Segmentation of the spider
Achaearanea tepidariorum
investigated by gene expression
and functional analysis
of the gap gene hunchback

Inaugural-Dissertation

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<tr>
<td>A</td>
<td>abdominal segments</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>C</td>
<td>cumulus</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy DNA</td>
</tr>
<tr>
<td>Ch</td>
<td>cheliceres</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>d</td>
<td>dorsal area</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>doublestranded RNA</td>
</tr>
<tr>
<td>gz</td>
<td>growth zone</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>L</td>
<td>walking leg</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
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</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>O</td>
<td>opisthosomal segments</td>
</tr>
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<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Pp</td>
<td>pedipalp</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>sec</td>
<td>seconds</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl-transferase-mediated</td>
</tr>
<tr>
<td>dUTP</td>
<td>dUTP–digoxy-genin nick-end labeling</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microliter</td>
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<tr>
<td>μM</td>
<td>micromolar</td>
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1. General introduction
1.1. Evolution of segmentation

The question of the origin and the evolution of segmentation has been one of the key themes of evolutionary developmental biology (Evo-Devo) during the last decade – and continues to be a subject of dispute (Damen, 2007; Davis and Patel, 1999; De Robertis, 2008; Peel et al., 2005; Seaver, 2003; Tautz, 2004). Three animal groups - chordates, annelids and arthropods - display a clear metameric organization of their body plans called segmentation (Figure 1). It is commonly assumed that the metameric organization along their anterior-posterior body axis made these three phyla highly successful during the course of evolution by allowing them to adapt by differentiating different body regions. In recent phylogenies (Aguinaldo et al., 1997; Dunn et al., 2008; Halanych, 2004), the three segmented phyla are not closely related to each other, but instead they are deeply separated and each of them is more closely related to groups that do not obviously display segmentation. For example the unsegmented echinoderms are closely related to vertebrates, the unsegmented nematodes group together with arthropods in the ecdysozoan clade and the unsegmented flatworms form the lophotrochozoan clade together with the annelids. Davis and Patel (1999) have proposed three different possible scenarios for the origin and evolution of segmentation: First, body metamerization might have evolved independently in all three lineages and might therefore be an example of convergence (Figure 1, green). Second, segmentation could have been acquired separately in chordates and in the protostome ancestor – this possibility would then require several losses of segmentation in both the ecdysozoan and lophotrochozoan unsegmented phyla (Figure 1, orange). Finally, segmentation could have evolved only once, already before the protostome-deuterostome split in a hypothetical animal that has been termed “Urbilateria” (De Robertis and Sasai, 1996) (Figure 1, red). Segmentation must then have been lost in several unsegmented groups that are also descendants of this animal.
Figure 1: **Origin of segmentation in the animal kingdom.** Simplified tree of bilateral symmetric animals based on a figure presented in Davis and Patel (Davis and Patel, 1999). Circles represent gains of segmentation, squares stand for losses of segmentation. The green, orange and red colors symbolize the three different theories for the origin of segmentation as presented by Davis and Patel (1999) and explained in the text above.

To solve this question of the origin of segmentation, Evo-Devo research has been comparing the developmental and molecular mechanisms governing the segmentation process in the three phyla. As highlighted in the sections below, remarkable similarities between the segmentation mechanisms in vertebrates, annelids and arthropods have been found, but at the same time it became more and more obvious that there is also a lot of divergence in the mechanisms studied within each of these groups. Thus, before we can make inferences about the relatedness of segmentation in the three groups, we first have to define the hypothetical ancestral state of the segmentation process within each group, a task that is far from being solved since only a limited number of model and non-model organisms have been studied so far.
The *Drosophila* paradigm

The fruit fly *Drosophila melanogaster* is the animal in which the segmentation process is understood best, and where it was also examined first. Therefore, *Drosophila* segmentation became a model, to which all other animal segmentation mechanisms have since been compared. However, it soon turned out that *Drosophila* segmentation is highly derived and probably adapted to the fast development of the fly. Unlike most other insects and arthropods and also unlike annelids and chordates, dipterans like *Drosophila* pattern all their segments simultaneously in the syncytial blastoderm. This process is governed by a hierarchical gene cascade that successively subdivides the embryo into smaller units. Long-range gradients formed by diffusion of maternally supplied gene products at the anterior and posterior poles of the embryo constitute the first level of this gene regulatory cascade. Regulated by these so-called maternal effect genes, the gap genes are the first zygotically expressed genes of the cascade. They are expressed in defined broad domains along the anterior-posterior axis and their proteins form short-range gradients by repressing each other. In combination with the maternal factors they control the pair rule genes, which show the first periodic expression patterns in the fly embryo and specify alternating segments. The pair rule genes then regulate the segment polarity genes, which delineate the segment borders. Gap genes in concert with segment polarity and pair rule genes also control the Hox genes, which define segment identities (for reviews see e.g. Ingham, 1988; St Johnston and Nüsslein-Volhard; Pankratz and Jäckle, 1993)

Segmentation in chordates

Chordate segmentation has been studied mostly in vertebrates. Vertebrate segmentation is different from arthropod and annelid segmentation in that only mesodermal derivatives are segmented. Transient structures, epithelial mesodermal blocks called somites, are rhythmically produced from the presomatic mesoderm in an anterior to posterior progression. Only about ten years ago, the molecular oscillator driving somitogenesis in vertebrates was identified (Palmeirim et al., 1997). This oscillator, the so-called vertebrate segmentation clock mainly involves the Wnt, FGF and Delta/Notch pathways. Genes that are parts of these three intercellular signaling pathways, show cyclical expression patterns in waves across the presomatic mesoderm
(PSM) of mouse embryos. In zebrafish, the chick and a snake so far only members of the Notch pathway have been found cycling in the PSM. However, a pacemaker behind this cyclic gene expression has not been identified so far (for recent reviews see Özbudak and Pourquié, 2008; Dequéant and Pourquié, 2008).

**Segmentation in annelids**

Annelid segmentation is superficially very similar to that of arthropods and therefore a common ancestry of segmentation in these two phyla had been suggested (Articulata hypothesis, reviewed by Scholz, 2002). On a molecular basis though, data remains to be inconclusive. Not only is there no functional data on annelid segmentation, but there are also conflicting results regarding an involvement of genes used in either Drosophila segmentation or vertebrate somitogenesis. For example, in the polychaete Platynereis dumerilii segment polarity gene orthologs are expressed in a way reminiscent of a role in segmentation (Prud’homme et al., 2003) while in other annelids they are not (Bely and Wray, 2001; Kang et al., 2003; Seaver and Kaneshige, 2006; Seaver et al., 2001; Seaver and Shankland, 2001).

Although similarities to vertebrate segmentation have been analyzed much less in annelids, there is inconclusive data on a Delta/Notch pathway member, a hairy/enhancer of split gene, and on Notch expression in the leech Helobdella robusta (Rivera et al., 2005; Song et al., 2004). Nevertheless, recently the expression of several Delta/Notch signaling genes in a way that could pinpoint to a role in segmentation was reported from the polychaete Capitella capitata (Thamm and Seaver, 2008).

Recently an expression pattern analysis of a group of homeobox genes belonging to the NK cluster proposed that these NK genes are involved in segmentation of Platynereis dumerilii. It is however unknown, whether these genes play a role in the segmentation process of the other segmented groups of animals (Saudemont et al., 2008).

**Segmentation in arthropods**

As noted above, the syncytial mode of development of Drosophila is not typical of the arthropod phylum. In contrast to the long germ dipterans that form all segments simultaneously from a germ rudiment that consists of the whole blastoderm, most other
insects and arthropods form only a limited number of segments in a syncytial environment or even form all their segments under cellular conditions. The germ rudiment of these short germ arthropods occupies only a small part of the blastoderm and the other segments are added sequentially from a posterior structure that is most commonly called “growth zone” (However exactly how and where growth is taking place in this region is not known. Nonetheless in the following I will use this term to describe the posterior zone from which segments are generated in arthropods.). This short germ type of development where segmentation is mainly taking place in a cellularized environment is thought to represent the ancestral state within arthropods (Davis and Patel, 2002; Tautz, 1994).

Nevertheless some levels of the Drosophila segmentation gene cascade are still conserved between the “higher” insects and other arthropods, this is particularly true for the lower levels of the cascade. Segment polarity genes like engrailed (en) orthologs display a remarkable degree of conservation and are expressed in segmental stripes in all arthropod classes examined so far (e.g. Damen, 2002b; Hughes and Kaufman, 2002a; Patel et al., 1989; Scholtz et al., 1994) and also the function of segment polarity genes seems to be comparable at least between Tribolium and Drosophila (Choe and Brown, 2008; Oppenheimer et al., 1999). It has also been proposed that the orthologs of the genes acting at the next higher level of the Drosophila segmentation gene cascade, the pair rule genes, are at least conserved in so far that they are expressed in a pattern that is suggestive of a role in segmentation, albeit the exact details differ between different arthropods (Damen, 2007). The more upstream levels of the segmentation gene cascade, maternal effect genes and gap genes, seem to be less conserved between Drosophila and other insects, however especially for maternal effect genes very little data is available. This applies also to the gap genes in non-insect arthropods, which will be more closely introduced later (see section 3).

During the last five years, there has been increasing evidence that short germ arthropods employ pathways during segmentation that are also used by vertebrates. First, Delta/Notch signaling has been found to be crucial for segment generation in the spider Cupiennius salei (Schoppmeier and Damen, 2005b; Stollewerk et al., 2003) and subsequently the dynamic expression of Notch-pathway genes has been reported also from the centipede Strigamia maritima (Chipman and Akam, 2008) and functional studies in a second spider species Achaearanea tepidariorum support the results in
Cupiennius (Oda et al., 2007). However only very recently the involvement of Delta/Notch signaling has been shown also in an insect, the cockroach Periplaneta americana (Pueyo et al., 2008). Moreover, Wnt signaling, which is also utilized during vertebrate somitogenesis, seems to be involved in the generation of segments in both the spider Achaearanea tepidariorum and the short germ beetle Tribolium castaneum (Bolognesi et al., 2008; McGregor et al., 2008b).

The use of similar genetic networks for segmentation indicates that the formation of segments in arthropods and vertebrates may have shared a genetic program in their last common ancestor. However, it still remains possible that Wnt and Notch pathway were independently co-opted for segmentation in arthropods and vertebrates. Only the (functional) examination of the segmentation process of a broader range of taxa within each of the segmented groups, but also of outgroups like onychophorans and tardigrades, and also functional studies in annelids might facilitate solving the question of the evolution of segmentation. With this thesis I want to provide a further piece of the puzzle by a closer examination of the segmentation mechanisms used by a chelicerate, the common house spider Achaearanea tepidariorum.
1.2. Spiders as model systems

Chelicerates are a group of basally branching arthropods (Friedrich and Tautz, 1995; Giribet et al., 2001), which therefore constitute an important clade for Evo-Devo research (McGregor et al., 2008a).

The first detailed embryological studies in spiders date back to the 19th century (Claparède, 1862). So for almost 150 years, every now and then spider embryos have been the focus of embryological research. From the beginning, comparative embryological work to explain evolution was one of the main goals of spider embryology (summarized in Anderson, 1973). Initially spider embryos were observed live immersed in oil and later spider development was examined by sections and histological staining. In the 1950s, Holm published amazing embryological experiments with living spider embryos, amongst which he identified the spider cumulus as an organizing center of dorsoventral axis determination (Holm, 1952). These findings were only recently explained by molecular means (Akiyama-Oda and Oda, 2006).

While purely descriptive embryological work is still being done with spiders nowadays (Chaw et al., 2007), it has been around the turn of the millennium that molecular work on spiders has revolutionized several ideas about the evolution of segmentation (Damen et al., 1998; Stollewerk et al., 2003) and spider research steadily continues to contribute to our understanding of arthropod evolution (McGregor et al., 2008a).

First, the wandering spider Cupiennius salei has been the main spider model organism and it was the first non-insect arthropod in which functional gene knockdown studies via embryonic RNAi had been made possible (Schoppmeier and Damen, 2001). However, recently another spider, the common house spider Achaearanea tepidariorum has emerged as a model organism. In contrast to Cupiennius, which has a long generation time (approximately 9 months), this spider has a generation time of only three months. It also produces cocoons (egg sacks) more regularly than Cupiennius (up to twice a week as opposed to once a month). Furthermore, in Achaearanea, knockdown of gene function can be facilitated by parental RNAi, which is much less tedious, but also
allows for an earlier impact of the knockdown effect since Cupiennius embryos could only be injected at blastoderm stages. Most importantly however is the fact that much earlier stages of Achaearanea embryos can be fixed for expression pattern analysis. Due to technical reasons, embryos of Cupiennius have never been accessible for in situ hybridizations in stages before segmentation of the prosoma had already taken place. Thus, if we want to understand both anterior and posterior segmentation in spiders, we need to study the segmentation process in an animal that allows us to functionally examine the whole segmentation process – a prerequisite that is perfectly met by Achaearanea tepidariorum.
1.3. Development of *Achaearanea*

Already in 1909, Montgomery (Montgomery, 1909) has described the embryogenesis of *Achaearanea tepidariorum* (then still called *Theridium*) on the basis of histological sections. Almost a century later Akiyama-Oda and Oda (2003) provided a description of *Achaearanea* development using live embryos, and they also introduced a staging system for *Achaearanea* embryos. This is summarized in Figure 2 below. The first syncytial cleavages take place in the center of the egg (stage 1). At stage 2 the cleavage energids rise to the periphery of the egg and divide synchronously until the 32-cell stage (stage 2). Cellularization probably takes place at the 16-cell stage, similar as in the related species *Achaearanea japonica* (Suzuki and Kondo, 1995). During stage 3, the energids migrate to one side of the egg to form a radial germ disc. Now the future anterior end of the embryo is the outer ring of the germ disc and the future posterior end is in the center. At stage 4, the blastopore forms in the center of the germ disc, visible as a white spot (primary thickening). In other spiders, cells invaginate through the blastopore to form mesenchymal cells (e.g. Chaw, 2007). Next to the blastopore, the cumulus arises (stage 5). This signaling center, consisting of mesenchymal cells, starts to migrate to the periphery of the germ disc during stage 5, thereby breaking the radial symmetry of the embryo. During the migration of the cumulus, the blastopore vanishes. At the beginning of stage 6, the cumulus starts to disappear where it has reached the rim of the germ disc. Now the embryo starts to "open" at this point, forming an area of extraembryonic cells also called the dorsal area. During stage 6 and 7, major re-shaping events take place while the embryo transforms from a germ disc to a germband. Cells at the sides of the dorsal area move circumferentially and anteriorly away from the dorsal area, which thereby expands. At the same time the former center of the germ disc becomes the posterior end of the embryo and forms the growth zone. At stage 8, the germband formation is completed and segmentation of the prosomal segments becomes visible. Next, posterior segments are sequentially added from the growth zone. Stage 9 is defined as the stage where the limb buds appear and start to grow outwards. At stage
10, the appendages get segmented and segmentation of the embryo is completed. At stage 11 a spider-specific process called reversion starts, the embryo opens along the ventral midline and the two embryo halves first perform dorsal closure before they later close ventrally again. A suggested “fate map”, based on expression patterns of a few genes, to visualize the developmental origin of the spider body regions is shown in Figure 3.

<table>
<thead>
<tr>
<th>stage 1</th>
<th>stage 2 early</th>
<th>stage 2 late</th>
<th>stage 3</th>
<th>stage 4</th>
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<td><img src="image2.png" alt="stage 2 early image" /></td>
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Figure 2: Early development of *Achaearanea tepidariorum*. This figure is modified from two figures presented in Akiyama-Oda and Oda (2003) and Yamazaki et al. (2005). Upper pictures show live embryos observed in oil, below are schematized drawings of corresponding stages. All drawings are lateral views, the embryo micrographs are lateral views in stage 1-3 and 8 and top (ventral) views in stages 4-7. The future anterior is down in all pictures. Details see text. Abbreviations: Ab abembryonic pole, Em Embryonic pole, CM cumulus mesenchymal, A anterior, P posterior, D dorsal, V ventral, Ex extraembryonic area, gz growth zone.
Figure 3: **Segmentation of the *Achaearanea* embryo.** This figure is taken from (McGregor et al., 2008a). Regions forming the anterior prosoma ("head") are colored in grey, the area that gives rise to the cheliceral and pedipalpal segment is shown in red, the walking leg region is colored in blue and opisthosomal segments are green. The cumulus is depicted as a yellow asterisk and white dots represent extraembryonic cells. Abbreviations: A anterior, P posterior, D dorsal, V ventral, Ce cephalic lobe, Ch cheliceral segment, Pp pedipalpal segment, L1-L4 leg bearing segments 1-4, O1-O9 opisthosomal segments 1-9.
1.4. Aims of this thesis

The aim of this thesis is to describe the segmentation process in *Achaearanea tepidariorum*, especially with respect to early stages and anterior segmentation, which up to now have been understudied in spiders. In the first part of this thesis I therefore will present the analysis of the expression of several segmentation genes. This data not only helps to better understand *Achaearanea* segmentation, but also provides us with a set of marker genes for functional analyses. In the second part of the thesis I want to contribute new insights into a specific class of segmentation genes, the gap genes, by a functional analysis of a spider ortholog of the *hunchback* gene, which has previously been suggested to only play a role in insect segmentation and not in other arthropods.
2. Segmentation gene expression in *Achaearanea tepidariorum*
2.1. Introduction

Before I start describing the expression patterns of segmentation genes in *Achaearanea* embryos, I briefly want to summarize what we presently know about segmentation gene expression during spider segmentation. As highlighted before (see section 1), most of our knowledge is based on gene expression in the segmentation process in the opisthosoma of *Cupiennius*, and due to the inaccessibility of early *Cupiennius* embryos we hardly know anything about anterior segmentation in spiders.

Like in other arthropods, the expression of segment polarity genes is well conserved in *Cupiennius* (Damen, 2002b). This analysis showed that, as in insects and other arthropods, *engrailed* and *wingless* orthologs define the parasegmental boundaries. Moreover, parasegmental boundaries are also respected by the posterior Hox genes, which was one of the lines of evidence for the parasegmental organization of the spider embryo and the ancestry of parasegmental organization in arthropods (Damen, 2002b).

Orthologs of pair rule genes that act at the next higher level of the segmentation gene cascade of *Drosophila* also seem to be involved in segmentation of the spider. This notion was only based on the dynamic expression of these genes in the growth zone before segment formation, and not on functional experiments (Damen, 2004; Damen et al., 2005; Damen et al., 2000).

It is presently unresolved if any of the higher levels of the *Drosophila* segmentation gene cascade participate in spider segmentation. During his PhD thesis, Michael Schoppmeier showed that RNAi for a gap gene ortholog, *Cupiennius Krüppel* (*Cs-Kr-1*) resulted in a breakdown of segmentation after four or five opisthosomal segments (Schoppmeier, 2003), but whether this phenotype can be accounted as a gap phenotype or a posterior elongation phenotype was not determined yet, as attempts to reproduce this phenotype in both *Cupiennius* and also *Achaearanea* remained unsuccessful (Kruck, 2008; own observations).
So far, the most exciting insights into spider segmentation derive from a comparison with vertebrate somitogenesis. Four orthologs of Delta/Notch pathway genes (*Notch, Delta, Suppressor of Hairless and Presenilin*) have been found to be involved in this process. One of these genes, *Cs-Delta-1*, is expressed dynamically in the spider growth zone in a way that is reminiscent of the cyclic expression of this gene in the presomitic mesoderm of vertebrates. Moreover, like in vertebrates, knockdown of these genes also results in defective segmentation and in disrupted expression of an *hairy/Enhancer of split (E(spl))* ortholog (Schoppmeier and Damen, 2005b; Stollewerk et al., 2003). These results have been confirmed in *Achaearanea*, where *Delta* and *Notch* orthologs are required for growth zone formation (Oda et al., 2007).

Recently, a second signaling pathway involved in vertebrate segmentation has been shown to be required for growth zone formation in *Achaearanea*. It is even the same ligand, *Wnt8*, that results in segmentation phenotypes in both vertebrates and the spider (McGregor et al., 2008b). Thus two signaling pathways that are indispensable for the generation of somites in vertebrates also play a major role in spider segmentation.

In conclusion, spider segmentation seems to involve elements of both vertebrate and *Drosophila* segmentation – but it is still unclear, how and if these two mechanisms are linked during segment formation from the spider growth zone. Furthermore, all work on spider segmentation focused solely on the opisthosoma, and hardly any data is available on the early stages when the prosomal segments are patterned. These I will try to add in the following chapter.
2.2. Results
2.2.1. Segment polarity gene expression

**Appearance of engrailed stripes in *Achaearanea***

The *Achaearanea engrailed* ortholog (*At-en*) has been described by Akiyama-Oda and Oda (2006) but they did not describe a detailed analysis of the early appearance of the *At-en* stripes. An expression pattern analysis of the *At-en* stripe appearance was also done by Matthias Pechmann (2007). For this thesis I re-examined the expression of this gene in the prosomal region in more detail to better understand the prosomal segmentation in *Achaearanea*.

*At-en* is first expressed as a stripe in the presumptive L1 segment (Figure 4A). Next, the stripes in the Pp and L4 appear (Figure 4B), and then the stripes in the cheliceral segment as well as L2 and L3 are added (Figure 4C). In contrast to the prosomal segments, the *At-en* stripes of the opisthosomal segments are added one after the other in a strict anterior to posterior order (Figure 4D-I).

The appearance of the *At-en* stripes in the opisthosoma of *Achaearanea* is very similar to the expression of *Cs-en* in *Cupiennius salei* that has been described in much detail for these later stages of spider embryo development (Damen, 2002b) (Figure 1D-I and not shown).
Figure 4: **Appearance of At-en stripes.** All panels show lateral views. (A-C) Proosomal At-en stripes appear rapidly and without a strict anteroposterior order. (D-I) Opisthosomal stripes are added sequentially.
**Achaearanea hedgehog expression**

The *Achaearanea hedgehog* ortholog (*At-hh*) has also first been described by Oda et al. (2007), but the authors do not describe the appearance of the segmental stripes of *At-hh*.

*At-hh* is expressed already very early in *Achaearanea* embryos (Natália Feitosa, unpublished observations), but here I will only concentrate on the appearance of the striped *At-hh* pattern. *At-hh* stripes that later are located in L4 and O1 are already present at stage 7. Also a triple segmental stripe that later splits into stripes in the pedipalpal and the cheliceral segment plus a stripe in the ocular region is present in the anterior embryo at this stage (Figure 5A). Next the stripe in the pedipalpal segment splits off (Figure 5B) and then at stage 8 early the stripe in the L1 segment becomes visible (Figure 5C). The remaining stripes of the prosoma, the stripes in the L2 and L3 segments, appear simultaneously at stage 8. During this stage, also the ocular/cheliceral stripe splits into two separate stripes (Figure 5D). *At-hh* stripes in the opisthosoma are added sequentially from the growth zone in a dynamic manner, similar to the expression of pair rule genes in this spider (see 2.2.3) (Figure 5C-H).

![Figure 5: Appearance of At-hh stripes](image)

All embryos are shown laterally, except for A, which is a ventrolateral view. (A-D) The cheliceral stripe forms as a double stripe together with a stripe in the ocular region (black arrowheads). Of the other prosomal segments, the L4 stripe forms first (white arrowhead), followed by a stripe in the pedipalpal segment. Then L1 expression appears and the L2 and L3 stripes are added least. (E-H) Opisthosomal stripes are added sequentially and are forming in the growth zone, starting from stage 7. The O1 stripe (black arrow in A and B) appears already together with the L4 stripe. The dark staining in the growth zone region of the embryo in B is unspecific.
**wingless expression in Achaearanea**

The *Achaearanea wingless* (*At-wg*) sequence was available from GenBank (accession AB167808). Here I describe its early expression in *Achaearanea* embryos.

*At-wg* expression appears as a stripe only in the L1 segment at stage 8 (Figure 6A) and slightly later also in the L2 segment (Figure 6B). These stripes never extend across the full width of the segments, the most dorsal parts of the germband remain free of expression. In the other prosomal segments, *At-wg* is not expressed in stripes, but in two dots per segment, which emerge simultaneously with the appearance of the limb buds. These dots are first located at the anteroventral base of the limb buds. As the limb buds extend, the *At-wg* expression forms stripes in the anteroventral portions of the legs. Only about mid stage 9 (in embryos with around 7 opisthosomal segments), *At-wg* expression is also visible in the opisthosoma. Here it is expressed in dorsal dots in the O2 and O3 segments, while it is absent from the other opisthosomal segments (Figure 6E). Later at stage 11, these dorsal dots extend to stripes in the dorsal regions of O2 and O3. In contrast to *Cs-wg* expression in *Cupiennius*, no other dorsal stripes are formed in the opisthosoma and also no dorsal dots appear in the prosomal segments (Figure 6F). Moreover, unlike *Cs-wg*, *At-wg* is never expressed in the growth zone, but it is only expressed at the posterior end when segmentation has already stopped (Figure 6F).

