisms have shown that members of the Hox gene family (the 'homeotic' genes) play a key role in specifying the morphological differences of segments in different parts of the body: different Hox genes are expressed in distinct regions of the body and mutations in these genes cause 'homeotic' transformations of one type of segment into another (Lewis 1978, McGinnis & Krumlauf 1992, Hughes & Kaufman 2002b). Classic examples in insects include the transformation of antennae into legs, the transformation of halteres (hindwings) into forewings, or the transformation of leg-less abdominal segments into leg-bearing thoracic segments. This well documented role of Hox genes led to the expectation that changes in their function might be responsible for morphological changes in segment specialization that are observed during the evolutionary history of arthropods. Early hypotheses proposed that changes in the number and type of Hox genes might directly reflect the changing patterns of segment specialization in each of the arthropod lineages. Thus, it was predicted that animals with a high degree of segment specialization (e. g. insects, decapod crustaceans) will turn out to have a larger number of Hox genes with specialized functions than animals with more homonomous body plans (e. g. myriapods). Studies by a number of labs in the 1990s showed this not to be the case (reviewed by Averof 1997, Hughes & Kaufman 2002b) – the major extant classes of arthropods are now known to have the same basic set of 8–9 Hox genes in their genome, namely orthologues of the *Drosophila* genes *labial (lab)*, *proboscipedia (pb)*, *Hox3*, *Deformed (Dfd)*, *Sex combs reduced (Scr)*, *Antennapedia (Antp)*, *Ultrabithorax (Ubx)*, *abdominal A (abdA)* and *Abdominal B (AbdB)*. This finding indicated that the full complement of Hox genes found in extant arthropods was already present in the common ancestor of these groups, prior to the Cambrian.

Comparisons of expression patterns then revealed significant differences in the expression domains of Hox genes among insects, myriapods, arachnids and crustaceans, suggesting that changes in the regulation of Hox gene expression may be the key genetic correlate with segment specialization at the morphological level (Averof & Akam 1995, Damen et al. 1998, Telford & Thomas 1998, Abzhanov & Kaufman 2000a, Hughes & Kaufman 2002a, 2002b). In parallel, the ability of Hox genes to regulate downstream effector genes appears to have changed in important ways – for example, the Hox genes *Ubx* and *abdA* acquired the ability to repress leg development in abdominal segments in the insect lineage (by repressing key genes involved in leg development, such as *Distal-less*), while their counterparts in crustaceans continued to be associated with the development of leg-bearing trunk segments (Palopoli & Patel 1998, Ronshaugen et al. 2002, Galant & Carroll 2002).

While comparisons among the major arthropod lineages revealed extensive differences in Hox gene expression, most of these studies did not provide concrete examples where specific changes in Hox gene expression could be linked to specific changes in patterns of segment specialization; in most cases the evolutionary distances under consideration were too large and the intermediate steps not obvious. An interesting exception was the evolution of maxillipeds, a new type of specialized appendage that

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emerged repeatedly in crustacean lineages and appears to be associated with a recurrent change in Hox gene expression (AVEROF & PATEL 1997).

2. Are maxillipeds partial homeotic transformations?

As their name implies, maxillipeds ('jaw-legs') are appendages with a hybrid morphology and function – they arise in anterior thoracic segments, but display mixed characteristics of thoracic (locomotory) and gnathal (feeding) appendages. They are functionally integrated with the more anterior gnathal appendages (maxillae 2, maxillae 1 and mandibles) and they are used primarily for the manipulation of food. Maxillipeds evolved several times within the crustaceans – different crustacean lineages have 0, 1, 2 or 3 pairs of maxillipeds. Studies of Hox gene expression reveal that maxillipeds are consistently associated with a shift in the expression of the Hox gene *Ubx* (AVEROF &

PATEL 1997): throughout the crustaceans, *Ubx* is expressed in thoracic segments that develop a typical thoracic-locomotory identity and is consistently excluded from thoracic segments that bear maxillipeds (Fig. 1). In some cases this shift is accompanied by the posterior expansion of the expression of *Scr*, the Hox gene that is normally associated with the more anterior maxillary segments (ABZHANOV & KAUFMAN 1999). Bearing in mind the conserved role of Hox genes in specifying segmental identities, it was attractive to propose a causal link between these changes in *Ubx* and *Scr* expression and the evolution of maxillipeds in crustaceans.

