CONTENT *Studies in the vinegar[1] fly* **Drosophila melanogaster** *indicate that head development differs significantly from trunk formation, since anterior head segments lack pair rule and Hox gene activity. However, despite the belief that head segmentation mechanisms have been conserved during evolution, they have not been identified in any organism so far. Due to the headless appearance of a fly maggot,* **Drosophila** *does not easily lend itself to the study of embryonic head development. To identify genes crucial to head segmentation, we are therefore currently isolating and functionally characterizing homologous candidate genes in two different arthropod species, the red flour beetle* **Tribolium castaneum** *and the amphipod crustacean* **Parhyale hawaiensis**. *Moreover, we aim to identify head-specific genes in an unbiased manner by a transposon mutagenesis screen in* **Tribolium**. *Since head structures of insects and crustaceans can be easily compared to each other, these analyses will enable us to draw conclusions on the evolution of head development over a period of about 450 million years.*

Beetle a-head

Investigating embryonic head formation using a novel model organism

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• The last common ancestor of men and flies lived about 500 million years ago, probably hidden in the mud layer at the bottom of the sea[1]. Because this species gave rise to all bilaterally symmetric animals, it has been named the »Urbilateria«[2]. Unfortunately, we have no direct evidence of its nature, since it probably died out without leaving any fossil traces. Nevertheless, we can assume that the Urbilateria already had a »head« that could be distinguished from the trunk, since all its descendents from flies to mice show such a subdivision in the body plan.

In all bilaterian organisms, the trunk is regionalized by the genes of the Hox cluster, i.e. the specific combinations of Hox gene activity give the different segments their particular identity[3]. By contrast, the anterior segments of the head are established in a zone free of Hox gene expression. This region is controlled by Orthodenticle (Otx in vertebrates), a paired-type homeodomain-containing transcription factor, whose function is strikingly conserved between arthropods and vertebrates[4]. In mice and flies, *orthodenticle* is expressed anteriorly during early embryogenesis, and the respective knockout animals do not develop anterior head structures. Moreover, the *Drosophila* protein is able to rescue *Otx* mouse mutants, and the mouse protein can rescue the fly mutant phenotype[5-7]. In addition, homologous genes of mice and flies are important for the establishment of the boundary region separating head from trunk[8]. For example, the *Drosophila* gene *buttonhead* and its vertebrate homologues play a particularly important role in the integration of the head and trunk segmentation systems at this boundary[8-11].

However, in spite of the high phylogenetic conservation of the involved genes, very little is known about the mechanisms underlying regionalization and segmentation of the anterior head. Does the use of homologous genes imply that some principles of head development are similar in mice and flies? This interesting question is impossible to answer at present, given that we do not even understand head development in the prime model system *Drosophila melanogaster*.

No head is alike

• When we say »head«, we usually mean a composite structure at the anterior end of an animal. The head is often separated from the trunk by a neck, includes the brain and represents the centre for sensation and feeding. This definition focuses mainly on functions of the head and therefore includes all the morphological structures used to perform these functions. The »head« in one group of animals may therefore comprise a different set of segments or body regions than in another. Indeed, different arthropod lineages vary in this respect. The fossil remains of Trilobites, an extinct group of arthropods, display three or four appendage-bearing head segments[12], and the shorter version has been proposed to be the original »construction plan« of arthropods[13]. Myriapods (millipedes and centipedes), crustaceans, and insects have added one more posterior segment to their heads, called the second maxillary segment or labial segment in insects. Spiders are a further exam-

[1] *Usually, but improperly, called fruit fly*
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ple of the plasticity of head composition. They display a fused structure, known as the prosoma, which contains both the head and the locomotory body section. In fact, the segment with the first walking legs corresponds to the insects' mandibular segment[14,15].

In vertebrates, segmentation is still believed to be a prominent component of head patterning, even though we do not understand how the segments initially develop. One important process during vertebrate head formation is the migration and differentiation of the cranial neural crest cells that give rise to most of the skull. The migration of these cells adds a further difficulty to the investigation of head formation in vertebrates. Embryonic head segmentation can be more easily addressed in arthropods than in vertebrates. Insects are therefore a good starting point for gaining a better mechanistic understanding of head patterning, even though the exact composition of the insect head is still a matter of some dispute.