Expression of *At-wg* in a stripe anterior to the labrum and in several dots in and adjacent to the opisthosomal limb buds is similar to the expression of the *wg* ortholog in *Cupiennius*. However, the remarkable differences to the *Cupiennius* expression pattern (no growth zone expression, no dorsal expression in every segment) suggest that either the function of the spider *wg* genes has diverged significantly between the *Achaearanea* and *Cupiennius*, or that maybe the two genes are not true orthologs and so far unrecovered duplicates might exist in both spiders. In *Cupiennius* though, an extensive search for another *wg* ortholog had been unsuccessful (Damen, 2002b).
Figure 6: Expression of **At-wg**. Panels A-C show lateral views, D and E also display ventral views of the same embryo in the center and on the right. F additionally shows a ventral view of the head region and the posterior end. **At-wg** forms a stripe only in the L1 segment (A) and the L2 segment (B). Both stripes do not extend to the dorsal sides of the germ band. In all other prosomal segments, **At-wg** is only expressed in one dot per hemisegment (C). The dots are located at the ventral bases of the limb buds. No expression is seen in the opisthosa (D). With the outgrowth of the appendages the **At-wg** expression forms a stripes in the anterior of the appendages. At this stage, expression is also visible in a few opisthosomal segments (O2 and O3) (E). These dots in O2 and O3 extend to stripes in the dorsal regions of the respective segments (black arrowheads). A complex pattern is seen in the opisthosomal limb buds and O1. **At-wg** is also expressed in a stripe anterior to the labrum (black arrow) and at the posterior end (white asterisk).
2.2.2. Hox gene expression

Hox genes are important developmental regulators of regional identity along the anteroposterior (AP) axis and are thought to underlie the evolution of the diverse bodyplans of bilaterian animals. All Hox genes contain the highly conserved 60 aa DNA binding domain called homeodomain (McGinnis et al., 1984). Another shared characteristic of Hox genes is their localization in physically linked clusters in most animal genomes and moreover they exhibit the phenomenon of spatial colinearity – Hox genes are expressed at a specific position along the AP axis of the embryo which is associated with the order of the genes on the chromosome (Carroll, 1995). Additionally some animals (mainly vertebrates) even exhibit temporal colinearity of Hox gene expression, where anterior Hox genes are also earlier expressed than posterior ones (Kmita and Duboule, 2003).

Chelicerate Hox gene expression has been studied in detail in the spider Cupiennius salei (Damen et al., 1998; Damen et al., 2005; Damen and Tautz, 1998; Damen and Tautz, 1999; Schwager et al., 2007). This data showed that ten classes of Hox genes are present in Cupiennius, which is most likely the ancestral condition in arthropods (Cook et al., 2001; Grenier et al., 1997; Hughes and Kaufman, 2002c). Compared to other arthropod Hox genes, the Cupiennius Hox genes exhibit two peculiarities: First, at least three of the Cupiennius Hox genes are duplicated, and, second, it seems that the tagma border between prosomal and opisthosomal segment is an important boundary as the expression of most Cupiennius Hox genes does not cross this border.

In 2001, Abzhanov et al. (2001) conducted a small PCR screen for Hox genes in Achaearanea. They obtained short fragments of two proboscipedia (pb), one Hox3, two Deformed (Dfd), two Antennapedia/Sex combs reduced like, one abdominalA and one AbdominalB gene ortholog. Of three of these genes (At-pb1, At-Hox3, At-Dfd2) the authors cloned slightly longer sequences (around 220 bp), which allowed initial expression pattern analysis, but the expression patterns of the other genes were not investigated. Additionally the protein expression of Scr and Ubx/abdA was examined using cross-reactive antibodies. However, the expression analysis was only carried out on older embryos (older than stage 9), as was the case for the Hox gene surveys
undertaken in *Cupiennius*. To gain a more complete understanding of Hox gene expression in *Achaearanea*, especially with respect to early embryogenesis, I obtained larger fragments of these and additional *Achaearanea* Hox genes to analyze the expression patterns of the Hox genes in more detail. An additional reason to do this analysis was that the Hox genes were required as marker genes in functional analysis of segmentation genes (e.g. see section 3).
Identification and expression of two Achaearanea labial orthologs

Two Achaearanea fragments similar to the Drosophila Hox gene labial have been identified in our lab. One fragment, At-labial-1 (At-lab-1), was isolated by Matthias Pechmann (Pechmann, 2007), and the other one, At-labial-2 (At-lab-2) has been identified among ESTs (Alistair McGregor, unpublished data). Unfortunately the two fragments were not overlapping (Figure 7), so it could not be decided by a sequence alignment if these two fragments belong to the same At-lab ortholog. However, a limited in situ hybridization experiment in older embryos (Pechmann, unpublished data) revealed the two clones had different expression patterns, which suggested they represent two distinct orthologs of lab.

At-lab-1 is first expressed in a broad transverse stripe in stage 6 embryos (Figure 8A). This stripe broadens at stage 7, while the anterior half of the stripe shows higher expression levels than the posterior half (Figure 8B). At stage 8 early, a new separate stripe of expression comes up posteriorly to this stripe (Figure 8C). At mid stage 8 segmental grooves become visible and the At-lab-1 expression domains can be mapped to corresponding segments: The anterior stripe splits into a stronger domain, which is now located in the pedipalpal segment and a weaker domain that is visible in the L1 segment. The most anterior Pp stripe exhibits the strongest expression and the most posterior L2 stripe shows the weakest expression (Figure 8D). These characteristics are retained throughout the rest of the development while the segmental expression of At-lab-1 resolves into a pattern in the ventral neuroectoderm and the legs of the respective segments, except for the L2 domain, where only very weak expression can be seen in the legs, but not in the ventral ectoderm. The sharp anterior border between the cheliceral and pedipalpal segments is maintained as well as the fact that expression is not observed posterior to L2 (Figure 8E-H).

| Cs–Lab     | GSGRTNFTQTELEKEFYNKYLTRARRIEIATALQLNETQVKWFQNSMGKRMKE |
| At–Lab-2   | G........SS..................A...L.D                   |
| At–Lab-1   | .T.....................F....                        |

Figure 7: Alignment of the homeodomain of Cs-Lab and At-Lab fragments. The two Achaearanea Labial fragments do not overlap. Identical aa are represented as dots, gaps introduced for alignment purposes are shown as hyphens.
Figure 8: Expression of *At-labial-1*. No expression is seen before stage 6 (A). The stripe of *At-labial-1* expression gets broader during stage 7, while the posterior half of the stripe (arrowhead) shows less strong expression (B). At stage 8, a new stripe (asterisk) appears in the prospective L2 segment (C). At stage 9, the segmental expression is transformed into a pattern in the legs and in the neuroectoderm (E-F). The arrow in F points at the ventral neuroectoderm of the pedipalpal segment and the arrowhead at the ventral neuroectoderm of L1. These expression domains are maintained during further development (G-H). Embryos on the left are viewed laterally and embryos on the right represent ventral views, except for the embryo in (A), which is viewed posteriorly.

*At-labial-2* is expressed at late stage 3 and thus much earlier than *At-labial-1*, but the expression is so weak that the staining is hardly visible (not shown). In stage 4 embryos, *At-labial-2* is expressed in a one cell wide ring around the germ disc (Figure 9A). This expression domain broadens in stage 5 and 6 embryos and also new cells that do not express *At-labial-2* appear at the rim of the germ disc (Figure 9B,C). At stage 7, like the *At-labial-1* expression at this stage, the broad stripe shows stronger expression in the
Segmentation gene expression in *Achaearanea tepidariorum* - Results

anterior of the stripe than in the posterior and a new domain appears posterior to this stripe (Figure 9D). In stage 8 embryos, strongest expression of *At-lab-2* is found in the pedipalpal segment and much weaker in the four walking leg segments. In the walking leg segments, the expression also decreases towards the posterior, just like *At-lab-1* does in L1 and L2 (Figure 9E). At stage 9 and 10, *At-lab-2* is expressed in the legs and the ventral neuroectoderm (Figure 9F-H), but in a pattern that is different from *At-lab-1*.

The different expression patterns of non-overlapping fragments of *At-lab-1* and *At-lab-2* imply that these fragments correspond to two different orthologs of *labial* in *Achaearanea*. The expression pattern of *At-lab-2* more than the expression of *At-lab-1* resembles the expression of *Cupiennius labial (Cs-lab)*, which is also expressed from Pp to L4 in the legs and the neuroectoderm (Damen et al., 1998). Thus, if I may infer homology of genes from their expression patterns, *At-lab-2* is probably homologous to *Cs-lab*. *At-lab-1* is then a paralog, which, if it exists in *Cupiennius*, has not been isolated yet.
Figure 9: **Expression of At-labial-2.** Expression appears at stage 4 (A) in a ring around the germ disc (arrow), the asterisk shows the blastopore. At stage 5 (B), the ring has become broader and a few cells that do not express At-lab-2 appear at the rim of the germ disc (arrowhead). Even more cells show no expression in the anterior of stage 6 embryos (C). The broad stripe shows stronger expression in the anterior (arrow) than in the posterior (arrowhead) and a very weak new stripe appears posterior to the initial stripe (asterisk) at stage 7 (D). The initial domain can be mapped onto the pedipalpal segment in stage 8 embryos (arrow) (E). Weak expression is found in the four walking leg segments, while expression is stronger in L1 (arrowhead) and weakens towards the posterior (asterisks). From stage 9 onwards, expression of At-lab-2 is found in the legs and the ventral neuroectoderm, the anterior border lies in the pedipalpal segment (arrow in F) and the posterior border of expression in L4 (F-H). Embryos on the left are viewed laterally, and embryos on the right ventrally.
Expression of an *Achaearanea Hox3* ortholog

*Hox3* genes have dramatically changed their expression pattern in arthropods; from an ancestral Hox gene like expression in non-insect arthropods to extraembryonic expression and function of the *Hox3* ortholog *zerknüllt* (*zen*) in insects (reviewed in Panfilio et al., 2006). Most likely, *Hox3* evolved into *zen* only within insects, since in the firebrat *Thermobia domestica*, a basal wingless insect, *Hox3* is expressed both Hox- and Zen-like (Hughes et al., 2004). Furthermore the only study in crustaceans, which are the closest relatives of the insects, revealed only the canonical Hox-like expression of *Hox3* in *Daphnia pulex* (Papillon and Telford, 2007). *Hox3* orthologs have also been cloned from chelicerates (*Cupiennius, Achaearanea* and the mite *Archegozetes longisetosus*). The Hox-like expression patterns of these genes had initially led to the interpretation that this was the ancestral state in arthropods (Abzhanov et al., 2001; Damen and Tautz, 1998). However, in these studies, as a result of practical problems, only later developmental stages had been examined and early extraembryonic expression were not investigated or reported. Therefore I extended the initial fragment of *At-Hox3* cloned by Abzhanov et al. (2001) via RACE-PCR and obtained the full coding sequence of the gene.

Like *Cupiennius Hox3* (Damen and Tautz, 1998), the *Achaearanea* ortholog is also expressed in the pedipalpal and the four walking leg segments. Expression of *At-Hox3* is not observed before stage 8, where 5 broad segmental stripes are located in the respective segments (Figure 10A,B). At stage 9, staining is predominantly found in the appendages, but the expression extends ventrally from the limb buds into the ventral ectoderm and forms small triangles anterior-lateral to the limb buds of Pp-L4 (Figure 10C). The pattern in the pedipalpal limb buds (a broad ectodermal ring) is different from the expression in the walking leg limb buds, where *At-Hox3* is expressed in the limb mesoderm (Figure 10C,D). At stage 10 an additional domain of expression appears in the ventral neuroectoderm, where *At-Hox3* is expressed in a few neurogenic cells of each segment between Pp and L4 (Figure 10D). This expression expands to a few more cells during stage 10 (Figure 10E). At stage 10 late yet another expression domain appears, this time outside of the original Hox3 domain, in the opisthosoma. Expression can be seen in one dot per hemisegment in the neurogenic ectoderm and also in a dot in the limb buds of the second opisthosomal segment (the future genital opening) (Figure 10F).
Figure 10: Expression of *Achaearanea Hox3*. *At-Hox3* is not expressed before stage 8. At this stage it is expressed in broad segmental stipes (most probably mesodermal) from the Pp to L4 at stage 8. The expression is strongest in L1 and L4 (A,B). In stage 9 and 10 embryos, *At-Hox3* is expressed in the mesoderm of the outgrowing limb buds, except for the Pp. These show a broad ring of expression (arrow in C) that later refines into to rings and additional expression at the tip of the Pp (D-E). Additionally expression extends anterior-ventral to the limb buds in triangular shape at stage 9 (arrowhead in C). This expression is gone at stage 10, but now *At-Hox3* is also expressed in the pedipalpal and walking leg segments in a group of neurogenic cells in the neuroectoderm (white arrows in D-F). In late stage 10 embryos, *At-Hox3* is also expressed in dots in the ventral neuroectoderm of every opisthosomal segment (black arrows in F). Furthermore a dot of expression can be found in the first opisthosomal limb bud (arrowhead in F). Embryos in A and B are shown laterally, embryos C-E are shown laterally on the left and ventrally on the right. F shows ventral views of the head region (left) and the opisthosoma (right) of an embryo at a similar stage as the embryo in E.
Expression of two *Achaearanea Deformed* orthologs

Abzhanov et al. (2001), previously described fragments of two *Achaearanea Deformed* orthologs, *At-Dfd-1* and *At-Dfd-2*. Of these orthologs, they reported only the late expression pattern of *At-Dfd-2*. Matthias Pechmann has extended the original fragment of *At-Dfd-1* via RACE-PCR to use it as a marker gene for RNAi phenotype analysis (Pechmann, 2007). Here I describe the expression of *At-Dfd-1* and the early expression of *At-Dfd-2*.

*At-Dfd-1* is first expressed at stage 4, where its expression covers almost the whole germ disc, except for a 1-3 cell wide ring at the rim of the germ disc and cells within the blastopore (Figure 11A). During stage 5 and 6 the number of cells not expressing *At-Dfd-1* at the rim of the germ disc increases, so that at late stage 6 there is an about 15 cells wide ring at the rim of the germ disc that does not show expression of *At-Dfd-1*. Furthermore, tissue at the posterior end (in the center of the opening germ disc) does not exhibit *At-Dfd-1* staining (Figure 11B,C). As a result, a wide ring (or stripe) of expression has formed. Expression in this stripe is not uniform, however, but in fact the anterior and the posterior of the domain are stained more strongly than the center (Figure 11C). These two domains with higher expression form two stripes at the anterior and posterior of the *At-Dfd-1* domain at stage 7. The posterior stripe then splits into two separate stripes and an additional stripe is inserted in between the original two stripes (Figure 11D). At stage 8, these four stripes encompass the four walking leg segments. The expression is stronger in the dorsal parts of the segments where the limb buds will arise. The sharp anterior border lies directly behind the Pp/L1 segment border and the *At-Dfd-1* expression domain ends posterior to L4. These borders are maintained throughout the rest of development, as *At-Dfd-1* is expressed in squares in the neurogenic ectoderm and in the outgrowing limb buds of the four walking legs (Figure 11F-H). The expression is strongest in the tips of the legs (Figure 11G,H).
Figure 11: **Expression of Achaearanea Deformed-1.** Each panel (except for A) shows the same embryo, once viewed laterally on the left and once as a ventral view on the right. The black arrow in A-C points at cells at the rim of the germ disc that do not express At-Dfd-1. The arrowhead in these pictures shows cells without At-Dfd-1 expression first in the blastopore (A) and later at the posterior end of the embryo at stage 6. The uniform broad stripe of expression visible at stages 4-6 early, subdivides first into two domains at stage 6 late (white asterisks in C) and then at stage 7 the posterior domain splits into two more stripes and in between the anterior and posterior domains a new stripe gets inserted so that there are four stripes of At-Dfd-1 expression at stage 7 (black asterisks in D). These 4 stripes are later located in the four walking leg segments at stage 8 (E). In stage 9-10 embryos, At-Dfd-1 is strongly expressed in the outgrowing walking legs with strongest expression in the tips of the legs (white arrows in G and H) and in the neuroectoderm (white arrowheads in F and G).
The expression of *At-Dfd-2* starts later than *At-Dfd-1* and is first visible at stage 7 in a broad transversal stripe in approximately the anterior medial portion of the forming germband (Figure 12A). At stage 8 this stripe splits into two stripes that are located in the L1 and L2 segments, and the anterior border of *At-Dfd-2* expression lies just behind the Pp/L1 segment border (like the *At-Dfd-1* expression). Very weak expression also appears in the L3 and L4 segment (Figure 12B). During stage 9, *At-Dfd-2* is predominantly expressed in the neuroectoderm of the L1 and L2 segments, weakly in the neuroectoderm of L3 and L4, and in the outgrowing limb buds.

![Figure 12: Expression of *Achaearanea Dfd-2*. Each picture shows the same embryo viewed laterally (left) and ventrally (right). The bracket in A depicts the weak broad stripe of *At-Dfd-2* expression. This stripe can be allocated to the first two walking leg segments at stage 8 (B). The anterior of the segments is stained stronger than the posterior. Additional, but much weaker expression can be seen in the L3 and L4 segments. At stage9 (C), *At-Dfd-2* is predominantly expressed in the ventral neuroectoderm. The anterior and posterior expression domain of this Hox gene is marked by arrowhead and arrow in B and C respectively.](image)
We have previously shown by sequence comparison that the two *Achaearanea Dfd* genes and two *Cupiennius* orthologs *Cs-Dfd-1* and *Cs-Dfd-2* must have undergone duplication before the two species diverged (Schwager et al., 2007). This sequence comparison, however, included only the small homeodomain fragment of At-Dfd-2. Using the additional 3’ sequence that I recovered, additional aa substitutions only shared between Cs-Dfd-1 and At-Dfd-1 or substitutions shared only between Cs-Dfd-2 and At-Dfd-2 are evident at the C-terminal ends of the proteins (Figure 13). These gene orthologies are corroborated by the comparison of the expression patterns of *At-Dfd-1* and *At-Dfd-2* with the two *Cupiennius* paralogs. Both *Cupiennius* and *Achaearanea Dfd-1* are expressed equally strong in all four walking legs, whereas *Cs*-and *At-Dfd-2* are more strongly expressed in L1 and L2 but weaker in L3 and L4.

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<td>..........................................................</td>
<td>/56/ ...S...................</td>
</tr>
<tr>
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<td>/35/ CTP-IEE.DE...........</td>
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<td>At-Dfd-1</td>
<td>..........................................................</td>
<td>/41/ L.C---IEE.DE...........</td>
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</tbody>
</table>

Figure 13: Alignment of *Cupiennius* and *Achaearanea Deformed*. Only two fragments of the C-terminal ends of the Dfd proteins are shown in the alignment. Dots represent identical aa, hyphens show gaps introduced for alignment purposes. Aa shared only between Cs-Dfd-2 and At-Dfd-2 are highlighted in dark grey and aa shared only between Cs-Dfd-1 and At-Dfd-1 are highlighted in light grey. Since the central part of the alignment did not align very well, it was omitted and the numbers display the number of aa between the two parts of the alignment.
Expression of an *Achaearanea Sex combs reduced* ortholog

Short homeodomain fragments of two *Achaearanea Antennapedia/Sex combs reduced-like* (Antp/Scr-like) orthologs have been described by Abzhanov et al. (2001). With only a small fragment of homeodomain sequence at hand, it was not possible to determine whether the fragments were *Antp* or *Scr* orthologs. I was able to extend one of these fragments (GenBank AF151999) by RACE-PCR and obtained the full coding sequence. An alignment with *Scr* and *Antp* orthologs of *Cupiennius* and other arthropods shows that this gene is undoubtedly an *Scr* ortholog (Figure 14). I failed so far to recover RACE-PCR fragments for the other *Antp/Scr-like* gene (GenBank AAF73214). In the following I will refer to the gene I have completely sequenced as *At-Scr-1*. In *Cupiennius*, *Scr* is one of the duplicated Hox genes. Unfortunately for both Cupiennius Scr genes only limited partly overlapping sequence information is available (Schwager et al., 2007). Nevertheless *Cs-Scr-1* and *At-Scr-1* share one specific amino acid substitution, so this is a first hint that these two genes might be orthologs.

<table>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<tr>
<td>Dm-Antp</td>
<td>SCL....-...-.FR..G.QT .......-......T .......-......T</td>
</tr>
</tbody>
</table>

Figure 14: Alignment of *Sex combs reduced* and *Antennapedia* proteins. Identical aa are represented as dots and gaps introduced for alignment purposes by hyphens. One aa substitution shared only between At-Scr and Cs-Scr-1 is highlighted in dark grey. At-Scr is a clear Scr ortholog. The orthology of another Antp/Scr ortholog isolated by Abzhanov et al. (2001) remains unsolved. At *Achaearanea tepidariorum*, Cs *Cupiennius salei*, Es *Endeis spinosa*, Nv *Nasonia vitripennis*, Dm *Drosophila melanogaster*. 

*At-Scr-1* is first expressed at stage 5 in a cap-like domain in the center of the germ disc (Figure 15A). The cap-like expression expands during stage 6, while the outer (or anterior) border of the cap forms an open ring (approximately 10 cells wide) of stronger expression and the rest of the cap expresses *At-Scr-1* at lower levels (Figure 15B). The new tissue that is added posteriorly during stages 6 and 7 also shows this low level expression (Figure 15C). At stage 7 the higher level ring-like expression splits into
two stripes (Figure 15C,D), while later during stage 7 and 8 two more stripes appear posterior to them. These At-Scr-1 stripes, however, are much weaker (Figure 15D). It becomes clear at stage 8 that the strong At-Scr-1 stripes are located in the anterior of L2 and L3 (Figure 15E,F). Still, all tissue between the stronger stripes and all posterior tissue shows weak At-Scr-1 expression.

Figure 15: Expression of Achaearanea Scr-1, stages 5-8. Each panel shows lateral (left) and ventral (right) views of the same embryo. At-Scr-1 is first expressed in the center of the germ disc at stage 5. The position of the cumulus in the embryo in A is marked with a “c”. The posterior cap expression widens during stage 6 (B). At-Scr-1 now forms an open ring, localized roughly halfway between anterior rim of the opening germ disc and the future posterior center of the germ disc. The cells posterior to the ring also express At-Scr-1, but at a much lower level. At stage 7, the ring splits up into two stripes (arrows, C) and a bit later two new stripes appear (arrowheads, D). The most anterior stripe of expression lies between L1 and L2 at stage 8 (E and D), the second anterior stripe lies between L2 and L3. The two posterior stripes cover L3 and L4. The weak expression of At-Scr-1 continues posterior to these stripes.
The sharp anterior expression border of At-Scr-1 directly posterior to the L1 segment is maintained from stage 9 onwards by almost ubiquitous expression in the ventral neuroectoderm (Figure 16A). In addition, expression can be found in the growing L2-L4 appendages, where expression is strongest in L3. In the legs the At-Scr-1 expression refines into a pattern consisting of multiple rings. The overall pattern is similar in all three legs, but the expression level is always higher in L3 compared to L2 and L4 (Figure 16A-D). Expression of At-Scr-1 also continues weakly in the opisthosomal segments, with an additional stronger expression domain in the growth zone (Figure 16A-C). This growth zone expression persists, until the end of posterior growth (Figure 16D). Starting at late stage 9, further expression can be seen in the ventral neuroectoderm of the opisthosomal segments (Figure 16B). The anterior expression border of At-Scr-1 at stage 8 and 9 is very similar to the expression of Cs-Scr-1 and moreover very different from Cs-Scr-2 at comparable stages. Together with the shared sequence similarity (Figure 14) this implies that At-Scr-1 and Cs-Scr-1 likely are orthologs.
Figure 16: **Expression of Achaearanea Scr-1, stages 9-10.** *At-Scr-1* is predominantly expressed in the limb buds and the ventral neuroectoderm of stage 9 embryos (A). The anterior expression border (arrow) is directly posterior to the L1 limb bud. Expression of *At-Scr-1* continues in the opisthosoma, but is much weaker, except for a domain in the growth zone (arrowhead). This expression in the growth zone continues to the end of segmentation (B,C). No more expression is visible at the posterior end of the embryo at stage 10 late (arrow in D). *At-Scr* expression forms similar patterns consisting of multiple rings in the legs (B,C), but it is much more strongly expressed in L3 compared to L2 and L4. At stage 9 late the expression of *At-Scr* in the neuroectoderm can also be found in every segment of the opisthosoma (arrow in C).
Expression of an *Achaearanea fushi tarazu* ortholog

Like *Hox3, fushi tarazu (ftz)* is one of the Hox gene orthologs, which has diverged much among arthropods (Damen, 2002a; Papillon and Telford, 2007; Telford, 2000). In *Cupiennius*, as well as in the mite *Archeozetes*, *ftz* is expressed in a Hox like fashion (Damen, 2002a; Telford, 2000). To investigate whether this Hox gene like expression is conserved in *Achaearanea*, I cloned a fragment of an *Achaearanea ftz* (*At-ftz*) ortholog. The 658 bp fragment of *At-ftz* codes for the C-terminal half of the homeobox and the remaining C-terminal part of the protein. An alignment of the homeodomain with other arthropod Ftz proteins is shown in Figure 17. Apart from the homeodomain, the two spider sequences are not very similar to each other (only 34% identity outside of the homeodomain, alignment not shown).

<table>
<thead>
<tr>
<th>Homeodomain</th>
</tr>
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<tbody>
<tr>
<td>At-Ftz</td>
</tr>
<tr>
<td>Cs-Ftz</td>
</tr>
<tr>
<td>Al-Ftz</td>
</tr>
<tr>
<td>Es-Ftz</td>
</tr>
<tr>
<td>La-Ftz</td>
</tr>
</tbody>
</table>

Figure 17: **Alignment of Fushi tarazu homeodomains.** Identical aa are represented by dots. At *Achaearanea tepidariorum*, Cs *Cupiennius salei*, Al *Archeozetes longisetosus*, Es *Endeis spinosa*, La *Lithobius atkinsoni*.

Expression of *At-ftz* is first visible at stage 6 late, when it is expressed strongly in the forming growth zone. Unlike spider pair rule gene orthologs (see 2.2.3), however, the expression in the growth zone never clears from the very posterior end. Instead, the posterior domain emanates stripes of expression towards the anterior. For every new segment, one new stripe of *At-ftz* expression buds off from the anterior growth zone. This stripe then fades, so that maximally two stripes are visible; one that is just forming in the anterior growth zone and one that is fading at the anterior border of the afore generated segment (Figure 18E,G and Figure 19A,C,D). This expression in the growth zone persists until no more segments are formed at stage 10 late (Figure 19E).

