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The $Wnt$ gene family in evolution
and development:
insights from Chilopoda

A thesis submitted in partial fulfilment of the requirements
of the National University of Ireland, Galway
for the degree of Doctor of Philosophy

by
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Submitted December 2012
Supervised by Prof. Wallace Arthur and Dr. Gerhard Schlosser

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Abstract

The importance of a relatively small number of highly conserved genes and signalling systems, which can be co-opted to many developmental functions over evolutionary time, is a central finding in the field of evolutionary developmental biology. As the genes of the Wnt family are prominent members of this “genetic toolkit”, the study of how these genes operate in various taxa has the potential to yield useful insights into how development and evolution interact. This thesis tackles such issues by examining the development of centipedes, focusing on the developmental roles played by Wnt genes.

First, a comparative study of limb development, segmentation and Wnt1 expression in three chilopod species was performed. Second, an analysis of Wnt expression in the forcipular segment of Strigamia maritima, a unique feature and an interesting case study for evolution in body patterning, was performed. Third, the roles of Wnt genes in the linked developmental processes of posterior development and segmentation in Strigamia maritima were investigated. This analysis included both detailed expression studies and newly-developed functional techniques based on chemical perturbation of the Wnt signalling system. Fourth, a census of Wnt genes present in Strigamia maritima and thorough analyses of their expression were performed, paying particular attention to the combinatorial action of these genes.

Several interesting findings have resulted from this study, including the conservation of posterior development functions in Strigamia, the presence of non-canonical segment boundary formation in centipedes and the involvement of Wnt genes in many developmental functions, constituting a mosaic of conserved and novel ones.
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Declaration

This thesis has not been submitted in whole or in part to this or any other University for any other degree and is, except where otherwise stated, the original work of the author.

Signed

Luke Hayden
Chapter 1: Introduction

Adult *Strigamia maritima* female
1.1 Theoretical Background

1.1.1 The role of development in evolutionary theory

Conceptions of the origin of biological organisms have changed greatly over time. Though earlier theories of common ancestry of life-forms and of change from one form to another existed, the theory of evolutionary change mediated by natural selection proposed by Darwin and Wallace revolutionised the biological sciences (Darwin, 1859). By the mid-twentieth century, additional theoretical contributions, built upon this foundation and incorporating a better understanding of heredity, had crystallised as the “Modern Synthesis”. This synthesis envisaged evolution in statistical terms, often making use of the assumption that macroevolution is not different in process from microevolution and greatly emphasising the importance of natural selection (Kutschera & Niklas, 2004). However, despite the work of some early proponents of evolution, most notably Ernst Haeckel and his associates, little attention was played to developmental biology in the formulation of the modern synthesis (Hoßfield & Olsson, 2003).

Evolutionary developmental biology, evo-devo for short, can be seen as an attempt to address this imbalance, based on the recognition that the evolution of organisms cannot be fully understood except in the context of the developmental processes by which evolutionary change must be mediated (Raff, 2000). This interest in the interface of developmental and evolutionary biology arose in the 1980s, with the most important driving factor behind it being the discovery of the conservation of the homeobox (Arthur, 2002a). Evo-devo can be conceptualised either as a radical departure from the modern synthesis (Müller, 2007) or, more modestly, simply as a shift of emphasis (Pigliucci, 2007).
1.1.2 Evo-devo and the “genetic toolkit”

In evo-devo, empirical work is performed across a wide range of scales and contexts and with a range of investigative approaches. Together with purely theoretical work, this diversity of methodology has produced intriguing results and opened new lines of enquiry that had been neglected under the modern synthesis. Examples of research programmes derived from evolutionary developmental biology include the origin of “evolutionary novelties”, the effects of developmental bias and constraint on evolution, the role of modularity in evolution and the evolutionary origins of complexity (Arthur, 2004, Müller, 2007). However, one particular finding has assumed a central place in this nascent field (Baguñà & Garcia-Fernàndez, 2003). This is the discovery that the same limited “developmental toolkit” acts to underpin developmental processes across the animal kingdom and that elements of this “toolkit” have been co-opted multiple times in different lineages in the development of an immense array of different structures (Pirez-DaSilva & Sommer, 2003, Carroll, 2008).

This “toolkit” includes the Hox genes, the BMP pathway, the Notch signalling system and the Wnt signalling system, among other elements. The Hox genes, for example, are generally involved in regionalisation of the body along the anteroposterior axis, but have also been co-opted into a range of other processes, the characteristic horns of some beetles being a salient example (Wasik et al., 2010). Given the fact that some of these “toolkit genes” are members of multi-gene families, it is essential to consider the evolution of developmental systems in the context of the evolution of these gene families (De Robertis, 2008a). The Wnt gene family is an excellent example of such an evolutionarily and developmentally important group of genes, playing crucial roles in
shaping the development of a wide range of animals (Van Amerongen & Nusse, 2009). The arthropods are an eminently suitable taxon upon which to focus a study of this gene family, as they display a large degree of morphological variation and have diverse developmental systems. Accordingly, the study of these genes and their roles in the arthropods offers an opportunity to gain a better understanding of how developmental processes evolve.

1.2 Wnt Signalling with Particular Reference to Arthropoda

1.2.1 The basics of Wnt signalling in arthropods and other animals

The Wnt gene family derives its name from the wingless (wg) gene of Drosophila melanogaster and the INT-1 (mouse mammary tumour virus integration site 1) gene of Mus musculus. Since the discovery of the important developmental roles of members of this gene family and of their presence across the animal kingdom, much research has been focused on attempts to understand the signalling pathways that they participate in. After giving a brief overview of Wnt signalling mechanisms, this review will examine the developmental roles of Wnt genes in the arthropods. The Wnt genes code for secreted glycoproteins. These contain 22 characteristic cysteine residues, which ensure proper protein folding and secretion, and usually consist of 350-400 amino acids (Cadigan & Nusse, 1997). After being palmitoylated via Porcupine, these glycoproteins are secreted and diffuse as classical morphogens (Gonzalez et al., 1991, Strigini & Cohen, 2000, Mikels & Nusse, 2006). They then play roles in multiple signalling pathways (Logan & Nusse, 2004). The best understood of these, known as the canonical Wnt pathway, was first identified by mutant screening in Drosophila.

Wnt protein is first secreted from cells, a process requiring the functions of both
gooseberry and wntless (Noordemeer et al., 1995, Banziger et al., 2006). It then binds to
the transmembrane Frizzled (Fz) receptors of target cells, which, along with the
cofactor/receptor Arrow/LRP, transduce downstream events (Yang-Snyder et al., 1996,
Wodarz & Nusse, 1998). Downstream transduction is done by means of Dishevelled
(Dvl), which is differentially phosphorylated by this process (Yanagawa et al., 1995).
This leads to the binding of Axin to Dvl, preventing Axin from playing a role in the
“destruction complex” of cells not receiving Wnt signalling. This destruction complex
usually causes the breakdown of Armadillo/β-catenin (Arm) via its phosphorylation. In
Wnt target cells, the destruction complex does not function and β-catenin is localised to
the nucleus. Here, it affects the action of Groucho in repressing transcription factors of
the pangolin/TCF/LEF family (Akiyama, 2000, Brunner et al., 1997). Thus, canonical
Wnt signalling allows for the activation of TCF/LEF target genes.

Figure 1.1: Simplified view of canonical Wnt pathway in cells not receiving and cells
receiving Wnt signal.
1.2.2 Additional complexities of Wnt signalling

Simple linear representations such as that given in the previous section are oversimplifications. There are additional, less well-understood mechanisms by which Wnt signalling can function, many of which operate via the same receptors as canonical signalling (Tolwinski et al., 2003). One of these is via the so-called planar cell polarity pathway. This involves the induction of JNK activity by Dvl via Rac, with downstream effects on the cytoskeleton (Veeman et al., 2003, Rosso et al., 2005). Wnt signalling can also act by means of a calcium-dependent pathway, involving calcium flux increases caused by Wnt binding to Fz. These calcium ions can then alter the activities of CamKII, activating TAK1, thus stimulating NLK and resulting in the phosphorylation of TCF/LEF transcription factors, thus antagonising the canonical pathway (Hayward et al., 2008). Wnt signalling via Siah2 and APC can also antagonise the canonical pathway (Topol et al., 2003).

Additionally, Wnt proteins can bind to other cell surface receptors (Gordon & Nusse, 2006, Van Amerongen et al., 2008). The binding of Wnt to Ryk can activate Src and thus influence other signalling pathways. Wnt-Ror binding can cause inhibition of the canonical pathway and interact with the planar cell polarity pathway via JNK. The binding relationships of Wnt and Fz molecules are not simply one-to-one (Wu & Nusse, 2002, Carmon & Loose, 2010) and have been suggested to be context-dependent (Beermann et al., 2011). Additionally, there is a redundancy of Wnt ligands in some situations (Thorpe et al., 2005), whereby multiple genes seem to play the same role and can cause similar intracellular effects (Kühl, 2004).

Beyond Wnt ligands and Fz receptors, certain elements of different Wnt pathways are
shared with each other, with calcium flux being one example (Kohn & Moon, 2005). Other molecules that take part in Wnt signalling pathways also have entirely different biological functions. The adherence functions and the involvement in hedgehog signalling of β-catenin are good examples of this (Van de Wetering et al., 1997, Wu & Pan, 2010). Wnt signalling is also commonly intertwined with the Notch signalling pathway (Hayward et al., 2008). All of these interactions and complexities combine to create a picture of a complex and non-linear system, one with wide-reaching effects on animal development and other processes.

1.2.3 Evolution of the Wnt gene family

The Wnt gene family is present across the animal kingdom, with representatives not only in Bilateria, but also in basally branching taxa such as the Porifera (Windsor & Leys, 2010). Of note is that the common ancestor of the Metazoa seems to have possessed a large complement of Wnt genes, a situation that has been preserved in some cnidarians (Kusserow et al., 2005), but many taxa possess a much smaller complement of Wnt genes. Reconstructions of Wnt gene evolutionary history suggest that the ancestral bilaterian (Urbilateria) possessed a large number of Wnt subfamilies (Prud'homme et al., 2002, Cho et al., 2010). This initial number has been secondarily reduced in many taxa, with secondary duplications further complicating the picture (Konikoff et al., 2010). This trend of secondary gene loss is readily apparent within the arthropods, for example in holometabolous insects such as Drosophila (Murat et al., 2010). The pattern of initial diversity and secondary loss seen in Wnt genes mirrors that seen for other important groups of developmental genes, such as the Hox genes (Lemons & McGinnis, 2006).
Such patterns of evolutionary change in important developmental factors are very interesting from the perspective of evolutionary developmental biology. As Wnt genes are crucial to normal animal development, the study of how their functions have evolved may shed light on the genotype-to-phenotype relationship and how it may change over broad scales. The great disparity and diversity of the arthropods (referring to morphology and species numbers respectively) make them an excellent taxon in which to examine such questions. Thus, the study of the Wnt genes in arthropods, dealing not only with what genes have been lost in what taxa, but also with the description of the different expression patterns and functions that these genes possess in different arthropod taxa, may shed much light on the relationship between phenotypic and genetic evolution mentioned previously. A summary of the current state of knowledge of the roles of each of these genes in the arthropods is given as follows.

1.3 Wnt Genes in Arthropods: Expression and Function

Wnt1

This gene, first identified as the segment polarity gene wingless in Drosophila, has lent its name to the entirety of the Wnt gene family. The Wnt1 subfamily (all Wnt1
orthologues) is significantly better understood than the other subfamilies of Wnt genes, as expression data have been obtained from a wide variety of arthropods and extensive functional analyses have been performed in Drosophila. The presence of Wnt1 is highly conserved across the group.

Most arthropods surveyed display certain similarities in their expression patterns for this gene. In a fly, Drosophila melanogaster, Wnt1 is expressed as many bands of expression, appearing simultaneously, prior to formation of segmental furrows, each corresponding to a single segment. These bands of expression are immediately anterior to bands of en expression, with the en-Wnt1 boundary eventually forming the parasegmental boundary (Baker, 1987, Sampedro et al., 1993). Additional expression is present in the neural precursors and in eye and wing imaginal discs. Wnt1 expression in a moth, Bombyx mori, another long-germ insect, is different at very early stages, with expression in a large posterior domain covering much of the embryo, but later resolving into a pattern which closely resembles that of Drosophila (Nakao, 2010).

In a short-germ insect, the beetle Tribolium castaneum, similar patterns are observed, but with the stripes of expression, discontinuous at the ventral midline, appearing gradually, in an anterior-to-posterior fashion, preceding the formation of segmental furrows and resolving into more limited domains in the developing limbs. Additional expression zones are present at the posterior of the embryo and in the optic primordia (Nagy & Carroll, 1994, Choe & Brown, 2009, Bolognesi et al, 2008a).

Four other short-germ insects, a cricket, Gryllus bimaculatus, a grasshopper, Schistocerca americana, a bug, Oncopeltus fasciatus, and a mayfly, Ephoron leukon,
display a highly similar expression pattern, albeit with minor differences in expression
surrounding the stomodeum and in the optic lobes (Dearden & Akam, 2001, Liu et al.,
2006, Miyawaki et al., 2004, Angelini & Kaufman, 2005, Dong & Friedrich, 2005,
O'Donnell & Jockusch, 2010). Expression of Wnt1 in eyes and in head development is
quite conserved among insects (Friedrich, 2003).

In a branchiopod crustacean, *Triops longicaudatus*, Wnt1 is expressed in segmental
stripes, but restricted to the ventral region, with additional expression in the limbs
(Nulsen & Nagy, 1999). In a mysid crustacean species, *Mysidium columbicae*, Wnt1 is
expressed as segmental stripes, but dorsally only. Additional expression is present in
heart, muscle and retina, similar to in *Drosophila* (Duman-Scheel et al., 2002).

*Wnt1* expression has also been examined in myriapods. In a centipede, *Lithobius
atkinsoni*, Wnt1 expression during germ-band growth closely resembles that of short-
germ insects, with some minor differences in anterior expression (Hughes & Kaufman,
2002a). In a millipede, *Glomeris marginata*, Wnt1 is also expressed as a single stripe in
each segment, but this expression is not present in dorsal tissue. As in other taxa, the
expression gradually retreats into the limbs. The gene is also expressed in the optic
lobes and at the embryo’s posterior (Janssen et al., 2004, 2006). In a spider, *Cupiennius
salei*, Wnt1 is also expressed as segmental stripes, but the pattern differs from that seen
in insects in certain details (Damen, 2002). However, another spider, *Parasteatoda
tepidariorum*, displays a more divergent expression pattern for this gene, as Wnt1
transcripts are not detected in the posterior region and segmental expression is restricted
to developing appendages. There is also later expression in the labrum and hindgut
(Janssen et al., 2010).
In *Drosophila*, multiple functions of Wnt1 have been revealed (Gonsalves & DasGupta, 2009). Perhaps the best known of these is its function as a “segment polarity” gene, establishing segmental boundaries via interactions with engrailed and hedgehog signalling (Siegfried et al., 1994, Baker, 1987). In leg imaginal disc development, it both provides a ventralising signal and influences cell proliferation (Wilder & Perrimon, 1995). This gene is also involved in dorso-ventral patterning of the developing wing (Neumann & Cohen, 1997). It plays roles in neuronal patterning (Patel et al., 1989, Packard et al., 2002), heart development (Wu, 2010) and tracheal development (Llimargas & Lawrence, 2001). Additionally, it operates in eye development, a role which is thought to be conserved across the insects (Friedrich & Benzer, 2000, Friedrich, 2003).