For many years it was not possible to test the significance of this correlation by directly examining the functions of these Hox genes in crustaceans. During the last decade, however, techniques for knocking down genes were developed in a number of arthropod species, including a few crustaceans (COPF et al. 2004, OZHAN-KIZIL et al. 2008, LIUBICICH et al. 2009). Our lab also established
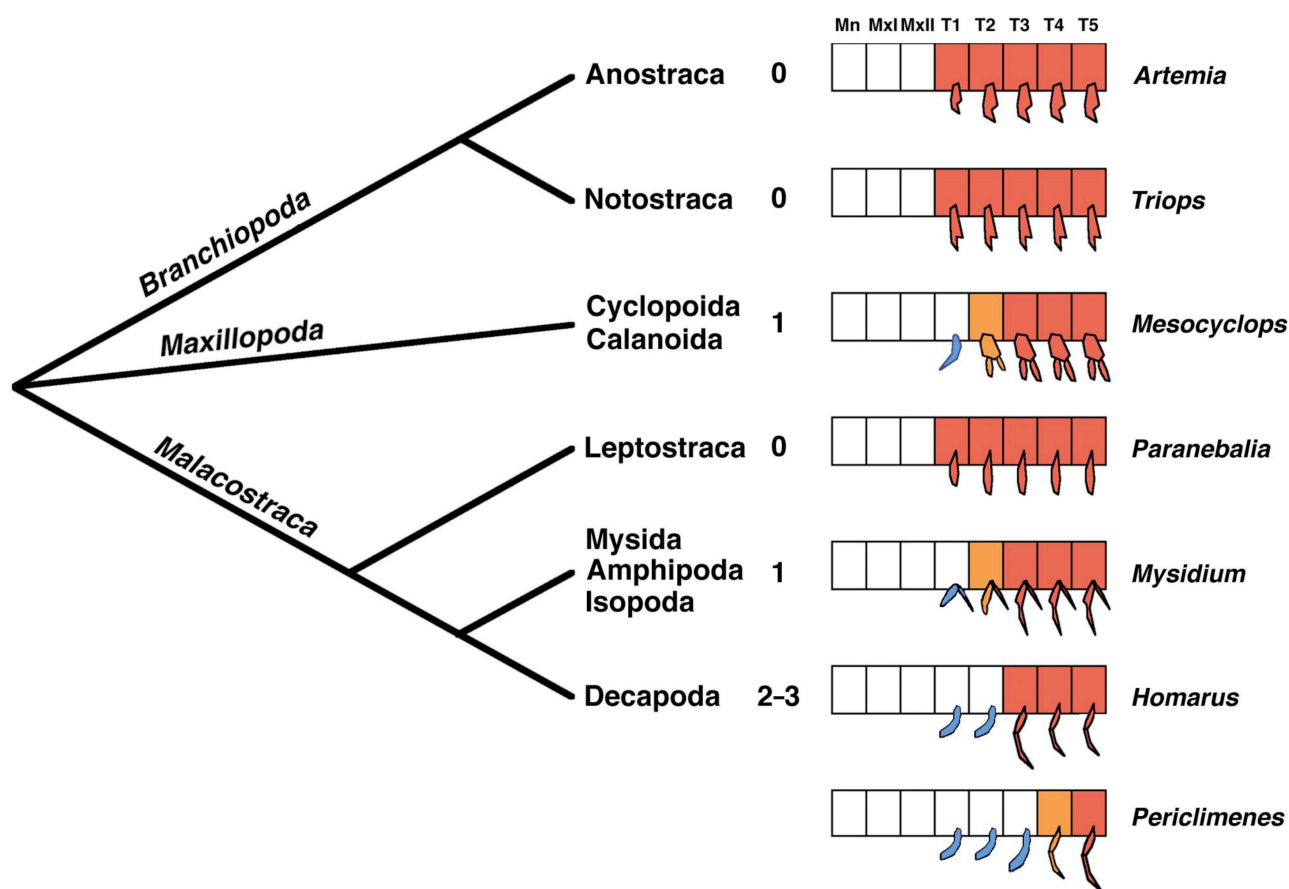

Fig. 1. Phylogenetic distribution of maxillipeds and corresponding changes in *Ubx* expression. Cartoons depict gnathal and anterior thoracic segments in crustacean species that bear 0, 1, 2 or 3 pairs of maxillipeds at hatching (maxillipeds shown in blue). Embryonic *Ubx* expression domains are shown in light and dark orange, corresponding to weak and strong *Ubx* expression. Reprinted from AVEROF & PATEL (1997).

tools for genetic manipulation via transgenesis in the amphipod *Parhyale hawaiensis*, which allow us to express genes ectopically in this species (PAVLOPOULOS & AVEROF 2005, PAVLOPOULOS et al. 2009). Many years after the original observation that *Ubx* expression correlates with the distribution of maxillipeds in crustaceans, it became possible to test experimentally the capacity of *Ubx* to control thoracic versus gnathal segmental identities.