Segmental composition and subdivision of the insect head

• Morphologically speaking, the insect head is comprised of several segments and may also include portions of non-segmental origin at its anterior end. The number of head segments is still unclear, the suggestions ranging from five to seven[16,17]. There is general agreement on the posterior five segments: the antennal, intercalary, mandibular, maxillary, and labial segments (*Figure 1*). These are regarded as homologous to trunk segments since they share at least some of the features defining a segment: constriction of the epidermis, ganglia, appendages, and the paired expression of the segment polarity genes *engrailed* and *wingless*. It is less clear, however, as to whether the portion anterior to the antennae is also composed of homologous segments. Suggestions for this region range from no or only one ocular segment to two segments (labral and ocular). Depending on the number of proposed segments, a more or less extensive region is assigned to be of non-segmental origin, called acron. This debate has not yet been settled because the anterior head region lacks unequivocal morphological similarities to trunk segments. One reason for this may be that head structures are highly adaptive features, and are therefore prone to fast evolutionary change, which might blur ancestral similarities.

Morphological features are most easily recognized and reflect the functional subdivision into different head parts. However, they do not help to gain a better understanding of the underlying developmental mechanisms. To achieve the latter, we need to focus on differences in gene and gene network activities between anterior and posterior head segments. Based on different morphological or molecular characteristics, the head can be differently subdivided into diverse sections:

(i) Sensing and biting. Functionally, the head can be separated into the procephalon and the gnathocephalon (*Figure 2*)[18]. The anterior procephalon contains three neuromeres that correspond to the ocular (eye) region (protocerebrum), the antennal segment (deutocerebrum), and the intercalary segment (tritocerebrum). These three neuromeres fuse to form the supra-oesophageal ganglion, and are the major sensory centre of the head. The gnathocephalon is composed of three segments that bear feeding appendages, namely mandibles, maxillae, and labium.

(ii) Hox or no Hox. As mentioned above, a boundary concerning genetic patterning mechanism is defined by the regionally restricted activity of Hox genes, which specify segment identity. This boundary divides the head into an anterior Hox-free region that includes the labrum, eyes, and antennae, and a posterior region that expresses genes of the Hox cluster comprising the intercalary segment and the gnathocephalon. Accordingly,

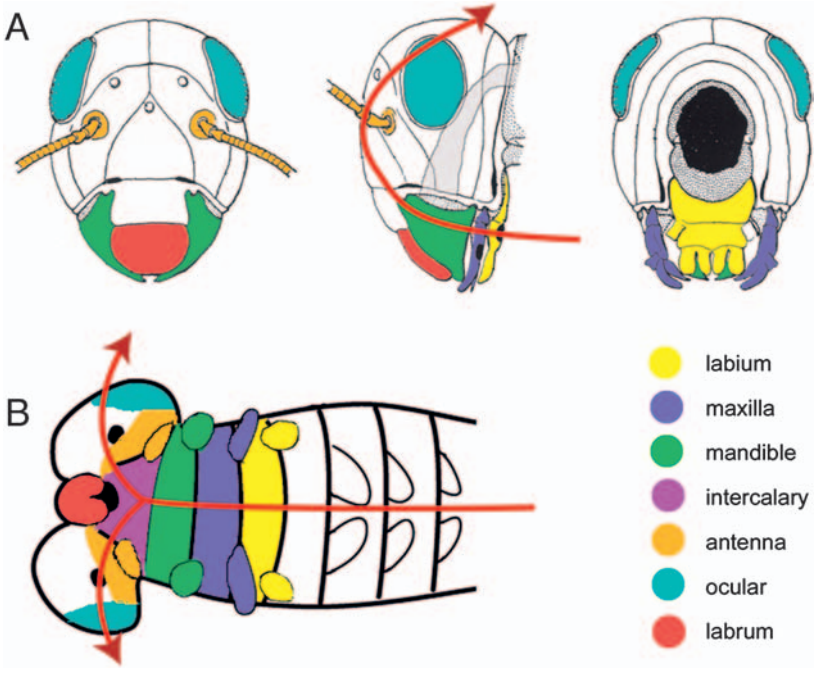

FIG. 1: *Segmental composition of the insect head. (A) A typical adult insect head is shown (left to right) frontally, laterally, and from the posterior. (B) The anterior portion of an insect embryo (anterior to the left). The segments giving rise to the structures shown in (A) have the same colour code as the structures themselves. Note that a strict posterior to anterior correlation of segments and structures is observed only for the posterior head segments (compare red arrow in A and B) (Modified from Tuxen, SL, 1963, Zool. Anz. 70, 468-471)*