Less abundant transcription in a broad transversal stripe is located at about half of the embryo length (Figure 18A). This stripe gets even weaker during the following stages 6-8, but the anterior border of the expression can be mapped onto the L1/L2 border at stage 8 (e.g. Figure 18E). This border corresponds to the anterior boundary of weak *At-ftz* expression seen in the neuroectoderm of later stages (Figure 19A-C). In these stage 9 embryos, the posterior expression border is located between the L4 and
O1 segments, while expression is strongest in the neuroectoderm of L2. Even though the anterior and posterior expression border of this Hox-like expression domain closely resembles the one from Cs-ftz in Cupiennius, the expression pattern in the neuroectoderm is different from Cupiennius. In Achaearanea, ftz is expressed more uniformly compared to Cs-ftz, which is only expressed in a few specific cells per hemisegment in the Cupiennius neuroectoderm. At stage 10 late, the uniform expression in the neuroectoderm has concentrated into one patch of staining per hemisegment (Figure 19E). At-ftz is also expressed in the legs, but only in L2. This is in contrast to Cupiennius, where it is expressed in the legs of L2-L4. The pattern in the Achaearanea legs is also different; At-ftz is expressed only in one stripe near the tips of the appendage, with weaker staining distally to the ring, whereas in Cupiennius, ftz expression is found in two rings and a domain near the distal tips.

However, the biggest and also most interesting difference between ftz in the two spiders is the expression of At-ftz in the growth zone of Achaearanea embryos, which is not present in Cupiennius. Based on the sequence differences and the expression differences ftz must have either diverged significantly between these spiders, or the genes are not each orthologs and a second ftz paralog that has not been recovered so far might be present in both spiders.
Figure 18: **Expression of Achaearanea ftz in stage 6-8 embryos.** Every panel shows the same embryo viewed laterally on the left and the posterior end of the embryo is shown ventrally on the right. (A) *At-ftz* is first expressed at stage 6 in a semicircle at the posterior end where the growth zone forms. The brackets mark an anterior broad stripe of *At-ftz* expression. This stripe of expression gets weaker during stages 7 and 8, where mostly only the anterior border of the stripe in L2 (arrow in E) is visible. The stripe is almost invisible when it is viewed ventrally (not shown). The expression in the posterior growth zone persists, and does not clear from the posterior end (A-G). It emanates stripes at the anterior end of the growth zone (white arrow in B, C, E, G). After budding off from the anterior growth zone, stripes stay visible at the anterior border of the last formed segment (white arrowhead in E-G) until the next stripe of *At-ftz* expression buds off.
Figure 19: **Expression of Achaearanea ftz during stages 9 and 10.** Every panel shows lateral (left) and ventral views of the central (middle) and opisthosomal region (right) of the same embryo. (A-C) The growth zone expression continues to bud off stripes towards anterior. White arrows point at new stripes being formed, white arrowheads point at stripes localized at the anterior border of the segments formed last. At stage 9, the anterior border of the Hox expression domain is located between L1 and L2 and the posterior border lies between L4 and O1 (black arrows in A-C). This expression is very weak and quite uniform in the ventral neuroectoderm. Even overstaining At-ftz in situ hybridizations does not make this domain stand out more from background staining (data not shown). (D,E) At-ftz expression in a ring near the distal tip of L3 at stage 10 (black arrow-heads in D and E). Lower levels of At-ftz expression are visible distal to the ring. (E) The legs on one side of the embryo were dissected off to show the expression in the ventral neuroectoderm, which has concentrated to one spot per hemisegment (black arrows) within the L2-L4 domain. The At-ftz expression at the posterior end has vanished (asterisk).
**Antennapedia isolation and expression in Achaearianea**

Since one of the Antp/Scr-like fragments isolated by Abzhanov et al. turned out to be a Scr ortholog, I attempted to isolate a fragment of an Antp ortholog by degenerate PCR. I obtained two different fragments, but only one of them I could extend via 3’ RACE-PCR. An alignment of this At-Antp ortholog with the Cupiennius Antp ortholog is shown in Figure 14.

As one of the posterior Hox genes in spiders, At-Antp is not expressed before stage 7, before opisthosomal tissue emerges from the growth zone. At this stage, At-Antp expression is visible at the posterior end of the embryo in a circular domain (Figure 20A). As the germband extends, the circular domain expands to a ring-like expression and cells, which show only very weak expression, follow the At-Antp expressing cells in the posterior (Figure 20B). Then a new domain of expression appears at the posterior end (Figure 20C), also transforming into a second stripe later at stage 8, which is again followed by cells with less At-Antp expression (Figure 20D). This way, segment by segment, a new stripe of expression gets added (Figure 20E-H), until segmentation is complete and At-Antp is no longer expressed in the growth zone (Figure 20H). The anterior expression border lies within the posterior limit of the L4 segment. This is also obvious from expression of At-Antp in two rings near the tips of the legs.

From stage 8 on, the expression is slightly stronger in O1, O2 and the growth zone compared to the other opisthosomal segments (Figure 20F), but this difference in the expression levels becomes more pronounced in older embryos of stage 10. The posterior border of the strong At-Antp expression lies within O2, in a way that the posterior portion of O2 is without strong expression of At-Antp (Figure 20G,H). Starting at stage 9 At-Antp is also expressed in a longitudinal stripe of cells in the neurogenic ectoderm of each opisthosomal hemisegment (Figure 20F-H).

**Figure 20: Expression of Achaearianea Antp.** Each picture shows lateral (left) and ventral posterior (right) views of the same embryo in A-F and additionally a lateral view of the anterior opisthosoma in G-I (middle). Expression of At-Antp arises in semicircles at the posterior end of the growth zone (arrowhead, A). This expression transforms into a stripe and is followed by cells that show only weak expression of At-Antp (arrow, B), before a new semicircle of expression appears at the posterior end of the growth zone (asterisk, C). The first two stripes are located in the opisthosomal segment O1 and O2 at stage 8 (D). New stripes (arrow, E) keep appearing from the growth zone (arrows, G and H) until the end of segmentation (I). At stage 9, the anterior border of At-Antp expression lies at the posterior end of L4 (arrowhead, F). Expression is now also seen in the neurogenic ectoderm, where it forms longitudinal rows in each hemisegment (white arrowheads, also in G and H). White arrows in H and I point at the very posterior part of O2, which is free from the strong At-Antp expression.
Isolation and expression of an *Achaearanea Ultrabithorax* ortholog

A short fragment of an *Achaearanea Ultrabithorax* ortholog was cloned from a degenerate PCR with primers directed against the *Antp* homeobox. This initial fragment was extended to the complete mRNA sequence by 5’- and 3’-RACE-PCR. An alignment of the homeodomain is shown in Figure 21.

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<th>Cs-Ubx-2</th>
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<th>Am-Ubx</th>
<th>Tc-Ubx</th>
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<td>ND</td>
<td>N.</td>
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</table>

Figure 21: Alignment of Ultrabithorax homeodomains. Identical aa are represented as dots. No significant aa substitutions to resolve the orthology of At-Ubx with Cs-Ubx-1 and -2 are present. At *Achaearanea tepidariorum*, Cs *Cupiennius salei*, Es *Endeis spinosa*, Am *Apis mellifera*, Tc *Tribolium castaneum*, Dm *Drosophila melanogaster*.

*At-Ubx* expression is first observed at stage 8, when a semicircular expression domain appears in the posterior growth zone and a faint stripe of expression becomes visible anterior to the growth zone (Figure 22A). This stripe becomes stronger, while the posterior domain enlarges (Figure 22B and C). Thus, every new segment that is generated anterior to the growth zone expresses *At-Ubx* (Figure 22D-H). At stage 8, the anterior stripe of *At-Ubx* expression can be assigned to the O2 segment, but the very anterior part of the segment is free from expression (Figure 22D-F). From late stage 9 on however, *At-Ubx* is also expressed in the anterior of O2, but only at the dorsal sides of the germ band and in the neuroectoderm. Posteriorly, *At-Ubx* expression continues to the posterior end of the embryo.

In *Cupiennius salei*, two orthologs of *Ub* have been isolated. *Cs-Ubx-1* expression at a stage comparable to stage 10 has its anterior border at the anterior edge of O2 and *Cs-Ubx-2* expression starts only in the posterior of O2. Like *Cs-Ubx-1* (Schwager et al., 2007), *At-Ubx-1* is not expressed uniformly in O2 and O3. As a result of the shared expression borders at stage 10, the general appearance of the expression pattern as well as sequence similarity, I suggest that *At-Ubx* is an ortholog of *Cs-Ubx-1*.
Figure 22: Expression of *Achaearanea Ubx*. Each picture shows lateral (left) and ventral (right) views of the same embryo. (A) Expression of *At-Ubx* appears at stage 8 in the growth zone (arrowhead) and in a weak stripe anterior to that (arrow). This stripe gets stronger and the posterior domain enlarges (B, C) so that all new tissue added posteriorly expresses *At-Ubx* (D-H). The anterior stripe is located in O2, while the anterior end of O2 does not express *At-Ubx* (segmental boundaries in C-H indicated by black lines) until stage 9 late, when it is expressed dorsally (white arrow in G) and in the neuroectoderm also in the anterior part of O2 (white arrow in G and H).
Expression of *Achaearanea abdominal-A*

Again, a fragment of this Hox gene was previously isolated by Abzhanov et al. (2001) and I extended this fragment by 5' and 3' RACE-PCR. Expression of the *Achaearanea abdominal-A* ortholog (*At-abdA*) begins at stage 9 and is located in the growth zone. This was unexpected because in both *Cupiennius* and *Achaearanea*, *abdA* is later expressed from the posterior part of O3 onwards. This implies that this Hox gene changes its expression border during development. However, the expression pattern in the segments anterior to O6 (which would correspond to the former growth zone expression in the stage 9 embryo shown in Figure 23A) is different from the posterior expression pattern. In O4 and O5, *At-abdA* is only expressed in the limb buds of these segments at stage 10 and in a stripe in each posterior hemisegment of O3. Posterior to O5, expression in the dorsal ectoderm is ubiquitously (Figure 23B). Later at stage 10, *At-abdA* expression can also be found in the dorsal ectoderm of O3-O5, and in the ventral ectoderm the expression border even extends into the posterior of O2.
Figure 23: **Expression of Achaearanea abd-A.** Each panel shows the same embryos viewed laterally on the left, and viewed ventrally in the middle and on the right. (A) No expression is seen anterior to the growth zone at stage 9. (B) In stage 10 embryos, the anterior border of At-abdA expression is in the posterior part of the third opisthosomal segment (arrowhead). (C) At stage 10 late, At-abdA is also expressed in the anterior part of O3 and the posterior part of O2, but only in the ventral neuroectoderm (arrow). In the more dorsal tissue of the segment, the border is still in the posterior part of O3 (arrowhead).
2.2.3. Pair rule gene expression

In *Drosophila*, pair rule genes are the first genes of the segmentation cascade to exhibit a periodical pattern and they define the segmental expression of the segment polarity genes. The pair rule genes themselves are initially expressed in a double segmental periodicity – one stripe in every second segment. Accordingly, alternating segments are deleted in *Drosophila* pair rule gene mutants. While it is still unclear to what extent this level of the *Drosophila* segmentation cascade is conserved in other arthropods, it seems apparent that pair rule gene orthologs are involved in segmentation of all arthropods examined to date. Many pair rule gene orthologues have already been described in *Cupiennius*, and all of these genes are expressed in stripes that emerge from the growth zone. However, the early expression patterns of these genes have not been characterized in this spider, and therefore it remains possible that pair rule genes might be expressed in the anterior earlier in development. Therefore I analyzed the expression patterns of these genes in detail to shed some light on early pair rule gene expression in the spider. Except for one, all of the *Achaearanea* pair rule gene orthologs described below were isolated by Natália Feitosa, and she kindly provided me with her clones and sequence information for this expression analysis.
Expression of Achaearanea *runt*

Fragments of three *runt* orthologs have been recovered from *Achaearanea*. Only one of these, which also proved to be the most similar clone to *Cupiennius runt*-1, was expressed in a pattern that reflected a role in segmentation. Therefore only the expression of *Achaearanea runt*-1 (*At-run-1*) is described below.

*At-run* is not expressed in the anterior regions of the embryo that contribute to the prosoma, and thus does not play a role in segmentation of the prosoma. *At-run* expression is first visible in a circular domain in the center of the germ disc at stage 6 (Figure 24A), when the growth zone is starting to form there. Towards the end of stage 6, expression retracts from the center of the circle, so that a ring of expression is formed (Figure 24B). This ring then opens at the posterior end to form a stripe, because the growth zone itself at this stage is no longer circular. De novo expression then appears in a few cells in the center of the opening circle (Figure 24C). This domain first expands, and then the posterior end again starts to clear (Figure 24D), so that a second stripe of *At-run* is formed. After the third *At-run* stripe has formed in the same way, the first stripe starts to fade (Figure 24E). This diminishing first stripe is located at the anterior border of segment 01 (Figure 24F). For each segment that is sequentially added from the growth zone, one stripe of *At-run* expression is generated. The dynamic expression only stops, after all segments have been added (Figure 25E). So, surprisingly *At-run* is not expressed during segmentation of the prosoma, and dynamic *At-run* expression only precedes the formation of the opisthosomal segments from the growth zone.

*At-run* is expressed later during embryogenesis in the head of the spider, most likely in the nervous system. One triangular domain appears dorsolaterally in each headlobe of stage 8 embryos. Another triangular domain is added anterior to the first one. These four domains in the head spread out over almost the whole of each head lobe during stages 9 and 10. Only the most ventral regions of the head, like the labrum and the stomodeum, remain free from *At-run* transcripts. Furthermore, *At-run* is also expressed in the neurogenic ectoderm. This expression starts at early stage 9, anterolateral to the limb buds (Figure 25A) and later, during stages 9 and 10, located in the anterior ventral neuroectoderm of each hemisegment. Towards the end of stage 10 *At-run* is expressed throughout the developing central nervous system (Figure 25E). Finally, *At-run* is expressed in rings in the legs, which start out as a single broad ring and later become refined (Figure 25A-E).
Figure 24: **Expression of At-run in stage 6-8 embryos.** Each panel shows a lateral (left) and a ventral view of the growth zone (right) of the same embryo. F and G additionally show a ventral view of the head region in the center of the panel. Expression of At-run starts in a circular domain in the middle of the opening germ disc (black arrowhead in A). At-run expression then clears from the center of the circle and forms a ring (black arrowhead in B). (C) New expression appears as a spot at the center of the ring (black arrow), while the ring transforms into a stripe as expression in what are now extraembryonic cells (black arrowhead) fades. (D) The second domain enlarges, while the posterior end clears of expression (white asterisk). (E) The second domain has transformed into a stripe during stage 7 and a third domain appears at the posterior end (black asterisk). (F,G) The At-run stripes end up at the anterior border of segments, starting with the first stripe in O1 (black arrowhead in F) before they fade. New domains that refine into stripes are added sequentially from the growth zone (e.g. white arrow in G). At-run is also expressed in the developing head lobes at stage 8 (white arrowheads in F and G).
Figure 25: **Expression of At-run in stage 9 and 10 embryos.** Each panel shows views of the same embryo. From left to right: a lateral view, head region, walking leg region as well as posterior end are shown ventrally. Stripes of At-run expression are added from the growth zone, until the end of segmentation, after which no further expression can be detected at the posterior end of the embryo (black asterisk in E). Two (C,D) or three (A,B) stripes can be visible at the same time, before the most anterior one disappears (black arrowheads). (A,B) At stage 9, secondary expression of At-run can be detected in (black arrow) and anterior (white arrow) to the limb buds. **At-run** is also expressed in new domains in the head (white asterisks), in addition to the domains that appeared at stage 8 (white arrowheads). (C-E) At-run expression in the legs is first found in a broad ring (C). Later the pattern develops into multiple rings. At-run is also expressed in the neurogenic ectoderm from stage 9 onwards (black arrows). The expression in the head spreads until it covers most of the head lobes (E) apart from labrum and stomodeum.
Expression of Achaearanea even-skipped

An *Achaearanea even-skipped* (*At-eve*) ortholog has been recovered by Alistair McGregor, who kindly provided me with this clone for expression analysis.

No *At-eve* expression was detected in embryos younger than stage 6 late. *At-eve* expression is first visible in a ring-shaped domain in the growth zone. It appears that this ring-shaped domain is already opening at the posterior end at this stage. A second domain of *At-eve* expression is also visible as a dot in the center of the ring. (Figure 26 A). While the ring opens and forms stripe 1 of *At-eve* expression, the second domain enlarges (Figure 26B,C). Then this domain shifts anteriorly and forms a stripe. This dynamic process of stripe formation in the growth zone continues until the end of segmentation (not shown). A maximum of three stripes of *At-eve* expression are visible at the posterior end. They end up at the posterior border of segments, before their expression fades (Figure 26F-H) and then completely disappears.

*At-eve* is secondarily expressed in the ventral neuroectoderm of stage 9 embryos. It forms one narrow ventral stripe per segment across the ventral midline. This segmental expression starts in O1 and then extends posteriorly and anteriorly, before it later fades again (Figure 27A-C). Ultimately, *At-eve* expression is also found in dorsolateral patches in the opisthosomal segments (Figure 27C). These cells expressing *At-eve* will later be forming the spider heart. The association of an *eve* ortholog with heart development in spiders has already been shown in *Cupiennius* (Janssen and Damen, 2008).

Thus, like *At-run*, *At-eve* is not expressed during segmentation of the prosoma in pre-growth zone formation embryos. It is only secondarily expressed in the prosomal region of the embryo later during development.
Figure 26: **Expression of At-eve in stage 6-8 embryos.** Each panel shows the same embryo viewed laterally (left) and ventrally posterior (right). *At-eve* expression appears at the posterior end of the embryo, forms a stripe and clears from the posterior end (e.g. asterisk in H) until a new stripe of expression comes up. To allow tracing of every new stripe, a different arrow labels each stripe: The black arrowhead in A-H marks the first *At-eve* stripe, the black arrow (A-H) marks the second stripe, and the white arrowhead points at the third stripe. The stripes end up at the posterior ends of segments, starting with the first *At-eve* stripe in O1 before they fade (F-G).
Figure 27: **At-eve expression in stage 9 and 10 embryos.** Each panel shows (from left to right) a lateral view, a ventral view of the anterior and a ventral view of the posterior opisthosomal region. *At-eve* stripes are added from the growth zone throughout posterior segmentation (white arrowheads), maximally 3 stripes are visible as the anterior ones fade. Expression of *At-eve* appears in a narrow, only a few cells wide stripe in the ventral ectoderm (black arrowheads). This secondary segmental expression appears first in O1 and then the expression extends to segments more anteriorly and to the newly formed segments posteriorly. Later in stage 10 the anterior ventral stripes fade again. Expression is also seen in patches along the dorsal sides of the germband (white arrows), which will later contribute to the heart.
Expression of Achaearanea pairberry

The *Drosophila* pair-rule gene *paired* (*Dm-prd*) and its paralogs the segment polarity gene *gooseberry* (*Dm-gsb*) and the *gooseberry-neuro* gene (*Dm-gbn*) all encode paired domain (PD) and an extended homeodomain (HD) containing proteins, which belong to the Pax3/7 subgroup of Pax (paired box) transcription factors. Other members of this subgroup are the vertebrate orthologs *Pax3* and *Pax7* (Balczerek et al., 1997).

A partial sequence (lacking the 5’ part of the coding region) of an *Achaearanea* Pax3/7 ortholog has been recovered, but this sequence cannot be clearly assigned to any of the three *Cupiennius* genes, nor to any of the three *Drosophila* or *Tribolium* Pax3/7 genes as the alignment in Figure 28 shows. As with *Cupiennius* and the cricket *Schistocerca*, the mite *Tetranychus* and the myriapod *Glomeris*, the orthology of the Pax3/7 genes remains unclear (Davis et al., 2001; Dearden et al., 2002; Janssen, 2004; Schoppmeier and Damen, 2005a), and in accordance with the terminology used by Davis et al. this gene was named *At-pairberry* (*At-pby*).

The expression of *At-pby* is very similar to the *Cs-pby-3* pattern in *Cupiennius* embryos, at least in the stages that have been analyzed in in this spider (comparable to stage 8 and older). *At-pby* expression starts already at early stage 6, when the germ disc is just opening (Figure 29A). This is before run and eve expression, which begin at late stage 6. *At-pby* expression is first in a circular domain, which then opens up and moves anteriorly as non-expressing cells appear at the posterior end of the embryo and forms a semicircular stripe (not shown). A second domain also appears during stage 6, and subsequently transforms into a stripe. Like the other pair rule genes, some of the staining is reiterated in the forming dorsal area, but later this expression in the extraembryonic cells fades (Figure 29B,C). The first stripe gets narrower by fading in the anterior half of the stripe (Figure 29D). While a third and fourth domain of *At-pby* expression emerges in the growth zone to form a stripe, it is possible to map the first stripe to the posterior L4 segment, before it completely fades (Figure 29E,F). Subsequently, for each new segment being generated, one new stripe of *At-pby* expression is dynamically formed in the growth zone (Figure 29G,H and Figure 30A-C) and like *eve* and *run*, *At-pby* expression in the growth zone stops only after formation of all segments (Figure 30D).

At stage 8 late, secondary expression of *At-pby* simultaneously appears as segmental stripes in all prosomal segments. These stripes are located in the posterior
segments and they are restricted to the ventral neuroectoderm. They never cross the embryo midline. In the opisthosoma, the newly formed segmental stripes restrict dorsally so that also in the opisthosoma *At-pby* is expressed in ventral neuroectodermal stripes in every segment. Like *Cs-pby-1*, *Cs-pby-3* and Pax3/7 protein expression in another spider *Schizocosa ocreata*, only the *At-pby* stripe of O1 extends to the dorsal sides of the germband (Damen et al., 2005; Davis et al., 2005).

In contrast to *At-eve* and *At-run*, *At-pby* expression is also detected in the anterior embryo (Figure 31). However, this expression is very weak and cannot easily be visualized. Only after developing the staining of the in situ hybridizations for a long time, this expression becomes weakly visible. It is first seen at stage 6, in a broad stripe that is located at the anterior of the embryo. The embryo in Figure 31Fig 8 A is slightly older than that, but in principle shows similar expression. The pattern also appears very weak because not all cells within the striped domain express *At-pby* and the patterns looks speckled. Later this broad stripe narrows to down to a single stripe covering the L1 segment (Figure 31B,C). After this, the staining is barely visible in L1 (not shown) and disappears during stage 8.

Figure 28: **Alignment of At-Pby with Cupiennius, Drosophila and Tribolium Pax3/7 proteins.** Alignment includes the paired domain, octapeptide and the partial extended homeodomain. Identical aa are shown as dots, gaps introduced for alignment purposes are indicated as hashes. Abbreviations: Pby Pairberry, Prd Paired, Gsb Gooseberry, Gbn Gooseberry-neuro, At, *Achaearanea tepidariorum*, Cs *Cupiennius salei*, Dm *Drosophila melanogaster*, Tc *Tribolium castaneum*.  

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Figure 29: Expression of At-pby in stages 6-8. All panels show lateral views on the left and ventral views of the posterior region of the embryo on the right. Stripes of At-pby are forming from the growth zone. To allow tracking of stripes, different arrows label each new stripe (A) At-pby is expressed in a circular domain (black arrow) in the center of the opening germ disc. (B) The domain has opened to form a semi-circular stripe, and a new circular domain appears (black arrowhead). (C) The second domain has also opened to a stripe. (D) A new spot of At-pby expression (black asterisk) appears at the posterior end. The most anterior stripe is fading first in the anterior half of the domain. (E) Before the first stripe fades completely it can be allocated to the posterior border of the emerging L4 segment. (F-H) New stripes (white arrow, white arrowhead) are appearing at the posterior end. The second At-pby stripe is found in the first opisthosomal segment and the third stripe in the second opisthosomal segment. In anterior segments, segmental expression in the ventral neuroectoderm, not extending to the dorsal sides of the germband appears (white asterisks).
Figure 30: **Expression of At-pby in stage 9 and 10 embryos.** All panels show (from left to right), a lateral view, a ventral view of the walking leg region and a ventral posterior view. *At-pby* is expressed in segmentally iterated stripes at the posterior borders of the segments in the ventral neuroectoderm. The stripes do not extend across the ventral midline. Only the O1 stripe extends dorsally (black arrow in B-C). Newly formed stripes also cover the complete width of the germ band. Up to three of these newly formed stripes can be visible (arrowheads in A-C). With the end of segmentation at stage 11, no more stripes are being formed posteriorly (asterisk). The O12 stripe of *At-pby* is only transiently visible (not shown).
Figure 31: **Expression of At-pby in the anterior.** Upper pictures are bright field images merged with the DAPI channel and lower pictures show the bright field image only. A and C are lateral views, B is a ventral view. (A) *At-pby* is expressed in a broad stripe (bracket) at the anterior end of the embryo. (B) The stripe is no longer located at the very anterior edge. (C) The stripe has narrowed down and is now located in L1.
Expression of Achaearanea sloppy-paired

Expression of At-slpa first appears as a triangular expression domain in each headlobe of early stage 8 embryos (Figure 32A). These two expression domains enlarge slightly during stage 9 (Figure 32B-D) and later at stage 10 cover about a third of the headlobes in an anterolateral position. Segmental expression of At-slpa appears simultaneously in the pedipalpal and the L1-L4 segment (Figure 32B), then also a further stripe in the cheliceral segment is added (Figure 32C) and opisthosomal stripes appear sequentially from anterior to posterior. The stripes are located only in the ventral portion of the segments. Initially the stripes are slightly broader, do not possess defined borders and they are located in the center of the segments. This changes during stage 9, when the stripes develop very sharp anterior and posterior boundaries and are restricted to the anterior of the parasegmental boundaries (Figure 32D). At-slpa is not expressed across the midline (Figure 33A). At stage 10, the stripes of At-slpa in the hemisegments become slightly U-shaped when the expression extends towards somewhat anterior at the dorsal and ventral edges of the stripes (Figure 33B,C). In late stage 10 embryos, expression is also found in the stomodeum and At-slpa expression forms a stripe in the central nervous system anterior to the cheliceral segmental stripe (Figure 33B,C).