Amphipods develop one pair of maxillipeds, in the first thoracic segment (T1), in the absence of *Ubx* expression. In *Parhyale*, *Ubx* was found to be expressed from the second thoracic segment (T2) backwards, in the segments that develop appendages with typical thoracic morphology (LIUBICICH et al. 2009). Using the tools available for gain-of-function and loss-of-function genetic studies, we could test whether we can recover leg-like thoracic morphologies in T1 by mis-expressing *Ubx* in that segment, and conversely, if we can transform additional thoracic appendages into maxillipeds by removing *Ubx* from its normal expression domain. These experiments were carried out in parallel by us (mis-expression of *Ubx*) and by the lab of NIPAM PATEL (*Ubx* knockdown using RNAi), and gave the anticipated results: ectopic *Ubx* transformed maxillipeds (and head appendages) into leg-like appendages (Fig. 2), while suppression of *Ubx* transformed additional thoracic appendages into maxillipeds (PAVLOPOULOS et al. 2009, LIUBICICH et al. 2009). The suggestion that maxillipeds represent partial homeotic transformations resulting from evolutionary changes in *Ubx* expression is therefore supported both by comparative expression studies and by experimental analyses of *Ubx* function.

3. Is there a 'maxilliped identity' and how can it be achieved?

Besides corroborating our hypothesis on the evolution of maxillipeds, the experiments in *Parhyale* have provided further insight into the mode of maxilliped evolution. One of the most puzzling observations to come out of the *Ubx* mis-expression experiments was that low levels of ectopic *Ubx* are capable of transforming second maxillae (Mx2) into maxillipeds rather than legs (PAVLOPOULOS et al. 2009). This was surprising because, as discussed earlier, maxillipeds normally develop in the absence of *Ubx*. But the transformations were frequent and almost complete, leaving no room for doubt. After considering several possibilities, we realized that these transformations correlate with the ability of *Ubx* to repress the more anterior-acting Hox gene *Scr*, even when *Ubx* is expressed at low levels (PAVLOPOULOS et al. 2009). Thus, we proposed that low levels of ectopic *Ubx* caused Mx2-to-maxilliped transformations by down-regulating the high levels of *Scr* present in Mx2 to the lower levels normally associated with maxillipeds. We expect that *Scr* RNAi will confirm this explanation. A second line of evidence suggesting that maxillipeds can arise in the presence of low levels of *Ubx* comes from the results of the RNAi experiments carried out by the Patel group (LIUBICICH et al. 2009); when the expression levels of *Ubx* were reduced, the appendages on T2 and T3 acquired characteristics that are typical of maxillipeds, such as the presence of endites. These changes occurred in the absence of *Scr*.
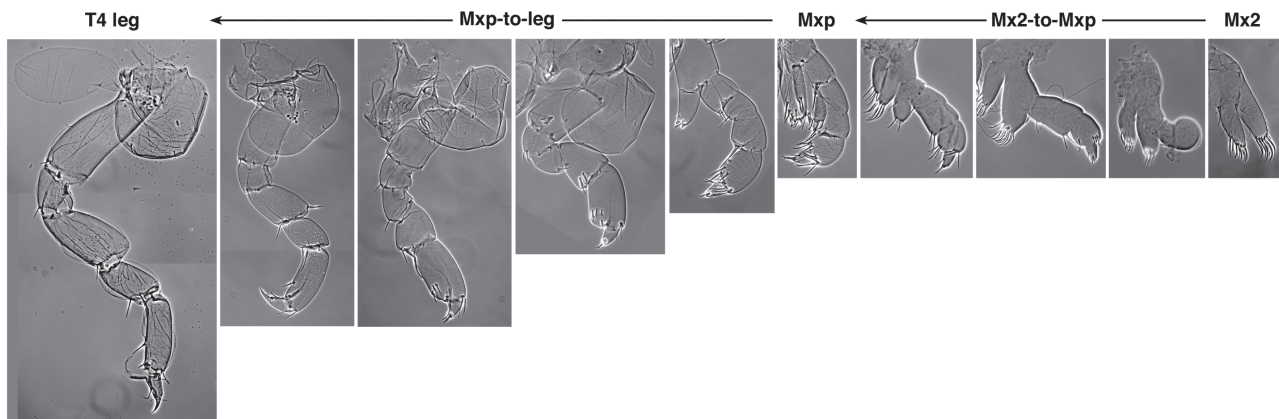
Fig. 2. Mis-expression of *Ubx* gives rise to Mx2-to-maxilliped and maxilliped-to-leg homeotic transformations, with a range of intermediate morphologies. Normal maxilla 2 (Mx2), maxilliped (Mxp) and T4 limbs are shown for comparison. Adapted from PAVLOPOULOS et al. 2009.
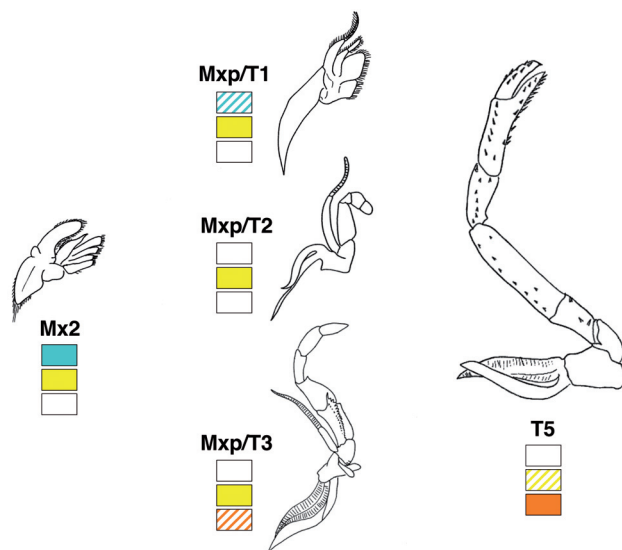
Fig. 3. The term 'maxilliped' embraces a range of appendages with hybrid, part gnathal and part thoracic morphology. This is illustrated by the maxillipeds of decapod crustaceans (redrawn by Richard S. Fox, after HERRICK 1909). During development these appendages express different levels and combinations of Hox genes (ABZHANOV & KAUFMAN 2000b), represented here by coloured boxes; cyan for *Scr*, yellow for *Antp*, orange for *Ubx*; solid colour for high/persistent expression, striped for weak or transient expression.