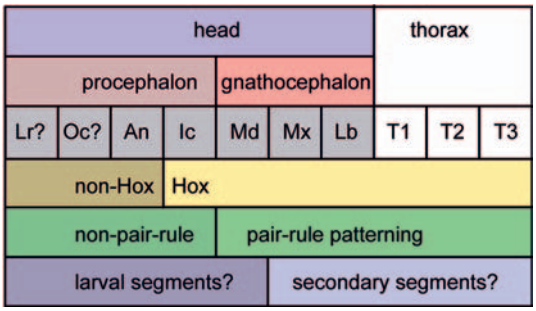

FIG. 2: Subdivisions of the insect head. The segments are shown schematically as boxes. Since the status of both the labral and the ocular segments are controversial, these are marked with a question mark. The top panels show functional subdivisions in pro- and gnathocephalon. The lower panels mark some of the subdivisions that are based on molecular observations. See text for further details. (Lr: labral segment; Oc: ocular s.; An: antennal s.; Ic: intercalary s.; Md: mandibular s.; Mx: maxillary s.; Lb: labial s.)

in a mutant of the red flour beetle *Tribolium castaneum* that lacks the complete Hox cluster, all segments become antennal, which represents the posterior-most, non-Hox-derived segment specificity[19]. This Hox expression border might actually be the vestige of an ancient »real« subdivision between different patterning mechanisms, as indicated in fossil Trilobites, where – with the notable exception of the antenna – all other head appendages are extremely similar[12].

(iii) Where pairs don't rule. The Hox genes are responsible for segment specification. However, for the actual establishment of segments, a cascade of segmentation genes is required. In the *Drosophila* trunk, metamerization is established by the activity of gap genes that control the periodic expression of pair rule genes. These then regulate the segment polarity genes, a highly conserved class of genes, which set up the segment borders. By contrast, the anterior head is metamerized without the activity of pair rule genes. Head gap-like genes seem to directly regulate the segment polarity genes there. This distinction between head and trunk segmentation mechanism has no counterpart in terms of a morphological boundary. In fact, the development of the gnathocephalic segments is indistinguishable from trunk segments. Gnathocephalic pattern formation essentially follows the well understood trunk segmentation cascade, including the periodic activity of pair rule genes. Since this class of genes is not required for the establishment of procephalic segments, there must be a clear difference between the segmentation mechanisms patterning pro- and gnathocephalon. In *Drosophila*, the mandible forms a hinge region where both the pair rule genes involved in trunk segmentation and the head gap-like genes co-operate to pattern the segment[9].

(iv) Larval *versus* post-larval head segments. There is also a difference in the regulation of the segment polarity genes, whose interactions differ for each of the anterior (procephalic and mandibular) segments, while their interaction is identical in the maxillary and all posterior segments[23]. This suggests a unique establishment of each of the anterior head segments and a common generation of the posterior segments. Interestingly, these different modes of regulation by segment polarity genes in anterior and posterior head segments may be due to their independent evolutionary origin. Classical embryology has revealed that the subdivision of the coelom (one hallmark of segmentation) occurs in two different ways. Anterior segments arise by concomitant subdivision of one large coelom, giving rise to so-called primary or larval segments. Coelomic sacs of the more posterior segments, by contrast, are usually formed one by one from a posterior growth zone. These latter segments are known as secondary segments. Such a bimodal segmentation is easily observed, for example, in some crustaceans: the nauplius larva forms three larval segments, namely first and second antennal segment (the latter corresponds to the intercalary segment in insects), and the mandibular segment[17]. In this respect, the procephalic segments and the mandibular segment are correlates of larval segments, while the remaining gnathocephalic and trunk segments are of the post-larval type. Differences in the regulation of segment polarity genes between anterior and posterior segments in the insect head could therefore reflect this ancestral subdivision in primary and secondary segmentation[20-22].

Open head questions

• As mentioned above, there are major differences between the mechanisms patterning anterior and posterior head regions. However, how the anterior is patterned, and where the transition between the different patterning mechanisms lies, remains uncertain. The main goal of our research of arthropod head development is therefore to determine the principle of pattern formation in the procephalon. This includes several developmental issues: what components and interactions comprise the gene network that patterns the anterior cellular field? What relevant boundaries must be specified? How are such boundaries established and maintained? Do they define compartments of cells that do not intermingle? What specifies the segment identity in the absence of Hox gene activity? How are the cells instructed to form the remarkable head lobes typical for arthropod embryos? How does ectodermal patterning influence the patterning of the brain?

