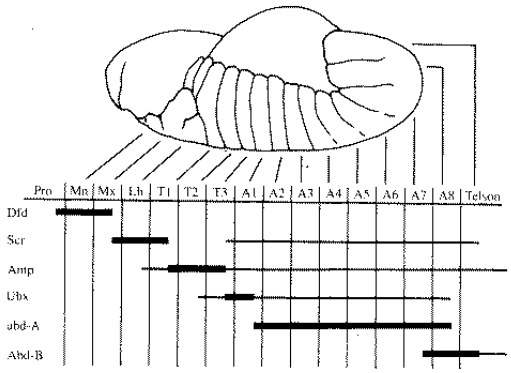
The molecular Bauplan



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‘Was ist mir geschehen?’ dachte er.

Franz Kafka, *Die Verwandlung*

Contents

1 Introduction

In the 30s of the nineteenth century a famous biological debate was held between the two French zoologists Cuvier and Saint-Hilaire (reviewed in references [18, 49]). According to Cuvier, the animal kingdom was subdivided in four branches: the radiates (coelenterates and echinoderms), the molluscs, the articulates (insects, crustaceans and annelids) and the vertebrates. Thinking about evolutionary relationships between species of separate branches was senseless; if one of the parts of an animal would be exchanged for a comparable part of a species of another branch, the organisation of the organism would collapse. All the parts of an organism were dependent on all the other parts, a situation that was called "integrated function" by Cuvier. Organs such as legs and eyes had a similar appearance in different phyla, just because they were used for similar functions, not because they really *were* similar.

Saint-Hilaire's view was different. In his view animal anatomy was a continuum, the different forms and functions found all over the animal kingdom were mere variations on a theme. "[Nature] were restricted to the same primitive ideas, one sees her always tend to cause the same elements to reappear" [1].

Until recently, a view similar to Cuvier's ideas has been widely held in developmental biology. Only with the greatest caution could results obtained in experiments with one species be applied to other species. Names of embryological structures were often given separate names (for instance Spemann's organiser in amphibians, Hensen's node in chicken and simply *node* in mice are in fact all the same structures) [2]. The same was true for genes. For instance, long after experimental evidence had strongly suggested their orthology, and the "general name" had already been used by many authors, scientists would still scowl if the *Drosophila* HOM-C complex was simply called *Hox*-complex.

The last decennium evidence is accumulating suggesting that Saint-Hilaire may have been right after all. In the search for the genetic basis of develop-

[1] E. Geoffroy Saint Hilaire (1807) Citation taken from reference [18], p. 623

[2] Illustrative for this Cuvier-like paradigm, or perhaps "habit" is that in the Hubrecht Laboratory, an important institute for developmental biology in Utrecht, the groups are circled — to say it too black-and-white — around the model animal that is studied. To nuance this immediately: often this is done for practical reasons (other experimental techniques are needed for frogs than for zebra fish and mice), and the groups study most of the time (but not always!) separate subjects.

ment, a general picture has emerged that links basic developmental processes in very different animal phyla, such as chordates and arthropods. More and more transcription factors have been found that seem to initiate the development of similar organs (like eyes, or limbs) in animals that were believed to be completely dissimilar. The similarity between these transcription factors often comprises nothing more than a similarity in the DNA binding domain. More interestingly, a growing number of transcription factors is known that appear to be "compatible" between species. Striking is the case of *Pax-6*, a transcription factor known to be important in eye development in many different phyla. Each of the *Pax-6* orthologues isolated from mice, from bag pipes and from squids is able to induce ectopic eyes in the fruit fly (for citations, see section 4).

The identification of more and more conserved patterning mechanisms has led to the proposal of several hypothetical ancestors to the animal kingdom, and a mere abstract notion of the defining character, or synapomorphy of the animal kingdom.

Since a class of patterning genes, the *Hox* genes, may "[...] encode relative position in all animals" Slack and others [56] proposed that the patterning system, formed by the Hox genes and some other genes, "[..] should be adopted as the defining character, or synapomorphy, of the kingdom Animalia". A common ancestor, the *Urbilateralia* of the animal phyla was proposed by De Robertis and Sasai [10], which would have dorsoventral and anteroposterior polarity, a central neural system, photoreceptors and a circulatory system. A genome ancestral to the genomes of the animalia, the Cambrian *pananimalia* genome was proposed by Ohno [45]. The *pananimalia* genome would contain a gene for the formation of ligaments and tendons, a gene coding for hemoglobin, a *Pax-6* gene for the formation of sensory organs and series of *Hox* genes. In this paper, I will discuss some of the genetic similarities of the animal taxa. Several of these genetic similarities are challenged. I will therefore conclude with a proposal extending the zootype idea of Slack et al [56].

The data available at present contributing to a picture of the *Urbilateralia* ancestor and to the more abstract notion of the *zootype* are reviewed and discussed. The subject of this paper is restricted to the *transcription factors* that are thought to be conserved over the animal kingdom. Hence, this text will *not* cover the "structural" genes like hemoglobin and lysloxidase that are included

in Ohno's notion of the pananimalian genome.

The reader might feel distracted from this main subject in two sections. The first of these is the discussion of Garstang's hypothesis on the origin of the chordata. The second one is the section discussing eye regression in the blind cave fish *Astyanax*. However, these two sections have been included to criticise some hypotheses coming up in the light of the many molecular similarities found in the animal kingdom.

In the next chapter the patterning of the anteroposterior axis by the *HOM-C/HOX* cluster will be described. The *HOM-C/HOX* cluster was the first system in which molecular homologies between mice, flies and frog were identified. It will be discussed how "tinkering" with the *HOM-C/HOX* cluster may have resulted in the diverse *Baupläne* of the vertebrates and the arthropods. The third chapter will discuss the evidence and criticisms on a recently revived hypothesis of Saint-Hilaire as to that the dorsoventral body axis would have been inverted in a chordate ancestor. The fourth chapter will discuss the data available at present supporting the notion of a possible conserved system involved in eye development. This system involves the highly conserved transcription factor *Pax-6*, which is believed to be the "master gene" that would turn on a regulatory cascade resulting in the development of eyes. This idea is criticised on several points. Furthermore, the "caveats" of the idea that a single gene could turn on or off a regulatory cascade are discussed in the light of adaptive eye reduction in the blind cave fish. In chapter five, an extension to the idea of a zootype [56] will be proposed and discussed. This zootype is built up from the information reviewed in this paper.

Sometimes the lists of genetic similarities between metazoan phyla are exhaustive. In these cases it has mostly been tried to refer to the important review articles on the subject and to sketch the general picture instead. It could however not be avoided that the reader is sometimes overwhelmed by the enormous list of genes regulating each other upstream and downstream. Please keep in mind in these cases that "it is not difficult, it is just very complex" (Ben Hesper, personal communication).

2 The anteroposterior axis: conservation of the Hox-complex

2.1 Hox genes specify position in the embryo

In 1969, Wolpert proposed his *french flag* mechanism [64] by which a cell would get information about its position in the developing embryo and express the appropriate genes for a cell in this position. The proposed mechanism consisted of two stages. First, a field, for instance a morphogen gradient had to be generated, from which the cell could "know" about its position in the embryo. Wolpert called the information about a cell's position in the embryo "positional information". Second, the cells had to *interpret* this information, for instance by means of dose sensitive promotors sensitive for the concentration of morphogen, in order to express the appropriate genes.

More and more is known about how "positional information" is generated in a number of species. For instance in *Drosophila* positional information is built up in a cascade specifying more and more specific regions of the embryo (for an excellent review see [33][1]. In the frog *Xenopus laevis* most of the positional identities seems to be specified during the process of neural induction (see for a non molecular review [44]). The general picture emerging from this is that the way positional information is generated is fairly variable over the animal kingdom. In contrast, the last decennium it appears that the way in which "positional information" is *fixed* once it has been generated is strikingly similar among many different types of organisms, including arthropods and chordates.

Position was first known to be fixed by the transcription factors belonging to the HOM-C/HOX-C class, which are more and more collectively referred to as the *Hox* genes[2].

[1] Lawrence warned the reader that his book "[...] **should not be quoted for matters of fact**" (author's boldface). However, the book presents a good overview of the early embryonic processes in *Drosophila* ending in the expression of the *Hox* genes. It is referenced here as such, a good introductory text.

[2] Originally, the term Hox-genes was exclusively reserved for vertebrate orthologues of the *Drosophila HOM-C* complex (see for example reference [40] or reference [12]) However, recently the term is used more and more to indicate the four vertebrate *Hox* clusters and the genes orthologous to these in invertebrate species (among which the *Drosophila HOM-C* complex) (see for instance references [56, 35, 4, 55, 21, 53]). In [6] even the words "[...] homeotic (or *Hox*) genes

Hox proteins are part of the larger class of transcription factors, that all contain a highly conserved DNA binding domain. This DNA binding domain, the *homeodomain* is encoded by a 180 base-pair long conserved DNA sequence, the *homeobox*[3].

NMR and X-ray studies of the homeodomain of several Hox-genes have revealed that the homeodomain has a very characteristic tertiary structure, although the protein sequence (primary structure) may differ strikingly among different the *Hox* genes. It consists of an N-terminal arm, followed by three α-helices. The N-terminal arm binds strongly to the minor groove of the DNA. The third helix is essential for specific and successful DNA binding and fits completely or loosely into the major groove (reviewed in reference [53, 35]).

The core DNA sequence to which *Hox* proteins bind is only four base pairs long. The binding specificity is influenced by the bases surrounding this core. The DNA sequences to which the homeodomain binds are very simple and therefore a gene can easily gain or loose regulation by a *Hox* gene (reviewed in [6]).