These results suggest there are many ways in which maxilliped-like appendages can be specified: they can arise with *Scr* and no *Ubx* (normal maxillipeds in *Parhyale* and in T1 of other crustaceans), with low *Ubx* and *Scr* (Mx2-to-maxilliped transformations in the gain-of-function experiments), or with low *Ubx* and no *Scr* (T2/3-to-maxilliped transformations in the loss-of-function experiments). This, in turn, raises the question of what 'maxilliped identity' really means. Is there a specific combination of Hox gene activities – a unique 'Hox code' – that is capable of eliciting the suite of morphologies that we associate with maxillipeds, or are there several distinct ways of achieving this? We propose that maxillipeds represent a range of appendages with a hybrid, part gnathal and part thoracic morphology, and that this hybrid identity can be achieved in a number of ways, by shifting the balance between Hox genes that specify 'thoracic' and those that specify 'gnathal' fates (e. g. Fig. 3). In most crustaceans, this distinction is probably effected by the Hox genes *Ubx* and *Scr*.

The phylogenetic distribution of maxillipeds suggests that this appendage type has evolved multiple times in crustaceans. Each time, this event must have been effected by a different sequence of genetic changes, shifting the balance in different ways. For example, maxilliped evo-lution may have been guided by changes in the expression patterns, in the levels and in the temporal dynamics of *Ubx* and *Scr* expression, as well as changes in the ability of Ubx and Scr proteins to compete on target promoters. Only the endpoints of these different evolutionary paths can be traced in today's species.

A second important question is how these morphological changes can be achieved in natural circumstances, without having to invoke 'hopeful monsters' – sudden morphological transitions that would be highly unlikely to survive in nature (GOLDSCHMIDT 1940, AKAM 1998). Mutations in Hox genes usually bring to mind homeotic transformations with dramatic morphological consequences induced in a single step. However, mutations that fine-tune Hox gene expression in terms of quantity, timing and cell-type specifity are capable of producing a wide range of subtle morphological changes (e. g. STERN 1998, 2003). When compounded over extended evolutionary periods such changes can lead to dramatic morphological transitions that are induced and selected in multiple small steps.

Our experiments in *Parhyale* show that a large number of intermediate morphologies can be produced even by relatively crude uniform mis-expression of *Ubx* (Fig. 2). Regulatory changes that modulate the levels, temporal and spatial patterns of Hox gene expression at a finer level would have much more subtle effects. Therefore we believe that the phenotypic space sampled by natural selection during the evolution of maxillipeds was much larger and smoother, and that multiple paths of gradual change were available for transforming one type of appendage into another.

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