In *Tribolium*, Wnt1 also seems to be required for the establishment of segmental boundaries and in leg development (Bolognesi et al., 2008b, Grossmann et al., 2009). The most severely affected Wnt1-perturbed embryos are highly compacted, indicating that this gene may also play roles in anteroposterior extension (Ober & Jockusch, 2006). In both *Tribolium* and *Drosophila*, the functional relationship between Wnt1 and en is seen to be conserved (Oppenheimer et al., 1999). However, in *Gryllus*, embryos are still capable of forming segment boundaries in the absence of Wnt1 transcripts, and it has been proposed that other Wnt genes are also involved in this function in this species, and perhaps in the wider group (Orthoptera) to which it belongs (Miyawaki et al., 2004). In *Glomeris*, it has been suggested that segment boundary formation is, in at least some regions, Wnt1-independent (Janssen et al., 2008). Evidence has also been presented that changes in Wnt1 expression underlie differences in head morphology between insect groups (Liu et al., 2006).
**Wnt2**

This gene is less well known than most other arthropod Wnt genes and seems to have been lost in the lineage leading to the insects. In addition, there is some evidence (based on an inability to amplify it with degenerate PCR) that the gene may also be absent from *Glomeris* (Janssen et al., 2010). The only arthropod for which expression data have been obtained for Wnt2 is Parasteatoda, where it is seen to be expressed late in embryogenesis, in the ocular region of the cephalic lobes only (Janssen et al., 2010). No functional data are available for this gene in arthropods.

**Wnt3**

No Wnt3 orthologues have been retrieved from arthropods to date, and this gene is thought to have been lost in the lineage leading to the protostomes.

**Wnt4**

This gene has been lost in Tribolium and Drosophila; it has been hypothesized to have been lost in the lineage leading to insects. It may also be absent in Glomeris (Janssen et al., 2010). The only arthropod from which expression data are available is Parasteatoda, where it is expressed in a small number of cells at the posterior of the germ band during late embryogenesis (Janssen et al., 2010). No functional data are available for this gene in arthropods.

**Wnt5**

The presence of this gene is widely conserved across the Arthropoda. Its activities have been quite extensively examined across the taxon. In Drosophila, Wnt5 is expressed in the gnathal segments, in each neuromere of the ventral nerve cord, the labrum, the
mesoderm and the limb primordia (Fradkin et al., 1995). In Tribolium, Wnt5 is again expressed in a segmentally reiterated fashion, first as stripes in new segments, then resolving into more limited expression domains. Expression is also present at the posterior of the embryo (Bolognesi et al., 2008a). In Parasteatoda, medial segmental stripes, with a midline break, are present, along with additional expression in appendages, at the stomodeum and in each optic lobe (Janssen et al., 2010). In Cupiennius, segmentally reiterated ventral medial stripes are present, along with expression in the appendages, posterior growth zone, head and labrum (Damen, 2002). Functional analyses in Drosophila have determined that this gene is involved in TCF/LEF-independent formation of axon tracts (Wouda et al., 2008) and that it plays a role in salivary gland migration with derailed (Harris & Beckendorf, 2007). Although it does not seem to play a role in segmentation in Drosophila (Fradkin et al., 1995), it has been posited to fulfill part of the canonical role of Wnt1 in segmentation in Glomeris (Damen et al., 2002).

**Wnt6**

The presence of this gene is widely conserved, being present in representatives of the insects, crustaceans, myriapods and chelicerates (Janssen et al., 2010). In Drosophila, Wnt6 is expressed first in the foregut and midgut. It later shows Wnt1-like expression, including in some imaginal discs (Janson et al., 2001). In Tribolium, it is expressed in segmental stripes, with stronger expression in the limbs. There is anterior expression at the stomodeum and also a posterior expression zone (Bolognesi et al., 2008a). Glomeris also has a segmentally reiterated expression pattern for this gene, with expression as four patches in each segment, all anterior to en expression. Additional expression is present at the stomodeum, surrounding the head region and at the embryo’s posterior
(Janssen et al., 2010). Little is known about the functions of this gene, but based on its expression pattern, it has been proposed that it operates to fine-tune Drosophila Wnt1 function (Van Amerongen & Nusse, 2009).

**Wnt7**

The presence of this gene is also quite highly conserved. It is found in all four traditional major arthropod groups (Janssen et al., 2010). In Drosophila, where it is known as Dwnt2, its expression is segmentally reiterated, but present in only some of the embryo's segments. Additional expression is present in the gonads and around the tracheal cells (Kozopas et al., 1998, Llimargas & Lawrence, 2001). The expression of this gene is also segmentally reiterated in Tribolium, with two patches per segment (Bolognesi et al., 2008a). In Glomeris, expression also follows a segmental pattern, with a strong patch of expression distally and weaker expression more proximally, near the limb buds (Janssen et al., 2010). Parasteatoda possesses two Wnt7 orthologues. The first of these is expressed in the posterior only, but the second has a segmentally reiterated pattern, with two distal expression patches in each segment (Janssen et al., 2010). Functional analyses of this gene have been conducted only in Drosophila. Here, it has been shown to play a role in salivary gland development, along with Wnt9 and Wnt1 (Harris & Beckendorf, 2007). It also functions, most likely via the canonical pathway, in tracheal development in cooperation with Wnt1 (Llimargas & Lawrence, 2001). In addition, it operates in the development of certain sex-specific structures, as it is required to induce some sex-specific cell types (Kozopas et al., 1998).
**Wnt8**

This gene is known to play important roles in posterior development in vertebrates (Shimizu et al., 2005). There is good evidence that it has some similar functions in many arthropod taxa. The gene itself is not present in all arthropods studied, and was suspected to have been lost in the lineage leading to the insects until phylogenetic analyses revealed that the gene known as *WntD* in *Drosophila* and *Tribolium* was a member of this subfamily. In *Drosophila*, the expression patterns are highly divergent from those observed in other taxa, with early expression in both poles of the pre-cellular blastoderm, then later expression ventrally, abutting mesectoderm and ventral neuroectoderm (Ganguly et al., 2005). In *Tribolium*, *Wnt8* is expressed very early on in the posterior region, later resolving into slightly more limited expression zones in this part of the embryo (Bolognesi et al., 2008a). In *Parasteatoda*, early expression takes the form of a large posterior zone, along with an additional, more anterior ring-shaped domain. Later, the posterior expression resolves into a more limited domain, and additional expression zones emerge in developing segments (McGregor et al., 2008). In *Glomeris*, its transcripts are primarily found in two anterior domains in early embryos, along with an additional, weaker, posterior expression zone (Janssen et al., 2010).

Functional analyses lend strong support to the concept that this posterior expression zone, as found in *Parasteatoda, Tribolium* and *Glomeris* plays a role in posterior determination and growth. Knock-down experiments on *Wnt8* in *Tribolium* result in truncation of the embryo and the loss of abdominal segments (Bolognesi et al., 2008b). This posterior identity role seems to be mediated by canonical Wnt signalling (Bolognesi et al., 2009). In *Parasteatoda, Wnt8* has been shown to be required for the establishment and development of the opisthosomal (posterior) segments (McGregor et
In *Drosophila*, however, as might be predicted from differences in expression patterns in relation to the other arthropods studied, no evidence is found for a similar role for *Wnt8*, a finding which fits with the unusual nature of posterior development in *Drosophila*. In addition, this gene has been shown to play a role in specifying dorsal identity via non-canonical Wnt signalling mechanisms (Ganguly et al., 2005).

**Wnt9**

This gene may be absent from *Glomeris*, *Parasteatoda* and *Cupiennius*, but a full genome sequence would be required to verify this (Janssen et al., 2010). In *Drosophila*, *Wnt9* displays a segment polarity pattern, overlapping with the expression of *Wnt1*. Additional expression is found in the labrum, foregut, nerve cord and ocular segment primordia (Graba et al., 1995). In *Tribolium*, a segmentally reiterated pattern is absent, but expression is still found in the gut, specifically at the border of endoderm and ectoderm (Bolognesi et al., 2008a). Functional analyses of this gene in *Drosophila* have revealed its many developmental roles in this species. Consistent with its segmental expression, it is known to play roles in the segmentation process, operating as an antagonist of *Wnt1* (Gieseler et al., 1999). However, despite this antagonistic role in segmentation, *Wnt9* can rescue *Wnt1* loss-of-function phenotypes in the antennae and halteres (Gieseler et al., 2001). *Wnt9* also plays a TCF/LEF-dependent role in salivary gland development (Harris & Beckendorf, 2007). However, the role it plays in regulation of cell motility in ovarian morphogenesis appears to be TCF/LEF independent (Cohen et al., 2002). It is also involved in synapse formation and innervation of muscles (Inaki et al., 2007). The above indicates a broad range of functions and mechanisms of activity for this gene in *Drosophila*. Further data would reveal the degree to which this is reflected in other arthropod taxa.
**Wnt10**

The activities of this gene are not well-documented, with expression data available from only two taxa and no functional data available. The gene has not been confirmed to be present in either *Glomeris* or *Parasteatoda*. In *Drosophila*, it is expressed in parts of the mesoderm, the gut and the central nervous system, along with possible low expression levels in the imaginal discs (Janson et al., 2001). In *Tribolium*, it is expressed in a segmentally-reiterated pattern, with stronger expression in developing limbs and additional expression in the ocular segment. There is no posterior expression zone (Bolognesi et al., 2008a). No functional analyses have been performed on this gene in arthropods.

**Wnt11**

The presence of this gene is quite widely conserved, though with its absence from the genomes of *Drosophila* and *Anopheles*, it is likely to have been lost in the lineage leading to the Diptera. In *Tribolium*, it is primarily expressed in the tips of developing appendages, with additional expression in serosal cells and some antennal and labral cells. Expression in appendages later resolves into proximal rings of expression, along with a distal central patch (Bolognesi et al., 2008a). In *Glomeris*, early expression is in the form of a ring surrounding the posterior. Later, the gene is also expressed in the developing appendages (Janssen et al., 2010). In *Parasteatoda*, two paralogues of this gene are present. For the first of these, no embryonic expression was detected. The second is expressed in a posterior zone and in developing appendages (Janssen et al., 2010). No functional analyses are known to have been performed on this gene in arthropods.
**Wnt16**

This gene is absent from most insect genomes, with the aphid *Acyrthosiphon* being the sole known exception, though the sampling bias of insect genomes towards the Holometabola may partially account for the lack of other known insect taxa with a *Wnt16* orthologue (Shigenobu et al., 2010). In *Glomeris*, it is expressed as segmentally reiterated stripes, anterior to *en* expression, with additional expression in the distal tips of developing appendages (Janssen et al., 2010). *Wnt16* expression in *Parasteatoda* initially takes the form of a broad anterior domain. Later expression is somewhat similar to that in *Glomeris*, with stripes in newly formed segments and dots in appendages. However, unlike in *Glomeris*, a posterior expression domain is also present (Janssen et al., 2010). It is noteworthy that expression stripes of *Wnt16* in both species start as complete stripes without the midline break that is common to the segmental expression of other *Wnt* genes in a great number of arthropods. No functional analyses have been performed on this gene in arthropods.

**WntA**

This gene has been found to be present in representatives of all four arthropod subphyla, despite being absent in *Drosophila*. In *Tribolium*, it is expressed in very early development at the future posterior of the embryo. Later, it is expressed in a segmentally reiterated pattern, with one stripe per segment, including a midline gap, anterior to *en* expressing cells. Additional expression is found around the stomodeum and in a posterior zone (Bolognesi et al., 2008a). In *Glomeris*, *WntA* expression is not altogether dissimilar, and displays a segmentally reiterated pattern of expression zones in the ventral neuroectoderm, anterior to *en* expression. Additional expression is also present in a strong posterior domain and in the developing heart (Janssen et al., 2010).
In *Cupiennius*, no segmentally reiterated expression is present, but there is a posterior expression region, as in *Glomeris* and *Tribolium*. Additional expression is present in the chelicerae and spinnerets (Damen *et al.*, 2002). The high degree of conservation of this posterior expression zone may indicate that it plays important roles in the control of posterior identity and growth in many arthropods.

1. 4 *Wnt* Functional Themes in Arthropods

1.4.1 Posterior development

The role of *Wnt* genes in posterior growth and development is both well-documented and highly conserved. Many records exist of the specification of posterior identity by *Wnt* genes in non-arthropod systems (Peterson & Reddien, 2009, Martin & Kimelman, 2009). Within the arthropods, much of the evidence for roles of *Wnt* genes in these processes is either based on perturbation of the canonical Wnt signalling pathway or is specific to *Wnt8*. Evidence of the former sort includes support for such roles of Wnt signalling in *Tribolium, Oncopeltus* and *Gryllus* (Angelini & Kaufman, 2005, Bolognesi *et al.*, 2008b, Bolognesi *et al.*, 2009, Beermann *et al.*, 2011, Miyawaki *et al.*, 2004). Evidence of the latter sort has been presented for *Tribolium* and *Parasteatoda* (Bolognesi *et al.*, 2008b, McGregor *et al.*, 2008). Additional circumstantial evidence for a conserved role of *Wnt* activity in patterning the arthropod posterior is provided by the many records of posterior expression of various *Wnt* genes in many arthropod taxa, as detailed previously in this chapter. It has previously been suggested that, not only is *Wnt*-based posterior identity and growth common to most arthropods, but that this system is part of a conserved posterior network, including Notch signalling, that is shared with vertebrates (Damen *et al.*, 2007, McGregor *et al.*, 2009). Other authors have disputed this, however (Kainz *et al.*, 2011).
1.4.2 Segmentation

Discussion of the roles of developmental genes in segmentation should make a clear distinction between genes that play roles in the segmentation process itself and genes that are involved in the development of structures that are segmentally reiterated. This distinction is particularly important in the arthropods, in which segmentally reiterated structures abound (Minelli & Fusco, 2004). The role of Wnt1 in segmentation processes is well recorded, as mentioned previously; but the roles of other Wnt genes in these processes are less well understood. However, the abundance of segmentally reiterated Wnt expression patterns suggests roles in segmentation for many of these genes. Additionally, the differences between knockdown phenotypes of Wnt1 and those of Wnt pathway components, may be said to be suggestive of a role for other Wnt genes in segmentation (Bolognesi et al., 2008a). Additionally, many Wnt genes are expressed in segmental patterns after the segments have already formed. As has been hypothesized in the case of Wnt6 in Drosophila, it is possible that many of these genes act to fine-tune the expression and activities of Wnt1 (Van Amerongen & Nusse, 2009). It is also possible that genes with such segmentally reiterated patterns act to pattern segmentally reiterated structures, but not the segments themselves.