Hox genes are expressed in a specific overlapping domains along the anteroposterior axis. In general, anterior expressed *Hox*-genes have shorter expression domains, and are less overlapped by the expression of *Hox* genes in the same region than *Hox* genes expressed more posterior. Posterior *Hox* genes have longer expression domains and more *Hox* genes are expressed together in the same domain than anterior (reviewed in [35]. Xenopus: [13]).

Interestingly, *Hox* genes are clustered on the genome in the same sequence as they are expressed on the anteroposterior axis. This is called the *colinearity* of the Hox-complex. In *Drosophila* the *Hox* cluster is split up into two parts, the Bithorax complex (BX-C) and the Antennapedia complex (ANT-C).

In *Drosophila*, a knock out of a *Hox* gene often results in the transformation of a segment towards a more anterior type of segment. It is easy to see that this results from the overlapping *Hox* expression domains and from a second principle, *posterior prevalence*. Posterior prevalence means that if both an anterior and a posterior *Hox* gene are expressed in a segment, it will get the identity

[...]" (author's italics) were found.

[3]To prevent confusion about the terminology around *Hox* genes: the word *homeobox* refers to the 180 base pairs long DNA stretch that codes for the *homeodomain*, the DNA binding motif that is characteristic for all homeodomain transcription factors. The group of the homeodomain proteins, that are coded by *homeobox genes* contains many classes of transcription factors. [40]

of the most posterior gene. It is easy to understand that in an overlapping domain the identity of this segment would be transformed to a more anterior fate, if the posterior gene is knocked out. Similarly, in gain-of-function mutations, anterior segments are often specified as a more posterior type (reviewed by [35, 40]).

The homeoboxes of *Hox* genes are not only well conserved, the gene of one species is also active in other species. For example, the *Drosophila Hox* genes *sex combs reduced* and *Antennapedia* can substitute for *lin-39* and *mab-5* in *Caenorhabditis elegans* [28]. Secondly, it has been shown that human *Hox-4.2* can substitute for *Deformed* [39].

2.2 Building new "body plans" by shifting Hox expression domains

As we have seen above, a change in the expression of a Hox gene can result in a homeotic transformation, the respecification of a body segment. In many cases, the more posterior specifications override the more anterior specifications, a process that is called "posterior prevalence".

The diversification of body plans since the cambrian explosion is widely believed to be driven by Hox-gene duplication events. For example, in vertebrates four Hox-clusters are present. These are thought to have originated by duplication events, both of the whole cluster and of individual *Hox* genes (reviewed in [26]).

The phylum of the arthropods is enormous, containing such different organisms as centipedes and crabs. Especially, the enormous radiation of jointed appendages in the arthropods is remarkable. Ranging from myriapods (centipedes and millipedes) with a row of identical trunk segments, each of them bearing a locomotory pair of legs, to crustaceans, in which almost every body segment has a different set of appendages, used for feeding, locomotion, sensing, defence and grasping, the arthropod phylum has one of the most diverse body plans of the animal kingdom (reviewed in ref [55])

One would think that this enormous variation of body plans is driven by a large diversification in the Hox-cluster. The enormous diversification of the arthropod phylum would have been accompanied by 1the expansion of the Hox-gene cluster by duplication events (reviewed by [21]), such as it is be-

lieved to have happened in the evolution of the chordates [26].

However, recent research suggests that the *Hox*-cluster remained untouched during the diversification of the arthropod body plan. The whole cluster of eight *Hox* genes is present in centipedes as well as in *Onychophora* in which hardly any segment diversity is present. Centipedes are simple arthropods with homonomous trunk segments, each of them bearing two legs. *Onychophora* belongs to a phylum that is believed to be closely related to the arthropods, and also has homonomous trunk segments.

Especially, the presence of the *Ultrabithorax* (*Ubx*) and *abdominal-A* (*Abd-A*) genes that are unique to arthropods in the centipede and in *Onychophoran* is remarkable. In *Drosophila*, *Ubx* specifies the identity of the second thoracic segment, where it transforms the second wing pair in a set of halteres (reviewed in [33]. *Abd-A*, specifying the fate of the abdominal segment, suppresses *distall-less*, in this way preventing the formation of legs. Both the studied Onychophoran and the centipede lack wings and have appendages on all the trunk segments.

Study of the expression domains of *Ubx* and *Abd-A* in the centipede and in the Onychoporan suggest two interesting trends that may have taken place in arthropod evolution. *Ubx* and *Abd-A* are expressed in the centipede in all the segments with walking legs. The boundary of the *Ubx* and *Abd-A* expression coincides with the transition from walking legs to poison claws. In the *Onychophoran* however, expression of *Ubx* and *Abd-A* is restricted to the last lobopod bearing segment and the terminus. The expression of *Ubx* and *Abd-A* in the last lobopod segment is somewhat problematic since this segment would be expected to have another identity induced by the *Ubx/Abd-A* expression. However, in many *Onychophoran* species the last lobopod segment is vestigial, indicating that this boundary may correlate to *cryptic* transition in segment identity [21].

These results suggest two possible mechanisms by which the enormous segment diversity in the arthropods could have evolved. First, changes in the regulation of downstream targets of *Hox* genes may have occurred. An example of this could be the repression of *Distall-less* by *Ubx* and *Abd-A* gene products that occurs in insects, resulting in limbless abdominal segments. In centipedes *Distall-less* is not repressed [21], suggesting that the targeting of *Distall-less* by *Ubx/Abd-A* may have evolved only in the insect lineage. Second, the upstream

regulation of the expression of *Hox* genes could have changed during arthropod evolution, resulting in shifts of the different domains of *Hox*-expression. This, in turn, would result in a shift of the border between different appendage types. One could imagine, for example, that the terminal appendages of the *Onychophoran* would serve better as a locomotory organ than a lobopod. A simple shift of the expression domain of *Ubx/Abd-A* to more anterior position could result in the transformation of lobopods into terminal appendages.

Very recently, support has been found for the idea that shifts in *Hox* gene expression domains can be an important source in the evolution of segmental diversity [4]. Crustacea, to which species such as shrimps and woodlice belong, are a highly diverse group of arthropods. They have a variable number of often ramified appendages, that are used and specialised a great range of functions. In most arthropod species, the thoracic appendages are used for locomotory functions, but in a number of crustacean species the first two pairs of thoracic legs have a morphology resembling that of the maxillae. These appendages, called *maxillipeds*, are specialised for food manipulation (reviewed in [4]). The evolutionary change of the more posterior, thoracic appendages towards a more anterior, maxillary fate, resembles homeotic transformation characteristic of *Hox*-gene null mutants (reviewed in [40]). This suggests that *Ubx/Abd-A* expression is absent in the segments with maxillipeds. Indeed, in the thirteen crustacean species studied, the *Ubx/Abd-A* expression appear to run from the posterior trunk up to, but excluding the segments bearing maxillary or maxillipedal appendages.

2.3 Evolutionary consequences of the *Hox* data

Concluding, the *Hox* genes specify anteroposterior position in many animal taxa. The *Hox* complexes of the different animal phyla are likely to have evolved from a single, colinear *Hox* cluster. Within the chordate lineage, two duplications of the whole *Hox* cluster may have occurred. Primitive chordates — the urochordates and the cephalochordates — only have one *Hox-cluster*. One duplication may have taken place in the lineage leading to the vertebrate lineage. Of these vertebrates with two *Hox-clusters* only the hagfish, acranial vertebrates, are living today. A second duplication event is likely to have taken place in the ancestor of the craniate vertebrates. As a result of these duplications, redundancy was created. This redundancy may have freed several *Hox-*

genes from essential regulative functions. As a result, paralogous *Hox* genes may have drifted apart and have come to serve different functions in developing vertebrates. This vast increase of *Hox* gene function may have resulted in the enormous radiation of vertebrate species.

In the arthropods, the *Hox*-cluster is highly conserved. Only one *Hox*-cluster is present, and in most taxa, the colinearity is preserved. An exception to this rule is *Drosophila*, in which the *Hox*-cluster is likely to have split up in the Antennapaedia and Bithorax clusters. An interesting evolutionary mechanism in the arthropods is suggested by the work of Averof and Grenier [4, 21]. The radiation of body plans in the arthropods have occurred by "natural homeotic transformations". These natural homeotic transformations are not due to disfunction or ectopic expression of *Hox* genes, but by shifts of the *Hox* expression domains.

3 Conservation of dorsoventral patterning in bilateral metazoa

3.1 Dorsoventral patterning is conserved between insect and chordates

Not only the anteroposterior axis seems to be patterned by orthologous genes in many distantly related metazoan taxa. Recently, evidence has been found suggesting the existence of a conserved dorsoventral patterning system in bilateral metazoa. Roughly, this dorsoventral patterning system consists of two antagonistic patterning molecules, expressed at opposite sides of the developing embryo. A molecule which is expressed at the neural side of the embryo is antagonised by a TGF-β related protein, inhibiting neural induction, that is expressed at the opposite dorsoventral position. Intriguingly, the elements of this patterning system seem to be species independent: the dorsoventral pattern of an insect can be manipulated with vertebrate proteins, and vice versa.

Evidence for this "universal" dorsoventral patterning system has been found in *Xenopus laevis* and *Danio rerio* (both vertebrates: a frog and a fish respectively) and *Drosophila melanogaster* (an insect).