At-slpa is thus not expressed in the growth zone, but only in newly formed segments. This expression is similar to that of Cupiennius, which is also only expressed in a very weak stripe at the anterior end of the growth zone.
Figure 32: **Expression of At-slp in stage 8-9 embryos.** Lateral (left) and ventral views of the head (middle) and walking leg region of the same embryo. (A) Expression of At-slp is first seen in two spots in the head region in early stage 8 embryos (white arrowheads). (B,C) Later At-slp is expressed in a stripe in the ventral region of each segment. The stripe is located in the center of the segment (black arrow). Segmental borders of the L2 segment are indicated by black lines. (D) At-slp is not expressed in the growth zone (black arrowhead). The At-slp stripes are now located at the posterior parasegmental border, the arrow points at the L2/L3 parasegmental stripe. The borders of the At-slp stripes become more defined and sharp during stages 8 and 9 (compare B to D).
Figure 33: **Expression of At-slp in stage 9 and 10 embryos.** Each panel shows lateral views (left) and ventral views of the head region (middle) and walking leg region (right) of the same embryo. *At-slp* is expressed in stripes at the anterior parasegmental boundaries, but not across the ventral midline (arrowhead, A). The stripes form a U-shape within each hemisegment (black arrows). The expression domains in the head expand to cover the anterolateral parts of the head lobes (white arrowheads). At stage 9, additional expression of *At-slp* appears in the stomodeum (white arrow) and at stage 10 expression is also found in a stripe in the head nervous system (white asterisks B,C).
2.2.4. **Expression of head regionalization genes**

The tissue that will later give rise to the segments carrying the pedipalps and walking appendages of the spider (Pp-L4) appears to be specified in the early spider embryo (see 2.2.2 and 2.3.2). Other work in our group has shown that the formation of the head tissue further anterior to the pedipalps (head lobe and cheliceral segments) depends on the spider *otd* ortholog (*At-otd-1*) (Pechmann, 2007; Pechmann et al., submitted). The entire anterior of the embryo up to the pedipalpal segment is missing after *otd* RNAi. Although *At-otd-1* is first expressed in a ring around the germ disc in stage 5 embryos (Akiyama-Oda and Oda, 2003; Pechmann, 2007). Later, at stage 7, *At-otd-1* expression is no longer located at the very anterior rim of the spider embryo but in a more posterior position. To further investigate spider head regionalization, I analyzed the expression patterns of the spider orthologs of *Six3* and *Pax6*. These two genes are involved in eye and photoreceptor development in various metazoans (for reviews see Kawakami et al., 2000; Kozmik, 2005), and are thought to also play a role in head and brain regionalization (Lagutin et al., 2003; Lowe et al., 2003; Wallis et al., 1999). Whether *Six3* and *Pax6* also have a function in arthropod head regionalization is largely unknown, because expression is only reported for a few arthropod species (e.g. Prpic, 2005; Blackburn, 2008), and apart from *Drosophila* and vertebrates, no functional data are available. As part of his diploma thesis, Klaus Jobi has already analyzed the expression of these genes in *Cupiennius*, but again, no early expression patterns are available (Jobi, 2007).
Isolation and expression of an *Achaearanea Six 3* ortholog

A short fragment of an *Achaearanea Six3* ortholog (*At-Six3*) was recovered using degenerate PCR. The fragment was extended via 3’RACE-PCR to obtain a sufficiently long enough probe for in situ hybridization.

*At-Six3* is not expressed in embryos before germband formation at stage 8. The expression starts in two lateral patches at the very anterior edge of the germband. This expression sometimes appears asynchronously in the two halves of the germband (Figure 34A). In early stage 9 embryos, the two patches become linked by tissue expressing *At-Six3* in a stripe along the anterior rim of the germband. The ventral portion of the stripe will later give rise to the labrum, where *At-Six3* will also be expressed (Figure 34D,E). The *At-Six3* expression domain later forms a V-stripe in the anterior head, when the head lobes take shape (Figure 34F). At stage 10, *At-Six3* expression also appears posterior to the forming labrum and in two longitudinal stripes along the ventral midline of the cheliceral segment.

The expression of a *Six3* ortholog has been already studied in *Cupiennius salei* (Jobi, 2007) and is different to the expression of the *Six3* ortholog described here in *Achaearanea*. *Cs-Six3* extends much more posteriorly in the head lobes in *Cupiennius*, whereas in *Achaearanea* the expression has very sharp borders and is restricted to the very anterior part of the germband. Only the expression in and posterior to the labrum is similar in both spiders. However it cannot be excluded that additional *Six3* orthologs exist in these spiders.
Figure 34: **Expression of At-Six3.** (A) Expression is first seen in two spots (black arrowheads) at the anterior end of the germband at stage 8. One spot can appear slightly earlier than the other, as seen in this embryo. (B,C) These two domains of At-Six3 expression become connected by an anterior ventral stripe at stage 9 and the expression level increases. (D,E) At-Six3 is expressed in the anterior head as well as in the labrum (white arrow), and in longitudinal stripes along the ventral midline (white arrowhead) that are also visible posterior to the stomodeum (black arrow).
Isolation and expression of an *Achaearanea Pax 6* ortholog

A 632 bp fragment of an *Achaearanea Pax6* ortholog (*At-Pax6*) was isolated via degenerate PCR. Since some insects and also a myriapod exhibit duplicated *Pax6* genes, 30 subclones of the same PCR reaction were sequenced, but all sequences belonged to the same ortholog.

*At-Pax6* is expressed much earlier than *At-Six3*, at stage 6. The expression at this stage is very weak and located in a two to three cell wide ring at the rim of the germ disc (Figure 35A). As the ring transforms into a stripe during stage 6 and 7, the expression becomes stronger and the domain gets broader (around eight to ten cells wide). Furthermore, at stage 6 a few cells not expressing *At-Pax6* appear at the very anterior of the embryo. These cells form a narrow stripe with a width of only one to three cells. In contrast to the stripe of *Pax6* expressing cells, this stripe of expression-free cells does not widen during stage 6 (Figure 35B,C). Only at stage 7 more tissue not expressing *At-Pax6* appears at the anterior end (Figure 35D,E).

At stage 8 the *At-Pax6* expression in the ventral region of the stripe starts to disappear and two dorsolateral squares of expression are formed (Figure 35F,G). These domains first enlarge until they fill almost all of the headlobe, except for the anterior parts at stage 9 (Figure 35H, Figure 36A). Subsequently, the *At-Pax6* expression in the head refines during stages 9 and 10.

Starting from stage 9, with the appearance of the limb buds, *At-Pax6* is also expressed in the ventral neuroectoderm in one lateral dot per hemisegment. These dots first emerge in the pedipalpal and the L1-L4 segments (Figure 35H) and are located in the anterior portions of the segments. Later dots in the cheliceral segment form and similar *At-Pax6* expression appears sequentially in the opisthosomal segments (Figure 36A-C).

Expression of a *Pax6* ortholog has also been studied in *Cupiennius salei* (Damen, unpublished; Jobi, 2007) but only in later stages, and is highly similar to the expression of *Pax6* in *Achaearanea* at comparable stages of (late stage 8 and later).
Figure 35: **Expression of At-Pax6 in stage 6-9 embryos.** Panels show a lateral view (left) and a ventral view of the head region (right) of the same embryo, except for A, which shows a top view of the germ disc on the right. (A) *At-Pax6* is expressed in a two to three cells wide ring (black arrowhead) around the germ discs rim. (B-E) The stripe gets stronger and wider during stage 7. A few cells not expressing *At-Pax6* appear at the anterior edge of the forming germ band (white arrowhead). Initially this stripe of not *At-Pax6* expressing cells is only up to two cells wide (B,C) but then gets wider towards the end of stage 7 where it is about five cells wide (E). (F,G) The broad transverse stripe of *At-Pax6* expression splits into two patches (black arrowheads) during stage 8 when expression in the ventral cells fades (black arrow). More tissue not expressing *At-Pax6* has appeared at the anterior (white arrowhead). (H) Expression in the head lobes enlarges, and expression appears in two dots per segment in the neuroectoderm of Pp-L4 (asterisks).
Figure 36: **Expression of At-Pax6 in stage 9 and 10 embryos.** At-Pax6 expression in the head lobes (arrowhead) enlarges further and later refines to a complex pattern in the head lobe. At-Pax6 is also expressed in two dots in the ventral neuroectoderm of each segment. The dots are located anteriorly in the segments.
2.3. Discussion
2.3.1. Anterior segmentation

**Prosome patterning of Achaearanea and head formation**

Our lab has recently proposed an expression pattern based “fate map” of the *Achaearanea* embryo (McGregor et al., 2008a) (see also 1.3 and Figure 3). Most parts of this presumptive fate map are supported by the expression pattern gained as part of this thesis. This is for example true for the formation of the opisthosomal tissue at stage 5, or the patterning of the leg bearing segment region. However, a small part of this presumptive fate map has to be corrected. The presumptive fate map suggested a ring around the germ disc at stages 5-6 early, which would show invariant width and would later give rise to the cheliceral and pedipalpal segments of the spider. This suggestion is not supported by the conflicting expression data that we have right now.

If one accepts that Hox genes show static anterior borders even at these early stages, then the expression of *At-lab-2* shows that the red ring in Figure 3) must be getting broader already during stage 5. Initially a narrow (one cell wide) ring of *At-lab-2* expression surrounds the embryo at stage 4. This stripe, which will later give rise to the pedipalpal and L1 segment then gets broader during stage 5 (about 3 cells wide), while a one cell wide ring not expressing *At-lab-2* appears anteriorly, which will form the future cheliceral region. The cheliceral/pedipalpal region at the anterior further broadens during stage 6, before expression of pre-cheliceral genes like *At-Pax6* appears at the rim of germ disc.

However, this explanation is in conflict with another observed phenomenon during spider head formation: We have recently shown that at least two waves of dynamic expression of *At-h* and *At-hh* sweep across the spider head (Pechmann et al., submitted). This has been demonstrated by double in situ hybridizations. Therefore it is possible that also other genes expressed in the very anterior spider embryo exhibit such dynamics. To determine if there are more genes expressed in a dynamic fashion, one has to perform a range of double in situ hybridizations with the genes in question.

The spider head is only formed relatively late in comparison to the tissue of the leg bearing segments. The origin of the cells forming the head is to date unknown. Cell division patterns are scattered all over the *Achaearanea* embryos at stage 6/7 and no enhanced cell division is seen in the anterior (Pechmann et al., submitted), but earlier
cell division patterns have not been characterized. Furthermore, the embryo undergoes quite dramatic shape changes at these stages so that cell movements could also account for an increase of cells at the anterior. Some interesting cell labeling experiments by Holm, in the spider *Agelesena*, suggested that extraembryonic cells, initially not part of the germ disc, contribute to anterior regions of the embryo. However, the *Agelesena* embryo does not show such a clear-cut germ disc as *Achaearanea* and therefore has a much larger contingent of extraembryonic cells (Holm, 1952).

Cell labeling experiments using fluorescent dyes or 4D microscopy to trace cells in the anterior embryo might help resolving a fate map of the spider embryo. This however could prove to be difficult since spider embryos display a remarkable regulatory ability to overcome damages during development (Holm, 1952; Seitz, 1966) and cell-tracing experiments might not be reproducible.

I therefore propose that as long as the developmental origin of the most anterior tissue in the spider has not been resolved, the presumptive fate map should be changed so that there is no distinction between a red and a grey stripe, but instead there should be a continuously broadening stripe at the anterior end that has only one fate, and that is to be come anything between the anterior end of the embryo and the pedipalpal segments.
Proosomal segment appearance

The analysis of segmentation genes fits perfectly with previous morphological descriptions of spider segmentation, but also allows refining these. Clearly, the anterior (prosomal) segments get patterned before any of the posterior (opisthosomal) segments appear. This is best corroborated by the appearance of the At-en segment polarity gene expression. All prosomal en stripes emerge, before the posterior At-en stripes get sequentially added. This phenomenon has also been described for Cupiennius salei (Damen, 2002b). The formation of the prosomal part of the germband from the blastoderm has previously been proposed as being the primitive condition for all chelicerates (Anderson, 1973). The gene expression patterns of Hox genes presented here confirm this proposed fate map. The At-Dfd-1 gene is expressed in L1-L4, and in the stage 4 embryos its expression covers almost the whole germ disc, except for the blastopore and the anterior rim of the germ disc. Furthermore, none of the posterior Hox genes that are only expressed in the opisthosomal segments is expressed before the transition from a germ disc to the germband at stages 6 and 7 (see 2.2.2 and 2.3.2). In addition, those pair rule genes, which are dynamically expressed during the formation of the opisthosomal segments (like At-eve, At-run and At-pby) start to be expressed only at stage 6, when the growth zone starts to form (see 2.2.3 and 2.3.4).

More importantly, I could show here that the prosomal At-en stripes do not form in a strict anterior to posterior order, but they form very quickly in a stereotypic order. However, other segmentally expressed genes do not appear in the same order as At-en, as shown for a few examples in Figure 37. Therefore it is impossible to conclude from this data which of the prosomal segments is segmented first. Also Montgomery has stated that the prosomal segments in Achaearanea “develop in situ and almost if not quite simultaneously” (Montgomery, 1909).
Figure 37: **Appearance of segmental stripes in the prosoma.** Order of appearance of At-en, At-hh, At-h, At-lab and At-Dfd-1 stripes. Segments are depicted on the right, the x-axis is a relative timing of the appearance of the stripes (earlier appearing stripes extend further to the left); the subdivision of the x-axis is not comparable for the different genes due to lacking double stainings. Arbitrarily, the first appearance of a stripe has been taken as starting point for each gene. Larger domains that later subdivide are shown as broad vertical stripes.

Nevertheless, it can still be stated that in contrast to the opisthosomal segments that are added from the growth zone, the anterior segments appear by the subdivision of a pre-existing field of cells. This process is reminiscent of anterior segment formation in other sequentially segmenting arthropods like the millipede *Glomeris marginata* (Janssen et al., 2004), the centipede *Strigamia maritima* (Chipman et al., 2004) and a variety of short germ insects like the grasshopper *Schistocerca gregaria* (Davis et al., 2001), where stripes of *en* orthologs in the gnathal and thoracic region also appear rapidly and not in an anterior to posterior progression. Even in *Drosophila*, the 14 stripes of the En protein expression do not appear completely simultaneously, but they emerge in a stereotypic order (DiNardo et al., 1985; Karr et al., 1989).

The phenomenon of anterior segments not being formed from the growth zone but via patterning of a field of cells in various short germ insects and sequentially segmenting arthropods has provoked the hypothesis that anterior and posterior
segmentation might use different mechanisms for segmenting the anterior and posterior segments (Dearden and Akam, 2001; Peel and Akam, 2003; Peel et al., 2005). This hypothesis is also corroborated by the expression of the three pair rule gene orthologs *At-run*, *At-eve* and *At-phy* in *Achaearanea* embryos. These genes are only expressed dynamically in the growth zone during the formation of segments posterior to L4, which suggests that segmentation of the prosomal segments does not require the dynamic expression of these genes but a different mechanism.
2.3.2. Hox genes in spiders

**Duplicates Hox genes in spiders?**

The discovery of duplicated Hox genes in *Cupiennius* (*Cs-Ubx-1 and-2*) (Damen et al., 1998) and *Achaearanea* (*At-probosclipedia-1 and -2 and At-Dfd-1 and -2, and possibly At-Scr*) (Abzhanov et al., 2001) led to the question of whether only a few Hox genes have been duplicated in different spider species, or if the whole spider Hox cluster might have been duplicated. For the horseshoe crab *Limulus polyphemus*, Cartwright et al. described up to four fragments per Hox gene class in their PCR screen, which are probably due to genome duplications (Cartwright et al., 1993). In contrast, the genome of the tick *Ixodes scapularis* only contains one copy of each Hox gene and thus does not have duplicated Hox genes (Damen, 2009). In addition, there is no evidence of duplicated Hox genes in either a mite or seaspiders (Manuel et al., 2006; Telford and Thomas, 1998)

Recently we were able to show that in addition to *Ubx* (Damen et al. 1998) at least two other Hox genes (*Dfd* and *Scr*) are duplicated in *Cupiennius* (Schwager et al., 2007). Furthermore we showed by sequence comparison that *Cs-Dfd-1* and *At-Dfd-1* are orthologs, and that *Cs-Dfd-2* and *At-Dfd2* are orthologs. Therefore, at least the *Dfd* gene must have duplicated before the two species diverged. Here I showed that in *Achaearanea* an additional Hox gene (*lab*) is present as two copies.

Before I discuss possible scenarios for Hox gene duplication events I first want to compare the Hox gene expression data from *Achaearanea* with *Cupiennius* orthologs. In order to facilitate this, I assembled all known *Achaearanea* and *Cupiennius* Hox gene expression data in Figure 38 that also includes the assumed orthologies, as discussed below.
Figure 38: **Hox gene expression and gene duplication in spiders.** Modified after Schwager et al. (2007). Colored bars represent expression domains of Hox genes at a stage comparable to At stage 10, darker colors represent strong expression and light colors weak expression. The same colors are used for duplicated genes, which are displayed one below the other. Hatched bars indicate that a Hox gene is only expressed in the respective region in *Achaearanea*. The segments of the spider body are indicated above. The list on the right depicts Hox genes that have been isolated from the two spiders. The genes whose expression pattern analysis were subject of this thesis are highlighted in grey and genes that have been described first in this thesis are additionally boxed. Grey font color points out genes that have been isolated in a previous study, but have not undergone expression analysis. Abbreviations: Oc ocular, Ch cheliceral, Pp pedipalpal segments; L1-L4 walking leg segments 1-4; O1-O12 opisthosomal segments 1-12.

Figure 38 shows that at least five or six out of the ten Hox genes are present as duplicated copies in spiders. The assumed orthology of *At-lab-2* to *Cs-lab-2* is only based on expression pattern analysis (p. 26ff). In contrast, the prediction that *At-pb-2* is orthologous to *Cs-pb* is based on the sequence alignment shown in (Figure 39). The orthology of *At-Dfd-1* and *Cs-Dfd-1* on the one hand, and of *At-Dfd-2* and *Cs-Dfd-2* on the other hand has been previously demonstrated by us (Schwager et al., 2007) and are confirmed by the addition of the newly acquired 3’sequence of *At-Dfd-2* to the alignment (Figure 13). The speculative suggestion of the existence of a duplicated *ftz* gene in spiders is based on the significant differences in expression pattern between *Cs-ftz* and *At-ftz* and their dissimilar sequences (see also 2.3.3). The two other orthologous gene pairs (*Cs-Scr-1/At-Scr-1* and *Cs-Ubx-1/At-Ubx-1*) were assigned mainly after expression pattern comparison, although also the sequences of *Cs-Scr-1* and *At-Scr-1* showed one shared aa substitution (Figure 14). Only the isolation of the predicted duplicates *At-
*Ubx*-2 and *At-Scr*-2 from *Achaearanea* and *Cupienius* will finally resolve whether these genes are true orthologs of *Cs-Ubx*-2 and *Cs-Scr*-2, respectively.

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**Figure 39: Alignment of spider pb orthologs.** This alignment is based on the *Cupienius pb* sequence and a *At-pb* sequence (*At-pb-1*) published in the NCBI database (accession numbers AM419029 and AF151997) and another *At-pb* sequence fragment (*At-pb-2*) which was used in the alignment in the paper by Abzhanov et al. (2001), but never published in any database. The alignment clearly shows that the variable region between the hexapeptide and the homeodomain is most similar in *At-pb-2* and *Cs-pb*.

Gene duplications can occur by 1) duplications of single genes or 2) by duplicating a whole cluster as a part of e.g. a chromosomal region or 3) as whole genome duplications. The emergence of the eu-bilaterian Hox cluster is presumably based on tandem duplications of Hox genes because the nearest eu-bilaterian relatives, acoeol flatworms, possess only three Hox genes (Cook et al., 2004; García-Fernández, 2005). Apart from the chelicerates, as discussed above, duplicated classes of Hox genes have only been described in vertebrates, where the whole Hox cluster has duplicated multiple times probably by whole genome amplifications (Hoegg and Meyer, 2005).

Based on the growing data set summarized in Figure 38, I predict that probably every Hox gene might be duplicated in spiders, and therefore the whole Hox gene cluster was duplicated in the lineage to the spiders. Indeed, since we also find duplications of a large number of other genes in both *Cupienius* and *Achaearanea*, the duplication of the Hox gene cluster in spider might part of duplication events of larger genomic areas or even of a duplicated genome (Schwager et al., 2007). Further experiments testing for the arrangement of the Hox genes in clusters, for example, via long range PCR, screening of a genomic BAC library or chromosome fluorescent in situ hybridization (FISH), and tests for genome duplications are needed to shed light on the nature of the Hox gene duplications in the spider.
Colinearity

One of the features of Hox genes is that their expression is often correlated with the physical location of the genes in the cluster. Most prominently, this correlation is found in terms of spatial expression along the anterior-posterior axis, which is called spatial colinearity (Kaufman et al., 1990) but in some animals (mainly deuterostomes) there is also a temporal correlation, in that the anterior genes are expressed earlier than posterior genes (temporal colinearity) (Dollé et al., 1989; Dush and Martin, 1992). It is unknown, how these correlations are mechanistically realized (for reviews see Deschamps, 2007; Duboule, 2007; Kmita and Duboule, 2003). It is also discussed whether they are the cause or the result of Hox gene clustering (Deschamps, 2007; Duboule, 2007; Ferrier and Minguillón, 2003; Monteiro and Ferrier, 2006; Shippy et al., 2008), since species that do not exhibit tightly organized clusters, often, but not always, lack temporal colinearity (Arenas-Mena et al., 2000; Ferrier and Holland, 2002; Negre et al., 2005; Seo et al., 2004).

As in Cupiennius (Schwager et al., 2007), duplicated Hox gene paralogs in Achaearanea are also expressed in similar, but not identical domains along the anterior-posterior axis. One remarkable phenomenon of the duplicated Hox genes in Achaearanea is that the timing, in which the expression of the duplicated genes appears, differs substantially between the paralogs. For instance, At-lab-2 is first expressed at stage 3-4, which is much earlier than its paralog At-lab-1 at stage 6. The same is true for At-Dfd-1 (stage 4) and At-Dfd-2 (stage 8). In the cases where two paralogs have been recovered, the first appearance of expression of one of these paralogs is always significantly earlier than the other (Figure 40). Another phenomenon is that there also seems to be something comparable to a temporal colinearity of the Achaearanea Hox genes. If one examines when the first paralog of a particular Hox gene class is expressed, then it is clear that first a lab gene is expressed at stage 3/4 (i.e. At-lab-2), and then subsequently a Dfd gene (At-Dfd-1) at stage 4, then a Scr gene (At-Scr) at stage 5, a ftz gene (At-ftz) at stage 6 and an Antp gene (At-Antp) at stage 7 early (Figure 40A). So some level of transcriptional regulation must be preventing the more posteriorly expressed genes to be expressed at the earliest stages. These spider Hox genes thus seem to follow not only a spatial but also a temporal colinearity. This is also true for the posterior Hox genes, because due to the sequentially segmenting opisthosomal segments also their expression is initiated sequentially. This putative temporal...
colinearity, next to the spatial colinearity, forms a further hint towards clustered spider Hox genes. However, for the second group of spider Hox genes, the later expressed genes like *At-lab-1*, *At-Hox3* and *At-Dfd-2*, the phenomenon observed for the early expressed genes does not apply, since *At-Hox3* is expressed much later than *At-Dfd-2* (Figure 40), which would be expected to be located more upstream on a hypothesized cluster.

Of course the main caveat is that the above grouping of the first expressed paralogs is arbitrary, and that we do not know anything about the genomic organization of the spider Hox genes (Figure 40). Presently there is no existing genome data for *Achaearanea* and it is thus unclear whether the genes indeed are organized in one or two clusters and whether they are adjacent to each other in one cluster.

These speculations have to be further tested by examining the early expression patterns of those *Achaearanea* Hox genes that are still missing. This particularly applies to the two *At-pb* orthologs, the possible second *At-Scr* ortholog, and in addition it has to be verified whether a second *Hox3* ortholog exists. Additionally, and as pointed out above, it has to be tested whether the early paralogs all are located in one cluster. If these assumptions hold true, this would be the first example of temporal colinearity in an arthropod, because this phenomenon has so far only been observed in vertebrates.
Figure 40: **Sequence of Hox gene appearance in *Achaearanea***. Emergence of Hox gene expression in the anterior spider embryo until stage 7. (A) Earliest expressed paralog of each Hox gene class, (B) later expressed paralog of Hox gene class. The expression is shown in red, red writing gives an estimate to which regions the expression domains correspond. The column on the right indicates the stage at what the particular gene is first expressed. The expression appearance in group A is temporally colinear.
2.3.3. Evolution of *fushi tarazu*

Even though the *fushi tarazu* (*ftz*) gene is located within the Hox cluster in *Drosophila*, it is neither expressed like a Hox gene nor does it have a Hox gene function. Instead it was initially discovered as one of the pair rule genes of the fly (Wakimoto and Kaufman, 1981; Wakimoto et al., 1984). Later in the development of *Drosophila* it is also involved in neurogenesis (Doe et al., 1988). Some years ago, it was revealed that *Drosophila ftz* is derived from a lophotrochozoan Hox gene (Lox5) and that a mite ortholog is expressed in a Hox-like domain (Telford, 2000). Similar expression has subsequently also been described for *ftz* in *Cupiennius* (Damen et al., 2005).

*ftz* orthologs have been studied in all major arthropod groups, but to date it is still unknown at which point in evolution the Hox function of *ftz* has been lost and when the segmentation and neurogenesis functions have been gained. Conflicting data is available from myriapods, where on the one hand the centipede *Lithobius atkinsoni* ortholog of *ftz* shows both Hox-like expression and a transient stripe emanating expression in the growth zone (Hughes and Kaufman, 2002b) and on the other hand in the millipede only a Hox-like expression of *Glomeris marginata ftz* has been reported (Janssen and Damen, 2006). In the two crustaceans examined, the cirripede crustacean *Sacculina ftz* ortholog *Diva* is only expressed in the CNS (Mouchel-Vielh et al., 2002) but the *Daphnia pulex ftz* ortholog has retained the Hox-like expression (Papillon and Telford, 2007). In a basal insect, the firebrat *Thermobia, ftz* is expressed in the growth zone and in segmental stripes that originate from it, this being similar to the way in which segmentation genes are expressed in short germ insect embryos (Hughes et al., 2004). In the grasshopper *Schistocerca gregaria*, next to an expression in the developing nervous system, *Sg-ftz* is also expressed in the growth zone. This is similar to another pair rule gene ortholog in *Schistocerca, even-skipped*, but it is not known, whether this expression corresponds to a function in segmentation (Dawes et al., 1994). This is also true for the *Tribolium ftz* ortholog, which is expressed in the growth zone and in stripes in the blastoderm and the extending germ band as well as later in neurogenic cells (Brown et al., 1994). However, neither a deletion of the chromosomal region containing the *Tc-ftz* gene nor *Tc-ftz* RNAi leads to a pair rule phenotype (Choe et al., 2006; Stuart et al., 1991).
The most recent interpretations of all this data is by Papillon et al. (2007), who presented two evolutionary scenarios: Either ftz has lost its presumable segmentation function in crustaceans and millipedes, or the segmentation gene-like expression in myriapods is convergent to that in insects. The authors regarded the second scenario as more likely.