1.4.3 Other structures

At least in Drosophila, Wnt genes have been demonstrated to play roles in the development of many structures, including genitalia, heart, wings and salivary glands. However, with little comparative data available, the evolutionary origins of such Wnt functions are unknown.
1.5 The Chilopoda

1.5.1 The centipedes as a choice of study taxon

In order to be able to reliably build a better understanding of how Wnt signalling operates in development and how the developmental roles of Wnt genes evolve, it is necessary to investigate a group whose study can yield useful results. The Chilopoda, the centipedes, are an excellent candidate taxon for such an examination. This group is traditionally considered to represent a Class within the Subphylum Myriapoda. Despite being a generally under-studied taxon, the myriapods represent wide variation in morphology. Of the four classes within the myriapods, Pauropoda, Symphyla, Chilopoda and Diplopoda, the latter two contain the vast majority of species. In the Diplopoda, attention has already been paid to the developmental roles of the Wnt genes. This includes examination of the embryonic expression patterns of Wnt1, Wnt5, Wnt6, Wnt7, Wnt8, Wnt16 and WntA, as described previously (Damen et al., 2002, Janssen et al., 2004, 2006, 2010). These data allow for the possibility of fruitful comparisons between Wnt gene activity in Chilopoda and Diplopoda. Such comparisons allow not only for the identification of differences between the two groups, but due to the Chilopoda and Diplopoda being maximally divergent within the Myriapoda (Regier et al., 2008, 2010), common features of the diplopod and chilopod Wnt signalling systems can be reasonably considered to be representative of the ancestral myriapod state.

1.5.2 The biology of centipedes

Studies on a particular taxon need to be placed in the context of the biological diversity and natural history of that group, both in order to provide the necessary context and to identify what scientific questions can be answered by the study of the group in question.
The following account will be kept brief, because comprehensive analyses of centipede biology can be found in Lewis (1981) and Minelli (2011), with the relevant terminology reviewed by Bonato et al. (2010). The Chilopoda is a group of over 3,000 species, divided into six orders: Scutigeromorpha, Lithobiomorpha, Craterostigmomorpha, Devonbiomorpha (extinct), Scolopendromorpha and Geophilomorpha. Doubt has been expressed as to the phylogenetic relationship of scutigeromorphs to other centipedes, but molecular analyses have confirmed the monophyly of the group (Edgecombe & Giribet, 2007). All chilopods share a number of common features, including the unique venom-delivering forcipules, a head composed of five segments with three pairs of mouthparts and one pair of antennae; and a segmented trunk with one pair of legs per segment (see Figure 1.3). The centipedes are predatory animals, a fact which is reflected in their general morphology. However, great variation, albeit developmentally constrained, exists in the number of leg-bearing segments, ranging from 15 to 191 (Minelli et al., 2000). Spiracle position, leg morphology, body shape and forcipular morphology are also key traits separating the orders of centipedes (Dugon & Arthur, 2012).
Figure 1.3: Segment and appendage identity in insect, chilopod and arachnid body plan.

The fossil record of the centipedes, despite its relative poverty, also provides additional context in which to consider studies of this group (Shear & Edgecombe, 2010).

However, the fact that some samples have been mistakenly assigned to the Chilopoda make this record difficult to interpret (Almond, 1985). The oldest centipede fossils date to the Silurian period, coinciding with an early invasion of the land by this group (Jerram et al., 1990). Early stem-group scutigeromorphs, in the form of the Crussolum genus, are present from the Carboniferous period, and the scutigeromorph record continues on into the Mesozoic period (Wilson, 2001, Anderson & Trewin, 2003). The scolopendromorph fossil record is also quite lengthy, extending back into the Carboniferous period (Wilson, 2003). The Devonbiomorpha is represented by a single
Devonian species, *Devonbius delta*, identified as a separate order on the basis of the morphology of the maxillipod coxosternae (Shear & Bonamo, 1988). Most fossil records of centipedes, however, are from later periods and are recovered from Cretaceous or Cenozoic amber, which is where the emergence of the geophilomorph crown-group is recorded (Edgecombe *et al.*, 2009).

1.6 The Aims and Approach of This Thesis

The body of work presented herein will seek to answer a number of questions relating to the development of centipedes, with the intention that the approach used will both shed light on the particular questions asked in each case and will help to build a better understanding of the ontogeny of this group. Particular attention will be paid to developmental roles of Wnt genes in this group, examining these genes, their expression and their functions in relation to a number of issues. These include the interaction between, and the combinatorial action of, the Wnt genes, a comparative analysis of segmentation and limb development in the centipedes, the examination of forcipular development in the context of Wnt expression and the status of the forcipule as a unique structure and posterior development in the light of possible ancient segment generation mechanisms. This list of issues encompasses a range of developmental roles played by Wnt genes and allows for the examination of the complexities of the Wnt signalling system from multiple perspectives. Attention will be paid to developing new functional techniques, based on chemical perturbation of the Wnt signalling system, to examine the developmental roles of these genes, thus allowing for greater certainty in relation to what roles these genes play in development. Effort will also be expended in opening up previously unstudied groups of centipedes to developmental analyses. The study species chosen are described in detail in Chapter 2. Approaches making comparisons within the
Chilopoda and with non-centipede arthropods will also be adopted. This comparative approach will allow for a broad evolutionary perspective. Finally, the most salient points arising from this study will be synthesised, shedding light on how development and evolution, the twin creative forces of life, interact.
Chapter 2:
Study species and general materials and methods

Brooding Strigamia maritima females with egg clutches
2.1 Study Species

This study involved work on three different species of centipedes, each belonging a different order of the five extant within the Chilopoda. These species were *Scutigera coleoptrata* (Scutigeromorpha), *Scolopendra subspinipes mutilans* (Scolopendromorpha) and *Strigamia maritima* (Geophilomorpha). The internal phylogeny of the Chilopoda has been investigated by Murienne *et al.* (2010), Edgecombe & Giribet (2007), Giribet *et al.*, (2001, 2006) and Edgecombe *et al.*, (2000). A simple phylogenetic tree of internal centipede phylogeny is depicted in Figure 2.1.

![Internal phylogeny of the Chilopoda, after Murienne et al., 2010](image)

**Figure 2.1:** Internal phylogeny of the Chilopoda, after Murienne *et al.*, 2010
2.1.1 *Strigamia maritima* (Leach)

2.1.1.1 Description

*Strigamia maritima* is a member of the order Geophilomorpha, a group that is characterised by an extended trunk with many segments, highly modified, “pliers-like” forcipules, the absence of eyes and a dorso-ventrally flattened head capsule. A large degree of variation in segment number is present in this order, ranging from 27 to 191 leg-bearing segments (Minelli et al., 2000). Apart from the outgroup family Mecistocephalidae, geophilomorphs share the trait, unusual among arthropods, of intraspecific variation in segment number, a trait which has instilled interest in this group as a model for evolution of arthropod segment number (Kettle & Arthur, 2000, Arthur & Chipman, 2005, Vedel et al., 2008, 2009, 2010, Hayden et al., 2012). Unlike most geophilomorphs, *Strigamia maritima* is a coastal species, distributed along the littoral zone of shingle beaches in northern Europe. This species lays eggs once a year, in late May-June, when the females excavate brood chambers and lay a clutch of eggs (Figure 2.6A), which they will incubate until hatching, after several weeks.

Development in *Strigamia* is epimorphic, whereby all of the trunk segments are developed in the embryo, with the last formed as the embryo hatches (Brena & Akam, 2012). The ecology and life history of this species have been described in detail by Lewis (1960).
**Figure 2.2:** Adult female *Strigamia maritima*

**Figure 2.3:** Life cycle of *Strigamia maritima*
2.1.1.2 Sampling and husbandry

*Strigamia maritima* is characterised by a long life cycle of 3-5 years and by a protracted developmental period. Thus, this species has never been bred in captivity. Therefore, clutches of eggs were sampled from wild populations in Brora, Scotland (2010) and Salthill, Ireland (2011 & 2012). The Brora sampling site is on a shingle beach between the villages of Brora and Golspie (coordinates: 57°98'N 3°91'W). The Gentian Hill sampling site is a shingle beach on the outskirts of Salthill in Galway, Ireland (coordinates: 53°15'N 9°07'W). The clutches were collected by excavating the upper shingle layer, under which solitary females are found, coiled around a clutch of 2-30 eggs. The adult female was then removed and the eggs taken using a small spatula and placed between filter papers soaked with locust embryo saline (LES). Alternatively, the eggs were placed into tubes of mineral oil (Sigma catalogue number 8410). Eggs were then raised to the desired stage at 15°C.

![Brora sampling site](image.jpg)

**Figure 2.4:** Brora sampling site. Image is derived from satellite imagery provided by Google.com.
Figure 2.5: Gentian Hill sampling site. Image is derived from satellite imagery provided by Google.com.

Figure 2.6: Sampling site with *Strigamia* clutch with attendant female (A) and collection of *Strigamia* eggs into culture dish (B).

2.1.2 *Scolopendra subspinipes mutilans* (L. Koch, 1878)

2.1.2.1 Description

This species is a member of the order Scolopendromorpha, the group containing the largest of the centipedes. The members of this order are characterised by the possession
of 21-23 trunk segments (with one notable exception being *Scolopendropsis duplicata* (Chagas-Junior et al., 2008, Minelli et al., 2009)), dorso-ventral flattening of the body, the presence of multiple ocelli and often extremely large size. *S. subspinipes mutilans* is found in China, with other *S. subspinipes* subspecies being found across South-East Asia. *S. subspinipes mutilans* is up to 16cm in length, with a black trunk and yellow or red legs. It has been described in detail by Attems (1930). Sex cannot be determined by means of external morphology. Eggs are laid as large clutches of tens of eggs, which are cared for by females which brood them in a similar manner to in the Geophilomorpha. These eggs develop epimorphically, in a similar fashion to Geophilomorpha.

![Image of Scolopendra subspinipes mutilans](image)

**Figure 2.7:** Adult *Scolopendra subspinipes mutilans*

### 2.1.2.2 Sampling and husbandry

Live adults were imported from Hong Kong, in China, and transported via the EMS courier service, to An Post, via Parcel Force in the UK. No regulations prohibit such transport, either in the UK or in Ireland. The animals were captive bred and were
transported in plastic containers containing *Sphagnum* moss. Once received, the animals were housed separately in plastic containers containing soil and a water dish and fed with live crickets of the species *Achaeta domesticus* or *Gryllus bimaculatus*. Animals were checked weekly to ensure general health, to remove food debris and to refill water dishes. Egg clutches were removed from females after a brief period and were incubated separately, in order to best allow for determination of developmental stage. The clutches were incubated at 25°C and placed on a plastic mesh in order to ensure air flow. This was placed in a small petri dish in a box with water-saturated paper. Care was taken to maintain the integrity of each clutch, thus preventing microbial infection and desiccation. 2.1.3 *Scutigera coleoptrata* (L.)

### 2.1.3.1 Description

This species is a member of the order Scutigeromorpha, which is basally branching from the rest of the Chilopoda. In terms of morphology, this order shares many traits which greatly differentiate it from the other centipedes. Its status as a member of this group was formerly in question, but the monophyly of the Chilopoda has been confirmed by molecular analyses (Giribet *et al.*, 1999, Murienne *et al.*, 2010). The Scutigeromorpha are characterised by a fusiform body, elongated legs with many tarsal segments, the lack of a direct correspondence between tergites and sternites, less modified forcipular apparatus (Dugon & Arthur, 2012) and compound eyes (Müller *et al.*, 2003). The last of these traits has been hypothesized to be homologous to the compound eyes of insects and crustaceans (Müller *et al.*, 2003). *Scutigera coleoptrata* is, in many ways, a “typical” member of this group, possessing all of the traits mentioned above, and, in terms of model organism choice, possesses considerable advantage of being more well-known than most scutigeromorphs and being more readily available to sample. Unlike the other two study species, the eggs of *Scutigera*
develop anamorphically, with additional trunk segments being added sequentially after hatching, with each moult, eventually reaching a final total of 15 leg-bearing segments. Also, unlike the other species studied here, no brooding behaviour is present, with eggs being laid in soil and then allowed to hatch without maternal care.

**Figure 2.8:** Adult *Scutigera coleoptrata* feeding on *Achaeta domestica*. Image courtesy of Michel Dugon.

### 2.1.3.1 Sampling and husbandry

Live *Scutigera coleoptrata* adults were sampled from a site in Punta Licosa in Italy (coordinates: 40°25'N 14°91'E). This region consists of a small hill and its surroundings 130km south of the city of Naples. Animals were sampled by removing stones and subsequently catching the animal with plastic containers. These animals were then packed into 15 ml centrifuge tubes with damp tissue and sent via post to Ireland. The sex of the animals was then determined by examining the ventral posterior segment of
the animals, where differences in gonopod morphology can be used to distinguish males and females (see Figure 2.10). The animals were then housed individually in plastic boxes at 20°C, with each provided with a source of moisture in the form of damp tissue and fed weekly with juvenile crickets of the species *Achaeta domesticus* or *Gryllus bimaculatus* or with locally small collected spiders. Females were provided with a petri dish of slightly moistened soil. This was replaced weekly and checked for freshly laid eggs. If desired, these eggs were then incubated at 20°C, allowing them to reach a later developmental stage.

![Figure 2.9: Punta Licosa sampling site. Image is derived from satellite imagery provided by Google.com.](image-url)
Figure 2.10: Sexual dimorphism in posterior of *Scutigera coleoptrata*. The red arrows point to the claw-like gonopods at the posterior. Left: male. Right: female.
2.2 General Methods

2.2.1 Light microscopy and image processing

Mid-magnification observation, manipulation and photography were performed using an Olympus SZX 16 stereomicroscope with an attached DP25 or DP71 camera, equipped with UV illumination capabilities. High-magnification images were taken with an Olympus BX51 compound microscope, equipped with DIC and UV illumination capabilities. Images were taken with the Olympus Cell^B, Cell^D and Cell^F software packages. Images were later adjusted for colour balance, brightness and contrast with the GIMP software package.