In *Drosophila melanogaster short gastrulation* (*sog*) is expressed dorsally, at the neural side. It is antagonised by a TGF-β orthologue, *dpp*, expressed at the abneural side.

In *Xenopus laevis*, dorsoventral patterning seems to take place in a similar way. In dorsal ectoderm explants, neural tissue is induced by several protein factors, such as follistatin [24], noggin [57] and chordin [52]. All these protein factors are expressed in dorsal mesoderm, thus suggesting a role in neural induction and in the specification of dorsal fates.

Of these three proteins, chordin has a high sequence identity to — and has actually been identified because of its similarity to — the *short gastrulation* (sog) protein of *Drosophila*.

Some experimental results have suggested that also in *Xenopus*, a neuralising factor (chordin), is antagonised by a protein of the TGF-β family. Firstly, dorsal ectoderm is prevented from adopting a neural fate by a protein of the TGF-β family: bone morphogenesis protein 4 (BMP-4), closely related to *Drosophila*'s *dpp*. If the cells of an isolated piece of dorsal ectoderm — nor-

mally developing into ciliated epidermal tissue – are dissociated, they will express neural specific markers. This effect is antagonised by BMP-4: dorsal ectoderm is not neuralised in the presence of BMP-4 [63], suggesting that BMP-4 is an epidermal inducer. Moreover, these experiments suggest that neural induction might take place by the inhibition of an epidermal fate, rather than the induction of neural tissue.

Secondly, overexpression of chordin results in severe dorsalisation (strong development of dorsal tissues, such as cement gland and neural tissue at the expense of more ventral tissues). This effect can be rescued by the overexpression of BMP-4 in early embryos, resulting in more or less normal tadpoles. [52].

The strongest evidence for conserved dorsoventral patterning in bilateral metazoans was found when the *Drosophila* proteins *dpp* and *sog* were injected in *Xenopus* and, vice versa, when the *Xenopus chd* and *BMP-4* were injected in *Drosophila* embryos. *Sog* promotes a neural (dorsal) fate in ventral cells if its mRNA is injected in *Xenopus* embryos. Similarly, if slightly modified *chd* mRNA is injected *dorsally* in *Drosophila* embryos, again a neural fate is promoted, resulting in ventralised *Drosophila* embryos. The same is true for the proteins specifying *abneural* fates. *Dpp* promotes a ventral fate in *Xenopus*, whereas the *Xenopus* abneural factor *BMP-4* promotes dorsal fates in *Drosophila*. The antagonisticity between *dpp* and *sog* works as well in *Xenopus*. Injection of both *dpp* and *sog* in 4 to 16 cell *Xenopus* embryos gives nearly normal phenotypes [27].

3.2 Inversion of the dorsoventral axis in the chordates

The similarity between the sog/dpp and the chd/Bmp-4 system has resulted in the revival of a long forgotten hypothesis on the evolutionary origin of the chordate body plan [3]. The famous zoologist Geoffroy Saint-Hilaire studied the anatomy of a lobster. In stead of looking at it in its normal orientation, with its belly oriented to the table, he turned it upside down. In this orientation, the lobster's anatomy stroke him as very similar to the body plan of a vertebrate: a dorsal nerve cord, underlain by a gut, lined by two lateral "rows" of segmented muscles (Fig. 3.1). This observation made him to hypothesise that the dorsoventral axis of the "articulata" is the same as in "vertebrata", although it is inverted.

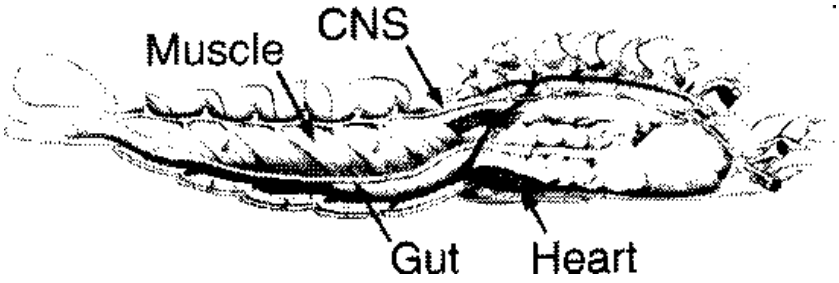

Figure 3.1: The drawing of Saint-Hilaire, showing that the ordering of the central nervous system, the muscles, the digestive tract and the heart in the lobster is the same as in vertebrates. (Taken with permission of the authors from reference [10])

This hypothesis has been picked up after the above described results on dorsoventral patterning had been found. The dorsally expressed gene of *Xenopus* induces a ventral fate in *Drosophila* and vice versa. This suggests that the ventral part of *Xenopus* corresponds to the dorsal part of *Drosophila* and the other way around. Therefore it was suggested that the dorsoventral axis was inverted prior to chordate evolution [3, 52].

3.3 Garstang's hypothesis: an alternative to dorsoventral inversion

Although much of the molecular data described above is in favour of dorsoventral inversion hypothesis, it has been challenged by alternative hypotheses.

The *auricularia* hypothesis of Garstang [16] was picked up by Lacalli et al [31, 32] as a less radical explanation for the inverted dorsoventral patterning system in chordates. As a common ancestor for the deuterostome phyla echinoderms (sea urchins and star fish), enteropneusts (hemichordates: acorn worms) and chordates (tunicates, lancelets and vertebrates) Garstang proposed an ancestor resembling an *auricularia* type echinoderm larva The *auricularia* possesses a ciliated ring, directly underlain by a nerve cord. This ciliated ring separates an aboral and an oral epithelial region. During the evolution of the

chordate branch, the ciliated ring would shift dorsally and fuse in the mid-dorsal region. The ciliated ring of the *auricularia* would be homologous to the vertebrate neural ridges. The aboral epithelial field, internalised if the neural ridges fuse, were to evolve into the neural plate. Evidence for this view was found in comparative microscopic anatomy studies between echinoderms, hemichordates and amphioxus. Ultrastructural correspondences were found between the so-called multipolar cells — part of the larval ciliated band — of the studied echinoderm and hemichordate species. These multipolar cells were related to similar cells found in the *Amphioxus* nervous system. [32].

Central to the hypothesis of Lacalli [31] is the fate of the aboral epithelial field. In protostomes, no counterpart of the oral field is present. The body surface of protostome would therefore be completely covered with a homologue of the aboral field. The epithelium in chordates, however, would be derived from the oral field. As the ciliated band shifted dorsally during chordate evolution, the aboral field — the neural plate now — would fold inwards. In this way the neural side of the aboral field would be directed dorsally in chordates, whereas it is directed ventrally in protostomes. In contrast, the side of the aboral field corresponding to the *dorsal* region in protostomes would correspond to the *ventral* part of the aboral field (the ventral side of the neural tube) in chordates.

4 Pax-6: *The* "master control gene" of eye development?

4.1 Eyes: of poly- or oligophyletic origin?

The various types of eyes that are found among metazoan phyla have long been seen as a classical example of convergent evolution. Based on a study on the ultrastructural properties of photoreceptive animal cells, eyes have been estimated to have evolved independently in forty to sixty-five phyla [51].

Indeed there are large differences between eye types. It would be hardly conceivable that the compound eyes of insects, consisting of many small eyes with separate lenses, could be in any way related to the camera like vertebrate eye. Similarly, the eyes of cephalopods and vertebrate have the same outside appearance, but their development is so different that they have long been thought to be convergent structures.

The last decades however, evidence is accumulating suggesting an oligophyletic origin of the metazoan eye. Metazoan eyes share a number of common structural properties. First, opsin, a protein excitable by light, is exploited in almost every known animal eye. Second, crystallins, a class of hydrophilic proteins with high refractive indices in dense solutions (reviewed by [59, 9] are found in many lenses.

Eakin (1979) challenged the arguments of [51] that photoreceptive cells would have independently evolved several times in an update of one his earlier papers [14]. Eakin argues that photoreceptive cells are diphyletic in origin. According to Eakin photoreceptors can be grouped in rhabdomeric and ciliary types. Rhabdomeric, or microvillic photoreceptors would be mainly found in protostomes and in echinoderms. Light sensitive structures settle in microvillic bends of the cell membrane. Ciliary photoreceptors would be found in deuterostomes with the exception of echinoderms (especially seastars). Photoreceptive pigments would reside at the base of the cilium. The forty to sixty-five different photoreceptor types would derive from either the ciliary or the rhabdomeric motive, by means of surface area increases of the cell membrane. For example, the membrane outgrowth of vertebrate rods, consisting of many superseded discoid structures, is thought to derive from a single cilium. As Eakin puts it: "[...] I am holding to the Darwinian principle of decent and

modification."

4.2 Pax-6 is important in eye development of many metazoan taxa

This idea of an oligophyletic origin of animal light sensitive organs has recently gained substantial support. The identification of a single gene that appears to be important in eye development in many distantly related species in fact suggests even a monophyletic origin of eye-like organs.

Pax-6, a member of a class of transcription factors containing at least a paired box DNA-binding domain and sometimes a homeobox, has been shown to be mutated in at least three vertebrate congenital eye abnormalities. Mice and rats heterozygous for the *small eyes* allele (reviewed in [48]) suffer from reduced eyes. Humans heterozygous for pax-6 mutations suffer from aniridia, absence of the iris, or Peter's anomaly, a congenital physical connection between the lens and the cornea.

Homozygous mutations in murine Pax-6 are lethal and result in the absence of eyes and nose and in brain abnormalities [22], suggesting that Pax-6 is important in the development of the prosencephalon, forming nose related structures, and in the diencephalon and the eyes. Similar homozygous phenotypes in humans have also been reported.