With the data added by this work from Achaearanea, the situation becomes a bit more complex, since in contrast to the mite and the spider Cupiennius (Damen et al., 2005; Telford, 2000), At-ftz shows both a Hox-like expression domain and expression in the posterior growth zone. The expression in the growth zone deviates from the one of the other examined Achaearanea pair rule genes since the expression persists in the growth zone and never clears from the posterior end (see 2.2.3). Very similar ftz expression patterns of stripes that are budding off from the growth zone are seen in the firebrat Thermobia domestica and the centipede Lithobius (Hughes and Kaufman, 2002; Hughes et al., 2004). Unfortunately, functional data for ftz is not available in any of these species. Nevertheless, the fact that the domain buds of one stripe of expression for every newly formed segment and that the expression stops after all segments are generated point towards an involvement of At-ftz in segmentation. This proposition of course has to be tested in functional experiments. However, given the proposed probability of a paralog of ftz in Achaearanea (see page 36), the functional analysis of At-ftz might be obscured by functional redundancy of the paralogs.

Taken together, expression data suggests that a role of ftz in segmentation is possibly ancestral for arthropods. Consequently, this expression must have been lost in at least mites, crustaceans and millipedes if no additional ftz paralog exists in these species. However, the latter is unlikely, as in contrast to Cupiennius, no Hox gene duplications have been found in these animals. Alternatively, a possible role in segmentation could have been evolved by convergent evolution multiple times, but this does not seem to be the most parsimonious explanation. At this point I can only come to one conclusion that is true for most unanswered questions in evolutionary developmental biology, which is that without more data from a wider range of animals and without solid functional data for ftz in multiple species, this question cannot be satisfactorily answered at the moment.
2.3.4. Pair rule gene expression in spiders

Pair rule gene patterning in the anterior?

Expression analysis in a range of insects and arthropods has shown that in principle there are at least two different ways in which pair rule gene orthologs can be expressed in arthropods: 1) The expression in initial double segmental pair rule stripes, which later refine or split into secondary segmental stripes; or 2) expression in segmental stripes. In sequentially segmenting arthropods, stripes of pair rule gene expression arise dynamically from a posterior growth zone. However in some organisms, pair rule genes can show double segmental patterns in anterior segments and segmental stripes in posterior segments. Furthermore, different pair rule gene orthologs in the same organism can show different patterns. Pair rule gene function has so far only been assessed in insects and this revealed even more ways of acting. Knockout mutants or knockdown phenotypes can display: 1) canonical pair rule gene phenotypes, mainly in holometabolous insects like Tribolium castaneum; 2) a posterior elongation phenotype, like in Bombyx (Liu et al., 2008); 3) gap-like phenotypes with the deletion of an adjacent set of segments like in Oncopeltus (Liu and Kaufman, 2005); or 4) a combination of the previously mentioned phenotypes as in Gryllus (Mito et al., 2006). Like the expression patterns, insect pair rule genes can also differ functionally in different body regions, for example the Tribolium h ortholog shows a pair rule function in anterior segments only (Aranda et al., 2008). For a more detailed recent review on the expression and function of pair rule gene orthologs in arthropods see Damen, 2007.

The only clear conclusion that can be drawn at the moment is that all examined pair rule genes in all species analyzed are indeed among the first genes that are expressed in periodic patterns and are involved in the transition from non-segmental patterns to segmental patterns. It was hypothesized that this is true for all segments (Damen, 2007). For the Achaearanea hairy pair rule gene ortholog At-h this assumption proved to be true, since this gene is expressed in broad domains also in the early spider embryo, while posterior stripes are added sequentially from the growth zone. Remarkable is the triple segmental pattern in the anterior embryo, which is generated by a broad domain that covers the L2-L4 segments. This domain first splits off the L4 stripe and then the remaining domain subdivides into an L2 and L3 stripe. A detailed
description of At-h expression was done by Matthias Pechmann (Pechmann, 2007), for the L2-L4 splitting event see also Figure 55. However the hypothesis that pair rule genes play a role in the segmentation of all segments (Damen, 2007) does not hold true after the analysis of the four additional pair rule genes described in this thesis. Only one of these, At-pby, is transcribed before segment formation in the anterior embryo, and just in one segment, L1. Nevertheless it is possible that other spider pair rule gene orthologs exist that are expressed early in the anterior embryo, but which we have not yet succeeded to isolate from Achaearanea. These could for example be the orthologs of odd-paired or odd-skipped, for which Cupiennius orthologs have already been described.

It is also interesting that At-pby is only expressed in L1 early on, because this is the only segment in which At-h is not expressed in before the actual segment formation. Here At-h is only expressed after the prosomal segmentation. Thus At-pby and At-h might complement each other in segmenting the anterior spider embryo. This of course remains to be tested by functional experiments, which unfortunately have not been successful for spider pair rule gene orthologs yet. In none of the attempted pair rule gene RNAi experiments a phenotype was obtained, nor was the expression of pair rule genes even reduced after parental dsRNA injections (Natália Feitosa, Matthias Pechmann, Alistair McGregor, unpublished observations).
Single segmental pair rule gene patterning in the growth zone

In addition to the four *Achaearanea* pair rule gene orthologs examined in this thesis and the *At-h* expression (Pechmann, 2007), a variety of pair rule gene orthologs have already been analyzed in later stages of the spider *Cupeinnius salei*. These include orthologs of *h*, *eve*, *run*, *odd*, *opa*, *slp*, and *prd* and tenascin-major (*ten-m*) (Damen et al., 2005; Damen et al., 2000; Jobi, 2007; Schoppmeier and Damen, 2005a).

Like *Cs-slp*, *At-slp* is not expressed in the growth zone at all, but only in segmentally iterated stripes that form anterior to the growth zone (see chapter 2.2.3, pp 64 and Damen et al., 2005). All other *Achaearanea* pair rule gene orthologs are expressed in a segmental periodicity in the growth zone. First expression appears at the posterior end and then emerges as stripes from the growth zone. The stripes later end up in newly formed segments, where most of them fade and only later get replaced by newly forming segmental expression. This is in contrast to *Cupeinnius*, where it was not possible to trace the expression register for other pair rule gene orthologs than *Cs-phy-3*, since, except also for *Cs-h*, all other genes fade before the new segments are formed. For those *Cupeinnius* genes that are expressed in the posterior growth zone, only maximally two (in the case of *Cs-eve* and *Cs-h* three) stripes are visible at the same time, while for both *At-run* and *At-eve*, three stripes are visible at the same time – at least during the early stages of posterior segmentation. This demonstrates that these two spider pair rule gene orthologs are also expressed in a single segmental rather than a double segmental manner, as has been already assumed (Damen et al., 2005).
A pair rule gene circuit in the spider?

Based on their expression patterns emerging posterior (before segment generation) or anterior (during or after segment generation) in the growth zone of Cupiennius embryos, the C. salei pair rule gene orthologs can be sorted three groups: Cs-eve and Cs-run are expressed only in the posterior part of the growth zone, Cs-odd-r1, Cs-opa, Cs-ten-m and Cs-slp are expressed in the anterior growth zone, and Cs-h and Cs-pby-3 are expressed in the entire growth zone. It was speculated, whether the early acting genes might be on a higher level in a regulatory hierarchy that regulates the later acting genes. It was argued that this might even reflect pair rule gene regulation in Drosophila, where the primary pair rule genes eve, h, and run, which receive input only from maternal and gap genes, regulate the expression of the secondary pair rule genes odd, opa, ftz, tenascin-major (ten-m), prd and slp (Damen, 2007; Damen et al., 2005; Jobi, 2007).

In the short germ beetle Tribolium there are also two functional levels of pair rule gene regulation. The primary pair rule genes Tc-eve, Tc-run, and Tc-odd regulate the transcription of the secondary pair rule genes Tc-prd and Tc-slp (Choe and Brown, 2007; Choe et al., 2006). However, it has been suggested that the primary pair rule genes in Tribolium are also organized in a regulatory circuit. In this pair rule gene circuit, Tc-eve activates Tc-run, which then activates Tc-odd. Tc-odd in turn represses Tc-eve to complete the regulatory cycle. This way by regulating the downstream secondary pair rule genes, double segmental units are sequentially patterned (Choe et al., 2006).

It is very interesting in this respect that the pair rule genes in Achaearanea seem to be activated successively in the forming growth zone. At-pby is expressed earliest, in early stage 6 embryos, then At-run expression is activated and At-eve expression is initiated last, very late at stage 6. Their exact positions with respect to each other need to be verified by analysis of double in situ hybridizations. The onset of expression is also correlated with the final location of the first forming stripe. The first At-pby stripe eventually locates in the posterior L4 segment, the first At-run stripe in the anterior O1 segment and the first At-eve stripe in the posterior of O1. Correspondingly, At-pby expression appears earliest and At-run expression appears last. Consistently with this, the At-h expression that corresponds to the later O1 stripe appears very early at stage 6 (Pechmann, 2007). It might therefore be possible that a regulatory circuit with pair rule genes activating each other to generate striped expression as proposed for Tribolium (Choe et al., 2006) also exists in spiders. However, as Peel (2008) also noted
recently, intracellular transcription factors in a cellular environment are likely to require information from cell-cell signaling pathways for their function. This role could be played by the Delta/Notch pathway, which has previously shown to be involved in spider segmentation, especially in the formation of posterior segments (Oda et al., 2007; Schoppmeier and Damen, 2005b; Stollewerk et al., 2003), and was now also demonstrated to be involved in segmentation of a hemimetabolous insect, the cockroach *Periplaneta americana* (Pueyo et al., 2008).
Cyclic gene expression or cell movements?

At this point, it remains still unclear how the dynamic stripe appearance in the growth zone of spiders is controlled. It has been suggested (Chipman and Akam, 2008; Damen, 2007) that the dynamic expression in the growth zone of arthropods is due to oscillatory gene expression, which would be reminiscent of the waves of gene expression in the vertebrate presomitic mesoderm. One possibility to prove oscillatory gene expression is to observe nascent gene expression in relation to protein expression of suggested cyclic genes at cellular and sub-cellular resolutions. Another alternative is to generate transgenic spiders that express fluorescent proteins under the control of the promoter of a supposedly oscillating gene. These can then be visualized by live fluorescent time-lapse videography, as has been done in e.g. mice (Aulehla et al., 2008). These possibilities, though technically challenging, should formally be possible to accomplish in the future.

Other ways to generate dynamic patterns in the growth zone could be either cell movements that occur by growth at the posterior end and successive “movement” or pushing of cells towards anterior. It has already been shown in Cupiennius that there are no reproducible or no accumulated cell division patterns in the spider growth zone (Schoppmeier, 2003), so the first possibility is very unlikely. Therefore, if no cyclical gene expression can be proven to exist in the growth zone this last conception may well be applicable to the spider growth zone. The spider embryo is initially wide and short and has a comparably large growth zone, which then during progressive development gets much narrower, while the embryo first elongates and only later gains width again.
3. *hunchback* acts as a gap gene in *Achaearanea tepidariorum*
3.1. Introduction

As described in the general introduction (see section 1), a hierarchic gene cascade controls the simultaneous formation of all segments at the blastoderm stage in the fruit fly *Drosophila melanogaster*. In most other arthropods a few anterior segments are also formed simultaneously, however the majority of their segments are generated sequentially from a posterior growth zone (Damen, 2007; Peel et al., 2005). It has therefore been suggested that anterior and posterior segmentation in arthropods might employ different segmentation mechanisms and that *Drosophila* segmentation is derived from such a dual system (Dearden and Akam, 2001; Schoppmeier and Damen, 2005b).

I have previously highlighted that in spiders the six anterior prosomal segments are segmented before the posterior opisthosomal segments are added in strict anterior to posterior order from the growth zone (see section 2). Posterior segmentation in spiders involves Delta/Notch and Wnt signaling (McGregor et al., 2008b; Oda et al., 2007; Schoppmeier and Damen, 2005b; Stollewerk et al., 2003) and is therefore similar to vertebrate segmentation (for a review see Dequéant and Pourquié, 2008). These pathways are not involved in *Drosophila* segmentation and they also do not seem to play a major role in anterior segmentation of the spider. This is most obvious in *At-Wntβ* RNAi embryos that completely lack opisthosomal segments, but form all prosomal segments (McGregor et al., 2008b).

While the posterior segments seem to be patterned in a vertebrate-like manner in the spider, hardly anything is known about the mechanisms that regulate formation of the prosomal segments. Since the prosomal segments get laid down in a field of cells, it is tempting to speculate that these segments might utilize parts of the *Drosophila melanogaster* segmentation cascade. In *Drosophila*, gap genes regulate the positioning of the downstream pair rule genes in metameric transverse stripes and are the key players in the transition from a non-periodic to a periodic pattern in the developing embryo. One of the most examined gap genes in insects is hunchback (*hb*). (Kraft and Jäckle, 1994; Liu and Kaufman, 2004; Marques-Souza et al., 2008; McGregor et al., 2001; Mito
et al., 2005; Patel et al., 2001; Pultz et al., 2005; Schröder, 2003; Tautz and Nigro, 1998; Wolff et al., 1998; Wolff et al., 1995).

The \textit{hb} gene encodes a zinc finger transcription factor, which is conserved only in protostomes (Kerner et al., 2006; Pinnell et al., 2006) and is indispensable for the segmentation process in insects. In \textit{Drosophila}, \textit{hb} mRNA is maternally provided and ubiquitously distributed, but due to the activity of \textit{nanos}, the protein forms an anterior to posterior gradient. Then \textit{hb} is zygotically expressed in a broad anterior domain and in a second posterior domain. Knockout of \textit{hb} results in canonical gap phenotypes with larvae lacking gnathal and thoracic segments as well as defects in the abdominal segments A7 and A8 (Lehmann and Nüsslein-Volhard, 1987; Tautz et al., 1987). \textit{hb} is also involved in segmentation in other insects, even though the exact functional details differ. It is not yet resolved whether the canonical gap function of \textit{hb} is conserved within insects, because in some insects the gap phenotype is obscured by homeotic transformations. Here \textit{hb} has been shown to repress posterior Hox genes in the anterior and knockdown causes the expression borders of these Hox genes to shift anteriorly. Then phenotypes look like they have lost a set of adjacent anterior segments, even though the same tissue has just been patterned differently by the shifted Hox genes expression (He et al., 2006; Liu and Kaufman, 2004; Marques-Souza et al., 2008; Mito et al., 2005).

Studies of \textit{hb} in non-insect arthropods are limited to gene expression analysis in the centipede \textit{Strigamia maritima} (Chipman and Stollewerk, 2006) and the crustacean \textit{Artemia franciscana} (Kontarakis et al., 2006), but no functional data was presented. In both cases \textit{hb} expression was associated with central nervous system differentiation and mesoderm development, but not with segmentation. Similar results have been obtained for some annelids. In these animals expression of \textit{hb} was found maternally, in the differentiating nervous system, extraembryonic membranes and the mesoderm (Iwasa et al., 2001; Kerner et al., 2006; Savage and Shankland, 1996; Shimizu and Savage, 2002; Werbrock et al., 2001). Fay et al. (1999) have shown that the \textit{hb} ortholog in the nematode \textit{C. elegans} is also expressed in the extraembryonic tissue and RNAi led to defects in morphogenesis.

While this expression data is informative, the lack of functional data outside insects means little is known about the evolution of the role of \textit{hb}. For example, it is still unclear whether \textit{hb} evolved a segmentation function only in insects or already before
the divergence of the arthropods. It has been hypothesized that *hb* acquired its segmentation role from an ancestral Hox gene regulating function. An alternative hypothesis is that *hb* has been recruited for segmentation from an ancestral role in the nervous system (Peel et al., 2005). Another proposed function of *hb* is in mesoderm development, though in *Drosophila*, *hb* is only expressed later in specific mesodermal cells where it is required for the function of specific cells in tracheal guidance (Wolf and Schuh, 2000). *hb* expression has also been observed in the mesoderm of various insects, a crustacean, a centipede and an annelid, which has led to the suggestion that *hb* might be involved in mesoderm formation (Chipman and Stollewerk, 2006; Kerner et al., 2006; Kontarakis et al., 2006; Patel et al., 2001). However, all these studies are based on expression data only and neither of them includes a functional analysis.

In this second chapter I will present expression patterns of *hb* in early embryos of a non-insect arthropod, the spider *Achaearanea tepidiorum*. I will demonstrate that *At-hb* parental RNAi (pRNAi) leads to a gap phenotype similar to the gap phenotype in *Drosophila* and that the gene surprisingly does not seem to regulate Hox genes in this spider. Moreover I will show implications for mesoderm development in *Achaearanea* and discuss the evolution of *hunchback* and its function in arthropods.
3.2. Results

3.2.1. Isolation of *Achaearanea* hunchback

The Hunchback protein (Hb) is a zinc finger protein for which true orthologs only exist in protostomes (Kerner et al., 2006). Hb can contain up to nine C\textsubscript{2}H\textsubscript{2} zinc fingers, two N-terminal fingers (NF1-2), four medial zinc fingers (MF1-4), one extra finger (Ex-F) and two C-terminal fingers (CF-2) (nomenclature after Patel et al., 2001). This assumed ancestral structure (Pinnell et al., 2006) has so far only been reported *C. elegans* and in the myriapod *Glomeris marginata* (Janssen, 2004; Patel et al., 2001).

The full coding sequence of an *Achaearanea* *hb* ortholog was obtained by an initial degenerate PCR and subsequent extension via 5’- and 3’-RACE-PCR. The 3541 bp cDNA of At-*hb* encodes for a protein comprising 914 aa. Alignment of this protein with Hb orthologs of various other species reveals that At-Hb retained the putative ancestral set of nine zinc fingers. Figure 41 shows that all insects lack the ExF domain. Hemimetabolous insects show derived NF domains and holometabolous insects have lost the NF domains completely. Thus, next to *C. elegans* and *Capitella* all other arthropod Hb orthologs apart from insects possess the ancestral set of zinc fingers.
Figure 41: Alignment of Hb protein zinc finger domains. The *Achaearanea* Hb protein contains the ancestral set of nine metal binding fingers. All insects have lost the ExF domain. Most holometabolous insects have lost the NF domain, while hemimetabolous insects still contain NF domains. Especially the NF-2 finger is more derived in insects compared to the sequences of chelicerates, the crustacean, the nematode and annelids. Alignments of the NF (A), MF (B), ExF (C) and CF domains (D). Structural C an H residues are highlighted by grey shading. The At-Hb sequence is shown in bold. Identical amino acids are displayed as dots and gaps introduced for alignment purposes are indicated as hyphens. (E) A structural overview of Hb proteins showing the position of the zinc fingers. The alignments are based on the complete Hb sequences of the three chelicerates *Achaearanea tepidariorum* (At), *Cupeniinius salei* (Cs), *Ixodes scapularis* (I), the myriapod *Glomeris marginata* (Gm), the crustacean *Daphnia pulex* (Dp), the hemimetabolous insects *Schistocerca americana* (Sa), *Oncopeltus fasciatus* (O), and *Acrhythosiphon pisum* (Ap), the holometabolous insects *Nasonia vitripennis* (Nv), *Apis mellifera* (Am), *Triboinum castaneum* (Tc), *Culex quinquefasciatus* (Cq), *Megaselia abdita* (Ma) and *Drosophila melanogaster* (Dm) the annelids *Capitella capitata* (Cc) and *Helobdella robusta* (Hr), and the nematode *Caenorhabditis elegans* (Ce). The Is, Dp, Ap, Hr and Cc sequences were obtained by BLAST searches of respective sequenced genomes or NCBI trace archives. The *Cupeniinius* sequence was isolated by Wim Damen. All other sequences are available from GenBank.
3.2.2. **Expression of *Achaearanea hunchback***

*At-hb* is first expressed maternally in developing oocytes (Figure 42A). The first detectable zygotic expression is observed in the blastopore region of stage 3/4 embryos, predominantly in the invaginated cells. (Figure 42C). This expression continues during stage 4, at which time additional expression of *At-hb* appears in a one cell wide ring around the germ disc (future anterior end of the embryo). During stage 5, cumulus mesenchymal cells also express *At-hb* as the cumulus migrates to the periphery of the germ disc. In the center of the germ disc (the previous site of the blastopore and presumptive posterior of the embryo) a few cells still express *At-hb* (Figure 42E,F). When the cumulus starts to disappear at stage 5 late, expression of *At-hb* at the posterior becomes stronger (Figure 42G). Also a third domain of *At-hb* expression now emerges as a broad stripe between the anterior ring of expression and the posterior domain (Figure 42G). The expression in this domain becomes stronger during stage 6 and merges with the anterior ring, while the embryo opens up dorsally and the growth zone is forming. At this stage, the most posterior cells no longer express *At-hb* so that the posterior domain also transforms into a broad stripe (Figure 42H). Later in stage 6, the broad anterior expression domain splits into two stripes so that now three stripes are observed (Figure 42I). In between these three broader stripes, two new narrower stripes of *At-hb* expression are inserted at stage 7 (Figure 43A,B) and the broad medial domain splits into two stripes (Figure 43B). In early stage 8 embryos, the broad anterior stripe covers the head and cheliceral region, the posterior expression domain is found in the presumptive L4 region, and the broad medial stripe encompasses the future L1/L2 region. The two narrow intercalated stripes are associated with the pedipalpal segment and the third walking leg segment, respectively (Figure 43C). Longitudinal sections reveal that all *At-hb* expression at this stage is both ectodermal and mesodermal, except for the L3 stripe, which is restricted to the mesoderm (Figure 43C). From stage 8 onwards, *At-hb* is expressed in the mesoderm of every new segment (Figure 43D,E), but never in the growth zone. Later the segmental *At-hb* expression fades concomitant with the appearance of expression in the nervous system, which resembles known *hb* expression patterns from other arthropods and which is also found in the opisthosoma (Figure 43E-G) (Chipman and Stollewerk, 2006; Isshiki et al.,

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Furthermore, *At-hb* expression is found in extraembryonic cells from stage 6 onwards. Initially the cells of the dorsal area reflect the *At-hb* pattern in the embryo and not all of these extraembryonic cells express *At-hb* (Figure 42H,I). Starting at stage 7 though, all cells of the former dorsal area show *At-hb* expression (Figure 43A-G). At later stages, some extraembryonic cells expressing *At-hb* are also found below the embryo, in the region where head and growth zone adjoin (Figure 43E,F). Holm has related these cells to midgut formation by cell labeling experiments in another spider (Holm, 1952). This theory is supported by the expression of markers for midgut (endodermal markers, *serpent* and *hepatocyte nuclear factor-4*) and fore- and hindgut (ectodermal gut marker, *forkhead*) in the extraembryonic cells (Natália Feitosa, personal communication). Alternatively, some extraembryonic cells that migrate below the germband (similar to these *At-hb* expressing cells in Figure 43E,F) were interpreted as differentiating blood cells by Montgomery in *Achaeareana* (Montgomery, 1909).
Figure 42: **Expression of *Achaearanea* hb in oocytes and stages 3-6.** (A) Expression of *At-hb* mRNA in young oocytes (black arrow). Older oocytes most likely are impenetrable for mRNA probes and show no staining (white arrow). (B) Oocytes hybridized with an *At-hb* sense probe are free from staining. Again, arrows mark oocytes of similar stages as the ones in A. Other panels show the same embryo, viewed laterally (left) and as a top view (middle/right). C,D and G additionally show median longitudinal sections of the same embryo on the right. G shows a ventral posterior view, and H displays a view on the dorsal area and the posterior end of the embryo in the right picture. (A,B) *At-hb* is first expressed in the blastopore (asterisk). The staining is predominantly found in invaginated cells. At stage 4, *At-hb* becomes expressed in a ring at the rim of the germdisc (black arrowhead). (E,F,G) Expression in the outer ring becomes stronger. Also mesenchymal cells (see section in G) of the cumulus (labeled by a "C") express *At-hb*. A few cells left behind at the site of the blastopore also express *hb* (white arrowhead). (G) *At-hb* is expressed in cells at the future posterior end (white arrowhead). At the same time a new expression domain appears in a broad stripe between outer ring and posterior expression domain (black bracket). (H,J) Outer ring and broad stripe merge to one domain, before they split into two broad stripes at the end of stage 6. The posterior end starts to clear from *At-hb* expression (grey arrow), so that the posterior domain now also forms a broad stipe (white bracket). *At-hb* is seen in some extra-embryonic cells that derive from the dorsal area, but initially not in all (white arrow).