2.2.2 Embryo fixation

Embryos were fixed in 4% w/v paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 24h at 4°C. They were then treated with successive washes of PBS, then dehydrated stepwise into 100% MeOH, as follows: PBS, PBS, 25% MeOH/75% PBS, 50% MeOH/50% PBS, 75% MeOH/25% PBS, 100% MeOH, 100% MeOH. The embryos were then stored at -20°C until use for in situ hybridisation, DAPI staining, or other procedures.

2.2.3 Nuclear staining with 4',6'-Diamidin-2-phenylindol (DAPI)

This stain was used to stain for nucleic acid in cleared embryos. A stock solution of DAPI at a concentration of 5 mg/ml in ddH₂O was created. This was diluted to 3:1000 in 70% glycerol/30% PBT. Embryos were incubated for 30 minutes at room temperature, then given successive washes of 70% glycerol/30% PBT.
2.2.4 RNA extraction

RNA was extracted from fresh tissue or from tissue preserved with RNAlater™ (Qiagen catalogue number 76104). Extraction was performed using the Qiagen RNeasy™ kit (Qiagen catalogue number 74104), as per manufacturer's instructions. RNA samples were stabilised with Ribolock™ ribonuclease inhibitor (Fermentas catalogue number EO0381) and stored at -70°C.

2.2.5 RNA gel electrophoresis

RNA quality was tested using RNA formaldehyde gel electrophoresis, with gels made with 1.5% agarose in 1X MOPS buffer and containing formaldehyde. All elements were maintained in a ribonuclease-free environment. 1 μg of each sample, mixed 1:1 with RiboRuler EtBr-containing loading dye (Fermentas catalogue number SM1821) was run for 30 minutes at 100 V. Gels were visualised under UV light.

2.2.6 cDNA synthesis

cDNA was synthesized from extracted RNA using the SuperScript™ II reverse transcriptase enzyme (Invitrogen catalogue number 18064-014) as per manufacturer's instructions. After reverse transcription, cDNA was treated with ribonuclease H (Fermentas catalogue number EN0201) to remove contaminating RNA. Samples were examined with agarose gel electrophoresis to ensure the quality of the resultant product.

2.2.7 DNA agarose gel electrophoresis

Gels were prepared as 1% agarose in 1X Tris-Acetate-EDTA (TAE), with 0.6X Sybr Safe DNA gel stain (Invitrogen catalogue number S33102) added for visualisation. Electrophoresis was performed in a standard set-up system and gels were imaged under
UV light.

2.2.8 Polymerase chain reaction (PCR) techniques

PCR allows for the amplification of DNA sequences of interest, and throughout this study, multiple variant PCR techniques were used. GoTaq DNA polymerase (Promega catalogue number M8301) was used for these assays. A wide array of oligonucleotide primers were used throughout, the sequence of each of these is provided in appendix B. Thermocycler programs utilised are included in appendix C. Temperature gradient PCR, with an annealing temperature gradient of 48-62°C was used to amplify fragments of relevant genes for cloning. Degenerate temperature gradient PCR, with the same annealing temperature range, was used to amplify relevant genes in *Scutigera* and *Scolopendra*. A shorter, conventional program was used to test bacterial clones for the presence of insert sequences of the correct size. Another conventional PCR approach was used to create templates for RNA probe synthesis.

2.2.9 DNA purification techniques

DNA sequences of interest, such as PCR amplicons, were purified prior to downstream applications. Products were purified after separation via agarose gel electrophoresis using the QIAquick gel extraction kit (Qiagen catalogue number 28704), with the addition of an isopropanol step. Other products were purified using the QIAquick PCR purification kit (Qiagen catalogue number 28104). The purity of resulting samples was tested via NanoDrop spectrophotometry.

2.2.10 TA cloning

Purified PCR products were cloned into bacterial cultures before sequencing. Two
vectors were used, pCR II-TOPO (Invitrogen catalogue number K4600) and pGEM-T easy (Promega catalogue number A1360), both of which have the advantage of containing binding sites for both SP6 and T7 polymerases flanking the insert site. After ligation of the purified PCR amplicon into this vector, they were then transformed into One Shot™ chemically competent cells (Invitrogen catalogue number K4600-01), screened for the presence of an insert of the expected size and sequenced.

2.2.11 DNA plasmid purification

Plasmids were purified via using the Promega PureYield™ system (Promega catalogue number A1223), with the protocol modified to replace the supplied elution buffer with nuclease-free water, in order to best accommodate downstream applications.

2.2.12 Sequencing

Sequencing of DNA plasmids containing the cloned sequences was performed by GATC Biotech (Konstanz, Germany), via the Sanger sequencing method.

2.2.13 RNA probe synthesis

RNA probes for in situ hybridisation were synthesized from DNA templates produced by PCR, by means of in vitro RNA transcription, with the inclusion of digoxigenin-ddUTP or fluorescein-ddUTP. The polymerases to be used for the sense (negative control) and antisense (positive sample) were determined via analysis of sequence data. Transcription was carried out using T7 RNA polymerase (Fermentas catalogue number EP0081) or SP6 RNA polymerase (Fermentas catalogue number EP0131). Use was made of the Roche premixed DIG RNA labelling mix (Roche catalogue number 11277073910) or Fluorescein RNA labelling mix (Roche catalogue
number11685619910). Ribolock™ ribonuclease inhibitor (Fermentas catalogue number EO0381) was added to ensure preservation of transcripts produced. The quantity and quality of the resultant products was tested by means of NanoDrop spectrophotometry and gel electrophoresis, with use of the former testing for the presence of contaminants and sample concentration, while the former was used to ensure that the RNA transcripts present were of the expected size.

2.2.14 In situ hybridisation

The basic in situ hybridisation protocol used here was adapted from that developed by Kettle et al. (2003) and further refined by Brena et al. (2006). This protocol was adapted to allow for multiple-probe, multiple-colour staining by means of hybridization with both fluorescein-labelled and DIG-labelled probes and further optimised for in situ hybridisation in Scolopendra subspinipes mutilans. The antibodies used for this purpose were anti-DIG-AP, an anti-DIG antibody coupled with alkaline phosphatase (Roche catalogue number 11093274910), and anti-FLU-POD, an anti-fluorescein antibody coupled with horseradish peroxidase (Roche catalogue number 11426346910). The two different chromogenic substrates utilised with these enzymes were NBT/BCIP for alkaline phosphatase, producing a purple colour (Roche catalogue number 11442074001) and diaminobenzidine for horseradish peroxidase, producing a brown colour (Roche catalogue number 11718096001). Detailed in situ hybridisation protocols are provided in Appendix D.

2.2.15 Embryo clearing and mounting

After in situ hybridisation, embryos were cleared into 30%, then 50% and finally 70% glycerol in PBT, each for two hours at RT. The embryos were then DAPI treated, as
described previously. Imaging was performed both on entire eggs and on flat-mounted embryos. Flat-mounting of embryos was performed by means of physical dissection of the yolk from the embryonic germ-band and the subsequent mounting of this germ-band on a microscope slide to allow for further microscopic imaging.
Chapter 3:

Analysis of *Wnt* gene complement and *Wnt* expression in *Strigamia maritima*

*Wnt4* expression in anterior of *Strigamia maritima* embryo
3.1 Introduction

As noted earlier, the Wnt gene family has multiple subfamilies, whose divergence has been dated to very deep within animal phylogeny (Kusserow et al., 2005, Windsor & Leys, 2010). However, despite the large number of Wnt subfamilies present within the ancestral arthropod, secondary loss of Wnt genes is very widespread, with many arthropods having much reduced Wnt gene complements (Konikoff et al., 2010, Murat et al., 2010). Interest has grown of late in the roles of Wnt genes in arthropods. The Wnt gene family has been quite extensively examined in Drosophila melanogaster (Murat et al., 2010) and also studied, albeit to a lesser degree, in Tribolium castaneum (Bolognesi et al., 2008a, 2008b, 2009). Additional work, most notably in the spiders Cupiennius salei (Damen, 2002) and Parasteatoda tepidariorum (McGregor et al., 2008, Janssen et al., 2010) and the millipede Glomeris marginata (Janssen et al., 2004, 2010), has analysed the genes of this family, focusing on their expression patterns and the pattern of gene loss. The examination of these data has paid particular attention to the roles of Wnt genes in segment generation, limb development and posterior development. A central theme running through much of this work, however, relates to the question of the functional relationships between the different Wnt genes.

In the context of a greatly variable Wnt gene complement between different taxa, such questions centre on the degree of redundancy present in this system. A number of hypotheses can be distinguished. These include the possibility that Wnt genes simply perform additional functions in species with many Wnt genes, the possibility that many of the Wnt genes are redundant and the possibility that the Wnt genes act in a combinatorial fashion, possibly with partial redundancy in some aspects of function. In order to distinguish between these different models for Wnt gene activity across the
arthropods, an approach that focuses only on a common model organism (like *Drosophila melanogaster*), to the exclusion of all other taxa, is not sufficient. It is instead necessary to take a comparative approach, which considers *Wnt* expression patterns across a wide cross-section of different taxa, taking care to include taxa with both intact *Wnt* systems and reduced complements of *Wnt* genes. The work here presented focuses on the centipede *Strigamia maritima*, an organism that is emerging as a useful system in which to examine questions of arthropod development. In this work, a census of *Wnt* genes present in the *Strigamia* genome and a comprehensive survey of their expression were performed. It is hoped that this work, when considered in the context of data from other taxa, may help to resolve the above questions regarding the developmental roles of *Wnt* genes and how these have evolved within the Arthropoda.

### 3.2 Materials and Methods

#### 3.2.1 Genome analysis and gene identification

The draft *Strigamia maritima* genome was searched using the Basic Local Alignment Search Tool (BLAST) available via the *Strigamia maritima* Genome Annotation Consortium website at [http://strigamia-annotation.org](http://strigamia-annotation.org). Searches were performed using amino acid sequences predicted on the basis of homologous *Wnt* genes from the most closely related taxon for which such a sequence was available on the publicly accessible NCBI protein database. The relevant genes were then annotated in the genome with the help of the sequenced cDNA sequences (see Appendix E) produced for *in situ* probe production. Annotation was performed using the Apollo annotation software package. The gene subfamily relationship of each identified gene was confirmed by the construction of a phylogenetic tree of amino acid sequences, using both the *Strigamia maritima* *Wnt* gene sequences and a large alignment of arthropod *Wnt* sequences.
obtained from Janssen et al. (2010). This tree was constructed using the neighbour-joining method with the Jones-Taylor-Thornton substitution matrix, performed by BioNJ software on the phylogeny.fr server (Gasquel, 1997). 100 bootstrap iterations were performed to test clade validity. This tree is presented in Appendix F.

3.2.2 Embryo sampling and preparation, in situ hybridisation and microscopy

Performed as described previously in Chapter 2.

3.3 Results

3.3.1 Census of Strigamia Wnt genes

*Strigamia maritima* is an exception to the common trend of secondary *Wnt* gene loss, with the present investigation having confirmed the presence of 11 *Wnt* genes in the *Strigamia* genome. Phylogenetic investigation has identified these genes as belonging to the *Wnt1, Wnt2, Wnt4, Wnt5, Wnt6, Wnt7, Wnt9, Wnt10, Wnt11, Wnt16* and *WntA* subfamilies (Figure 3.1). While the absence of *Wnt3* is in line with all other studied arthropods, *Wnt8* has also been lost, a character which is not shared by *Glomeris*, another myriapod (Janssen et al., 2010). One *Wnt* pseudogene, of unknown subfamily affinity, was also identified. No evidence of lineage-specific duplications has been found. All other *Wnt* genes examined possessed the expected *Wnt* domain and conserved open reading frame, indicating their functional status. Further evidence for their expression and functionality comes from the examination of transcriptome data and from the sequencing of fragments of these genes amplified by reverse-transcriptase PCR. The *Wnt* genes are also known to display a degree of synteny in many arthropod groups. Some conservation of this is also found in *Strigamia*, with *Wnt1* and *Wnt6* adjacent to each other on the same scaffold, as in other arthropod taxa. The status of the
*Strigamia* genome makes it impossible to determine the synteny relationships of other *Wnt* genes.

**Figure 3.1:** *Wnt* genes present in *Strigamia maritima* in comparison with other arthropods and three non-arthropod outgroups (after Janssen *et al.*, 2010).

### 3.3.2 Interpretation of expression data

The embryonic development of *Strigamia maritima* was first examined by Kettle *et al.* (2003). A staging system for this species was devised to cover the period from early cleavage until hatching (Chipman *et al.*, 2004a) and this staging system has been recently updated (Brena & Akam, 2012). The development of *Strigamia maritima* has been described in these publications in some detail, and so this will not be elaborated in any great detail here. In brief, the period of development examined stretches from cleavage to the sinking stage (stage 6 under the schemes described above). During this period, cleavage occurs, the blastoderm forms on the surface of the egg and coalesces to form a germ-band, which sequentially forms segments as it grows, then the embryo spreads laterally and sinks into the yolk towards the end of the segmentation process. Note that the sequential segmentation process means that anterior segments are more mature than posterior ones, creating a natural developmental series within each embryo.
3.3.3 Wnt1

This gene was first identified as the segment polarity gene wingless in *Drosophila* and has been examined in greater detail than other *Wnt* subfamilies in arthropods, in relation to both its expression and its functional roles. In a large range of arthropods, *Wnt1* acts as a segment polarity gene, expressed in stripes anterior to *en*-expressing cells, albeit with some variation in detail (Sampedro *et al.*, 1993, Nagy & Carroll, 1994, Nulsen & Nagy, 1999, Damen, 2002). *Wnt1* expression in *Strigamia* is consistent with its being involved in a segment polarity role (Figure 3.2). In early stage embryos, after the germ-band has been formed and the initial segments are being formed, *Wnt1* is expressed as stripes, with a medial gap, which appear in a stepwise fashion, one for each of the forming segments, preceding the formation of segmental grooves. These stripes are present anterior to *engrailed* expression stripes (Figure 3.2 D), as in the canonical model of arthropod segment polarity gene activity (von Dassow *et al.*, 2000). The extreme lateral-most regions of the *Strigamia* germ band, the presumptive future dorsal regions, do not display *Wnt1* expression. The degree to which this situation is comparable to that in *Glomeris*, where ventral and dorsal segmentation seem to be decoupled, is unknown (Janssen *et al.*, 2004). *Wnt1* expression stripes will later, after segmental furrows have been formed, retreat into smaller domains of expression, confined to the medial region of the forming appendages. Again, this is consistent with known conserved roles of this gene, as *Wnt1* is known to be involved in leg development in both *Tribolium* and *Drosophila*, to give just two examples (Wilder & Perrimon, 1995, Grossmann *et al.*, 2009). See Chapter 4 for more on *Wnt1* in segmentation and limb development. *Wnt1* is also expressed at the posterior of the embryo, surrounding the developing proctodeum (see Chapter 6). Additional expression is present in the pre-segmental region, with the early v-shaped regions resolving into three spots of expression on each side of the
embryo. This expression pattern bears some similarity to the expression zones of this gene that are involved in eye development of insects (Friedrich, 2003). In sum, \textit{Wnt1} expression in \textit{Strigamia} shows a remarkable degree of similarity to the expression of this same gene in other arthropods.