A similar mutant phenotype in the fruit fly *Drosophila melanogaster* is known: *eyeless*. *Eyeless* mutants are characterised by the complete or partial absence of eyes (reviewed by [23]). Interestingly, a Pax-6 orthologue has been shown to be affected in *Eyeless* phenotypes [48], suggesting that Pax-6 might be important in eye development in many different metazoan species. This view is supported by the great many of animals in *Pax-6* orthologues have been identified [48, 58, 5, 34, 19].

Experimental results have led to the idea that Pax-6 might be a "master control" gene, controlling eye development in many species. Using an ingenious experimental setup they were able to ectopically express the Pax-6 orthologue *eyeless* in *Drosphila*. Intriguingly the ectopic expression of *eyeless* switched on eye development in the wing, leg and antennae imaginal discs.

Ectopic eye expression in *Drosophila*, using the same technique appeared also to be possible if the murine Pax-6 orthologue *small eyes* was ectopically

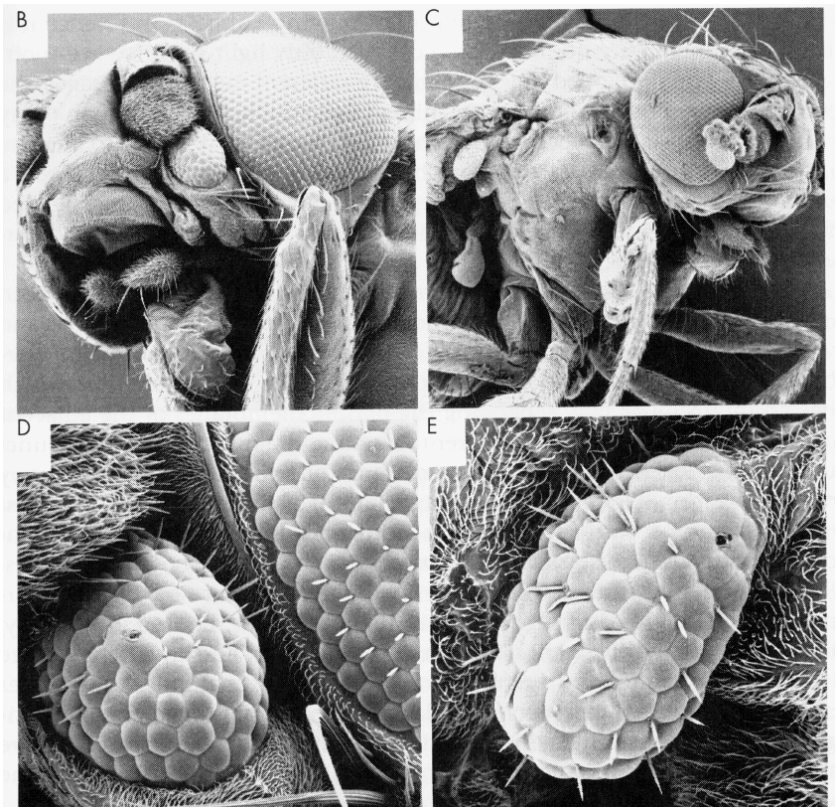

Figure 4.1: Ectopic eyes in *Drosophila*, induced by the ectopic expression of the Drosophila Pax-6 orthologue *eyeless*. Interestingly, non-Drosophila Pax-6 orthologues are also able to induce ectopic eyes in Drosophila. Among these are Pax-6 orthologues from mouse, squid and ascidian. (Taken with permission of the authors from reference [23])

expressed [23]. Additional pax-6 orthologues that could induce ectopic eye expression in *Drosophila*, were isolated from the squid *Loligo opalescens* [59] and the ascidian *Phallusia mammillata* [19].

Summarising, pax-6 is known to be involved in the development of vertebrate, insect, molluscan, ascidian and nemertean eyes. The pax-6 orthologues of *Drosophila*, mouse, the squid *Loligo* and the ascidian *Phallusia* can induce ectopic eyes in *Drosophila* imaginal discs.

These results have led to the now widely held view that pax-6 would be *the* metazoan "master control" gene of eye development. Pax-6 would stand at the top of a cascade of all the genes involved in eye development. The specific downstream genes would have changed during evolution, whereas the "switch", turning on the downstream genes, would have remained unchanged.

4.3 Pax-6 and adaptive eye reduction

How far the idea of "master control gene" for eye development has percolated in a short time into developmental biology is illustrated by research which was conducted by [5] on eye regression in the blind cave dweller *Astynax fasciatus*.

Astynax fasciatus, a small Mexican fresh water fish, has several cave dwelling populations that most probably have split off during the pleistocene. In some of these populations eyes have degenerated, whereas the nonvisual senses are very well developed (Fig. 4.2) (reviewed [61]).

From a certain developmental stage onwards, eye development in cave *Astynax* eyes seems to be arrested. The cave form's pigment epithelium is less well developed than the surface form's pigment epithelium. Moreover, the production of crystallin in the lens fibers is strongly reduced in the cave form. As it will be discussed below, crystallins are an important downstream target of Pax-6.

Since eye reduction in *Astynax* resembles in a way the mild eye anomalies that occur in heterozygous Pax-6 mutants, Behrens [5] hypothesised that in the evolution of the cave forms of *Astynax* relative to the surface forms, the expression of Pax-6 might have been lost, or at least altered.

Their results clearly showed that this is not the case. The predicted protein sequences of the Pax-6 gene of the cave dwelling and of the surface *Astynax* are identical. Moreover, the Pax-6 protein of *Astynax* was 98% identical to the zebrafish's (*Brachydanio rerio*) Pax-6. This suggests that there is no difference

in activity of the Pax-6 protein between cave and surface forms. Secondly, no differences in the expression of Pax-6 in the developing eye and brain were observed. It was suggested that an inability of *Pax-6* to bind to downstream targets due to alternative splicing could have caused the cave phenotype (the cause of a human congenital eye disease [15]. Nonetheless, the general picture seems to be that Pax-6 functions perfectly normal in the studied cave populations of *Astyanax*.

This negative result is not surprising. The study of murine homozygous mutants of *small eyes* suggests that Pax-6 plays a crucial role in vertebrates, not only in the development of eyes, but also in the development of the olfactory organ and part of the central nervous system[22]. Therefore, it is likely that an altered *Pax-6* expression will not only affect the eyes, but also the non-visual senses and the central nervous system. *Astyanax* cave forms even have *better* non-visual senses than their epigean conspecifics [61]!

It would be hard to believe that in the little time the *Astyanax* cave forms have evolved (since the pleistocene), such a basic transcription factor could be discarded, whereas it seems to have persisted all over the metazoan taxa since the precambrium. To say it in an "adaptionist" way: evolution could have chosen a safer way to get rid of the eyes.

Crosses between cave and epigean forms, that resulted in phenotypes ranging from completely reduced to nearly normal eyes in the F_2, led to the assumption that at least six loci would be responsible for the "eyeless" phenotype. It has been argued that these loci would more likely contain regulatory genes than structural genes since most of the components of the are developed in the cave form (reviewed in [5]).

However, one could also argue that it is more likely that the expression of a number of structural genes is affected during eye reduction. First, the risk of epistatic effects, like the reduction of the olfactory senses in the case of Pax-6, would be much lower for mutations in the regulation of downstream genes. Second, this scenario would be more likely when thinking of the "selectional advantages" an organism would get from the reduction of an unused organ. Several advantages would be feasible. One could think of the energetic advantage of not having to waist unused proteins, or of freeing the "brain capacity" normally allocated by the visual system for other (sensory) functions. In both of these scenarios one could imagine several "safe" mutational events knock-

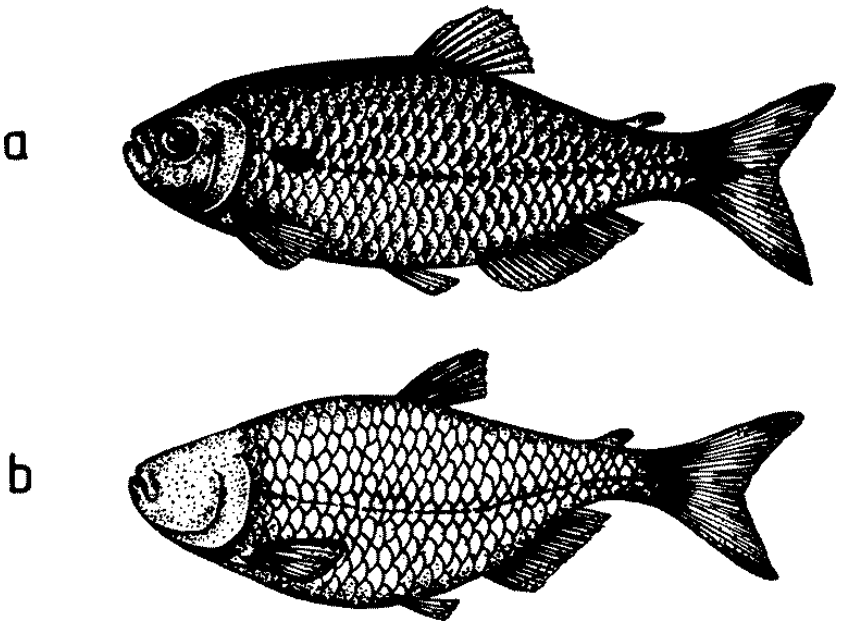

Figure 4.2: Epigean (a) and cave-dwelling (b) forms of *Astyanax fasciatus*. Some authors have hypothesised ([5]) that the absence of eyes in the cave-dwelling form of *Astyanax* might have been caused by a mutation in *Pax-6* (see text). (Taken without permission from reference [61])

ing out the production of a protein involved in eye development. For example, I would suggest that the loss of a neural cellular adhesion molecule (NCAM) involved in connecting neurons of the optical nerve to the visual cortex could result in the abortion of the differentiation of photoreceptor cells (reviewed in [5]). This process would be similar to the death of neural cells not being able to make any synapses to other neurons, such as it occurs in the development of the brain.