Once we have found answers to some of these issues, we shall come closer to solving the long-standing zoological dispute about the number of segments that compose the insect head. This issue could not be resolved unequivocally by morphological approaches, and almost as many theories have been put forward as there are scientists dedicated to the issue. A molecular approach, i.e. a comparison of the expression of marker genes during embryogenesis, may provide novel cues. A similar open question is the origin of the anterior median structure called the labrum (upper lip). It encloses the preoral cavity from the front (*Figure 1*). Suggestions as to its origin vary from the labrum being a simple non-segmental epidermal sac to it being an appendage. Similar expression patterns of genes

involved in proximo-distal appendage outgrowth and a *Tribolium* homeotic transformation of the labrum into maxillae strongly suggest that it is an appendage[24]. The debate on its segmental origin, however, revolves around three possibilities: it could be the appendage of an anterior-most labral segment, the ocular, or the intercalary segment. Identifying the genes required for specifying the labrum and placing its anlagen on a map of marker genes will provide new insight into this long-standing question.

Finally, we aim to determine whether insects really do have an anterior non-segmented region that corresponds to the prostomium of annelids, ringed worms such as earthworms or leeches. This hypothesis originates from the Articulata hypothesis that puts annelids alongside arthropods as sister groups, mainly on account of the striking similarity in the morphology of their segments. The anterior-most part of annelids, the prostomium, is clearly non-segmental and contains brain and eyes. Are the insects' counterparts of these structures part of an anterior non-segmental region called acron? And if so, what is this acron composed of? This question has forfeited some of its urgency, due to analyses in molecular phylogeny that deeply separate arthropods and annelids into the distinct protostome clades ecdysozoa and lophotrochozoa, respectively. However, when we assume that head segmentation evolved only once within the protostomes, the anterior non-segmental region again pleads for homologous structures.

FIG. 3: *The red flour beetle* Tribolium castaneum *– a new model system. Unlike* Drosophila, *it is suitable for studying embryonic head development (Reprinted from reference 25 by kind permission of Elsevier Press, Amsterdam, The Netherlands)*

Beetles making headway

• Unfortunately, *Drosophila*, the prime model organism for many developmental processes, is not particularly suitable for investigating embryonic head development. During embryogenesis, the anterior region of the fly embryo is pulled into the inside. This is known as head involution, and leads to the maggot's head being hidden within the thorax. Concomitantly, the respective segments are drastically reduced, rendering the assignment of specific mutant phenotypes to specific segments a difficult and tedious task. Furthermore, the derived head morphology raises the question of how conserved the underlying mechanisms actually are. Analyses of model systems which do not suffer from these *Drosophila*-specific difficulties, but which are still easily manipulated, are therefore crucial to an understanding of head development.

We propose that the red flour beetle *Tribolium castaneum* (*Figure 3*) is currently the arthropod model system best suited for studies on embryonic head development. First, its larval head displays all structures of a typical insect head (*Figure 4*). This also suggests that the genetic principle of head development is less derived than in *Drosophila*. Second, *Tribolium* is easy to rear and produces more than enough offspring all year round under laboratory conditions. Third, recent technical advances have rendered *Tribolium* amenable to powerful experimental manipulations using forward and reverse genetics[25]. The use of a non-species-specific transformation system based on broad-range transposons facilitates transgenesis in *Tribolium* with high efficiency[26]. This technique has been successfully used to analyse enhancers[27] and to produce new tools for insertional mutagenesis[28]. The GEKU (Göttingen, Erlangen, Kansas, United States Department of Agriculture) consortium is currently creating 20,000 transposon insertion lines that will be also screened for embryonic head defects.

Tribolium's special strength lies in its powerful reverse genetics: the combination of highly efficient RNA interference (RNAi)[29] with the availability of the full genome sequence. This enables the rapid isolation and functional analysis of candidate genes. The injection of a few female pupae with double-stranded RNA fragments of a candidate gene leads to knockdown or even knockout phenotypes in numerous offspring – a technique known as parental RNAi[30]. Moreover, these phenocopies can represent a complete phenotypic series of the respective gene ranging from hypomorphic to amorphic situations, which enables a more detailed analysis of a gene's function. Furthermore, the large number of knockdown embryos that can be collected allows for the analysis of changes in gene expression patterns in knockdown animals.