\textbf{Figure 3.2:} Expression of \textit{Wnt1} in flat-mounted embryos of \textit{Strigamia maritima} at stages 4.1(A), 4.3 (B) and 5 (C). Arrowhead indicates first leg-bearing segment. \textit{Wnt1} and \textit{engrailed} double \textit{in situ} at stage 5 in D.
3.3.4 *Wnt2*

The presence of this gene is not highly conserved; *Wnt2* has been lost in the insect lineage and may also be absent from *Glomeris*. Accordingly, little data are available with which to compare findings from *Strigamia*. *Wnt2*, in marked contrast to most other *Strigamia Wnt* genes, is embryonically expressed for a relatively brief period (only during late stage 4 to stage 5) in just two small regions of the embryo (Figure 3.3). *In situ* hybridization signal strength indicates the presence of low transcript levels in both regions. This expression is present at the anterior end of the embryo, as an expression domain at either side of the pre-antennal region; there is a notable similarity between this expression pattern and the expression of *Wnt2* in *Parasteatoda* (Janssen *et al.*, 2010). Additional expression is also present at the proctodeum (see Chapter 6).

**Figure 3.3:** Expression of *Wnt2* in anterior (A) and posterior (B) regions of flat-mounted embryos of *Strigamia maritima*. Arrowhead indicates stomodeum.
3.3.5 *Wnt4*

This gene is commonly absent from arthropod genomes and little is known about its roles in those species where it has been identified. In *Strigamia*, it is expressed in a segmentally reiterated pattern and in complex patterns in the pre-antennal region (Figure 3.4). In the segmented trunk, *Wnt4* transcripts are visible most prominently as two expression zones per segment, at the posterior of each segment, on either side of the midline of the embryo. Additional, fainter expression is present in more lateral zones. This expression arises after segmental boundaries are already formed. Additional expression of *Wnt4* is present in the anterior region of the embryo, firstly as a lateral expression zone in each of the pre-antennal lobes. Each of these initial expression zones resolves into multiple expression domains. Additional zones of expression around the head region arise later in development.
Figure 3.4: Expression of Wnt4 in flat-mounted embryos of Strigamia maritima at stages 4.1 (A), 4.3 (B) and 5 (C). Arrowhead indicates first leg-bearing segment.
3.3.6 *Wnt5*

The presence of this gene is well-conserved across the Arthropoda and its activities have been quite well-examined, providing useful context within which to interpret the data presented here.

*Wnt5* is expressed first as one stripe in each segment, near the anterior segmental furrow. These stripes then resolve into more defined patterns, with strong expression at the base of the developing limbs (Figure 3.5). The presence of expression in the limbs is in keeping with expression in a similar region in *Drosophila, Tribolium* and *Parasteatoda* (Fradkin *et al.*, 1995, Bolognesi *et al.*, 2008a, Janssen *et al.*, 2010). From stage 4 onwards, *Wnt5* is also expressed in a large, strong posterior region surrounding the proctodeum. This expression domain is dealt with in more detail in Chapter 6.

*Wnt5* is also expressed in the pre-antennal region. Here, expression initially takes the form of one patch on each side, each of which later resolve into two smaller expression zones. Additional expression is present around the stomodeum, inviting comparison with labral expression of this gene in *Cupiennius* and *Drosophila* (Fradkin *et al.*, 1995, Damen, 2002).
Figure 3.5: Expression of Wnt5 in flat-mounted embryos of Strigamia maritima at stages 4.1 (A), 4.2 (B) and 5 (C). Arrowhead indicates first leg-bearing segment.
3.3.7 Wnt6

The presence of this gene is well conserved, again providing vital context for interpretation. It is noteworthy that this gene has a conserved syntenic clustering next to Wnt1 in the arthropods (Nusse, 2001), an arrangement which is preserved in Strigamia and may indicate some degree of co-regulation.

In Strigamia, Wnt6 expression closely resembles that of Wnt1. Again, stripes of expression appear sequentially in the trunk, preceding the formation of segmental furrows. These stripes show the same form as those of Wnt1; with a single stripe per segment being located anterior to en-expressing cells, with a mid-line gap (Figure 3.6). The presence of these segmental stripes is somewhat similar to the situation in Tribolium and Glomeris (Bolognesi et al., 2008a, Janssen et al., 2010). As with Wnt1, these segmental stripes will retreat into smaller domains of expression in the developing limb buds. The small posterior expression domain also closely matches that of Wnt1. The only apparent difference between Wnt6 and Wnt1 expression is at the anterior end of the embryo. Here, the anterior expression zones of Wnt6, on either side of the pre-antennal region, differ from the anterior expression pattern seen in Wnt1 (Figure 3.12).
Figure 3.6: Expression of Wnt6 in flat-mounted embryos of Strigamia maritima at stages 4.1 (A), 4.3 (B) and 5 (C). Arrowhead indicates first leg-bearing segment.
3.3.8 Wnt7

The presence of this gene is quite highly conserved and its functions have been examined in a certain degree of detail in other arthropods. In Strigamia, expression commences at stage 3, in the form of stripes in a single segment periodicity, with a mid-line gap, as seen with Wnt1. However, the expression is strongest in four patches of each stripe, two lateral regions of weak expression and two regions close to the mid-line of the embryo. By stage 4, the expression stripes commence as short stripes and quickly retreat to small medial expression zones. This segmental expression will soon fade away, being almost absent by the latter period of stage 4 (Figure 3.7). This Wnt7 segmental expression pattern in Strigamia shows certain similarities with Wnt7 expression in Parasteatoda and Glomeris, where this gene is also expressed as similar segmentally reiterated patches. Additional expression is present in the pre-antennal region, as a large expression patch on either side of the future head region. Additional expression is present around the future stomodeum and very strongly in the developing antennal lobes. Of particular interest is the fact that this gene is expressed strongly in the developing forcipular limb buds. This unique expression pattern is of particular interest and is discussed further in Chapter 5.
Figure 3.7: Expression of $Wnt7$ in flat-mounted embryos of *Strigamia maritima* at stages 4.1 (A), 4.3 (B) and 5 (C). Arrowhead indicates first leg-bearing segment.
3.3.9 Wnt9

This gene is absent from many arthropod genomes, but has been studied to some degree in Drosophila and Tribolium. In the former, it is expressed in a segment polarity gene-like pattern, among other domains of expression (Graba et al., 1995), but is restricted to the gut region in Tribolium. In Strigamia, Wnt9 expression is much more restricted than most other Wnt genes present in this species (Figure 3.8). Its expression shows some resemblance to the expression pattern observed in Tribolium, in that Wnt9 transcripts are restricted to the region surrounding the proctodeum, during late germ-band development (see Chapter 6). While this expression may indicate some roles in posterior development, ones that may well share some commonality with those of Wnt9 in Tribolium, it seems likely that the most important roles played by this gene occur later in development, after the time period covered here.

Figure 3.8: Expression of Wnt9 in posterior of flat-mounted embryo of Strigamia maritima at stage 5.
3.3.10 Wnt10

The presence of this gene is not well-conserved in the arthropods. Accordingly, little data are available with which to compare the situation found in Strigamia. In some arthropods, Wnt10 is clustered with Wnt1 and Wnt6 (Nusse, 2001), but there is no evidence that this is the case in Strigamia.

Wnt10 expression patterns closely resemble those of Wnt1; a stripe for each segment, anterior to the en-expressing region, with a gap along the mid-line of the embryo (Figure 3.9). As with Wnt1, the appearance of these stripes precedes the formation of segmental furrows. Again akin to Wnt1, these segmental expression zones will then recede into smaller domains in the developing limb buds. This segmental limb-bud expression has some similarity to the expression pattern of this gene in Tribolium (Bolognesi et al., 2008a). Wnt10 is also expressed at the posterior of the embryo, surrounding the proctodeum, matching the expression of Wnt1 (see Chapter 6). Wnt10 expression does differ from that of Wnt1 and Wnt6 in one region, the anterior end of the embryo. In the pre-antennal region, this gene is expressed in a much smaller region initially (during stage 3) and expression then fades away to very low levels.
Figure 3.9: Expression of Wnt10 in flat-mounted embryos of Strigamia maritima at stages 4.1 (A), 4.3 (B) and 5 (C). Arrowhead indicates first leg-bearing segment.
3.3.11 Wnt11

The presence of this gene is conserved to a moderate degree in the arthropods, where it is generally expressed in developing limbs. In Strigamia, it is first expressed as a large posterior domain during very early development. This aspect will be discussed in further detail in Chapter 6. In addition, from stage 3 onwards, Wnt11 is expressed in the developing appendages of the head region. However, over time, the expression will recede from the anterior-most of these in a stepwise fashion. By stage 5, the expression is restricted to the developing forcipules only (Figure 3.10). This interesting expression pattern will be discussed in further detail in Chapter 5. It is worth noting here that the early limb expression bears some similarities to the situation in Tribolium, Glomeris and Parasteatoda (Bolognesi et al., 2008a, Janssen et al., 2010). Wnt11 is also expressed at the stomodeum from early stages, an expression pattern which persists into late-stage embryos.
Figure 3.10: Expression of Wnt11 in flat-mounted embryos of Strigamia maritima at stages 4.1 (A), 4.3 (B) and 5 (C). Arrowhead indicates first leg-bearing segment.
The presence of this gene is not very highly conserved, nor has it been examined to any great extent, increasing the difficulty of interpreting these data. In *Strigamia*, this gene is expressed in a segmentally reiterated pattern, at the posterior of the embryo and in the pre-antennal region of the head (Figure 3.11). In the developing trunk, *Wnt16* is first expressed in segmental stripes from early segmentation; these will later resolve into more restricted expression domains, most strongly as a single patch of expression crossing the mid-line of the embryo. Additional expression is present in the developing limb region. Despite many *Wnt* genes displaying expression in *Strigamia* segments, this is the only one of *Strigamia*’s *Wnt* genes whose segmental stripes of expression cross the mid-line of the embryo. This expression shows strong similarities to *Wnt16* expression in both *Glomeris* and *Parasteatoda*, as not only is *Wnt16* expressed as segmental stripes in those taxa, but the stripes of *Wnt16* expression also cross the midline of the embryo in these taxa (Bolognesi *et al.*, 2008a, Janssen *et al.*, 2010), something that is unusual in arthropod *Wnt* expression. Additional expression is seen at the embryo’s posterior, in a broad domain surrounding the developing proctodeum. This expression is of interest in the context of the roles of *Wnt* genes in posterior development and will be discussed further in Chapter 6. As with so many other *Wnt* genes, additional *Wnt16* expression is present at the anterior end of the embryo, in the pre-antennal region, as two expression domains on either side.
Figure 3.11: Expression of Wnt16 in flat-mounted embryos of Strigamia maritima at stages 4.3 (B) and 5 (C). Arrowhead indicates first leg-bearing segment.
3.3.13 *WntA*

The presence of *WntA* is relatively highly conserved and a reasonably wide range of detail is available from different arthropods on the activities of this gene. *WntA* expression is first seen as single-segment stripes, which appear sequentially at a single-segment periodicity, with a mid-line break (Figure 3.12). These appear superficially similar to those of *Wnt1*, but with the crucial difference that these expression stripes appear later than the stripes of *Wnt1* expression. As this expression does not precede the formation of segmental boundaries, *WntA* is not a candidate for roles in segmental boundary formation. Note that this segmental expression displays similarities to the expression of *WntA* in *Tribolium* and *Glomeris* (Bolognesi *et al.*, 2008a, Janssen *et al.*, 2010). The segmental expression stripes decrease in size as development proceeds. *WntA* is also expressed at the posterior of the embryo, in a broad domain, beginning from stage 4. This interesting posterior expression will be discussed in Chapter 6. *WntA* is also expressed in the pre-antennal region of the embryo, beginning with stage 3. This expression takes the form of a complex pattern on each side of the head region, which becomes more defined over time. From stage 4 onwards, expression is also present at the stomodeum.
Figure 3.12: Expression of WntA in flat-mounted embryos of Strigamia maritima at stages 4.1 (A), 4.3 (B) and 5 (C). Arrowhead indicates first leg-bearing segment.
3.4 Discussion

3.4.1 Wnt expression in developing segments

One of the earliest identified functions of a Wnt gene in arthropods was the role of Wnt1 (wingless) as a segment polarity gene in Drosophila. Other Wnt genes also have segmental expression patterns in a range of arthropods. Segmentally reiterated expression is also present in the great majority of Wnt genes in Strigamia maritima. However, in interpreting these results, it is important to consider that the highly segmented body plan of centipedes (and, indeed, arthropods in general) means that many body structures are segmentally reiterated, a configuration which would be expected to produce segmentally reiterated gene expression. In order for a gene to be a candidate for roles in segment generation, it is necessary that this gene is expressed as segmental stripes that precede the formation of the segmental furrows. Wnt1, Wnt5, Wnt6 and Wnt10 all exhibit expression patterns of this nature, possibly suggesting roles in segment formation.

Of note is the fact that segmental Wnt expression patterns tend to resolve from initial stripes to later smaller, more refined, expression domains. This resolution of expression corresponds to a decrease in the degree of overlap between the expression domains of various genes at later stages. Later segmental Wnt expression is also interesting, as considerable diversity exists in terms of the position of the segmentally repeated expression domains; the segmental expression domains observed are summarized in Figure 3.13. However, partial overlap of expression patterns is very common. This partial overlap and its consequences are discussed further in section 3.4.3.
Figure 3.13: Schematic view of the expression of *Wnt1*, *Wnt4*, *Wnt5*, *Wnt6*, *Wnt10*, *Wnt16* and *WntA* in a typical trunk segment of *Strigamia maritima* at stage 5 (ventral view, anterior to top).