A candidate for the first scenario, in which the synthesis of a costly protein is suppressed, would be the inhibition of crystallin synthesis. Crystallins are important proteins present in the eye lenses of many vertebrate and invertebrate species. They are important downstream genes of *Pax-6* [59, 9] and will be discussed in more detail later.

In the lens cells of the cave dwelling *Astyanax*, no crystallins are synthesised [5]. One could explain this by loss of function mutations in crystallin promotors (see for instance [58]). The loss of crystallin synthesis might be energetically favourable and could be followed by the suppression of other eye specific proteins. In this scenario the eye structures would be normally formed. I would suggest two possibilities for the sudden abortion of eye development that is observed in the cave dwelling *Astynax*. Firstly, the synthesis or normal binding of an important signalling molecule needed at this stage could be absent.

Secondly, in many developmental processes physical forces play a major role. The development of many anatomical structures, like tendons connecting a muscle to a bone or joints between two bones, is very much dependent on the physical forces acting upon them. If these interactions and forces change during development, the outcome of the developmental process can change completely. For example, in birds the tibia and fibula are normally connected via a bony bridge. If the tibia and fibula are separated by a piece of gold, both the tibia and the fibula develop a joint with the femur. This situations resembles that of reptiles. Processes like this have been suggested to play an important role in the evolution of novel morphological structures [43]. It is likely that the exact shape and stiffness of several eye structures such as the eye lens or the vitrial body is affected by the loss of a number of structural genes. Without the shape and stiffness of the normal eye, eye development might not complete.

Concluding, care should be taken with the idea of a “master control gene”.

Pax-6 is certainly very important in eye development. For, absence of *Pax-6* results in severe eye abnormalities. But, as illustrated above, it is dangerous to turn this observation around and to suggest that where eyes are absent, *Pax-6* will be absent. First, *Pax-6* is too important in the development of a large part of the central neural system (eye+diencephalon, nose+prosencephalon) to be missed, even in a blind species. Second, in a developmental process, many genes, structures and processes work and fit together. The loss of any of these many genes and processes might completely or slightly disturb the final structure. A number of transcription factors that could compete for a position in the "master committee" are described below.

4.4 The "master committee" of eye development

"[..] The all-powerful master controller may be [...] a member of a committee [...]" [50]. The position of Pax-6 as the "master control gene" is challenged from several sides. In this section several transcription factors, playing a similar role in eye development as *Pax-6*, are discussed.

Apart from *Pax-6*, many transcription factors are known to play a role in eye development. An exhaustive list of transcription factors expressed in the developing murine eye is given in [9]. Another homeobox gene has been found that may have conserved its role in eye development since the last common ancestor of insects and vertebrate. This gene is *Six-3*, the third identified vertebrate orthologue of *Drosophila sine oculis* [46]. *Six-3* and *sine oculis* belong to a subclass of homeobox containing transcription factors, characterised by the so-called six-domain, a highly conserved amino acid sequence domain flanking the homeobox. Null mutants of *sine oculis* are characterised by the complete lack of the visual system [8], suggesting that it is required for eye development. *Six-3* is expressed anterior in the neural plate and its derivatives. Importantly, it is also expressed in the ventral part of the diencephalon (the part of the brain from which the optic vesicles "sprout"), in the optic vesicles and in the olfactory placodes. The expression pattern of *Six-3* resembles that of *Pax-6*, suggesting that it could play a related role in eye development, or that it may interact with it [46]. However, no experimental results have been able to find any regulatory interactions between the two transcription factors. *Six-3* is expressed normally in *small-eye* mutants (mice defective of *Pax-6*). This could mean that it *Six-3* acts completely independent of *Pax-6*, but it could also mean

that *Pax-6* is acting downstream of *Six-3*.

The actual regulatory interactions between *Six-3* and *Pax-6* still wait for investigation, but what can be concluded is that "any genetic element that can become critical can be seen as a 'master regulator"', as it was posed by Kevin Moses in [50].

The position of *Pax-6* is severely challenged by another gene that can induce ectopic expression of eyes in *Drosophila*: *dachshund*[1] (*dac*) [36] *Dachshund* is a mutation both affecting the legs and the placement of the ommatidia in the compound eye[36]. Although the knock-out phenotype of *dachshund* does not affect eye development as severe as *eyeless* does, overexpression of *Dachshund* using the same method as in the overexpression of *eyeless* induces ectopic eyes in antennal discs in 20% of the cases, and less frequently in leg and wing discs [54]. These results could mean that *dachshund* is induced of *Pax-6* and thus is positioned lower in possible regulatory hierarchy. Several observations are consistent with this idea. First, *dachshund* is expressed ectopically if *eyeless* is ectopically expressed. Second, ectopic *eyeless* expression does not result in ectopic eyes in null-mutants of *dachshund*. This suggests *eyeless* positively regulates *dachshund* and that *dachshund* is operating downstream of *eyeless*. However, this appears not to be the case. *Eyeless* is expressed in some of the cells of the eyes induced by ectotopic *dachshund*-expression, whereas *dachshund* is expressed in all the cells expressing *eyeless*. This suggests that *dachshund* and *eyeless* are in a positive feedback loop. They may have evolved together and cooperate in the "master committee" of eye development.

Summarising, the role of *Pax-6* as a "master control gene" is severely challenged. Firstly, alternative "conserved" transcription factors, essential in eye development in both insects and vertebrates are being discovered. For example, of *Drosophila sine oculis* the murine orthologue *Six-3* has been identified. Although the exact role *Six-3* and *sine oculis* in eye development still needs much investigation, the evidence at the moment suggests "master" role for *Six-3*. Secondly, the role of *Pax-6* is challenged by the identification of *dachshund*. *dachshund* and *eyeless* might be in a positive feedback loop, facilitating each

[1] [50] shortly mentions that in Gehring's lab, a second "challenge" to *eyeless*, *twin-of-eyeless* (*toy*) would have been cloned. *toy* would show even more sequence homology to murine *Pax-6* than *eyeless.* Until the time of this writing however, cloning of *twin-of-eyeless* seems to have remained unreported. For a discussion on *twin of eyeless* also listen to Gehring's talk at the 13th International Congress of Developmental Biology [17]

other's synthesis. Thus, the picture emerging from the present experimental evidence suggests a role for *Pax-6* as a "committee" member, a network of transcription factors regulating eye development.

4.5 Ectopic expression of *Pax-6* in vertebrates

If *Pax-6* would really deserve to be called the member of a universal "master control gene committee" of eye development, *Pax-6* should also be able to induce ectopic eyes in other species than *Drosophila*. Recently, the first of such experiments were conducted. It was tested whether ectopic expression of *Pax-6* would result in the development of ectopic eyes in the frog *Xenopus laevis*. First, a *Xenopus* Pax-6 homolog was isolated [25]. This *Pax-6* homolog appears to be expressed in neural cells contributing to the eye, and to parts of the forebrain, hindbrain and spinal cord. This expression pattern resembles that of other vertebrates.

In this study, early misexpression of *Pax-6* in *Xenopus* embryos resulted in axis defects, but no eye structures were observed. If the expression pattern of *Pax-6* was changed, such that expression in the presumptive eye fields was reduced, no eye were formed. Hence, although *Pax-6* is necessary for the development of eyes, *Pax-6* alone seemed not to be sufficient to induce eyes in *Xenopus*.

However, in a second study, Pax-6 was overexpressed in early *Xenopus* embryos by injecting the mRNA of Pax-6 in the two cell stage embryo [2]. This operation resulted in the ectopic development of eye lenses in many embryos. This eye development occured in a cell autonomous way. In other words, all the extra lens tissue was derived from the cell in which the Pax-6 mRNA was injected. This indicates that Pax-6 induces the cells directly to develop lens tissue, instead of inducing cells that induce *other* cells to develop lens tissue.

These results again suggest a role for Pax-6 as a universal "eye inducer". Pax-6 seems to be involved in eye development in virtual all the animal phyla. Even, Pax-6 homologs of one species can sometimes induce eyes in an other species, as discussed above.

At the same time, however, we have to be prudent with the terminology. Pax-6 is certainly not the "master control gene" of eye development. As discussed above, many other genes have been found, and, probably *will* be found, in the same way impairing eye development if their expression is suppressed, and in

inducing eye development if they are overexpressed. Indeed, it will be better to look for a “master control committee” instead.

5 “Urbilateralian” or “zootype”?

5.1 The “Urbilateralian”

Based on the data discussed above, De Robertis and Sasai proposed a common ancestor to the arthropods and the vertebrates [52]. This common ancestor would have anteroposterior polarity, regulated by the *Hox* gene complexes (discussed in chapter 2). It would have dorsoventral polarity, determined by the *sog/chd* and *dpp/BMP-4* dorsoventral patterning systems (discussed in chapter 3). It would also have a subepidermal CNS, which is supported by the presence of the conserved axon-finding molecule *netrin-1* (undiscussed here). The Urbilateralian would have simple photoreceptors, supported by the conserved eye inducing function of *Pax-6* (discussed in chapter 4). Finally, the Urbilateralian was proposed to have a circulatory system with a contractive blood vessel. This is supported by the finding of vertebrate orthologues of two transcription factors involved in the development of the *Drosophila* contractive vessel.