Figure 43: **Expression of *At-hb* in stages 7-10.** Pictures of one panel are of the same embryo. (A,B) Embryos viewed laterally (left), and showing the posterior end from dorsally (right). Two new thin stripes (arrowheads) of *At-hb* expression insert between the initial three broad stripes (brackets, see Figure 42). All extraembryonic cells derived from the dorsal area strongly express *At-hb*. The growth zone is free from expression (black asterisks). (C) Lateral (left), ventral view (middle) and longitudinal section of the same embryo. The lateral view is shown without overlaid DAPI staining to allow visualization of mesodermal staining. The anterior broad domain covers the head region, the anterior thin stripe is in the pedipalpal segment, the central broad domain locates to L1 and L2, the posterior thin stripe is located in L3 and the posterior broad domain covers L4. Only the L3 stripe is purely mesodermal. (D) Lateral view and ventral views of head region and posterior end as well as posterior end without DAPI staining (from left to right). *At-hb* is expressed in the mesoderm of newly added segments of the opisthosoma (white asterisks). (E,F,G) Lateral view and ventral views of head region/posterior end, leg region and opisthosoma (from left to right). Segmental *At-hb* expression has faded and is now found in the ventral neuroectoderm as well as in extraembryonic cells and mesodermal stripes in newly added segments. Some extraembryonic cells expressing *At-hb* are located beneath the embryo in the growth zone/head region (black arrows). (G) *At-hb* expressing cells invaginate in the neuroectoderm, in more anterior segments the expression is weaker than in posterior segments. *At-hb* is also expressed in neurons in the legs (white arrow).
3.2.3. Parental RNAi leads to a loss of appendage bearing segments

The dynamic expression of At-hb in broad stripes before and during critical phases of anterior segmentation suggested that this gene might play an important role in spider segmentation. Therefore to investigate the function of hb during early Achaearanea development I applied parental RNAi to knock down At-hb expression.

As seen in Figure 44, the At-hb expression was significantly reduced in At-hb\textsuperscript{pRNAi} embryos compared to wildtype embryos. At later stages though, secondary expression of At-hb in the nervous system was not suppressed in many At-hb\textsuperscript{pRNAi} embryos (not shown).

![Figure 44: At-hb expression is downregulated after At-hb pRNAi](image)

First instars that hatch from At-hb\textsuperscript{pRNAi} cocoons show a reduction to three or even two leg pairs compared to the four leg pairs of first instars from control injections (Figure 45). Many first instars survive the next molt and are able to walk, despite having only four legs (see movies 1 and 2 on the attached CD).
Figure 45: **First instars show a reduced number of legs after At-hb pRNAi.** Brightfield images of phenotypes observed in live first instars (B, C) and offspring of dsgfp injected control spiders (A). Compared to control first instars (A), class I (B) and class II (C) At-hb<sup>pRNAi</sup> first instars look normal, except they lack one or two leg pairs, respectively. Upper pictures show lateral views except for a dorsal view in C, lower pictures show ventral views.

To analyze the At-hb<sup>pRNAi</sup> phenotype in more detail, I examined stage 10 embryos. A phenotypic series was detected (Figure 46 and Figure 47), and the phenotypes were classified into three classes: Class I phenotypes show a range of defects compared to controls, from a shorter L1 in the mildly affected embryos (Figure 46B) to complete reduction of these legs in more severe examples (Figure 46C and not shown). Class II phenotypes lack L2 and in addition show a reduction or complete loss of the first leg pair (L1) (Figure 2G). Class III phenotypes are missing L1 and L2 and in addition exhibit a reduction or loss of the fourth walking leg (L4) (Figure 46F-H), while some embryos show remnants of L1 (Figure 46G). The strongest class III phenotypes have fully lost L1, L2 and L4, but the third walking legs (L3) are never affected (Figure 2H). Asymmetric phenotypes with differences in the severity of the leg reductions were also observed (e.g. Figure 46F). Class III phenotypes also finish embryonic development, but fail to hatch from the egg. An overview of the phenotypic distributions in different cocoons of spiders is given in Figure 47.
Figure 46: **Phenotypic series observed after At-hb pRNAi.** (B,C) In stage 10, the weakest class I phenotypes of the phenotypic series show only a reduction in size of the second walking leg. (D,E) Class II phenotypes have completely lost the second walking leg (L2) and additionally show a reduction (D) or complete loss (E) of the first walking leg (L1). (F-H) Class III phenotypes moreover show a reduction (H) or complete loss (I,H) of the fourth walking leg (L4). Upper pictures show lateral views and lower pictures show ventral views.
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Figure 47: Embryonic phenotypes after gfp (controls) and At-hb dsRNA injections. Each chart corresponds to one Achaearanea female injected with either gfp dsRNA (A, B) or dsRNA directed against two non-overlapping fragments of At-hb, hb5' (C-E) and hb3' (F-H). Each bar displays the phenotype distribution of randomly chosen eggs of one cocoon, unfertilized eggs were not counted. Total numbers are shown in brackets. Cocoons from which embryos were fixed at too early stages where phenotypes are not fully visible or cocoons that contained mainly unfertilized embryos are not represented. Embryos were categorized into wildtype (blue), class I (yellow), class II (orange), class II (red) and unspecific (grey) phenotypes.

Both head and opisthosomal segments appear normal in At-hb 
RNAi embryos. For example, head marker genes like At-Six3, At-Pax6 and At-otd-1 (Figure 48) and posterior Hox genes (see for example Figure 58) show wildtype expression patterns in the head and opisthosoma respectively.
Figure 48: The head is formed normally in *At-hbpRNAi* embryos. The expression of the head marker genes *At-Six3* (A and B), *At-Pax6* (C and D) *At-otd-1* (E and F) is not changed after *At-hb* knockdown. (A,C,E) wildtype embryos, (B,D,F) At-hb knockdown embryos. All panels show ventral views on the left and lateral views on the right.
3.2.4. *At-hb* pRNAi causes a loss of segments

To determine the nature of the reduction of legs after *hb* pRNAi I used the expression of the segmental markers *At-en* and *At-pby* to investigate whether this is due to the loss of whole segments or if only the appendages are reduced. Embryos from *At-hb* pRNAi cocoons with class II and III phenotypes do not form the *At-en* or *At-pby* stripes in the L2 segment, or L2 and L1 segments, respectively (Figure 49). The *At-en* expression in the L4 segment is never fully gone, even in embryos from cocoons with strong class III *At-hb* pRNAi embryos (Figure 49C). At stage 10 though, the L4 *At-en* stripe is distorted in these embryos (Figure 49F). The distortion of the *At-en* stripe in the L4 segment happens only later during development, in late stage 9 and 10 embryos, because I never observed defects or strong deformation of the L4 *At-en* stripe in earlier embryos (e.g. Figure 49C), even if taken from cocoons in which embryos at later time points show distorted stripes. In contrast to *At-en*, *At-pby* is not expressed in the L4 segment of strong class III *At-hb* pRNAi embryos. These experiments imply that not only the appendages are missing in *At-hb* pRNAi embryos, but that also the complete corresponding segments do not form.

I never observed any abnormality in the *At-en* and *At-pby* stripes of the more anterior cheliceral and pedipalpal segment. Also, the *At-en* and *At-pby* stripes of the segments of the opisthosoma were never affected in *At-hb* pRNAi embryos. This includes O1 where *At-pby* shows a very characteristic pattern, as the stripe extends much more dorsally compared to other opisthosomal segments. While the L4 *At-pby* stripe is missing in class III *At-hb* pRNAi embryos, the O1 *At-pby* stripe is never affected by *At-hb* pRNAi (Figure 49G-I). The influence of *hb* thus is restricted to the central segments of the spider embryo.
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A - wildtype
B - hb pRNAi class II
C - hb pRNAi class III

At-en stage 9 early

At-en stage 10

At-pby stage 10

L1, L2, L3, L4, Pp, Ch

F - arrow
Figure 49: **Central segments do not form in At-hbRNAi embryos.** The segmental marker genes *At-en* (A-F) and *At-pby* (G-I) were used to analyze segmental defects after *At-hb* pRNAi in stage 9 (A-C) and stage 10 embryos (D-I). (A,D,G) wildtype embryos, (B,E,H) class II *At-hbRNAi* embryos, (C,F,I) class III *At-hbRNAi* embryos. (A) *At-en* is expressed in the posterior part of every segment. In class II phenotypes the stripe in L2 does not form (B) and in class III (C) the stripes in both L1 and L2 are missing. The arrows point at a few cells in the presumptive L1 region expressing *At-en*. There is a rather big gap between the L1 and L3 *At-en* stripe (B) and the Pp and L3 stripe (C) in class II and class III embryos, respectively. However, this gap gets reduced over time (compare early stage 9 embryos in B and C with stage 10 embryo in E and F, respectively). In class III stage 10 embryos (F), *At-en* expression in L4 is also distorted, but never fully lost. (G) *At-pby* is expressed in the posterior of every segment, but only in the ventral ectoderm and not in the legs. Only in the first opisthosomal segment (O1) *At-pby* expression expands more dorsally and is an excellent marker for O1 (marked by asterisks in G-I); the O1 *At-pby* stripes is not affected (H and I). In contrast to *At-en*, *At-pby* expression is not only gone in the L1 and L2 segment but is also lost from the L4 segment in class III embryos (I). In all panels: The upper pictures show lateral views and the lower pictures show ventral views of the same embryos.
3.2.5. The gap resizing process

Up to early stage 9 there is initially a rather large gap between the At-en stripes of the L2 and L3 segment in class II embryos (Figure 49B) or the pedipalpal and L3 segment in class III embryos (Figure 49C). In control embryos the At-en stripes of the L2 and L3 segment are located here (Figure 49A). During stage 9 and 10 this gap however reduces during embryo growth and a general compaction of all segments. This is obvious from observations of live embryos and by time-lapse microscopy of wildtype and At-hbRiRNAi embryos.

The microscopy setup in Michael Akam’s lab at the Institute for Zoology in Cambridge, UK, allows temperature-controlled live imaging and had previously been used by Matthias Pechmann and Alistair McGregor for movies of early Achaearanea development (McGregor et al., 2008a). Unfortunately, this microscope setup proved to be unsuitable to image later developmental stages in a satisfying way. For this reason I decided to only show a selection of still images from some of the filmed embryos in Figure 50 and Figure 51.

Visible signs of segmentation in the prosoma are seen in wildtype embryos of stage 8, while At-hbRiRNAi embryos apparently do not form morphologically visible segments in the L1/L2 region (Figure 51A-C). When the limb buds emerge at stage 9 early, a large gap is seen between the pedipalps and L2 or L3 respectively in these embryos (Figure 51E). But also the wildtype embryos initially show wide-spaced appendages (Figure 50G). During the later course of development and the sequential addition of segments at the posterior end, this space between the appendages is reduced, so that in late stage 10 embryos, the prosomal appendages are directly adjacent to each other. Similarly, the At-hbRiRNAi embryos reduce the space between their appendages and while this happens, also the gap between Pp and L2 or L3 shrinks. Some embryos die before completion of this process and show an arrested development at stage 9 late with a gap still present between Pp and L2 or Pp and L3 (not shown).

Thus the Pp-L3 gap in class II and III embryos might be shrinking during a general resizing process that is taking place also in wildtype embryos.
**Figure 50: Wildtype development.** Development of wildtype *Achaearanea* embryos as observed by time-lapse microscopy. Pictures were taken every 5 minutes, every 40th still frame is shown above, so between each image above there are 200 min of developmental time. The developmental series above was assembled from two different wild type embryos that turned around before or after the shown series. A-F are pictures from the first embryo and G-T are pictures from the second embryo. White arrowheads in A-C point at forming segmental grooves. The groove between L2 and L3 is the last to appear (C). Pp, L1, L2, L3 and L4 appendages have been marked by an asterisk at the appendage bases in G-T. The segments undergo a resizing process during stage 9 and 10, as visible by the reduced spaces between the asterisks. Embryos are viewed laterally, anterior is to the left.
Figure 51: **Development of an At-hbRNAi embryo.** Class II At-hbRNAi embryo as observed by time-lapse microscopy. Pictures were taken every 5 minutes, every 40th still frame is shown above, so between each image above there are 200 min of developmental time. White arrowheads in A-C point at forming segmental grooves. The groove between L2 and L3 does not appear and the grooves between Pp and L1 and L1 and L2 are not as pronounced as in wildtype (compare Figure 50) due to the lack of L1 and L2 in this embryo. Only 4 pairs of appendages form (Ch, Pp, L3 and L4), Pp, L3 and L4 are marked by asterisks at the appendage bases. It is clearly visible that the initially large space between Pp and L3 (e.g. in E-H) is reduced during stages 9 and 10 (I-X). All embryos are viewed laterally, anterior is to the left (and slightly down in Q-X).

In some *Drosophila hb* mutants, two large metameres also undergo a resizing process during development. It was shown that this process is aided by high levels of cell death. The dying cells are intermingled with surviving cells and are restricted to the two affected metameres (White and Lehmann, 1986).

To investigate whether cell death also plays a role in the Pp-L3 resizing process in *At-hb* knockdown embryos, I examined cell death by terminal deoxynucleotidyl transferase-mediated dUTP-digoxygenin nick end labeling (TUNEL) in *At-hbRNAi* embryos (Prpic and Damen, 2005). In both wildtype and *At-hb* knockdown embryos, no cell death is visible before stage 9 (not shown). In stage 9 wildtype embryos, occasional cell death is found in extraembryonic cells at the dorsal sides of the germband (Figure 52A,B). This pattern is enhanced in *At-hbRNAi* embryos, although the distribution of TUNEL stained cells is not symmetrical on both sides of the germband (Figure 53A,B). In stage 10 embryos, cell death is predominantly found first in a few cells in the appendages (Figure 52C) and then mostly in the distal parts of the appendages, as well as in the opisthosoma and in the head lobes in late stage 10 and 11 embryos (Figure 52D,E). In *At-hb* knockdown embryos, three differently severe types of cell death can be observed. Some embryos do not show increased levels of cell death (Figure 53C), while others display enhanced levels of cell death in the gap region between Pp and L3. This again is mostly found only in one hemisphere of the embryo and not symmetrically in both (Figure 53D). Finally there are a number of embryos that show enhanced cell death levels, but TUNEL labeled cells can be found all over the embryo and the pattern is not only restricted to the resizing and shrinking region (Figure 53E,F). All three types of embryos have been found at comparable rates. Thus no consistent cell death pattern was observed in *A-hbRNAi* embryos and it remains unclear, if cell death plays a major role during the resizing process.
Figure 52: **Cell death in Achaearanea wildtype embryos.** TUNEL staining in wildtype embryos. (A,B) During stage 9 only occasional extraembryonic cells show labeling (white arrows). (C,D) At stage 10, some cells in the appendages (black arrows) and the opisthosoma are labeled. (E) In stage 11 embryos, a high number of cells are labeled in the head and in ventral regions of the opisthosoma. Some labeling is also seen in the distal parts of the prosomal appendages. Panels A-C show lateral views (left) and ventral views (right) of the same embryos. D and E show (from left to right), a lateral view and ventral views of the head region, the walking leg region and the opisthosoma.
Figure 53: **Cell death in At-hb<sup>RNAi</sup> embryos.** TUNEL staining in *At-hb* knockdown embryos. Panels A-B show lateral views (left) and ventral views (right) of the same embryos. In all embryos, more TUNEL staining than in wildtype embryos is observed in extraembryonic cells dorsolateral to the germband (white arrows). Cell death is also found in appendages (black arrows), the head region and the opisthosoma (white arrowheads) and unlike in wildtype embryos, in some embryos TUNEL staining is also seen in the gap region between PP and L3 or L1 and L3 respectively (black arrowheads). C-F show (from left to right), a lateral view and ventral views of the head region, the walking leg region and the opisthosoma.
3.2.6. \textit{At-hb} is required for the expression of segmentation genes

To determine the influence of \textit{At-hb} on the initiation of segmentation gene expression I examined the expression of these genes in earlier \textit{At-hb}\textsubscript{pRNAi} embryos. As described in section 2, at stage 8, six segmentally iterated stripes of \textit{At-en} appear almost simultaneously in the prosomal region of wildtype embryos (see Figure 4 and Figure 54A). Only four \textit{At-en} stripes form in class III \textit{At-hb}\textsubscript{pRNAi} embryos because L1 and L2 \textit{At-en} stripes are not initiated (Figure 54B). Similarly, the segmental stripes of another segment polarity gene ortholog, \textit{At-hedgehog} (\textit{At-hh}), never form in L1 and L2 in class III \textit{At-hb}\textsubscript{pRNAi} embryos (Figure 54C,D). This data suggests that the L1 and L2 segment never become specified in these embryos. This is in contrast to the L4 segment that seems to become specified (\textit{At-en} and \textit{At-hh} stripes form), but then gets secondarily lost in class III embryos (see 3.2.4).

More interestingly, also the expression pattern of the pair rule gene ortholog \textit{At-hairy} (\textit{At-h}) is affected by \textit{At-hb} pRNAi. In wildtype embryos, at stage 7 \textit{At-h} is expressed in a broad stripe covering the presumptive L2, L3 and L4 area (Figure 55A). In early stage 8 wildtype embryos this stripe buds off one posterior stripe that eventually ends up in the L4 segment (Figure 55B). Slightly later, the remaining domain also splits, forming the L2 and L3 \textit{At-h} stripes (Figure 55C). The three distinct stripes of \textit{At-h} in the L2-4 segments therefore are the result of splitting of an initial single, broad \textit{At-h} domain. This splitting of the L2/L3 stripe does not occur in \textit{At-hb}\textsubscript{pRNAi} embryos and a broader \textit{At-h} stripe remains present in the L3 segment (Figure 55D). \textit{At-hb} thus is required for splitting the L2/L3 \textit{At-h} stripe.

The above results show that \textit{At-hb} controls other segmentation genes in the spider. It is required for the activation of the segment-polarity genes \textit{At-en} and \textit{At-hh} in the L1 and L2 segments. Furthermore, \textit{At-hb} controls the correct organization of the expression pattern of the pair rule ortholog \textit{At-h} in the central part of the spider embryo.
Figure 54: **At-hb regulates the expression of segment polarity genes.** Expression of *At-en* (A,B) and *At-hh* (C,D) in stage 8 wildtype (A,C) and class III *At-hb* pRNAi (C,D) embryos. Each panel shows lateral (left) and ventral (right) views of the same embryo. *At-en* and *At-hh* stripes do not form in the L1 and L2 segments of *At-hb* knockdown embryos.
Figure 55: *At-hb* regulates splitting of *At-h* stripe. Expression of *At-h* in three successive stages of wildtype embryos (A-C) and in stage 8 *At-hbpRNAi* embryos (D). Each panel shows the same embryo, viewed lateral (left) and ventrally (right). (A) *At-h* is initially expressed in a broad stripe covering the presumptive L2-L4 region. (B) The L4 stripe splits off the large domain. (C) The L2/3 stripe splits and an additional stripe appears in the mesoderm of L1. (D) The L2/3 *At-h* stripe does not split in *At-hb* knockdown embryos and expression in L1 does not appear.
3.2.7.  **At-hb does not regulate Hox genes**

It has been claimed that the ancestral role of *hb* was in regulating Hox gene expression and *hb* only secondarily acquired its role as a gap gene (Marques-Souza et al., 2008; Peel et al., 2005). In several insect species, *hb* prevents the activation of posterior Hox genes in anterior regions of the embryo. In these insects, *hb* RNAi leads to ectopic anterior expression of several Hox genes and causes homeotic transformations of gnathal and thoracic segments into abdominal identity. In the course of these transformations, the embryos also exhibit a loss of certain anterior appendages (Liu and Kaufman, 2004; Marques-Souza et al., 2008; Mito et al., 2005). To exclude that the loss-of-legs *At-hb* phenotype (Figure 45 and Figure 46) is due to a homeotic transformation that changes the identity of segments, I analyzed the expression of the *Achaearanea* Hox gene orthologs *labial (At-lab-1), Hox3 (At-Hox3), Deformed (At-Dfd-1), Sex combs reduced (At-Scr), fushi tarazu (At-ftz), Antennapedia (At-Antp), Ultrabithorax (At-Ubx) and abdominal-A (At-abdA)* in *At-hb*RNAi embryos. However, no ectopic Hox gene expression was observed (Figure 56, Figure 57 and Figure 58). All analyzed Hox genes are expressed in domains comparable to those of wildtype embryos. Thus there are no hints for an effect of *At-hb* on Hox genes, which leads to the conclusion that in contrast to insect *hb*, the spider *hb* ortholog is not involved in the regulation of Hox genes.

Moreover, the Hox gene expression patterns corroborate the interpretation that only the L3 segment is present in strong class III *At-hb*RNAi embryos. The remaining leg pair in class III RNAi embryos expresses the L3 marker *At-ftz* (Figure 57D-F), but neither expresses *At-lab-1* (Figure 56C) nor *At-Antp* (Figure 58C), which excludes a L1 or L2 identity, since L1 and L2 weakly express *At-lab-1* (Figure 56A) and excludes a L4 identity, since *At-Antp* is weakly expressed in L4 (Figure 58A). Finally, the expression of *At-Dfd-1* also shows that the space between Pp and L3 in class III *At-hb*RNAi embryos is taken up by an enlarged L3 neuroectoderm (Figure 56G-I).
Figure 56: *At-hb* does not regulate the expression of anterior Hox genes – part 1. Expression of anterior Hox genes *At-lab-1* (A-C), *At-Hox3* (D-F) and *At-Dfd-1* (G-I) in wildtype (A,D,G), class II (B,E,H) and class III *At-hb*<sup>RNAi</sup> embryos (C,F,I). All embryos are stage 10 embryos, upper pictures are lateral views and lower pictures are ventral views. (A) *At-lab* is strongly expressed in Pp and weakly expressed in L1 and L2 in wt embryos, (B,C) in *At-hb*<sup>RNAi</sup> embryos *At-lab-1* expression is only seen in Pp, L2 (C) and in class III embryos L1 (B) expression are missing. (D) *At-Hox3* is expressed in the Pp and in L1-L4. In *At-hb*<sup>RNAi</sup> embryos expression of *At-Hox3* is only in the Pp and L3 (F) and in class II embryos also in L4 (E). *At-Dfd-1* is expressed in one block per hemisegment (black arrowhead) in the neuroectoderm and the legs of L1-L4 in wt embryos (G), and in class III *At-hb*<sup>RNAi</sup> embryos only in L3 and the remaining neuroectoderm of L4 (I) or L3 and L4 in class II embryos (H). The space between Pp and L3 is only filled by one block of neuroectodermal *At-Dfd-1* staining.

Figure 57: *At-hb* does not regulate the expression of anterior Hox genes – part 2. Expression of anterior Hox genes in wildtype (A,D), class II (B,E) class III *At-hb*<sup>RNAi</sup> embryos (C,F). All embryos are stage 10 embryos, upper pictures are lateral views and lower pictures are ventral views. (A) In wt embryos, *At-Scr* is expressed in L1-L4, while the expression is strongest in L3. (C) In class III *At-hb*<sup>RNAi</sup> embryos *At-Scr* is expressed only in L3, while the expression resembles that in wt L3 legs. The remaining L4 ectoderm expresses *At-Scr* (white arrowhead). *At-ftz* is expressed at the tip of the L3 legs (black arrow) in wildtype (D) and *At-hb*<sup>RNAi</sup> embryos (E,F).
Figure 58: At-hb does not regulate posterior Hox genes. Expression of the posterior Hox genes At-Antp (A-D), At-Ubx (E-H), At-abdA (G-I) in wildtype (A,D,G), class II (E,H) class III At-hb\textsuperscript{RNAi} embryos (C,F,I). The embryo in B is a class I embryo. All embryos are stage 10 embryos, upper pictures are lateral views and lower pictures are ventral views of the opisthosoma. Embryos on the right (B',C',E',F',H',I') are ventral views of the prosomal region of the same embryos as in B,C,E,F,H,I. At-Antp is only expressed posterior to L4, in both wildtype (A) and At-hb\textsuperscript{RNAi} embryos (B',B',C'). Expression of At-Ubx is posterior to O1 and At-abdA expression starts at the posterior end of O3 in wildtype (D,G) and At-hb\textsuperscript{RNAi} embryos (E,F,H,I) and not in prosomal segments (E',F',H',I').
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3.2.8. Effects of At-hb on mesoderm formation

At-hb is also expressed in mesodermal cells of each segment in Achaearanea (3.2.2, Figure 43). This observation fits well with the suggestions that hb might be involved in mesoderm formation (Kerner et al., 2006; Kontarakis et al., 2006).

To determine if hb has a role in mesoderm development in the spider I investigated the expression of the mesodermal marker At-twist (At-tw1) (Yamazaki et al., 2005) in At-hb pRNAi embryos. The At-tw1 pattern is not disturbed in early At-hb pRNAi embryos (Figure 59B). In both wildtype and hb pRNAi early stage 7 embryos, At-tw1 expressing mesodermal cells are distributed all-over the embryo, however At-tw1 is stronger expressed in the future L4 region (Figure 59A,B). In wildtype embryos, starting at late stage 7, the twi-positive mesodermal cells become organized segmentally in the L1-4 region (Figure 59C,E), as has been described by Yamazaki et al. (2005). Surprisingly, this process is also observed in At-hb pRNAi embryos (Figure 59D,F), even though ectodermal segments L1 and L2 do not form. This is obvious in the embryos simultaneously stained for At-en and At-tw1 in a single color double in situ hybridization. These embryos express the mesodermal marker twi but not the ectodermal marker en in the L1 and L2 segments (Figure 59J). In wildtype embryos, during stage 8 the mesodermal cells proliferate and form broad mesodermal stripes (Figure 59E,G). However, in hb pRNAi embryos this proliferation does not take place, and the mesodermal rows of cells in the L1 and L2 region do not get any broader (Figure 59F,H) but instead they seem to disintegrate (Figure 59H).

These results suggest that At-hb is only required for the segmentation of the ectoderm of L1 and L2. Spider hb is not needed for the initial segmentation of the mesoderm, but instead for its maintenance in the region that is affected by ectodermal hb patterning defects.
Figure 59: Effects of hunchback on At-twι expression. Expression of At-twι in stage 7-8 wildtype (A,C,G,I) and At-hb pRNAi (B,D,F,H,J) embryos. (I) and (J) are additionally hybridized with an At-en probe (single color double in situ hybridization). All embryos are shown with bright field pictures only (left) and a bright field picture with DAPI fluorescent staining (right). Mesodermal At-twι stripes are labeled with corresponding segments L1-L4, the ectodermal At-en stripes in I and J are marked with asterisks. All At-hb pRNAi embryos were taken from cocoons that exhibited class III phenotypes in more than a third of embryos at later stages. At-hb pRNAi does not affect sorting of At-twι expressing cells into stripes, even in segments that are not segmented ectodermally (D,F,J).
3.3. Discussion
3.3.1. *hunchback* acts as a gap gene in a non-insect arthropod

I have demonstrated above that the spider *hb* gene is expressed before the onset of segmentation and that silencing of *At-hb* via pRNAi leads to a gap phenotype. This is surprising, because although *hb* is a true gap gene in the fruit fly *Drosophila*, in which it was described originally, data from other insects suggest that the ancestral role of *hb* presumably is in regulating Hox genes rather than in segment generation. Furthermore, data on gap gene orthologs in the centipede *Strigamia maritima* and the crustacean *Artemia franciscana* suggested no involvement of *hb* in segmentation either (Chipman and Stollewerk, 2006; Kontarakis et al., 2006). However, both of these non-insect arthropod studies focused on rather late developmental stages due to fixation problems with younger embryos, and a possible early *hb* expression might have been overlooked.