3.4.2 *Wnt* expression in other regions of the embryo

Expression of *Strigamia Wnt* genes in the pre-antennal region of the embryo is very common. This anterior expression bears some similarity to anterior *Wnt* expression seen in other arthropods. The conservation of pre-antennal *Wnt1* expression in other arthropods is noteworthy (Liu et al., 2006, Miyawaki et al., 2004, Angelini & Kaufman, 2005, Dong & Friedrich, 2005, O'Donnell & Jockusch, 2010); this conservation of anterior expression has been examined in the context of known roles for *Wnt1* in *Drosophila* eye development (Friedrich, 2003). Anterior *Wnt* expression of this sort is also seen in other arthropod taxa, for a number of different *Wnt* genes. However, despite the fact that pre-antennal *Wnt* expression is conserved, *Strigamia* is entirely eyeless. This eliminates the possibility that anterior *Wnt* expression may be involved in eye development in this species. Without knowing which developing structures the observed expression patterns correspond to, no clear hypotheses as to the functions of anterior *Wnt* expression can be proposed. However, two patterns are noteworthy. First, there is great variation in the *Wnt* expression observed in this region, more so than with
the expression of *Wnt* genes in other regions of the embryo. This variation is depicted in Figure 3.14. The divergent anterior expression of *Wnt1, Wnt6* and *Wnt10*, three genes that are expressed identically elsewhere in the embryo is noteworthy in this respect. Second, there is the common pattern of early expression in large expression zones resolving into smaller and more defined later expression zones. As the expression zones resolve in this manner, the degree of overlap in expression between different genes decreases.

Additional *Wnt* gene expression is present in the posterior of the embryo and in the developing forcipules. The roles of *Wnt* genes in posterior identity and development have elicited much interest, as this gene family has highly conserved roles in these processes (Niehrs, 2010). Apart from commenting that posterior *Wnt* expression is very common but exhibits considerable variation in its details, such issues will not be dealt with here, as they will be discussed in detail in Chapter 6. It is also interesting to note the differential expression of *Wnt7* and *Wnt11* in the developing forcipules. This aspect will be covered in more detail in Chapter 5.
3.4.3 Common themes of differential Wnt expression patterns

The expression of the Wnt genes of Strigamia maritima has been discussed above. Proceeding from this discussion and the data already presented, it is clear that much of the expression domains of Strigamia Wnt genes fit within three themes. These themes are trunk expression with a single-segment periodicity, expression in the pre-antennal region of the head and expression at the embryo’s posterior. All Strigamia Wnt genes
display expression that fits within at least one of these themes and few expression
domains that do not. It is common for expression in each these regions to start out as
large zones of expression or segmental stripes and to only later resolve into more
restricted expression domains. However, once the expression domains have shrunk into
these more limited domains, a large degree of diversity is present, so that *Wnt* genes are
only partially co-expressed at later stages. This is illustrated in more detail in Figures
3.13 and 3.14, which display comparative expression of *Wnt* genes in a trunk segment
and in the pre-antennal region of the developing head at stage 5, respectively. The
variation in *Wnt* expression is immediately apparent.

### 3.4.4 Support for combinatorial action of *Wnt* genes?

This pattern of partial co-expression (from an initial large degree of co-expression to
less co-expression in later developmental stages) is not consistent with a model of
complete *Wnt* gene redundancy, as such a model would depend on a much greater co-
expression of *Wnt* genes. Further evidence against this redundancy model is provided by
the conservation of *Wnt* gene presence as argued by Janssen *et al.* (2010). A possible
exception to this is found in the case of *Wnt1, Wnt6* and *Wnt10* co-expression in
developing segments, where the extreme similarity of expression observed may indicate
some level of redundancy. The directly opposite model, one of entirely separate and
unrelated functions of different *Wnt* genes, would not predict any great commonality of
themes of *Wnt* expression and would not predict the high incidence of partial co-
expression observed. However, a third model, whereby the different *Wnt* genes operate
in a combinatorial fashion, would predict the presence of both partial co-expression and
*Wnt* expression patterns fitting into common themes. This model is that of complex
combinatorial action of *Wnt* genes, whereby the combinatorial activity of multiple *Wnt*
ligands with their various receptors allows for the fine-tuning of Wnt activity (Van Amerongen & Nusse, 2009). This is allowed for by the great complexity present in Wnt signalling systems, as their many interacting pathways and receptors allow such systems to operate in complex fashions (Gordon & Nusse, 2006). Such a system of combinatorial action has already been identified in the case of leg development in Tribolium (Beermann et al., 2011). Other analyses have previously supported the concept of combinatorial Wnt activity in arthropods (Murat et al., 2009, Janssen et al., 2010). However, further examination of these issues would be greatly assisted by the use of functional techniques to probe the activities of Wnt genes in as broad a spectrum of arthropod taxa as possible.
Chapter 4: Comparative segmentation, limb development and $Wnt1$ expression in centipedes

Late germ-band stage *Scolopendra subspinipes mutilans* embryo
4.1 Introduction

4.1.1 Comparative development of the Arthropoda

Investigations within the field of evolutionary developmental biology must integrate attempts to (a) understand details pertaining to particular taxa (idiographics) and (b) reveal more general principles of biological systems (nomothetics) (Jenner & Wills 2007). The use and interpretation of data from varied model organisms rests at the intersection of these two programs. Much progress in developmental biology has resulted from the use of model systems (Anderson & Ingham, 2003), but the data obtained in such systems are often quite specific for the models analyzed and cannot necessarily be generalized for larger taxa, which can restrict our view of nature (Fields & Johnston, 2005). Reductionism, while necessary in all attempts to build general principles from scientific data, must be careful not to stray into the territory of “greedy reductionism”, where important distinctions (as between taxa) can be missed and sight of the complexity of variation present in nature can be lost (Dennet, 1995).

Within the arthropods, the most commonly studied model system is, of course, *Drosophila melanogaster* (an insect displaying long-germ development), but further efforts have been made to widen the range of taxa under examination, thus producing a clearer picture (Abzhanov et al., 2008). The principal additional taxa studied include *Tribolium castaneum* (a short-germ insect), *Parhyale hawaiensis* (an amphipod crustacean), *Parasteatoda tepidariorum* (an arachnid spider), *Glomeris marginata* (a glomerid millipede) and *Strigamia maritima* (a geophilomorph centipede). Though this broadening of the evidentiary base for comparative developmental studies is welcome, simply broadening the scope of studies of arthropod development to a single species as a representative of each large taxon (e.g. an order within the insects or a class
or subphylum elsewhere) is not sufficient. Firstly, any chosen species cannot be considered a perfect proxy for its entire group, especially when those groups exhibit considerable intra-taxon variation. The problems inherent to such assumptions are exacerbated when it is assumed that a species represents an ancestral condition based entirely on relative phylogenetic position (Jenner 2006). Secondly, the choice of model organism must also be informed by the questions being asked. The phylogenetic spread present should reflect the breadth of the timescale under examination: microevolutionary patterns will best be observed by comparing closely-related, lower-rank taxa such as genera and species (Abzhanov et al., 2008). The choice of model organism for evo-devo also depends on the presence of suitable variation (Milinkovitch et al., 2007). Overall, the taxa studied should be chosen in order to throw light on a particular question (Jenner & Mills, 2007). The use of the domestic dog (Canis familiaris) as a model for developmental potential is an excellent example of this (Neff & Rine, 2006).

Bearing these points in mind, the present study will analyse the development of centipedes by means of a comparative approach. It is hoped that such a study may shed light on the evolution of segmentation and limb development. This group includes variation in developmental mode: specifically, it includes both anamorphic taxa (developing additional segments with post-hatching moults) and epimorphic taxa (developing all segments embryonically). It also includes great variation in limb morphology and in the degree of modification of their unique, leg-derived forcipular appendage (Dugon & Arthur, 2012). The Chilopoda also show extreme variation in segment number, with interesting developmental constraints on this variation (Arthur & Farrow, 1999, Arthur, 2002b, Damen, 2004, Chipman et al., 2004b). It is this variation
in segment number and leg morphology within the taxon that makes the Chilopoda an interesting group in which to examine the evolution of varying arthropod forms.

4.1.2 Chilopod development

This chapter examines the morphological development of three species of centipede: *Scutigera coleoptrata* (order Scutigeromorpha), *Scolopendra subspinipes mutilans* (order Scolopendromorpha) and *Strigamia maritima* (order Geophilomorpha). Though early studies on the development of centipedes date back over a century (Heymons, 1901, Johanssen & Butt, 1941), the literature on chilopod development is, nevertheless, quite limited. For the Scutigeromorpha, the level of detail in descriptions does not extend beyond a basic outline of egg creation and the formation of the embryo (Dohle, 1970, Dugon, 2011). For the Scolopendromorpha, descriptions are somewhat more detailed (Whittington et al., 1991). For the Geophilomorpha, embryonic development is relatively well understood in *Strigamia maritima*. The investigation of embryonic development in this species, largely inspired by attempts to understand the evolution of segment number (Arthur & Chipman, 2005), has been well under-way for the past decade. Initial investigations have revealed the pattern of segment formation, with the help of *engrailed* expression analysis (Kettle et al., 2003) and have shed light on the molecular mechanisms generating those segments (Chipman et al., 2004a, Chipman and Akam, 2008). Additional work has also revealed the expression domains of some of the *Hox* genes (Brena & Akam, 2006) and has investigated neural precursor formation (Chipman & Stollewerk, 2006). It should also be noted that some authors have performed certain analyses of embryonic development in *Lithobius forficatus*, a member of a chilopod order not under investigation here (Kadner & Stollewerk, 2004, Hughes & Kaufman, 2002a, 2002b). Certain common features of centipede development can be
discerned. These include the sequential formation of segments in an anterior-to-posterior fashion, the presence of the membrana ventralis dividing the right and left halves of the germ-band and the sinking of the germ-band into the yolk after segmentation.

With regard to segmentation and limb morphology, the three species under investigation here show considerable variation. In terms of the former, there is great variation in segment number (from fifteen to fifty-five leg-bearing segments) and the mode of segmentation (with the presence of both anamorphy and epimorphy). In terms of the latter, there is great variation in leg morphology between the taxa; from the extended legs of *Scutigera* with their many articles to the much shorter legs of *Strigamia*.

### 4.1.3 *Wnt1* in arthropod development

Having examined the variation in the morphologically evident processes involved in segmentation and limb development, this chapter will then go on to examine these processes at a molecular level, examining and comparing the expression of a suitable candidate gene in two of these species. The gene chosen for these purposes is *Wnt1*, also known as *wingless*. This gene has been observed to play roles in both segmentation and limb development in a large array of arthropod taxa. It was first identified as having such roles in *Drosophila melanogaster*, where it is one of the segment polarity genes, expressed at a single-segment periodicity anterior to the *en*-expressing zone, with a role in establishing segmental boundaries; it also operates in limb development (Sampedro *et al.*, 1993, Noordermeer *et al.*, 1995). These roles seem to be largely conserved in *Tribolium* (Nagy & Carroll, 1994, Ober & Jockusch, 2006), but the data for *Gryllus* are less conclusive (Miyawaki *et al.*, 2004). The data are also somewhat variable in spiders,
with conservation of the segment-polarity type expression pattern in *Cupiennius* (Damen, 2002), but with expression seemingly restricted to appendages in *Parasteatoda* (Janssen *et al.*, 2010). In the millipede, *Glomeris*, the segment-polarity expression of *Wnt1* is only partially conserved, with *Wnt1* expression stripes being present in ventral tissues only (Janssen *et al.*, 2004, 2008). In the centipede *Lithobius*, *Wnt1* is expressed in a segment-polarity fashion and includes expression in the developing limbs (Hughes & Kaufman, 2002b). Overall, there seems to be a reasonable degree of conservation in these two basic patterns of segment-polarity type expression and developing limb expression, but several exceptions exist.

This chapter will first examine the morphological development of embryos of all three species, from development of the germ-band to segmentation and through to the development of limbs. The expression of *Wnt1* will also be examined in both *Strigamia maritima* and *Scolopendra subspinipes mutilans* across the same time-frame.

### 4.2 Materials and Methods

#### 4.2.1 Embryo sampling and preparation, *in situ* hybridisation and microscopy

Performed as described previously in Chapter 2.
4.3 Results

4.3.1 Development of *Scutigera coleoptrata*

The germ-band, when first forming, covers roughly one sixth of the embryo’s circumference. This germ-band consists of a pre-segmental region, followed by a number of segments, corresponding to the antennal segment, the intercalary segment, the mandibular segment, two maxillary segments, the forcipular segment and the start of the development of leg-bearing segments (Figure 4.1 A). Posterior to the leg-bearing segments is a post-segmental zone. As the segmentation process takes place, additional leg-bearing segments will be added and the embryo grows along the anteroposterior axis (Figure 4.1 A-B).

The developing segments will also take on more definition and limbs will start to develop after five leg-bearing segments have formed. Note that, in keeping with the fact that embryos of *Scutigera* hatch with only four pairs of legs (the fifth leg developing at the first larval moult), the last of these segments is significantly less developed than those anterior to it throughout development. Aside from the antennae and the mandibles, the appendages are not morphologically distinguishable from each other at early stages (Figure 4.1 C). However, the most posterior limbs (those most recently formed) are less well-defined than the more anterior ones, thus creating a developmental gradient from anterior to posterior. These limbs will develop quite substantially and will be protruding from the germ-band before the germ-band sinking process starts (Figure 4.1 C). The forming tergites will also start to become visible at this stage. As these boundaries form, the germ-band will expand laterally (Figure 4.1 D). Note that, in line with the adult segmentation of *Scutigera*, with an unequal number of sternites and tergites (Acosta, 2003), these forming tergites do not correspond directly to the forming sternites. After
sinking (Figure 4.1 E) the limbs will continue to develop and will lengthen very considerably, while the segments will greatly increase in volume and definition.

**Figure 4.1:** Embryonic development in *Scutigera coleoptrata* from early segmentation of the germ-band (A) to late development of the limbs post-sinking (E) in flat-mounted DAPI-stained embryos. The segments are labelled pre-antennal/ocular (oc), antennal (ant), mandibular (mnd), first maxillary (mx1), second maxillary (mx2), forcipular (fc) and first and last leg-bearing (LBS).
4.3.2 Development of *Scolopendra subspinipes mutilans*

The eggs of *Scolopendra subspinipes mutilans* take the form of an oblate spheroid, ~5 mm long and ~3 mm wide. After formation of the germ-band, the blastoderm will form, oriented along the longitudinal axis of the egg. The early germ-band of *Scolopendra* consists of a slight thickening in the blastoderm which will grow as segments form in an anterior-to-posterior fashion (Figure 4.2 A). From this early stage, the membrana ventralis, the mid-line cleft in the embryo is clearly visible. As the segmentation process continues, the existing segments will increase in definition and the germ-band will grow in length. The limb primordia will also begin to form, starting from the anterior of the embryo (Figure 4.2 B). By the time of limb primordia formation, the final number of 21 leg-bearing segments has been reached. A developmental gradient is visible, whereby the anterior segments are more well-defined than the posterior ones. Over time, the germ-band will continue to thicken and the segmented regions distal to the appendages, the future tergites, will form (Figure 4.2 C). During this period, the membrana ventralis will greatly increase in width, a process that seems to be analogous to the lateral expansion of the germ-band in *Strigamia* development. By the time of this lateral extension, the limbs are sufficiently voluminous to protrude from the germ-band. The germ-band will then sink into the yolk of the egg. This process will conclude with the anterior and posterior ends of the embryo in direct contact with each other. The limb primordia on all segments will be well-developed by this stage, with the anterior-most appendages showing signs of their own segmentation. By this stage, both sternites and tergites will be well-defined (Figure 4.2 D).
Figure 4.2: Embryonic development in *Scolopendra subspinipes mutilans* from early segmentation of the germ-band (A) to late development of germ-band immediately pre-sinking (C) in flat-mounted DAPI-stained embryos. The post-sinking development of the limbs is shown in D. The segments are labelled pre-antennal/ocular (oc), antennal (ant), mandibular (mnd), first maxillary (mx1), second maxillary (mx2), forcipular (fc) and first and last leg-bearing (LBS).
4.3.3 Development of *Strigamia maritima*

As the development of this species has been described in detail previously by Kettle *et al.* (2003), Chipman *et al.* (2004a) and Brena and Akam (2012), this description will remain brief and will focus on elements relevant to making comparisons with *Scutigera coleoptrata* and *Scolopendra subspinipes mutilans*.