The Urbilateralian hypothesis has been updated twice [30, 11] to include evidence for a segmented Urbilateralia. The first update appeared after the discovery of *hairy* in the first vertebrate orthologue of a pair ruled gene involved in segmentation. The second update followed the discovery of an orthologue of *engrailed*, involved in segmentation in the cephalochordate *Amphioxus*.

Although the data supporting the idea of a complex bilateralian ancestor of the protostome and the deuterostome lineage is becoming more and convincing, care should be taken not be convinced to early. In this paper, three of the arguments support the Urbilateralia theory have been challenged.

The *Hox* genes, that pattern comparable positions in vertebrates and insects, are all present in the *Onychophora*, but the *Ubx/Abd-B* genes are only expressed in a small posterior domain. Onychophora are believed to be a sister phylum of the arthropoda. To explain the fact that the *Hox* genes in *Onychophora* have such unusual expression domains, two alternative explanations would be possible. First, the expression of the *Ubx/Abd-A* cluster may have been shifted to the terminus of the *Onychophora* secondarily. Alternatively, the analogous expression domains of the *Hox* genes in vertebrates and in arthropods may be “semi-coincidental”. By this I mean that the similarity in expression may not be caused by descent from a common bilateral ancestor, but it may be the result of a less direct cause instead. As such an indirect cause I

would propose the colinearity of the *Hox*-cluster. The *Hox* genes are placed in the same sequence in the DNA as their expression domains on the anteroposterior access. This colinearity of the *Hox*-cluster is very well conserved (until now only *Drosophila* and *Caenorhabditis* are minor exceptions to this rule), suggesting that it has an important function in development [40]. It would be feasible that a colinear cluster of *Hox* genes in an non-bilateralian ancestor, would result in the development of two bilateralian lineages in which the *Hox* genes are expressed in the colinear sequence. This proposal is supported by the presence of *Hox*-cluster genes in *Hydra*, a member of the cnidarians, that is widely believed to be primitively radial symmetric.

A second challenge to the Urbilateralia hypothesis concerns the notion of *Pax-6* orthologue in the common bilaterialian ancestor. Although *Pax-6* is more and more pushed in a role as a gene involved in eye development (see chapter 4), it should not remain unnoted that is in fact expressed also in the anterior central neural system, as well as in the anterior non-visual sensory structures. It would hence be possible that *Pax-6* has been important in all kinds of sensory systems in a radial symmetric ancestor of the protostomes and the deuterostomes.

Considering these objections to the Urbilateralia theory of Sasai et al., it should be marked as too preliminary for the moment. Evolutionary scenarios can be thought up by which the protostomes and the deuterostomes would evolve from a common, primitively radial ancestor.

5.2 The extended “zootype”

The data could support an extension of the zootype theory of Slack and others. Slack proposed an abstract notion of the encoding of relative position in the embryo by the *Hox*-cluster genes. Every animal phylum studied until thusfar seemed to contain a common set of Hox-genes [1]. This abstract positioning system is considered a defining character, or synapomorphy, of the animal kingdom, and it was therefore called the “zootype” (Fig. 5.1). Additionally it was proposed that the zootypic stage was visible at the so called phylotypic stage.

Slack proposed already that other patterning genes could be incorporated in

[1] Recently a Cnidarian has been tested, and shown to lack some of the Hox-genes that are part of this “zootype”-cluster [37]

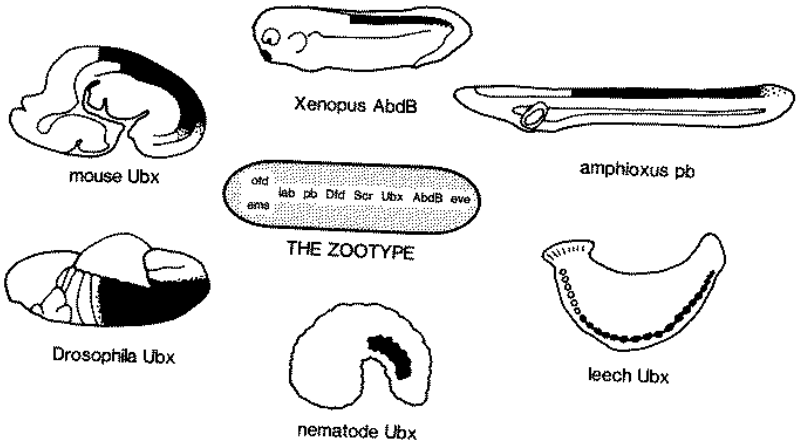

Figure 5.1: Slack and others proposed the “zootype” as the synapomorphy, the defining character of the animal kingdom. They argued that in all the animal phyla investigated until now, the same set of Hox-genes is present. Moreover, these Hox-genes are expressed in the same anteroposterior order. (Taken without permission from reference [56])

the zootype as well. I suggest that the following genes could be added to the zootype, provided that additional evidence supports the ubiquity of these patterning genes in the animal kingdom. (1) *Dpp/Bmp-4* and *sog/chd*, patterning the axis perpendicular to the relative axis patterned by *Hox* genes. (2) Genes positioning sensory systems, such as *Pax-6*. (3) Genes expressed at alternating positions in segment like structures. (4) Genes related to a circulatory system.

6 Regulatory evolution: rewiring the network.

6.1 Introduction

Evolution has long been seen as a process in which novel genes would gradually evolve, adding more and more properties to the evolving organisms, and gradually changing existing structures. For example Lewis hypothesised the evolution of a "wing suppressing gene" and a "haltere promoting gene" in Drosophila (reviewed in [6]).

Almost 20 years after "[Lewis'] landmark 1978 review" [6] the picture seems to be different. Leg-suppressing genes and haltere promoting genes have been isolated (*Ubx/Abd-A* and *Bc-x* respectively), but they are present in four-winged insects and millipedes alike [6]. They have even been found in *Hydra* [56]. It would be hardly conceivable that *Ubx* and *Abd-A* would be a "leg-suppressing gene" in Hydra, let alone that bithorax would have a "haltere promoting" function.

Hence it is clear that in insects no leg-suppressing genes and that in diptera no "haltere promoting genes" evolved, at least as far as *Ubx/Abd-A* and *bithorax* are concerned. But then what did evolve? More and more experimental evidence supports the idea that much of the evolution does not involve the acquisition of new *genes* but the acquisition of *new regulative interactions* between genes, a process I call "regulative rewiring" here.

Having discussed the concept of the "zootype" [56] in the preceding section, I propose here two kinds of regulative rewiring. First, rewiring upstream to the genes belonging to the zootype results in the change of the pattern of expression of positioning genes, such as the Hox-genes. Second, one can distinguish rewirings downstream to the positioning complement of genes. In regulative changes of this kind, the pattern of positioning genes remains the same, but the exact structures developing at the positions change.

So, *upstream* regulative rewiring change positional values, or "segment identities". After *downstream rewiring* the positional values remain unchanged but the actual structure developing at this position is altered.

Concerning regulative rewiring, several questions can be asked. First, are there examples known of upstream and downstream regulative rewirings? Second, how do regulative rewirings evolve, and in conjunction to this point, is this indeed "easier" mode of evolution than evolution of new genes? Third, it

will be discussed what will be the effect of regulative rewiring on evolutionary dynamics. Finally, it will be discussed what the concept of regulative rewiring means for the notion of genetic information content.

6.2 Examples of regulatory rewiring

Several examples are known of regulative changes downstream to the "phylotypic" positioning genes. In *Drosophila* the expression of *Distall-less*, a gene that is crucial for the development of legs, is suppressed by *Ubx* and *Abd-A* [1] [60]

However, *Distall-less* is not suppressed in the domain of *Ubx* and *Abd-A* in centipedes and Onychophorans (Arthropod related animals with non-articulating legs) suggesting that the repression of *Distall-less* by *Ubx* and *Abd-A* in the limbless abdomen of insects evolved only in the insect lineage.

A beautiful example of what could be called "upstream regulative rewiring" is the example of "expression-domain shifting" discussed in section 2.2. These examples showed that in the evolution of arthropods and in vertebrate evolution segments may have changed their identities as a result of a shift in the expression domain of Hox genes. Similar processes could be feasible for other transcription factors. For example, a change in the expression domain of Pax-6 might recruit more neuro-structures devoted to sensing functions.

6.3 Evolution of new regulatory interactions

An important question concerned with the evolutionary mechanism of regulative rewiring is how new regulative interactions evolve. Again, this question falls apart in roughly two questions: (i) How do new downstream targets of Hox genes evolve? (ii) How can the expression domains of "zootypic" genes be changed? I need to stress that this distinction is highly artificial and that it only makes sense in the light of the "zootype", discussed above.

[1] Akam [7] stresses that the suppression of *Distall-less* by *Ubx* and *Abd-A* is somewhat more complicated than it is generally assumed. At a certain stage *Ubx* is expressed together with *Distall-less* in T3. The expression of *Distall-less* is initiated in an earlier developmental stage, when *Ubx* is expressed only in part of T3 but not at the position of the future leg. Later, *Ubx* is expressed all over T3, *Distall-less* remains expressed as it induces itself in an autoregulatory loop.