This study not only demonstrates that *hb* is expressed in broad domains in the early spider embryo, but also provides functional evidence that *At-hb* is required for the generation of segments. Most likely the early *hb* expression domains cover the area of the prospective L1-L2 and L4 segments in the spider embryo. RNAi of *At-hb* results in the deletion of the two adjacent segments L1 and L2 and the disruption of L4. This phenotype can clearly be considered a gap gene phenotype, and as such it is more similar to *Drosophila* than most of the other insect *hb* phenotypes, especially because it is not obscured by homeotic transformations like in most insects (Liu and Kaufman, 2004; Marques-Souza et al., 2008; Mito et al., 2005) (see 3.3.4). This is very surprising, because in contrast to *Drosophila*, segments do not form in a syncytium in *Achaearanea* (McGregor et al., 2008a; Montgomery, 1909; Suzuki and Kondo, 1995), which impedes with the diffusion of transcription factors like Hb to form gradients, as happens in the syncytial *Drosophila* embryo. This could be one reason for the spider gap phenotype being less extensive and affecting only two adjacent segments in comparison to the *Drosophila* *hb* gap phenotype in which all gnathal and thoracic segments can be affected.

In the fruit fly *Drosophila* the short-range gradients of gap gene products are part of the hierarchic network of the segmentation gene cascade that controls segmentation. One of the most important functions of gap genes like *hb* is to control the activation of downstream genes in metameric patterns, which forms the first sign of
segmentation in the *Drosophila* embryo. *Dm-hb* is required for the appearance and the positioning of the expression boundary of particular stripes of the pair rule genes *even-skipped (eve) runt (run)* and *hairy (h)* (Gutjahr et al., 1993; Klingler et al., 1996; Small et al., 1996; Small et al., 1991; Stanoevic et al., 1991). In two other insects, the cricket *Gryllus* and the beetle *Tribolium*, *hb* also regulates the expression and positioning of *eve* stripes in the thoracic region, but this is not within the *hb* expression domain, which is more anterior in the gnathal segments. *hb* thus also regulates stripes of pair rule gene expression in these short germ insects, but this regulation presumably is not direct but indirect (Marques-Souza et al., 2008; Mito et al., 2005).

This aspect of a gap gene, the regulation of segmentation gene expression, is also observed for *hb* in the spider *Achaearanea*. I showed that *At-hb* is required for correct formation of the stripes of the pair rule gene ortholog *hairy* in the L2 segment. The L2/L3 *hairy* stripe does not split in *At-hb* pRNAi embryos and as a consequence no separate L2 stripe forms. *At-hb* thus is required for splitting of this stripe. Although the effects on *At-h*, as well as on the segment polarity gene expression of *At-en, At-hh* and *At-pby*, are within the expression domain of *At-hb*, it is unclear whether *hb* directly or indirectly regulates these target genes.

Most of the other pair rule gene orthologs that we have isolated from *Achaearanea* are not expressed in the anterior at the relevant stages and therefore are not subject to regulation by *hb*. Only *At-pby* might be a candidate for this task, but the expression level of *At-pby* in L1 is very low and I could not determine with certainty if the stripe was present in *At-hb* pRNAi embryos or not. Therefore it remains possible that another pair rule gene (which has not been recovered from *Achaearanea* so far) could be regulated by *At-hb* in the L1/L2 region, where *At-h* is not ectodermally expressed during segmentation.

Another, but indirect hint at a possible segmentation gene regulation of *At-hb* is given by the germband morphology of *At-hb* pRNAi embryos. Irvine and Wieschaus (Irvine and Wieschaus, 1994) showed that *hb* in *Drosophila* by regulating pair rule genes is also necessary for correct cell rearrangements during germband extension. During these reorganizations, cells intercalate between their neighbors and this leads to a narrower but longer germband. In *Gryllus hb* RNAi embryos, broader germbands were observed in the region of the thoracic segments that showed segmentation defects such as disrupted *Gb-wg* expression. Mito et al. (2005) concluded that this could also be
associated with disrupted cell rearrangements during germband morphogenesis. Germbands of \( At-hb^{RNAi} \) spider embryos were also typically broader and shorter than wildtype embryos and this often coincided with disturbed expression of segment polarity genes during the initiation of these expression patterns (e.g. Figure 4B,D).

Nonetheless the detected influence of \( At-hb \) on \( At-h \) and the segment polarity genes within the domain of \( At-hb \) expression explains the lack of segmentation of the L1 and L2 segment area after \( At-hb \) RNAi and confirms that \( At-hb \) acts like a gap gene in the spider.
3.3.2. Producing a gap via cell rearrangements?

It remains an open question what happens to the tissue of the presumptive L1/L2 area in *At-hbp*\textsuperscript{RNNAI} embryos. Although initially tissue of a presumptive L1/L2 area is present between the pedipalpal and the L3 segment at stage 9, this area does not get patterned in *At-hbp*\textsuperscript{RNNAI} embryos and is no longer present at stage 10 in embryos that have continued development and will later hatch (Figure 49, Figure 7). At stage 10 the pedipalpal and L3 segment are flanking each other and no additional tissue is present between them. Since the cell death analysis did not point to massive cell death specifically in this L1/L2 or L4 region, it remains unclear how the tissue between Pp is reduced, but probably cell rearrangements play a role in this process. The reduction of this large region again demonstrates the large developmental regulatory capacities of spider embryos that I already mentioned before (see 2.3.1).
3.3.3. No role of At-hb in posterior segmentation

A function that is clearly not conserved between the spider and Drosophila or other insects is the expression of hb in the posterior part of the embryo, and the resulting posterior effects in hb RNAi experiments or mutants. At-hb is not expressed in the opisthosomal segments, except for the mesodermal expression in newly formed segments and in the ventral neurogenic regions. In Drosophila the posterior domain is activated independently from the thoracic expression domain by the terminal system (Margolis et al., 1995). A posterior domain of hb expression is also present in most other insect species and it is also located in the A7-A9 region, comparable to Drosophila. However, in the sequentially segmenting short-germ insects hb expression is only later located in the prospective A7-A9 region. It is first expressed in the growth zone where it is thought to be responsible for the formation of posterior segments (Kraft and Jäckle, 1994; McGregor et al., 2001; Mito et al., 2005; Patel et al., 2001; Pultz et al., 2005; Tautz and Nigro, 1998; Wolff et al., 1995). Only in the milkweed bug, posterior hb expression is located just in the growth zone throughout germband elongation and similar to the other short-germ insects RNAi results in posterior compaction. In the two other non-insect arthropods, no posterior expression domain or expression in the growth zone was found (Chipman and Stollewerk, 2006; Kontarakis et al., 2006). Together with the data from Achaearanea this suggests that the posterior hb domain and its function in posterior growth or posterior segment generation has evolved only within insects.
3.3.4. *hunchback* does not regulate Hox gene expression in the spider

*hb* plays a major role in regulating Hox gene expression in insects. In *Drosophila*, Hb represses *Dm-Antp* and *Dm-Ubx* expression in the anterior part of the embryo, and some *hb* mutants show homeotic transformations superimposed on the deletion phenotype (Irish et al., 1989; Lehmann and Nüsslein-Volhard, 1987; Qian et al., 1991; White and Lehmann, 1986; Zhang and Bienz, 1992). Apart from *Drosophila*, *hb* function has been assessed in the wasp *Nasonia vitripennis*, the beetle *Tribolium castaneum*, the milkweed bug *Oncopeltus fasciatus*, the cricket *Gryllus bimaculatus* and the locust *Locusta migratoria manilensis* (He et al., 2006; Liu and Kaufman, 2004; Marques-Souza et al., 2008; Mito et al., 2005; Pultz et al., 2005; Schröder, 2003). In most of these insects, an anterior segment deletion phenotype is complemented or sometimes superimposed by homeotic transformations caused by ectopic expression of Hox genes. Marquez-Souza et al. (2008) even restrict the conserved function of *hb* and other gap genes in insects to Hox gene regulation. However, in the spider *Achaearanea*, *At-hb* does not seem to regulate Hox gene expression, as I did not observe ectopic expression of Hox genes in *At-hb*\textsuperscript{PRNAI} embryos. Consequently, spider *hb* has either lost this ability or else it has only evolved in insects. The latter case would contradict suggestions of evolution of the canonical gap function from an ancestral Hox gene regulating function (Mito et al., 2005; Peel et al., 2005). Which of these cases holds to be true, has to be tested by functional examination of the *hb* role in other non-insect arthropods.
3.3.5. Mesoderm development and segmentation in the spider

One of the unanswered questions related to the origin and evolution of segmentation is the role of the mesoderm in the segmentation process. It is not known in arthropods whether ectoderm and mesoderm are patterned independently, and to what extent these two germ layers influence each other during segmentation (Peel et al., 2005).

Yamazaki et al. (2005) proposed that mesoderm and ectoderm in the spider are patterned in parallel. I have shown here that at least in the leg bearing segments in spiders, the mesoderm is segmented first, since mesodermal cells sort out into stripes before ectodermal segmentation is evident from expression of segment polarity genes (see 2.2.1). Similarly, in the brine shrimp mesodermal cells are arranged in segments before ectodermal segmentation becomes obvious (Kontarakis et al., 2006). The possibility that the mesoderm is segmented first in Achaearanea is corroborated by At-hb pRNAi not affecting the initial segmentation of the mesoderm, even in segments that are not patterned in the ectoderm. Later, the two germ layers do seem to influence each other though, since the mesodermal cells of the ectodermally missing segments do not proliferate as in wildtype embryos, but instead the stripes of mesodermal cells expressing At-tw1 disintegrate. It remains unknown which mechanisms initially pattern the mesoderm in spiders. What makes the scattered mesodermal cells sort out into stripes, before the ectoderm is segmented? This data offers some insight into the interplay of ecto- and mesoderm in arthropods, but it is necessary to investigate these questions in a much larger sample of arthropods.
4. **General Discussion**
Distinct mechanisms pattern anterior and posterior arthropod embryos

There is a clear distinction in the mode and genetic mechanisms of segmentation between the anterior prosoma and the posterior opisthosoma in the spider. Previous work on spiders showed that opisthosomal segments are sequentially added from a posterior growth zone and their specification depends on Wnt signaling (McGregor et al., 2008b) as well as Notch-Delta signaling (Akiyama-Oda and Oda, 2006; Schoppmeier and Damen, 2005b; Stollewerk et al., 2003). Both signaling pathways are also used by vertebrates to sequentially pattern their somites from the unsegmented presomitic mesoderm (Dequéant and Pourquié, 2008). This thesis provides evidence that, in contrast, the prosomal segments are patterned via a mechanism more reminiscent of Drosophila segmentation. The prosomal segments do get not patterned sequentially but more or less simultaneously from a pre-existing field of cells. This is also obvious from how the stripes of segmentation genes like At-h and At-en appear in these regions respectively. For example, the L2-L4 At-h stripes form by splitting of an initial broad stripe, while the six prosomal At-en stripes appear in a stereotypic, but not strict anterior-to-posterior pattern shortly after each other. Most strikingly however, hb is required for the correct formation of these stripes like in Drosophila, and this gene controls the more or less simultaneous patterning of the leg-bearing segments in the prosoma.

The use of distinct upstream genetic pathways for segmentation of the prosoma and the opisthosoma is most obvious from the RNAi experiments. No segmentation defects were observed in the opisthosomal segments of At-hb RNAi embryos, while in At-Wnt8 RNAi embryos all prosomal segments form, but the opisthosoma was missing (McGregor et al., 2008b). Also in Cupiennius, embryonic RNAi against Cs-Delta or Cs-Notch resulted only in truncated opisthosomas, but the prosomal segments were properly segmented (Schoppmeier and Damen, 2005b; Stollewerk et al., 2003). The spider thus seems to utilize a “Drosophila”-like genetic mode to pattern its anterior segments and a vertebrate-like way to pattern its posterior segments. The spider data provide the first functional evidence for the hypothesis that short germ arthropods may employ two separate mechanisms to segment their anterior and posterior body regions (Dearden and Akam, 2001; Peel and Akam, 2003; Peel et al., 2005; Schoppmeier and Damen, 2005b).

This notion has important implications for our understanding of the evolution of segmentation in arthropods. Spiders are chelicerates, a basally branching arthropod group (Friedrich and Tautz, 1995; Giribet et al., 2001). I propose that also the last common ancestor of arthropods utilized different mechanisms for anterior and
posterior segmentation. The anterior segments of this ancestor were presumably patterned simultaneously via a mechanism depending on \textit{hb} and reminiscent of what is seen in the spider prosoma and in \textit{Drosophila}. The ancestral posterior patterning mechanism likely involved both Wnt8 and Notch-Delta signaling, since involvement of Wnt8 signaling and Delta-Notch signaling has also been shown for other arthropods (Bolognesi et al., 2008; Chipman and Akam, 2008; Pueyo et al., 2008). Simultaneous specification of all segments as seen in today’s long germ insects like \textit{Drosophila} then might be due to an expansion of the anterior specification mechanism to posterior during the course of evolution (Damen, 2007; Peel and Akam, 2003).

It may even be speculated that a segmented urbilaterian ancestor might have also used both patterning mechanisms that then diversified in the different lineages leading to today’s segmented animals.
5. Material and Methods
5.1. Molecular cloning

For standard molecular biology methods and reagents, protocols from Sambrook and Russel (1989) were followed. If not specifically noted, kits and reagents were used according to manufacturers’ protocols.

Total RNA extraction
A range of different stages (1-10) of Achaearanea embryos were collected and frozen at -80 °C. RNA was extracted using Trizol reagent (Invitrogen). Eppendorf phase lock gel heavy tubes were used for the phase separation process.

cDNA synthesis
First strand cDNA was synthesized using the Invitrogen SuperScript III First Strand Synthesis System for RT-PCR.
Degenerate PCR

For cloning of degenerate gene fragments from cDNA (see above), the following Primers were used:

**List of degenerate Primers:** Most primers were designed by Wim Damen (W), the Six3 primers were designed by Klaus Jobi (K).

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RACE template synthesis

For purification of PolyA RNA from total RNA preparations (see above) the Promega PolyATract kit was applied. Purified PolyA-RNA was then used as a template for the RACE (rapid amplification of cDNA ends) template synthesis with the Marathon kit by Clontech.

RACE-PCR

Nested 5’ and 3’ RACE-PCR reactions were performed using the Advantage 2 Polymerase mix together with Advantage 2 PCR buffer (Clontech). A reaction contained 20 µl H2O, 3 µl 10 x buffer, 3 µl 2 mM dNTPs, 0.75 µl 10 µM gene specific primer (GSP) 1, 0.75 µl 10 µM AP1 primer and 2 µl of a 1:250 dilution of RACE template (see above) as well as 0.6 µl Polymerase mix. For the nested reaction 1 µl of the first PCR was used as a template, the primers were exchanged for the AP2 and the GSP2 primer and the H2O
amount was reduced to 1 µl. The following thermocycler program was used: 5 cycles of 30 sec at 94 ºC followed by 4 min 72 ºC, 5 cycles of 30 sec at 94 ºC followed by 4 min at 70 ºC and 25 cycles of 30 sec at 94 ºC followed by 4 min at 68 ºC. A final elongation step of 5 min at 68 ºC was added and subsequently the RACE-PCR reaction was kept at 4ºC. For the primer design of GSP1 and GSP2 primers the manufacturer rules of the Marathon RACE PCR system were followed.

**Cloning**

After gel extraction (Zymo gel extraction DNA recovery kit, elution in 10 µl H₂O), PCR and RACE-PCR fragments were cloned using the TOPO TA Cloning Kit for Sequencing with pCR4 vector (Invitrogen). Fragments were transformed into chemically or electrocompetent TOP10 cells and transformations were selected on LB-Amp plates over night. Clones were tested with a colony PCR and plasmids with the right insert size were grown over night and plasmid mini preparations were done using the Eppendorf Fast Plasmid Mini Kit.

**Sequencing**

Cloned genes and gene fragments were sequenced on an ABI Prism 3700 at the Cologne Center for Genomics with Big Dye Terminator Mix v.3.1 chemistry. Sequence analysis and assembly was assisted by Codon Code Aligner software version 2.06.

**Alignments**

Alignments were generated using ClustalW with the standard parameters. Alignments were then hand corrected and edited with BioEdit version 7.0.9.
5.2. Embryological methods

*Achaearanea tepidariorum* culture

Adult spiders were kept separately in insect breeding containers capped with foam plugs (Greiner Bio-one) on moist coconut fibre substrate (supplied by local pet shops). Females were fed twice a week with crickets (*Achaeta domestica*) of 1 cm size and after mating produced one to two cocoons per week for up to two months. For breeding, cocoons were usually kept separate from the mother. Freshly hatched spiderlings were first fed with *Drosophila melanogaster* flies and two or three molts after hatching from the cocoons they were separated from each other and fed with either *Drosophila virilis* or small crickets and subsequently with successively larger crickets.

Parental RNAi

dsRNA was generated from PCR fragments of a 1168 bp 5’ fragment corresponding to nt 1-1168 of the *At-hb* sequence and a 583 bp 3’ fragment corresponding to nt 1192-1775 of the *At-hb* sequence as well as *gfp* using the Ambion T7 Megascript Kit without additional annealing of the RNA after the synthesis. A non-purified PCR product served as a template for the synthesis. The PCR product was generated using T7 and T3-T7 primers for genes that had been cloned into TOPO pCR4 vectors.

Female spiders were injected using an Eppendorf capillary holder equipped with a pulled glass capillary (Sutter P-97, using Hilgenberg capillaries), connected to a Microinjector (CellTram Oil, Eppendorf) and mounted on a micromanipulator. The needles were beveled using fine scissors and front-loaded with the injection liquid. For injection, spiders were kept in a 3 cm petri dish lined with a trimmed foam plug (leaving enough space for the spider opisthosoma) and secured by a mesh sealed to an open petri dish lid.

For RNAi, spiders were injected into a dorsolateral region on their opisthosoma every two days with 1.5 µl of a 2 µg/µl dsRNA solution for a total of 5 times. *dsGFP* was injected as a negative control.
Injected females were either mated after the first injections or after the whole injection cycle, which had an effect on the appearance of phenotypes. Usually phenotypes increased more slowly in severity over a longer period of time when they were mated after the first injection. Mating the spiders after the injection cycle resulted in very fast occurring strong phenotypes (e.g. in the second cocoon), but subsequent cocoons were soon free from phenotypes. The same effect was observed when spiders were starved after the injections as the spiders heavily depend on the availability of food for the production of offspring.

Injected spiders were reared exactly as described for normal, non-injected spiders (see above).

**Fixation of embryos**

After taking cocoons from the mothers, until fixation, eggs were kept in glass dishes, which were placed in 9 cm petri dishes together with moist tissue to prevent drying of the embryos. For staging, embryos were immersed in MeOH or mineral oil (embryo culture tested, Sigma). In mineral oil, embryos developed at the same speed as non-immersed embryos until hatching.

The fixation protocol was modified after a protocol that had been kindly provided by Hiroki Oda. For dechorionation, 2 ml sodium hypochlorite (Dan Klorix) was added to the embryos in the glass dishes and constantly sprinkled on them with a Pasteur pipette until the embryos floated to the sides of the dish. Then 1 ml of the bleach solution was taken off and replaced with 1 ml of de-ionized Water (dH2O), which caused the embryos to sink. After a 2 min incubation, all liquid was taken off and embryos were washed three times with dH2O. Then the fixative consisting of 4 ml heptane, 0.6 ml 37% formaldehyde (Sigma) and 3.4 ml PEMS (0.1 M PIPES, 2 mM EGTA, 1 mM MgSO4, pH 6.9) was added and the embryos were transferred to a 20 ml scintillation vial (Wheaton Science products). The vial was mounted on a test-tube rotator (Snijders) and embryos were fixed at RT for 2 days, but younger embryos (up to stage 7) were usually fixed for 3 days.

The fixative was then completely removed using a glass Pasteur pipette, and embryos were washed 3 times with PEMS-T (PEMS supplemented with 0.1 % Tween-20). Afterwards the embryos were gradually moved to 100 % MeOH, by carefully
adding MeOH over the course of at least half an hour. They were then left in 100 % MeOH for at least another 30 min before storage at -20 ºC.

**Fixation of ovaries**

Ovaries were dissected from adult female spiders. To obtain a range of different oocytes stages, two spiders were dissected, one that had recently (<2 days ago) built a cocoon and one that was just about to build a new cocoon (large opisthosoma, last cocoon at >3 days ago). Spiders were dissected in PBST and ovaries were directly transferred into 4% formaldehyde in PBST and fixed for 20 minutes at RT. Next they were washed 3 times in PBST and then gradually transferred into 100 % MeOH, similar to the embryo fixation protocol.

**Whole mount situ hybridization**

For in situ hybridization experiments, the vitelline membranes of the embryos had to be removed by hand using fine forceps (Dumont #5, biology tip). Ovaries were merely returned to PBST and not further dissected.

The whole mount in situ hybridization protocol in principle follows the protocol published for *Cupiennius salei* (Prpic et al., 2008), except for shorter blocking times (2 x 20 min) and a shorter antibody incubation period (3 h) and an additional post-fixation step at the end of the protocol to prevent embryos from darkening too much under UV light exposure during microscopy.

**Embedding stained embryos in Durcupan**

After in situ hybridization (embryos in PBST), embryos were dehydrated through an ethanol series to 100 % EtOH (10 min 50 %, 10 min 70 %, 10 min 90 %, 2 x 10 min 100 % EtOH). They were then transferred to a 1:1 mixture of Durcupan (Fluka) and acetone and incubated over night in this mixture, leaving the tubes open under a fume hood to allow evaporation of the acetone. Next the embryos were transferred into molds and fresh Durcupan was added to them. The molds were placed into a 65 ºC incubator and after 30 min the embryos were aligned within the molds and subsequently incubated over night at 65 ºC.
Sectioning
6 μm sections were cut using a Leica microtome and mounted on glass cover slips using durecupan. Slides were dried over night at 65 °C before microscopy.

Assaying cell death via TUNEL
For TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP–digoxigenin nick-end labeling) experiments, the exact protocol published for Cupiennius was followed (Prpic and Damen, 2005; Prpic et al., 2008)

DAPI staining
For nuclear staining with 4’,6-diamidino-2-phenylindole (DAPI) embryos were carefully washed 3x 10 min to remove all traces of formaldehyde from the postfixation, since this can cause background staining in the yolk. The embryos next were incubated in the dark with 1 μg/ml DAPI (Roche) in PBST for 30 min and several short washing steps (5 x 5 min) followed.
5.3. Microscopy

**Microscopy of whole mount embryos**
Embryos were analyzed as whole mounts using a Leica dissecting microscope equipped with a Zeiss Axiocam. Of each embryo, first an image under UV illumination was acquired to visualize DAPI staining, and then a brightfield image was acquired under the exact same magnification settings.

**Microscopy of sections**
Sectioned material was imaged on a Zeiss Axioplan-2 Microscope equipped with a Zeiss Axiocam using Zeiss Axiovision software release 3.6

**Movies**
Movies of 2nd instar spiders were taken on a Leica MZ16FA equipped with a Leica camera using Leica Application Suite version 2.8.1. Movies were assembled with Apple iMovie and Quicktime software.

**Timelapse microscopy**
For time-lapse microscopy, *Achaearanea* embryos were staged in MeOH or mineral oil (see above). To delay development of embryos if necessary, they were stored at temperatures as low as 16 °C. Embryos for time-lapse microscopy were dechorionated as described above and then aligned in grids of 4x4 embryos on 1,5 % agar plates using an eyelash tool. Embryos were glued to coverslips with heptane glue prepared from Scotch 3G tape dissolved in heptane over night. The coverslips next were placed into 3 cm petri dishes and were then immersed in Halocarbon Oil 700 (Sigma). This was found to be the only oil that does not dissolve the glue, but does not affect embryo development in a negative way. In Voltalev oil, which is used for embryonic RNAi in *Cupiennius, Achaearanea* development was delayed and embryos usually did not
complete embryonic development. Timelapse microscopy of spider embryos was carried out with a Zeiss Axioskop 2 mot plus equipped with an Axiocam and Zeiss Axiovision software release 4.6. Additionally the microscope was outfitted with an external temperature controlled table to keep the embryos at a constant temperature of 25 °C. Furthermore the microscope room temperature was set to 25 °C.