After the cleavage stage has been completed and the blastoderm has formed (see Brena *et al.*, 2012 for a detailed description), the germ-band starts to condense on one side of the egg, covering roughly one-quarter of its circumference. The initial segments form rapidly (Figure 4.3 A). After a brief period of no additional segmentation, the main period of segmentation begins and segments are added sequentially in an anterior-to-posterior fashion (Figure 4.3 B-D). As the segments are being added, the embryo extends anteroposteriorly, more than doubling in length. These segments will continue to develop, increasing in definition and with limbs starting to develop after roughly 20 leg-bearing segments have formed (Figure 4.3 C). A strong developmental gradient is present; with anterior segments being much more developed than posterior ones (Figure 4.3 D). After nearly all of the segments are formed, the germ-band will then spread laterally and the characteristic sinking process will occur (Figure 4.3 E). By the time that this occurs, most of the limbs have not formed and the existing limb-buds are small rudiments. Further appendage development proceeds after sinking.
**Figure 4.3:** Embryonic development in *Strigamia maritima* from early segmentation of the germ-band (A) to development of the limbs post-sinking (E) in flat-mounted DAPI-stained embryos. The segments are labelled pre-antennal/ocular (oc), antennal (ant), mandibular (mnd), first maxillary (mx1), second maxillary (mx2), forcipular (fc) and first and last leg-bearing (LBS).
4.3.4 Wnt1 expression in *Scolopendra subspinipes mutilans*

*Wnt1* transcripts are localised in stripes in developing segments, medially on the anteroposterior axis and with a gap along the membrana ventralis that separates the left and right sides of the embryo (Figure 4.4 B, C). However, examining the presence of these stripes of expression in relation to the formation of the segmental furrows reveals a surprising pattern: high levels of *Wnt1* transcripts are not present until the segments have already formed morphologically (Figure 4.4 A). This is not easy to reconcile with the hypothesis that *Wnt1* in *Scolopendra* is involved in segment boundary formation in a similar manner to the role played by its homologue in *Drosophila*, as such a hypothesis would require *Wnt1* expression stripe formation to precede segment formation in order for the former to play a causative role in the latter. In limb primordia, the expression patterns observed conform more closely to expectations based on other arthropod taxa, as expression stripes in segments will retreat into slightly smaller domains in the developing limb buds. This limb expression is compatible with *Wnt1* playing similar roles in the development of *Scolopendra* limbs as in other arthropods. Additional *Wnt1* expression is present near the developing proctodeum (Figure 4.4 A).
Figure 4.4: *Wnt1* expression in *Scolopendra subspinipes mutilans* embryos in embryo during germ band extension (A) and in forming segments (B), with the latter counter-stained with DAPI (C).
4.3.5 Wnt1 expression in Strigamia maritima

In relation to segmentation, Wnt1 transcripts were visualised from very early stages in the developing germ-band of Strigamia. Wnt1 is expressed in the form of stripes at a single-segment periodicity, anterior to the en-expressing zone, preceding the formation of segments (Figure 4.5 A, B). These stripes of expression are anterior to the en-expressing region, displaying a conservation of Wnt1 expression in Strigamia in relation to other arthropod taxa and possibly also a conservation of the role of Wnt1 as a segment polarity gene into myriapods (see section 3.3.3). These Wnt1 expression zones will later retreat into the limb primordia (Figure 4.5 D), again reflecting similarities with the situation in many other arthropod taxa. Again, this reflects possible conservation of function. The expression of this gene was discussed further in Chapter 3.
**Figure 4.5:** Wnt1 expression in flat-mounted *Strigamia maritima* embryos in embryo during germ band extension (A), in forming segments (B) and developing limbs (D). In C and E, the same region, counter-stained with DAPI, is shown.
4.4 Discussion

4.4.1 Differences in segmentation

Based on the aforementioned differences between the species examined in terms of the morphology of the trunk and the number of segments present in adults, it is to be expected that variation would exist in terms of the developmental process of segmentation. This is clearly observable in the increased limb length observed in *Scutigera* relative to the other species examined, in the variation in embryonic segment number and in the variation in germ-band length, correlating with body length relative to width. However, these aforementioned differences could be predicted based purely on the adult morphology of the different species. The data presented above also demonstrate certain differences that extend beyond these relatively obvious expected ones, including a number of examples of heterochronic differences. For all three species, more posterior (and thus more recently developed) segments are developmentally delayed relative to those anterior to them. Variation exists in the degree of this “developmental gradient” present; it is most readily apparent in *Strigamia* and least evident in *Scutigera*, with *Scolopendra* occupying an intermediate position in this respect. Variation also exists in relation to the timing of the formation of the tergites; this is most conspicuous in the case of *Scutigera*, where tergites form much earlier than in the other two species. The embryonic tergites of *Scutigera* also display the non-correspondence with the sternites that characterises the adult morphology of this species. Another crucial difference seen between the three species examined is in relation to *Wnt1* expression. The differences in timing of this expression between *Scolopendra* and *Strigamia* are quite striking. The expression of reiterated *Wnt1* stripes in *Strigamia*, preceding as it does the morphological segmentation process, is consistent with this gene operating in a segment-polarity fashion, but in *Scolopendra* the data do
not support this hypothesis of segment-polarity-like activity, as the segments seem to be already formed by the time that Wnt1 expression stripes appear. This difference in timing of Wnt1 makes it difficult to determine the ancestral state of this character, though it seems likely, given the wide range of arthropod taxa with repeated early Wnt1 expression, that the ancestral state is the presence of Wnt1 stripes acting in a segment-polarity fashion. It is possible that another gene of the Wnt family has assumed some of the segmental functions of Wnt1 in Scolopendra, as is thought to be the case in Glomeris (Janssen et al., 2004).

4.4.2 Differences in appendage development

Multiple differences were also identified between these three species in appendage development, many of which mirror the differences in segmentation previously documented. As with the development of the segments, there is a gradient within the germ-band in relation to the level of development of appendages: the most posterior segments are the most-recently developed ones and have less-developed limbs than more anterior segments. However, the degree of this difference between the posterior and the anterior segments varies from species to species, being very prominent in Strigamia and much less so in Scolopendra and Scutigera. A clear example of heterochrony can also be identified in the timing of appendage development relative to the sinking of the germ-band. This germ-band sinking acts as a useful temporal marker with which to make comparisons, as it is present and clearly identifiable in all three species studied. When comparing the onset of limb development in relation to this point, the limbs of Scutigera are well-developed by the time of sinking, while at this stage, most segments of Strigamia have no limb-buds and the most-developed of their limbs are still small outgrowths. As with many other traits, the situation in Scolopendra
is intermediate between that of the other two species. This difference is maintained into later development, as a similar pattern is seen if hatching is used as the temporal marker. Note that this pattern fits closely with the relatively precocious development of *Scutigera* in comparison to the altricial development of *Strigamia* and the earlier leg development that this would suggest for the former species. Despite the differences observed above, one factor seemed to be quite constant in the two species under examination: *Wnt1* is expressed in developing appendages in both species. This suggests a conservation of at least part of the genetic machinery underlying limb development, despite the heterochronic differences at multiple levels in limb development.
Figure 4.6: Simplified diagram of embryonic development in *Strigamia maritima*, *Scolopendra subspinipes mutilans* and *Scutigera coleoptrata* in the period under study.
4.4.3 Fundamental similarities in embryonic development

As noted previously, the Scutigeromorpha and Geophilomorpha are highly morphologically divergent, being at opposite ends of the variation present within the Chilopoda. It is in keeping with this that, despite the fact that Scolopendromorpha and Geophilomorpha are equally phylogenetically distant from Scutigeromorpha (Murienne et al., 2010), Scolopendra is observed to be intermediate in state between Strigamia and Scutigera for many developmental characters. Considering the Geophilomorpha as a highly derived group (Arthur & Chipman, 2005a), this is consistent with the view, based on fossil evidence, of scutigeromorphs as a less-derived clade (Shear & Edgecombe, 2010). Comparing the development of these three species reveals the presence of a number of points of commonality. These include sequential segmentation, germ-band extension accompanying segmentation, a developmental gradient along the germ-band, Wnt1 expression patterns and the sinking of the germ-band. The observed differences between these taxa include a number of clear examples of heterochrony; differences in the timing of essentially similar developmental events. Other developmental differences are quantitative in nature, relating to the relative size of various structures. Such findings are consistent with conceptions of evolution as a “tinkerer”, with small changes in existing processes causing the variation seen in nature (Jacob, 1977). It is noteworthy that the expression of Wnt1 is quite highly conserved across the taxa under examination, with the observed differences between chilopod taxa being heterochronic differences in the onset of expression. Accordingly, the morphologically observable details of development show many similarities to at least some of the molecular mechanisms underlying development. However, in order to gain a more detailed understanding of the molecular mechanisms underlying evolutionary differences between these taxa, it would be necessary to both widen the examination to
include a broad range of developmental markers and to deepen these studies to include
the use of functional techniques that can try to establish causative links between
changes in the activity of particular genes and actual changes in the morphology of the
organisms in question. This study has laid some foundational work for such
comparisons and it is hoped that future work can build upon this to provide a more
comprehensive understanding of the evolution of arthropod diversity.
Chapter 5:
The roles of $Wnt$ genes in forcipular development

Anterior end of juvenile *Strigamia maritima*
5.1 Introduction

5.1.1 The forcipular system

The centipedes possess a unique synapomorphic character in the form of the appendages of the first post-cephalic segment, modified to form a pair of venom-delivering claws called forcipules. These claws are used for prey capture and, to a lesser extent, for predator deterrence. The forcipular segment is considered to be a modification of the first leg-bearing segment, and has been examined previously in the context of the conceptual framework of evolutionary novelties (Dugon et al., 2012a, 2012b, Dugon & Arthur, 2012).

5.1.2 Defining evolutionary novelties

This concept is a slippery one; not only is there some uncertainty about whether a feature like the forcipule fits into it, but there is also great disagreement about how an evolutionary novelty can be defined and how this concept can be used in general. An attempt will be made herein to summarize the main efforts that have been undertaken thus far to define what an evolutionary novelty is and why such definitional problems are important.

A very simple and straightforward definition of an evolutionary novelty is provided by Arthur (2000), where novelties are equated with apomorphies. While this definition has the advantage of clarity and unambiguity, it is problematic in being too broad.

In an earlier endeavour to pin down the meaning of ‘evolutionary novelty’, Mayr (1960) cast his definition of it in functional terms, proposing to restrict the definition of novelties to features that allow for new functions. The definition employed by Pigliucci
(2008) is broadly similar in its emphasis on the importance of function.

Other authors have defined novelties as qualitative, as opposed to quantitative change in a character (Davis, 2012). However, the boundary between quantitative and qualitative is often unclear; complex characters that are widely acclaimed as novelties often involve integrated changes in multiple traits, which can be variously interpreted as qualitative or quantitative; (Moczek, 2008). So, such definitions may do little more than restate the problem in different terms.

An influential class of definitions focuses on homology, defining novelties as structures without homologies (Müller & Wagner, 1991, Müller & Newman, 2005, Wagner & Lynch, 2010). Such definitions run into problems, not only from the difficulties involved in trying to understand and identify homology and from the hierarchical nature of homology, but due to the fact that the manner in which features evolve, by the modification of existing features, meaning that all features have homologues on some level (Halgrímsson et al., 2012). Even the application of this definition to particular arrangements of features still leaves room for much subjective judgement as to what falls within the definition. If such a definition is strictly applied, all features are excluded from its remit. Consequently, it is the opposite of Arthur’s (2000) definition: that definition was too broad; the homology-based one is too narrow. Additionally, ignorance of the details regarding a particular feature may often be the cause of claims of non-homology (Hall & Kerney, 2012).

Other, less common definitions of novelty rest on it being the locus for further evolution (Rice, 2012) or the feature that breaks an evolutionary constraint (Halgrímsson et al.)
However, reliably identifying either the loci of evolution or evolutionary
constraints is problematic (Arthur & Farrow, 1999). So great is this variation in
definitions and approaches that the issue of evolutionary novelties has been
classified not as a single problem, but rather as a ‘problem agenda’ composed of
multiple issues, where (a) attempts to find formal definitions are doomed to failure and
(b) the issues must be approached without a unified theoretical basis, but instead within

5.1.3 Are novelties a unified group?
In order for a theory of evolutionary novelties to be capable of providing predictive
power, progress must be made towards uncovering generative principles underlying
evolutionary novelties (Müller, 2007). The non-linear relationship between the
underlying developmental network and the phenotype that is being produced must be
considered (Wagner, 2011). Even if a definition of novelty can be agreed on, there is no
particular reason to assume that all novelties are produced in a similar fashion. Such an
assumption would require that the processes producing morphologically novel features
differ greatly from those that produce quantitative changes. Such issues are problematic
for novelties as defined in multiple ways. For functional definitions, the fact that some
particular traits share the feature of allowing new functions is no reason to assume that
they share generative mechanisms; the enabling of new functions is an emergent
property of the feature and its organismal and ecological context, largely independent of
developmental factors. For qualitative change or homology based definitions, the non-
linearity of the genotype-phenotype map means that it cannot be assumed that novelties
are united by the sharing of developmental traits. Indeed, putative novelties are
extremely variable and it is doubtful how much they share at all. In the light of the
haphazard way in which features are considered as being an evolutionary novelty, it has been suggested that a putative novelty is classed as such due largely to its origins not being understood (Brigandt & Love, 2012).