The evolution of new Hox targets

In chapter 2 it was discussed that homeoboxes bind to short four-base core sequences of the DNA. There is quite a lot of tolerance for the actual sequence surrounding this core (reviewed by [6]). Little changes in this four-base sequence could easily result in changes in affinity to Hox genes, or could even result in the evolution of a binding site for another Hox gene. The sequence surrounding the four-base core could easily modulate the binding specificity of different homeobox genes (reviewed in [6]).

Apart from the evolution of new Hox-gene binding sites, one could also think of other mechanisms adding or removing downstream homeobox gene control. One of these mechanisms is suggested by a comparative study of the structure of Hox genes [53]. They found sequences inside but also outside the homeodomain that were characteristic for a paralogue group.

The fact that also conserved sequences *outside* the homeodomain are found, suggests that they may have an important function. Sharkey and co-workers [53] suggest that these conserved domains are evolved in protein interactions. Allosteric regulation might prohibit Hox-protein–DNA binding or enhance it.

Upstream regulative rewiring

Many different mechanisms are conceivable to change the expression domains of homeobox genes. Akam recently estimated at a conference the number of genes regulating hox-genes between several tens to several thousands of genes [1].

Thus, the genetic regulatory circuit upstream to a *Hox*-gene provides many opportunities by which its domain of expression could be readily modified. One of ther mechanisms by which the domain of expression of a *Hox*-gene could be shifted is suggested by the differential inducability of *Hox*-genes by retinoic acids (RA) in *Xenopus laevis* [13, 47]. Retinoic acids are related to the steroid hormones. They are bound by nucleic receptors, that binds pairwise to the DNA, in this way influencing the expression of other genes.

The expression of *Hox*-genes is induced by retinoic acids in a differential way. More anterior expressed *Hox*-genes are better RA inducable than more posterior expressed *Hox*-genes [13, 47]. In this way modulations of RA-gradients

— or at least inhomogeneous RA-distributions — over the embryo could result in shifts of *Hox*-expression domains. Modulation of RA-distribution could occur for example by inhomogeneous distribution of proteins interacting with retinoic acids like xCRABPs and RA receptors (RARs and RXRs). Another mechanism might be modulation of RA diffusion by the intracellular pH [41].

Summarising, body plans may be modified by evolutionary rewiring of genes. In the case of homeobox genes, two extreme ways of rewiring can be distinguished, upstream and downstream rewiring. Downstream DNA binding sites of homeobox genes are easily gained and lost. Protein-protein interactions can modulate binding of homeobox proteins to DNA as well. In the order of tens to thousands of genes may influence the expression of homeobox genes. Expression domains might be modulated by distribution of "morphogens", such as retinoic acids.

Regulative rewiring and evolutionary dynamics

It has been discussed by many authors how evolutionary dynamics can be influenced if evolution is seen as a process where regulative pathways are modified, rather than structural genes. The importance of regulatory mutations is stressed by Wilson (1985) [62] and by Carroll (1995) [6].

Gould (1980) [20] suggested that the "bursts" of evolutionary change that he observed in the fossil record might be explained as "disruption of regulation", "[while] structural gene substitutions control most small scale [...] variation".

The mechanism of "punctuated evolution" as a result of "disruption of regulation" suggested by Gould has recently gained support by observations done in a paradigm system of the evolution of multicellular animals that I developed as a master's project under supervision of Paulien Hogeweg [42]. In this paradigm system the development of simple, oligocellular creatures is driven by a simple regulatory network present in each cell. During evolution, the genetic networks are mutated in two ways: individual Boolean functions can change and connections between genes evolve. The creatures are selected for the number of cell types they develop in their ontogeny. Three "types" of genes evolve: regulatory genes, downstream genes and housekeeping genes.

Interestingly, the evolution shows metastable behaviour. Periods of stasis, in which a characteristic type of development prevails, are intermitted by short

periods of change in which the population is taken over by “animals” exhibiting a new type of development. We have been able to track down these “key innovations” on changes in the “regulatory feedback loops”. Small changes in the ontogeny can be tracked down to changes downstream to the “regulatory feedback loops”. The changes in regulatory parts of the network result in new responses of the cells to inducing signals. These new responses can result in fundamental changes in the developmental program of the organisms. Downstream changes on the other hand result in slight modifications or improvements of the outcome of the developmental program, while the actual developmental program remains essentially the same.

Genetic information and regulative rewiring

The information content of a DNA molecule is often assumed to be equal to its length, i.e. the number of bases of the molecule. For example, the concept of the “information threshold” states that there is a maximum amount of information a replicating RNA molecule can contain expressed in the length of this molecule. This amount of information depends on the mutation rate during replication (an excellent discussion on the “information threshold” is given in [38]).

However, one could say that the amount of information stored in a genome is not only dependent on the number of base pairs by which it is coded, but also on the complexity of the interaction structure between the genes. This certainly true for “epigenetic” information, the information that is built up during developmental process. A genome in which the genes do not interact can only have a single pattern of gene expression. If the connectivity of the genome slowly increases, more and more epigenetic states are possible, while for very high connectivity the system “percolates” and different epigenetic states “merge” (see for instance reference[29]).

One could extend the notion of epigenetic information and consider the *potential* of genetic network to build up epigenetic information as part of the information content of a genome. In other words, a genome capable of “remembering” ten possible epigenetic states has a higher information content than a genome coded by a DNA string of the same length that is capable of storing only one epigenetic state.

This idea is nicely illustrated by the paradigm system of the evolution of multicellular development which was shortly described above. The genetic network of these creatures consists of a Boolean network of 24 nodes, or *genes*. Each of these genes gets two inputs (*regulatory interactions*) from other nodes. These numbers do not change over evolutionary time. However, the number of epigenetic states or "cell types" that the organisms, driven by the networks, develop increases during evolution. Although the "length" of the genome does not increase at all, I would still say that the information content of the genome increases during the evolutionary process, because the number of epigenetic states the networks can attain increases.

6.4 Regulatory rewiring: a source of variation

For a long time, the evolutionary process has been seen as a process in which variation is generated by the recombination and random mutation of genes, each of these genes more or less directly coding for a trait of the animal.

Now, the picture of evolutionary processes may have been changed. Looking at animal genomes for an developmental biological point of view, it seems as if they are all built up from the same elements. Regulatory factors often have the same functions in animals that are separated by a trough only bridged by more than 500 million years of evolution. Evolution might be a process of the gradual change of connections between existing elements, more than just the process of gradual change of the genetic units themselves. Note however, that this does not withstand the fact that the change of these genetic units is a vitally important process as well.

This doesn't make the evolutionary process less *Darwinian*, though. The picture has changed, not on the level of the selection and fixation of new genetic traits, but moreover, on the source of the variation on which selection can act.

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Bibliography

[1] AKAM, M. Hox genes, homeosis and the evolution of segment identity. In *6th ESEB conference* (Arnhem, The Netherlands, 24-24 August 1997), European Society of Evolutionary Biology.

[2] ALTMANN, C. R., CHOW, R. L., LANG, R. A., AND HEMMATI-BRIVANLOU, A. Lens induction by pax-6 in *xenopus laevis*. *Developmental Biology 185* (1997), 119–123.

[3] ARENDT, D., AND NÜBLER-JUNG, K. Inversion of dorsoventral axis? *Nature 371* (september 1994), 26.

[4] AVEROF, M., AND PATEL, N. H. Crustacean appendage evolution associated with changes in hox gene expression. *Nature 388* (14 August 1997), 682–686.

[5] BEHRENS, M., LANGECKER, T. G., WILKENS, H., AND SCHMALE, H. Comparitive analysis of *pax-6* sequence and expression in the eye development of the blind cave fish *astyanax fasciatus* and its epigean conspecific. *Molecular Biology and Evolution 14*, 3 (1997), 299–308.

[6] CARROLL, S. B. Homeotic genes and the evolution of arthropods and chordates. *Nature 376* (1995), 479–485.

[7] CASTELLI-GAIR, J., AND AKAM, M. How the hox gene *ultrabithorax* specifies two different segments: the significance of spatial and temporal regulation within metameres. *development 121* (1995), 2973–2982.

[8] CHEYETTE, B. N. R., GREEN, P. J., MARTIN, K., GARREN, H., HARTENSTEIN, V., AND ZIPURSKY, S. L. The *drosophila sine oculis* locus encodes a homeodomain-containing protein required for the development of the entire visual system. *Neuron 12* (1994), 997–996.

[9] CVEKL, A., AND PIATIGORSKY, J. Lens development and crystallin gene expression, many roles for pax-6. *BioEssays 18*, 8 (1996), 621–630.

[10] DE ROBERTIS, E., AND SASAI, Y. A common plan for dorsoventral patterning in bilateralia. *Nature 380*, 37 (March 1996).

[11] DE ROBERTIS, E. M. The ancestry of segmentation. *Nature 387* (May 1997), 25–26.

[12] DEKKER, E.-J. *Regulation of early Xenopus pattern formation by retinoid signalling*. PhD thesis, Universiteit Utrecht, November 1993.

[13] DEKKER, E.-J., PANNESE, M., HOUTZAGER, E., TIMMERMANS, A., BONCINELLI, E., AND DURSTON, A. Xenopus *hox-2* genes are expressed sequentially after the onset of gastrulation and are differentially inducible by retinoic acid. *Development supplement* (1992), 192–202.

[14] EAKIN, R. M. Lines of evolution of photoreceptors. In *General physiology of cell specialization* (New York, 1963), D. Mazia and A. Tyler, Eds., McGraw-Hill, pp. 393–425.

[15] EPSTEIN, J., GLASER, T., CAI, J., JEPEAL, L., WALTON, D., AND MAAS, R. Two independent and interactive dna-binding subdomains of the *pax-6* paired domain are regulated by alternative splicing. *Genes and Development 8* (1994), 2022–2034.