To eventually determine the principles of head development, two strategies should be combined. A candidate gene approach can rapidly identify the function of several genes important for head development. This approach is necessarily biased, since it is based on previous findings in other model organisms. It therefore should be supplemented by an independent forward genetic approach, such as insertional mutagenesis. Only such a hypothesis-independent screen is able to identify novel, unexpected players in head development. The results should then be compared to other model systems that hold crucial positions in the phylogenetic tree. For instance, the hemimetabolous milkweed bug *Oncopeltus fasciatus* and the cricket *Gryllus bimaculatus* are amenable to parental RNAi, the latter also to transgenesis[31-33]. These two species could provide comparisons within the insects. To have an outgroup to insects, i.e. a group closely related to insects but which are not insects, we also analyse head gene function in the crustacean beach hopper *Parhyale hawaiensis*, which is

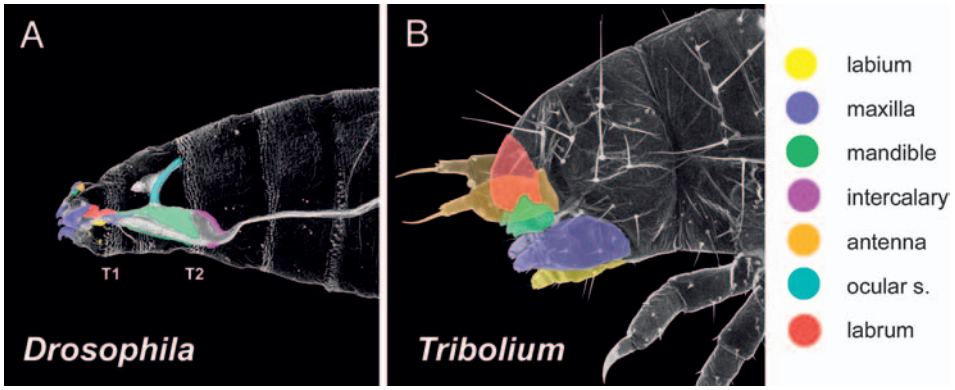

FIG. 4: Comparison of head structures of (A) Drosophila *with (B)* Tribolium. *The corresponding features have been colour-coded. (A) The Drosophila head is involuted during embryogenesis. The entire head thus comes to lie within the thorax and the structures are highly derived. This has hampered the analysis of mutants affecting head development. (B) The* Tribolium *head displays all structures typical of an insect head (compare with* figure 1A*). The effects of mutations or RNAi treatment are therefore readily interpretable.*

amenable to transgenesis and RNAi[34]. A comparative functional analysis of head development processes in a series of differently related arthropod species will help us to determine which mechanisms are evolutionarily conserved and which have undergone group-specific environmental adaptations or were generated anew.

Peliminary results on the function of gap and gap-like genes in *Tribolium* head development indicate that there are major functional changes in comparison to *Drosophila*. These results also demonstrate the ease of analysing the function of known genes in *Tribolium*, but raise the question of how variable such gene functions can be between closely related species. It will take extensive comparative approaches to ascertain which functions are species-specific and which are phylogenetically conserved.

Moreover, one has to bear in mind that the comparative molecular approach outlined above depends on the assumption that similar expression patterns indeed indicate homology of a particular structure. Unfortunately, aspects of gene expression frequently change in the course of evolution, and structures fuse or separate. If such a comparison is to be reliable, it must therefore be based on multiple genes or even on complete gene networks.

Reconstructing the Urbilateria

• Once we have obtained a deeper understanding of head development in one insect species, we can investigate how conserved these mechanisms are within insects, arthropods, protostomes, and even all bilateria. This may reveal a core network of players and conserved interactions responsible for the patterning of the anterior head. Indeed, the strikingly similar expression patterns and functions of several anterior genes nurture our hope of finding such similarities. The identification of conserved genetic circuitries may provide a framework for studies in other animals, such as mice, that are more difficult to investigate. Moreover, since such phylogenetically conserved gene networks were probably present in the last common ancestor of all bilateral symmetric animals, comparisons between the different animal phyla will provide some insight into the nature of our extinct ancestor, the Urbilateria.

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