**Image processing**

Brightfield and UV channel images of whole mount embryos were merged using Photoshop CS3, which was also utilized to adjust brightness and contrast settings of other images and to assemble figure tables.
6. Bibliography


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7. Appendix
7.1. Sequences

**At-abdA**

Incomplete 5’RACE-PCR fragment and 3’RACE-PCR fragment, assembled with the degenerate *At-abdA* fragment (underlined) isolated by Abzhanov et al. (2001). The 5’RACE-PCR fragment lacks the transcription start, sequence coding for presumably 12 more Aa is missing.

```
PTSSYGQHRMYPYSLSQPSPS
CCAACCTCTAGTTACGGGCAACACAGGATGTATCCCTATGTATCACTCTCCCCCCAAAGT
60
SMSVSGNLTTYTSNADFASKC
AGCATGTCCGTCTCAAGGAAACCTCTACTATACGTCGAATGCCGATTTTGCAAGTTG
120
RYNSTQALDGPGYSGFLQ
CGTACAACACAGCACCAGCGAGAATCTGAACTGGGTCATCTGGTCTGCAACACC
180
NAGPGATPTMTMPGQFFTHP
AATGCGGGGCCCCGGAACCCACCAACCAGATGAGATCCTCTGGATTTCTACATGCTGG
240
ASTDLSSITSNCNLGMRQL
GCATCGACGGACCTCAAGGAAACCTCTACTATACGTCGAATGCCGATTTTGCAAGTTG
300
DPIDVPDPWMSIALANFA
GATCCCGGCCCCGGAACCCACCAACCAGATGAGATCCTCTGGATTTCTACATGCTGG
360
GPNCPRRRGROQTYTTRFQTL
GGACCGAATGGCTCTCTCCCGCGGAGAAGACCAATCTACCCCCAGATGAGACCTGG
420
ELEKEFHFHNHYLITRRRRIEI
GAACCTTGAAGGAATTTCACCCATCACCCTCTTAAACCCGGCGGATATGAGATTG
480
AHACLTERQIKIFQNRRRM
GCTCACGGCCCTGTGCTACCGGAGCCAAATAAGATATGTTCCAAATCTGGAAGT
540
KLKEMRAVKIEQARMES
AAGCTCAAGAAAGATGAGCGCGGCCAAAGAAATCAACGAACAGCTCGATGGAATCC
600
SKVEDEDKDKNVDHQAKE
TCCAAAGTGCAAGAGGAGGCCAAAGAAATCAACGAACAGCTCGATGGAATCC
660
DRKSESHEPPS IITAPTSLLI
GACAGGAGATCCCTCAATCGAACCACCCACCTCCATCTGCTCCACATCTCTCAT
720
LEDKIRNPKP *
TTAGAGGCAAAAAATCGAAATCCGAAATCCGAAATCCGAAATCCGAAATCCGAAATCC
780
GTAACTAGTGATGGTGACGAGGAAACATCAATTACATTATACATTATACATTATACATT
840
AGTTGCAATAGCTATTTCAACAGACATTTACAAATCAATAGCTATTTACAAAT
900
TATCGGATGACAGATCTACTAAATATACATTATACATTATACATTATACATTATAC
960
TAAACGCAATGCATCTCAATCGGCAATCCGCAATCCGCAATCCGCAATCCGCAATCC
1020
GTTTTCGAGTTGTTTCAATACCCGCAAACCTCTGAAATCCGAAATCCGAAATCCGAAATCC
1080
AAGTATGATCATATTATATGAGATTTCTTGAGATTTCTTGAGATTTCTTGAGATTTCT
1140
```
**At-Antp**

Partial mRNA sequence of *At-Antp*, isolated by degenerate PCR and following 5’- and 3’ RACE-PCR. The 3’RACE-PCR fragment is incomplete.

\[
\begin{align*}
\text{CGATTCCC} & \text{AAAGCCCCACCAGACACTATTTACACCCCAGTGCAGTCAATCTCAGGGCAACAT} \\
\text{GGCCCA} & \text{ACGCGCAATTCAGACACCACAACACACACCTTTAACACATCCTAACGCCTATGT} \\
\text{GACAACAT} & \text{CTGTCACCTTCGCAAAACATTTCAACACAGCAATCGGATGATGCA} \\
\text{GGATCCCAACGGAGTACATCGGCCTGTAATCCGGATTGTGCTGCCAACAATATGCATCC} \\
\text{GCAACACTGTCAAAGTCCTGTACATTCCCCGCAACAAATGTACCCACCGAATCACGTACA} \\
\text{ACAACAGCCTCCCAATCCACAGAACGTCAACCAAGTTCAACCAGCTAGTGGTGGACCTAG} \\
\text{TCCTTTGTATCCATGGATGCGGAGTCAGTTTGAGAGGAAACGTGGTCGGCAGACCTACAC} \\
\text{TCGCTACCAGACCCTTGAATTAGAGAAGGAATTTCATTTCAATCGCTACTTGACGCGTCG} \\
\text{GCGTCGGATCGAGATCGCTCACACTCTCTGCTTGACGGAGCGACAGATCAAGATCTGGTT} \\
\text{CCAGAACCGACGCATGAAGTGGAAAAAAGAAAACAAGTCAAAGAG} \\
\text{YPWWMKKKXHXTVAAANGNFPG20} \\
\text{TACCGTGGATGAAGAAAGTNCATNTTGGGACAGTTGCTGCCAATGGAAATTTCCCTGGA} \\
\text{VEPKRQRTAYTRHQILEK40} \\
\text{GTAGAACCCTAAAAGGCCAAAGGACAGCCTATACTGCACCACAGATCTGAATTTTGGAAA} \\
\end{align*}
\]

At-Dfd-2

Incomplete mRNA sequence of *At-Dfd-2* obtained by 3’-RACE-PCR and assembly with initial fragment by Abzhanov et al. The initial fragment is underlined.

\[
\begin{align*}
\text{YPWMKKXHXTVVAAANGNFPG20} \\
\text{TACCGTGGATGAAGAAAGTNCATNTTGGGACAGTTGCTGCCAATGGAAATTTCCCTGGA} \\
\text{VEPKRQRTAYTRHQILEK40} \\
\end{align*}
\]
At-ftz

This 3’ fragment of At-ftz was cloned as a byproduct of a 3’-RACE-PCR directed against At-Antp2.

CGTCGTTGAAATAGCTCATAACTACCTGTTGCTTTACAGAAGAGCAAATTAGATATGGTTT 60
RRV E I A H T L G L T E R Q I K I W F 20

CAGAATGCCAGAGTGAAGCTAAGGAAAGGAACAAAATTTCCAAAATCTGTTTTCCCAACA 120
Q N R R V K A K K E N K F P N P V P S T 40

AGTCCATGGAACATCCAAATGGCATGATCCTGCTTCTCCACA 180
SP L S N P N C D P D L P E I L Q N K Q 60

ACTCTTAAGCTATTTTCCTGTTTTACATATTGCAGCAGACACAGAAAAGATGGATTTTT 240
TL M H I S G F T L S H D S S K D G M F 80

TTTCTTCCACAACACGAAAGAAACTATAAATACCTAAAGTTGGTTAAGCAATATACTGATA 300
FLPQQ RETILT 91

ATCTTATAACATTATATATCAGGAGGAAATTACCTTTCTGATGATTGTTACT 360
ATGATATAGGTAAATTTGGAATTTGUATACCTGAGGTGTAATTGCTCAGAAAGTTAATTTGCTCAAA 420
GATTGTGCGAAGATAGTTACGTAGAGTGATGAGTCAACAGAATAGTGCAGTCT 480
TTATGATAATATATCAGGAAATGGGTTGAGAGACAGTAAATACTACGAAACAGGAGGAGCTGAA 540
TTTATATATATGAGAGGAAACAAATCTGCTCAGAATCAATATACATGAAATTTGATTCTTA 600
AAGACAAGTTTATACATTAAATTTTAAAAACACGAAAAAaaaaaaaaaaaaaaaaaaaaaaa 658
At-hb

Complete coding sequence of At-hb.

GCAGTGTGTGATACGTGTTGGGATGTTCAATGCAAGTCAACAAGTTTTTTTTTAGTTTT 60
TTCCCTTCTGAGAAATAGAGAGTGGCTACTTTTTGAGAAGAACCTGTGAGCGTGTTTGATGG 120
ATGAAAAGAATTTGTCTTCTTGTGAAAAATTTGGAATATAAAGAGAGAAATCTGATN 180
M S V E C Q T 7
AATTCTATTGCGCGATAAACAACTTTACAGGCCTCATAAATGAGCGTCGAGTGTCAGACT 240
R T F I Q S S N N P A D L S F N M A P L 27
CGTACATTCTCATCAATCAATCAAAACAAACTCCGCTAGATTGAATTGCTAAATGATACATC 300
G D M Q Q D M Q K H S E S V I Y H P H S 47
GGAGACATGCAACAGGACATGCAGAAGCACTCAGAAAGTGTGATATACCATCCCCATTCA 360
A K N C Q S T P V S S P M T S M G S G S 67
GCAAGAATTGTCGAAGATACCTCTGACTGTTCTCTGCTGATTCCAGGTGATTCCAGAT 420
S P R Y T E D P M S E D N L D P A I 87
TCCCCTGAGATCAACGGGAAATCCCTGAGATGGAAGCAATCTGCATCTGCTGCCGATT 480
D L S D D S C L Y S E D E M Q N M S S 107
GATTGTCTTCTCGAGTACGCTACTGGGAATATGGAATATGCAATCTGCTGCTGCCGATT 540
N P L N E L Q N S L D K N G F L G Q S L 127
AATCCCTTGAACAGTGTTGCAAATTTCTGCGACAAAACGGATTTTTAGGGCAGGCTGTG 600
S A R P N G T N G R D S A E D S D S M 147
TCTGCGAGGCCCCCAATGGAAACACCGGCTGGGATTCTGCCGAGATCTGGATTCTGATG 660
N K G V E T L E C H L C N F Q A V S P E 167
AACAAAGGGGTCGAAACCCTACGATGCTGACTGACTTATTTTCAAGCTGGGTTTCTCCGAA 720
E Y S H H I D G H F D H K C P I C D Y 187
GAATACGTCATCAATCATATGATGTCGTTTTGACCATCTACGGCGATTGATTTGATACACG 780
S R T E G R L N R H I Q D F H S E V P P 207
TCACGAACTGGGAGCCGTTGAGCTTGGGATTCCCCTGAGCTGAGTGCGCTCCGAA 840
E S W A G K K K P N Q V R D D K P E N S 227
GAAAGCTGGGAGGGAAGAAAAGGGCTTGACAAATGCGGATTGATGATTATACACG 900
S S S P A S Q K A K I N R C K Q C N F 247
AGTTCATTCTCTCTTCGCCCTCGAGCGACAAATATGCAAAACTGCACTTTCC 960
S C D N K P E Y Y K H L Q E V H M K K D 267
AGCTGGGACAAATAAACCCTGAGATTAATATCTCTCTCTGATCGACGTGAGATGGAAGAT 1020
K I L Q C P N C C F P V T E Y K H H F E Y 287
AAAATCTTGGCAATGCTCCTGACATTGTCCTTGGTACAGGTACAAAACATCTACTTTCGAAAT 1080
H L R N H V G S K P F R C D K C N Y S C 307
CATCTTGGCAACCGGTTGGTCCAACCCCTTCTGAGATGCGACAAATGCAATTATAGGTG 1140
V N K S M L N S H E K S H S K I Y P Y R 327
GTTGACCAAAATCGATGTTGGAATGCCGAGAAGAAAATCTCATTCCAAAAGATTTATACGATCAACG 1200
C A N C K Y A T K Y C H S L K M H L R K 347
TGTCAGTCATGTTAGTCCCTGACGAATTTGGAATGAGATGCAACCTCGGAAA 1260
Y G H S Q G Q I L N P D G T P N H N P I 367
TACGGCCACAGTCCAGGGACAGATTCTCAATCCAGATGGCACCCCGAACCATAATCGATA 1320
I D V H G R R R G P K G K S G G K N K Q 387
ATTGATGGTCATGTAAGAGAAGGGTTCCTCAAAAGGAAAATCGGGGTTAAAATAAACAA 1380
K C L T Y S D I S P N I C N A K T E M 407
AAGTGGTTAACCTACAGCAGATATTTCTCTCTTCCAAACTTGGATGCAAACAGGAGT 1440
S T Q P F L Y P T P H F P P K T F S S M 427
TCCAGCAACCGGTTCTTCTATATCCAAACACCTATTTCCCTCTCTTAAACCTTCTTCCAT 1500
G M P T S L D L Q I P S F S V W P R P 447
CTTATGGGACCACCTTCTTGGAACATTACAAATTCCTTCCATGTTTTCGTTGGCAGAC 1560
F K C H Y C D F M T E S L D L Y Q Q H A 467
TTCAAAGTGCATCTGCAGTTCTATGACAGGATCCCTGCAGATCTTTTTACAAACACAGTC 1620
V A H A V K E N S D L M R T C N I N P D 487
GTAGCCCATCTGGCTTAAAGAATTCTGATTTGATGCGCACCTGCAACATCAATCCGGAC 1680
I F H L Q A Q H L T F N N Y P Q N S I H 507
ATATTTCCATTTGCAAGCTAGCATACTGACCTTTTTAATAACTCTATCTCAAAATTCCTAT 1740
H V D E V S R S M H K N E I E K K I V C 527
CATGTTGACAGAAGTCTAGTCATGAAAATGAAAAATGAAAAAGATCAGTGGC 1800
S T A S E N A R S H S I H S E N A I S 547
AGCACTGCTTCGGGAAGCAGCTCGAAGTCAATGCATTTTTAACTTCAACATGCGAAGAC 1860
G N S I Y S S S A E N K I P L T N S V 567
ACTGGCAACAGGCAATTCTCCGCAGAGAAAAATGAAATGAAAAATGAAAAAGATCAGTGGC 1920
P Q I P D S K T Y S P V S I S S D G T K 587
CCTCAGATTTCCTGATTCCCAACATATTTTCTCCCTCCACGAGCAAAGGCTTCCGAC 1980
N I S D N H K P S S D Y P E I L P R Y S 607
AAATTTTCAGATAATCAAAACACATCTCCGAGATTACCTGAGATCCTCTCTATGACT 2040
P P A R T H T S P V H F S T Y N E N G N N 627
CCTCCTGCTGCTATACCTTGCCGGCTGATTATTTTTCTATCCTAATGAAAAATGAAAAAT 2100
H T S S D K Q S L T S P Q E A L S I S E 647
CATACATCAAGGAGAACAAGAAAAAGCCTAATCTTCAACCCCAAGAGCGCCCTTTCATCTCAGAA 2160
C S P K S S S F N S D E N A R N S F E C 667
TGCTCACCACAGTGAGTTCTTCTCGGAGAAGCTAGAAATCCTGAGATCTCGAGATGACA 2220
Q N E H S P L D L S G P K I S E P I S 687
CAAAATTGAGGAGCATCCTCCTGGATTTTTAAGTGAGCCTAAAATCTCTGAGGCCCCATACC 2280
R G V K I E H H T A K R K L S P N D V A 707
CGAGGAGCTCAAAATTCAGCAGCATCAACCTGAGAAGGAAAAACTTGTCAATCAGGCTAG 2340
S T N Q D S Q S R N R R K G K A F K L D 727
TCTACAAATCGAGCAAGCATACTCCGAGGACAGCAGAAAGGAAAAAGCTTTTTAATACGAC 2400
H Y I Q C E D S P I K T D L D A A S D F 747
CACTACATCAGTGCGAAGATCTCGCCCATCAAAACCGATCTGGATGCTCGATCCAGATTC 2460
K Y F V G S E D T A K R E A S Q L L D S 767
AAATCTTCTGAGGCTCTCAGAGACATGCTAAAGAGAAAGCGAGATCGAAATCTTCTAGACT 2520
At-Hox3

Complete coding region from 3’ and 5’ RACE-PCR, based on homeodomain fragment by Abzhanov et al. The initial fragment is underlined.

```
CTAAGGTTTCTGATATGGGCTCTCTTGCACAGCTGCTGAGGAGGAGGAGGAGGAGGAGGAG
AAAGCTTTATATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT
AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG
60
AAAGCTTTATATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT
AAAGCTTTATATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT
120
TTGCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCTCGCT
180
M C H T S K W K S S T S K N K K L Y R 20
TGCTGACGTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
240
T V M P F K T P L S P Y I N N N S C Y A 40
CTGTGACGTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
300
T A S P D L V A K G F D G G G S D Q N 60
CTGTGACGTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
360
D H P H D E D S S G N S Q V I L D I E 80
TGCTGACGTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
420
T S N H D E G S Q L D E E Q Q K L L L L 100
CTGTGACGTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
480
S R S L C Q E Q N S P P V A P S T Y T S 120
CTGTGACGTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
540
```
At-Pax6

Degenerate fragment of At-Pax6.

A H S G A R P C D I S R I L Q V S N G  19
TTGGCAAGGCGGCCTCCGGCGCCCGACAGGTATCTCCGGATCTCAGGTTCTAAGCTGGCCT  60
C V S K I L G R Y Y E T G S I R P R A I  39
GGGTGCTCTAGATCTCCGCTTCAGGTCAGGAGAAGGCTGCGGTCTCTCTAAGGGTGTTCT  120
G G S K P R V A A P D V V A K I A H F K  59
GGGACGTCATGAGCTCCTCAGTGACGTCGCTCTGGTGCCGCAAGATGCCATACACGCTCT  180
R E C P S I F A W E I R D R L L S E G A  79
GGGATGAGTCCCCCTCCATTGGTCTGGGAAATGGGAAATCGGATGATGGCTCTGCTGAGTGCT  240
C T N D S V P S V S S I N R V L R N L A  99
GCAACTACGATAGGCTGGTCAAGGTTCTGCTCCTAAGGACTGCTCTGCTCTTGGGCTCT  300
A Q K E Q A Q V Q A Q D A V Y D K L R M  119
CGGCAAAAAGACGACGCGCACGCTACGGGCAGCCCCAGAGCTACACAGGATGACCTGCCGCT  360
L N G Q G W P P N P W Y P G T G T  139
TCAATGTCAGGTTGCGCTGCCCTGCTACCTCCCTGCTCTGGTGGCGACGCTTTGGGAG  420
G I A P S Y I A P V T T P A T P L E N G  159
GAATGACGAGTTATATAGGCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT  480
L N P K R E S G V E A S T P S D Q S G  179
TCAACCCTAAAAGAAGAGAAAGGAGCCTAGGAGGCTACAGCAAGCAAGCAAGCAAGCAAG  540
S G E E D S A A R L R L R L K R L Q R N R  199
GTGTTGGAGGGAGGTTCAGGGCCGAGGTTAGGTGGTAAGGGAGGGAGGGAGGGAGGGAGGG  600
T S F T P E Q I E A L  219
CATCCTTCTACACACAAGAAATAGAAGCTTT  632
**At-Scr**

Incomplete mRNA sequence of *At-Scr* gained by assembling a 5' RACE-PCR fragment with the fragment isolated by Abzhanov et al. (2001) (underlined).

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  CATAATTCATCAAATATGGGAGGTGTTTTCTGCGGGAAAAATTATAGCTGCTCCACAAA  60
    M S S Y Q F V N S L T S C Y S Q  16
     TCAAAACGGGACTTATGCTAGTCTGATGATGAGTGCACTCGTGTGATTAGCT  120
    A R Q D P T A Q D Y Y T P P V Q A Y G N  36
    AGCAGGGAGCCACCCGTCAGGCTACTACGCCGCGCTCCAGCTGCGGTTAGGGGCCCCCTATTCTCA  180
     C Y N G P S S P V Q P Q T Y G A P Y S Q  56
     CTGTTATAAAGGCGGACCTTCCCGCAGCGACAGGTATATGGGGCCCCCTATTCTCA  240
    H L Q N Q N G D P H F S S C S Q Q Q R  76
     ACATTTGCAAAATCGAATGGGACCCCTACATTATTTGATTGTTTCTCAACACAGAG  300
    L G H S L S S Q S S R T P T P S A T P V  96
     ACTTGGCATCTCCCCTGATTAGCTAGGCTGGGACCCCAACACATCGCCGACGCGT  360
     P S C K Y A E P T V H A A S P Q D L L  116
     ACCCGAATGCAGGTTACCGGGACTTTATGTTCTGATGCGGCCCTTATTCCCA  420
     T T S S S Q P P E S P E Q G D S P T P  136
     GACCAACAACGAGCTACTTCATGCGAGTTAGGGCCACGGAGATTCACCTTCAACACTCC  480
    P T S K S S T S R H L S P D S S P Q E S  156
     ACCCACTTTAAATTCGACTGCTGCCGACTATCGAGGGAGTAGCTTCAACACTTCAAAAGGCAATGCTGC  540
     S P Q Q S P S P Q S S T S Q K G N A A  176
     CAGTCTAGCAAGAGTTACCGGGACTTTATGTTCTGATGCGGCCACGGAGATTCACCTTCAACACTCC  600
     F Q G N P P Q I Y P W M R K V H V G Q N  196
     TTTTCAGGGCAAATCTGCTGCCGACTATCGAGGGAGTAGCTTCAACACTTCAAAAGGCAATGCTGC  660
    G V N S M G T K R Q R T S Y T R Y Q T  216
     TGGTGTAATCCGCTGGAGGAACAAAGCGCCAGAAGACATCTACCGCCCTTACGCAAC  720
    L E L E F H F N R Y L T R R R R I E  236
     ATGGAGGCTCGAGAAGAGATTACGTCTTACGAGGAGTAAGGTGTGTCGATTTCATGCGGCCCTTGT  780
     I A H A L C L T E R Q I K I  250
     GATAAGCTCATGCCCCCTGCTGCTGACAGAAGACAAATATACCA  823
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**At-Ubx**

Complete coding region plus additional 5' and 3' UTRs of *At-Ubx*, isolated by degenerate PCR and following 5' and 3' RACE-PCR.

```
    TAGGGTGAGAAAAATATGCACCCGGCTTGGACGTAACAAATATCATCACCCTCCCTTGCA  60
    TTCTCTCGGTGCTGCTGACTCAGCATCAACCCTCTGGGTTGATAAGGTGTGTCGATTTCATGCGGCCCTTGT  120
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Summary

During the last decade, the molecular examination of the spider segmentation process has provided exciting new insights into the question of the origin and evolution of segmentation. Spiders have been shown to utilize two signaling pathways to generate their segmental pattern that are also involved in segmentation in vertebrates. This has been interpreted as an indication that there might be a common origin of segmentation that dates back to the last common ancestor of bilateral symmetrical animals, the “Urbilateria”.

However, this data was so far only based on the segmentation process in the posterior body part of the spiders, the opisthosoma. The earlier segmental patterning process in the anterior, the spider prosoma, has remained unstudied. It is therefore the aim of this thesis to provide some insights on the prosomal segmentation mechanism by studying segmentation genes in the early embryo of *Achaearanea tepidariorum*. The first part of this thesis demonstrates that the anterior patterning mechanism is fundamentally different from the posterior patterning mechanisms, since the prosomal segments are not generated sequentially, but more or less simultaneously. The second part of this thesis takes a closer look at the role of one particular segmentation gene, the gap gene *hunchback (hb)*. In insects, *hb* is required for the formation of an adjacent set of segments through the regulation of downstream target genes of the pair rule and segment-polarity class. In addition, *hb* is a major regulator of Hox genes in insects and it has even been suggested that this is the ancestral role of *hb*. However, to date *hb* function has only been analyzed in insects. The here presented work surprisingly shows that *hb* acts like a gap gene during anterior segmentation of the spider, a non-insect arthropod. The leg-bearing segments L1, L2 and L4 are missing after down-regulation of At-*hb* via RNAi and *hb* is required for the correct organization of segmentation genes in this region of the embryo. Even more surprisingly, At-*hb* does not control Hox gene expression in the spider, which thus does not support the assumption that this is the ancestral role of *hb*.

These findings suggest that anterior spider segmentation utilizes a *Drosophila*-like genetic mode, in that a field of cells is subdivided into segmental units, while a vertebrate-like mechanism involving *Wnt8* and Notch/Delta signaling is used to pattern posterior segments. This supports the assumption that short germ arthropods employ two distinct mechanisms to segment their anterior and posterior body parts.
Zusammenfassung

Im letzten Jahrzehnt hat die molekulare Erforschung der Spinnensegmentierung zu erstaunlichen neuen Ansichten zur Frage nach dem Ursprung und der Evolution des Segmentierungsprozesses geführt. Es wurde nachgewiesen, dass Spinnen zwei Signalwege zur Erzeugung ihrer Segmente verwenden die auch an der Segmentierung in Vertebraten beteiligt sind. Dies wurde als Hinweis auf einen möglichen gemeinsamen Ursprung der Segmentierung gedeutet, welcher zu dem letzten gemeinsamen Vorfahren der bilateralsymmetrischen Tiere zurückreicht, dem „Urbilateria“.


Der erste Teil dieser Arbeit weist nach, dass der anteriore Musterbildungsprozess fundamentale Unterschiede zum posterioren Musterbildungsprozess aufzeigt, da die prosomalen Segmente nicht sequenziell gebildet werden, sondern mehr oder weniger gleichzeitig. Im zweiten Teil dieser Arbeit wird die Rolle eines bestimmten Segmentierungs-gens, die des Gapgens hunchback (hb), näher betrachtet. In Insekten wird hb für die Ausbildung einer Reihe benachbarter Segmente durch die Regulation von nachgeschalteten Zielgenen der Pararegelgen- und Segmentpolaritätsgenklasse benötigt. Zudem ist hb ein wichtiger Regulator von Hoxgenen, und es ist sogar vorgeschlagen worden, dass dies die ursprüngliche Funktion von hb ist. Bis heute wurde die Funktion von hb jedoch nur in Insekten untersucht. Die hier vorgelegte Analyse von hb in der Spinnensegmentierung zeigt überraschenderweise, dass hb in der Rolle eines Gap Gens auch an der anterioren Segmentierung außerhalb von Insekten beteiligt ist.

Die laufbeintragenden Segmente L1, L2 und L4 fehlen nach Knockdown der Genfunktion von At-hb durch RNAi und zudem scheint At-hb für die ordnungsgemässe Expression von Segmentierungsgenen in dieser Region benötigt zu werden. Im Gegensatz zu Insekten reguliert hb jedoch nicht die Hoxgenexpression in der Spinne. Diese Ergebnisse legen nahe, dass die anteriore Spinnensegmentierung einen genetischen Modus ähnlich Drosophila verwendet, wo ein Zellfeld in segmentale Einheiten aufgeteilt wird. Die posteriore Spinnensegmentierung involviert dagegen
ähnlich wie in Vertebraten Wnt8 und den Delta/Notch Signalweg. Dies unterstützt die Annahme, dass Kurzkeim Arthropoden zwei unterschiedliche Mechanismen für die Segmentierung ihrer anterioren und posterioren Körperregionen verwenden.
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Erklärung


Köln, November 2008

Evelyn Schwager