[16] GARSTANG, W. Preliminary note on a new theory of the phylogeny of the chordata. *Zoologischer Anzeiger 27* (1894), 122–125.

[17] GEHRING, W. J. The master control genes for morphogenesis and evolution of the eye. In *The 13th internation congress of Developmental Biology* (1997), no. `http://sdb.bio.purdue.edu-/SDBNews/97IntCongress/97IntCongressProgr.html`, Society for developmental biology.

[18] GILBERT, S. F. *Developmental Biology*, fourth edition ed. Sinnauer Associates, Sunderland, Massachusetts, 1994.

[19] GLARDON, S., CALLAERTS, P., HALDER, G., AND GEHRING, W. J. Conservation of pax-6 in a lower chordate, the ascidian *phallusia mammillata*. *Development 124* (1997), 817–825.

[20] GOULD, S. J. Is a new and general theory of evolution emerging? *Paleobiology 6*, 1 (1980), 119–130.

[21] GRENIER, J. K., GARBER, T. L., WARREN, R., WHITINGTON, P. M., AND CAROLL, S. Evolution of the entire arthropod *hox* gene set predated the origin and radiation of tye onychophoran/arthropod clade. *Current Biology 7* (4 July 1997), 547–553.

[22] GRINDLEY, J. C., DAVIDSON, D. R., AND HILL, R. E. The role of *pax-6* in eye and nasal development. *Development 121* (1995), 1433–1442.

[23] HALDER, G., CALLAERTS, P., AND GEHRING, W. J. Induction of ectopic eyes by targeted expression of the *eyeless* gene in *drosophila*. *Science 267* (March 1995), 1788–1792.

[24] HEMMATI-BRIVANLOU, A., KELLY, O. G., AND MELTON, D. Follistatin, an antagonist of activin, is expressed in the spemann organizer and displays direct neuralizing activity. *Cell 77* (1994), 283–295.

[25] HIRSCH, N., AND HARRIS, W. A. Xenopus pax-6 and retinal development. *Journal of Neurobiology 32*, 1 (1997), 45–61.

[26] HOLLAND, P. W., AND GARCIA-FERNÁNDEZ, J. *Hox* genes and chordate evolution. *Developmental Biology 173* (1996), 382–395.

[27] HOLLEY, S. A., JACKSON, P. D., SASAI, Y., LU, B., DE ROBERTIS, E. M., HOFFMAN, F. M., AND FERGUSON, E. L. A conserved system for dorsal-ventral patterning in insects and verebrates involving *sog* and *chordin*. *Nature 376* (july 1995), 249–253.

[28] HUNTER, C. P., AND KEYNON, C. Specification of anteroposterior cell fates in *caenorhabditis elegans* by *drosophila hox* proteins. *Nature 377* (september 1995), 229–232.

[29] KAUFFMAN, S. A. *The origins of order*. Oxford University Press, 1993.

[30] KIMMEL, C. B. Was *urbilateria* segmented. *Trends in Genetics 12*, 9 (1996), 329–331.

[31] LACALLI, T. Dorsoventral axis inversion. *Nature 373* (january 1995), 110–111.

[32] LACALLI, T., AND WEST, J. A distinctive nerve cell type common to diverse deuterostome larvae: comparative data from echinoderms, hemichordates and amphioxus. *Acta Zoologica 74*, 1 (1993), 1–8.

[33] LAWRENCE, P. A. *The making of a fly: the genetics of animal design*. Blackwell scientific publications, Oxford, 1992.

[34] LOOSLI, F., KMITA-CUNISSE, M., AND GEHRING, W. J. Isolation of a *pax-6* homolog from the ribbonworm *lineus sanguineus*. *Proceedings of the National Academy of Sciences USA 93* (April 1996), 2658–2663.

[35] MANAK, J. R., AND SCOTT, M. P. A class act: conservation of homeodomain protein functions. *Development Supplement* (1994), 61–71.

[36] MARDON, G., SOLOMON, N. M., AND RUBIN, G. M. *dachshund* encodes a nuclear protein required for normal eye and leg development in *drosophila*. *Development 120* (1994), 3474–3486.

[37] MARTÍNEZ, D. E., BRIDGE, D., MASUDA-NAKAGAWA, L. M., AND CARTWRIGHT, P. Cnidarian homeoboxes and the zootype. *Nature 393* (June 1998), 748–749.

[38] MAYNARD SMITH, J., AND SZATHMÁRY, E. *The major transitions in evolution*. W.H. Freeman, Oxford, 1995.

[39] MCGINNIS, N., KUZIORA, M. A., AND MCGINNIS, W. Human *hox-4.2* and drosophila *deformed* encode similar regulatory specifities in drosophila embryos and larvae. *Cell 63* (1990), 969–976.

[40] MCGINNIS, W., AND KRUMLAUF, R. Homeobox genes and axial patterning. *Cell 68* (24 January 1992), 283–302.

[41] MERKS, R. Pattern formation in the Xenopus laevis anteroposterior axis. Master's thesis, Utrecht University, 1995.

[42] MERKS, R. Evolving "metazoan" development. Master's thesis, Utrecht University, 1997.

[43] MÜLLER, G. B. Developmental mechanisms at the origin of morphological novelty: a side-effect hypothesis. In *Evolutionary Innovations* (Chicago, U. C. P., 1990), M. H. Nitecki, Ed., pp. 99–130.

[44] NIEUWKOOP, P., JOHNEN, A., AND ALBERS, B. *The epigenetic nature of early chordate development*. Cambridge University Press, Cambridge, 1985.

[45] OHNO, S. The notion of the cambrian pananimalia genome. *Proceedings of the National Academy of Sciences USA 93* (August 1996), 8475–8478.

[46] OLIVER, G., MAILHOS, A., WEHR, R., COPELAND, N. G., JENKINS, N. A., AND GRUSS, P. *Six3*, a murine homologue of the *sine ocultis* gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. *Development 121* (1995), 4045–4055.

[47] PANNESE, M., DEKKER, E.-J., GODSAVE, S., HOLLING, T., DURSTON, A., AND BONCINELLI, E. Xenopus Hoxa and Hoxb complex comparison: differences in their spatio-temporal expression patterns and inducibility by retinoic acid during embryogenesis.

[48] QUIRING, R., WALLDORF, U., KLOTER, U., AND GEHRING, W. J. Homology of the eyeless gene of drosophila to the small eye gene in mice and aniridia in humans. *Science 265* (5 august 1994), 785–789.

[49] RAFF, R. A. *The shape of life*. The University of Chicago Press, Chicago, London, 1996.

[50] ROUSH, W. A "master control" gene for fly eyes shares its power (editorial). *Science 275* (January, 31st 1997), 618–619.

[51] SALVINI-PLAWEN, S. V., AND MAYR, E. On the evolution of photoreceptors and eyes. *Evolutionary Biology 10* (1997), 207–263.

[52] SASAI, Y., LU, B., STEINBEISSER, H., AND DE ROBERTIS, E. M. Regulation of neural induction of the chd and bmp-4 antagonistic patterning signals in *xenopus*. *Nature 376* (july 1995), 333–336.

[53] SHARKEY, M., GRABA, Y., AND SCOTT, MATTHEW, P. *Hox* genes in evolution: protein surfaces and paralog groups. *Trends in Genetics 13*, 4 (April 1997).

[54] SHEN, W., AND MARDON, G. Ectopic eye development in *drosophila* induced by directed *dachshund* expression. *Development 124* (1997), 45–52.

[55] SHUBIN, N., TABIN, C., AND CARROLL, S. Fossils, genes and the evolution of animal lims. *Nature 388* (14 August 1997), 639–648.

[56] SLACK, J. M. W., HOLLAND, P. W. H., AND GRAHAM, C. F. The zootype and the phylotypic stage. *Nature 361* (February 1993), 490–492.

[57] SMITH, W., AND HARLAND, R. Expression cloning of noggin, a new dorsalizing factor localized to spemann organizer in *xenopus* embryos. *Cell 70* (1992), 829–840.

[58] TOMAREV, S. I., DUNCAN, M. K., ROTH, H. J., CVEKL, A., AND PIATIGORSKY, J. Convergent evolution of crystallin gene regulation in squid and chicken: the ap-1/are connection. *Journal of Molecular Evolution 39* (1994), 134–143.

[59] TOMAREV, S. I., AND PIATIGORSKY, J. Lens crystalllins of invertebrates: Diversity and recruitment from detoxification enzymes and novel proteins. *European Journal of Biochemistry 235* (1996), 449–465.

[60] VACHON, G., COHEN, B., PFEIFLE, C., MCGUFFIN, M., BOTAS, J., AND COHEN, S. Homeotic genes of the bithorax complex repress limb development in the abdomen of the Drosophila embryo through the target gene distal-less. *Cell 71* (1992), 437–450.

[61] WILKENS, H. Evolution and genetics of epigean and cave *astyanax fasciatus* (characidae, pisces). *Evolutionary Biology 23* (1988), 271–367.

[62] WILSON, A. C. the molecular basis of evolution. *scientific American 253*, 4 (1985), 148–157.

[63] WILSON, P. A., AND HEMMATI-BRIVANLOU, A. Induction of epidermis and inhibition of neural fate by bmp-4. *Nature 376* (July 1995), 331–333.

[64] WOLPERT, L. Positional information and the spatial pattern of cellular differentiation. *Journal of theoretical biology 25* (1969), 1–47.