Despite the theoretical morass that is the attempt to unify various conceptions of evolutionary novelty, empirical studies have made a certain amount of progress in understanding various features that have been classed as evolutionary novelties. The eyespots of butterfly wings (Saenko et al., 2008, 2010), the tetrapod autopod (distal limb) (Shubin 2002, Shubin et al., 2009) and the feathers of birds (Brush, 1996, Prum, 1999) have been examined in this way, with the re-deployment of existing pathways found to be a prominent feature in their production in each case. Indeed, some authors have noted that the acquisition of new functions by existing genes is central to the generation of novelties (Ganfornia & Sanchez, 1999, Shubin & Marshall, 2000). However, this redeployment of existing pathways is a general theme in evolutionary developmental biology and cannot be seen as the basis of a proposed developmental distinction between novelties and other evolutionary change.

5.1.4 Body pattern evolution as an alternative theoretical context

It may well be more useful to break the morass of problems and complexities associated with evolutionary novelties into a number of smaller problems. Part of this approach involves acknowledging that it may well be the case that the generative mechanisms underlying any one class of novelty do not provide much in the way of meaningful insights into others. Such an approach, more humble in scope, could make meaningful progress in understanding each of the problems separately.
Within this framework, a useful context in which to consider the forcipular apparatus is as part of the broader issue of the evolution of body patterning, the process by which the identity of a particular part of an organism’s body changes through evolution. As the venom claw segment is derived from the first-leg-bearing segment of an ancestral myriapod, it is an example of one unit out of many homonomous parts acquiring its own identity and differentiating from the other leg-bearing segments. Questions of evolution of body patterning come with their own interesting theoretical discussions. While a historical debate in this area might be focused on the possible importance of saltational evolution, a more recent debate is over the relative primacy of changes in body pattern and in the roles of the genes at the base of the developmental systems building that body pattern (Budd, 1999, Akam 2000). Two models can be distinguished: (i) that body patterns first evolve by changes in the downstream elements of developmental systems which are then integrated and stabilised by changes in the upstream elements of the developmental hierarchies, or (ii) that body pattern changes begin via changes in the regulation of these upstream genes.

Analysis of Wnt gene expression in the forcipular segment of centipedes may provide useful insights into such questions. Firstly, Wnt genes are implicated in both body patterning processes (Niehrs, 2010) and in limb development (Grossmann et al., 2009), so they sit at the intersection of these issues. Secondly, the availability of Wnt expression data from other myriapod taxa allows for useful comparisons to be made (Damen et al., 2002, Janssen et al., 2010). This chapter will seek to examine Wnt expression in the developing forcipules in Strigamia maritima and will then describe in detail the relevant details of forcipule-specific Wnt expression.
5.2 Materials and Methods

5.2.1 Embryo sampling and preparation, in situ hybridisation and microscopy

Performed as described previously in Chapter 2.

5.3 Results

5.3.1 Identification of Wnt genes with forcipule-specific expression

The expression of all Wnt genes present in Strigamia was examined. Two of these, Wnt7 and Wnt11, were identified as displaying forcipule-specific expression patterns. These are examined further here. The interpretation of the data on other Wnt genes is dealt with in Chapters 3, 4 and 6.

5.3.2 Forcipule-specific Wnt11 expression in Strigamia

One of the two Wnt genes with a forcipular segment-specific expression is Wnt11. Note that the expression of this gene is further discussed in Chapter 3 (in relation to segmentation, combinatorial action and other issues) and in Chapter 6 (in relation to posterior growth and development). Initial segmental Wnt11 expression is present in the developing appendages of the head, the antennae (weakly), the mandibles (weakly), the first set of maxillary appendages, the second set of maxillary appendages and finally, in the forcipules (Figure 5.1). The expression in the more anterior of these appendages then gradually fades away, leaving expression in just the forcipular appendages. The expression in the forcipules then fades away from much of the initially broad region of expression, becoming almost entirely restricted to the proximal part of the developing forcipules (Figure 5.1 E). Much weaker expression is seen in other appendages. This is a unique forcipular expression pattern, as the forcipule is the only appendage with Wnt11 expression in its proximal region and the only appendage with high expression
levels. The pattern of an early commonality of expression patterns seen between the forcipules and the appendages of the head is quite interesting in the context of the morphology of these organisms. The forcipules, despite being derived from a modification of the first pair of legs, have many traits in common with the appendages of the head and, like these, are orientated under the cephalic shield (Dugon et al., 2012a). Thus, here we see a strong parallel between the morphological and the molecular aspects of development.
Figure 5.1: Wnt11 expression and visualisation with DAPI staining in the anterior region of developing embryos of Strigamia maritima at stages 4.1 (A, B), 4.3 (C, D) and 5 (E, F). The segments are labelled mandibular (mnd), first maxillary (mx1), second maxillary (mx2), forcipular (fc) and first leg-bearing (LBS1).
5.3.3 Forcipule-specific Wnt7 expression in Strigamia

This is another Wnt gene for which a forcipular segment-specific expression pattern was identified. Expression of Wnt7 in the developing segments is present from early stages and takes the form of stripes of expression on either side of the mid-line of the embryo in each segment. This expression will fade away from most of the trunk segments over time (Figure 5.2 A, C). By stage 5, appendage-specific expression is restricted to the antennae, the mandibles and the forcipules, with much weaker expression remaining in the legs. In the forcipules, expression is present in the medial part of the appendage on the proximo-distal axis. As expression in the antennae is in a large distal domain and expression in the mandibles also takes the form of a distal domain, this forcipular expression pattern is unique, as the forcipule is the only appendage with Wnt7 expression in its medial region (Figure 5.2 E).
Figure 5.2: Wnt7 expression and visualisation with DAPI staining in the anterior region of developing embryos of Strigamia maritima at stages 4.1 (A, B), 4.2 (C, D) and 5 (E, F). The segments are labelled mandibular (mnd), first maxillary (mx1), second maxillary (mx2), forcipular (fc) and leg-bearing (LBS1).
5.3.4 Differential expression in the forcipules of *Strigamia*

Though both *Wnt7* and *Wnt11* are expressed in the forcipules, they are differentially expressed in this region. While in earlier embryos they are expressed as broad domains covering much of the appendage, the expression domains of each decrease over time. Initial forcipular *Wnt11* expression covers much of the appendage (Figure 5.3 A). Over time, this expression will fade from much of this domain, retreating to two smaller expression domains, an expression zone at the proximal end of the appendage and an expression zone near the proximo-distal mid-point of the appendage (Figure 5.3 C). The latter of these two expression zones then fades away (Figure 5.3 E). The expression of *Wnt7* follows a different pattern, as differential forcipular expression does not commence until well into stage 4, by which time the forcipules are already morphologically distinct from the limbs (Figure 5.3 B). This takes the form of a zone of expression somewhat near to the proximal end of the forcipular appendage and extending out to the proximo-distal mid-point of the forcipule (Figure 5.3 D). It is noteworthy that by stage 5, *Wnt7* and *Wnt11* are highly differentially expressed (Figure 5.3 E, F).
Figure 5.3: Comparative expression of *Wnt11* (A, C, E) and *Wnt7* (B, D, F) in the forcipular area of flat-mounted embryos of *Strigamia maritima* at stages 4.1 (A, B), 4.3 (C, D) and 5 (E, F). The arrowhead indicates the developing forcipular appendage.
5.4 Discussion

5.4.1 Re-deployment of existing genes in forcipular development

In the patterning of the different segments in the development of an arthropod, two separable processes can be distinguished: the conferring of differential identity on the different segments and the subsequent development of these segments in varying ways (Akam, 1995). The expression of Wnt11 can be interpreted as consistent with either of these functions, but in the case of Wnt7 expression, the appearance of differential expression after the forcipules are already noticeably larger than the leg-bearing segments excludes this gene from operating in the establishment of forcipular identity. However, though there are no functional data presented here that provide a definitive link between Wnt activity and forcipular development, based on the strong and highly specific gene expression, it is very likely that Wnt7 and Wnt11 play important roles in forcipular development.

This differential Wnt expression in the forcipules is not the first instance of gene expression patterns unique to the forcipular segment, as a unique combination of Hox genes (Scr, ftz and Antp), including both head and trunk genes, are expressed in the forcipular segment of Lithobius (Hughes & Kaufman, 2002a). However, no Hox gene has its expression confined to the forcipular segment, in the way that two Wnt genes do at late germ-band stages in Strigamia, as shown here.

In examinations of the evolution of animal body plans, much attention has already been paid to the role of the Hox genes as ‘high-level executives’ in development (Pearson et al., 1995). The expression domains of these genes along the anteroposterior axis underlie the differential identity of both vertebrate skeletal units (Mallo et al., 2010) and
arthropod segments (Averof & Patel, 1997, Abzhanov & Kaufman, 2000, Deutsch & Mouchel-Vielh, 2003, Pavlopoulos et al., 2009). As a very flexible developmental module, the Hox genes can be utilised to allow body plan evolution (Gellon & McGinnis, 1998, Hughes & Kaufman, 2002c). The genes of the Wnt family are also a flexible developmental unit, capable of operating in many contexts (Cadigan & Nusse, 1997, Nusse, 2005). The use of both Wnt genes and Hox genes can be considered in the same context, as examples of existing developmental modules taking on new functions.

5.4.2 Diverse developmental changes underlying evolution of body patterning

The establishment of forcipule-specific Wnt7 and Wnt11 expression patterns and the establishment of a forcipule-specific Hox code accompanied the body patterning changes required in the evolutionary change of the first leg-bearing segment to a forcipular segment. However, these changes in gene expression display crucial differences in terms of the evolutionary changes from the probable ancestral state before forcipular evolution. In Glomeris, a millipede, and Parasteatoda, a spider, Wnt11 is expressed in multiple developing trunk appendages (Janssen et al., 2010). Thus, the evolutionary change seen here in Wnt11 expression is a reduction of the region of expression of this gene during appendage development. In contrast, Wnt7 expression in the developing forcipules seems to be a case of a new region of expression arising de novo in the developing forcipules. Though Wnt7 is expressed in multiple segments in Tribolium, Glomeris and Parasteatoda (Bolognesi et al., 2008a, Janssen et al., 2010), this expression seems to closely resemble the early segmental expression of Wnt7 in Strigamia. The later forcipular expression of Wnt7 seems to be a new expression zone entirely. Finally, the unique Hox code of the forcipules observed previously in Lithobius (Hughes & Kaufman, 2002a) is a different type of change in expression patterns, the
shifting of the boundaries of existing expression domains. So, each of the three observed expression changes observed to be correlated with the morphological patterning changes associated with the evolutionary origin of the forcipules is a different type of evolutionary change.
Chapter 6:
A functional analysis of *Wnt* gene activity in posterior growth & development

Posterior of LiCl-treated *Strigamia maritima* embryo
6.1 Introduction

6.1.1 The Wnt genes in posterior development

The Wnt genes play important roles in the control of posterior development in a wide range of animals, functions that can be divided into (i) the conferring of posterior identity on a part of the embryo and (ii) the control of posterior growth and development in this defined region (Peterson & Reddien, 2009). Posterior Wnt gene expression and posterior development functions are conserved across the Animalia (Ryan & Baxevanis, 2007).

In poriferans, Wnt expression is present at the posterior pole of the developing larva (Adamska et al., 2007) and functional studies support a role for Wnt signalling in anteroposterior patterning (Windsor & Leys, 2010). In cnidarians, many Wnt genes are present, being expressed in different domains, in either the ectoderm or endoderm, towards the posterior of the embryo (Kusserow et al., 2005, Lee et al., 2006). Functional studies in cnidarians have confirmed the importance of Wnt signalling in posterior development (Momose et al., 2008, Duffy et al., 2010). Wnt signalling is also involved in the anteroposterior patterning of the planarian Schmidtea mediterranea (Gurley et al., 2008) and the nematode Caenorhabditis elegans (Maloof et al., 1999, Silhankova & Korswagen, 2007). In the vertebrates, an abundance of evidence attests to the importance of Wnt signalling in first establishing anteroposterior polarity and then contributing to posterior development (Lekven et al., 2008). Both the former (Liu et al., 1999, Kiecker and Niehrs, 2001, Lekven et al., 2001) and the latter (Bang et al., 1999) functions are attested in several taxa. Within the Arthropoda, the importance of Wnt signalling in posterior identity and development is also widely reported, including Wnt8 being required for posterior development of a spider, Parasteatoda (McGregor et al.,
Additionally, multiple Wnt genes, signalling via the canonical Wnt pathway, are involved in the posterior development of *Tribolium* (Bolognesi *et al.*, 2008b, 2009).

### 6.1.2 Segmentation processes and posterior development

In the case of taxa like the vertebrates and the arthropods, where very many body systems are segmented, posterior development can only be fully understood when it is considered in the context of this segmented body plan and the development of the segments. In the vertebrates, the segments develop via a “clock and wavefront” generative mechanism, based on dynamic waves of oscillating gene expression in the posterior of the embryo (the clock) which become fixed into permanent stripes of expression as they reach a determination front a certain distance from the posterior (the wavefront) (Pourquié, 2003). Operating together with the FGF and Notch pathways (Jiang *et al.*, 2000, Jouve *et al.*, 2000), Wnt signalling is vital in this process, both to regulate the oscillatory gene expression of the “clock” and in the gradients underlying the “wavefront” of determination (Aulehla *et al.*, 2008, Gibb *et al.*, 2009). Based on studies in *Drosophila melanogaster*, the segmentation processes of arthropods were initially thought to be entirely dissimilar to those of vertebrates (Peel & Akam, 2003). However, studies of segmentation in a broader range of arthropod taxa have cast doubt on this interpretation. In *Cupiennius salei* and *Parasteatoda tepidariorum* (two spiders), dynamic *Delta* expression, along with other gene expression data, suggests a role for a Notch-signalling based oscillator in segment generation (Damen *et al.*, 2000, Stollewerk *et al.*, 2003, Oda *et al.*, 2007). Functional studies have confirmed the importance of such mechanisms in patterning segments (Schoppmeier & Damen, 2005). In *Tribolium castaneum*, recent evidence based on dynamic gene expression suggests the activity of a clock-like segment generation mechanism (Sarrazin *et al.*, 2012, El-Sherif *et al.*, 2012).
In *Oncopeltus fasciatus* and *Periplaneta americana*, other short-germ insects, additional evidence is present for the activity of a Notch-based oscillator in segmentation, resembling that of vertebrates (Angelini & Kaufman, 2005, De Robertis, 2008b, Pueyo et al., 2008). However, in *Gryllus bimaculatus*, yet another short-germ insect, the mechanism of segment generation seems to have a different basis, with Notch-Delta signalling not being required (Kainz et al., 2011). Some research has also been conducted into such questions in *Strigamia maritima*, and this species has been found to display dynamic gene expression consistent with a clock-like mechanism underlying segmentation (Chipman & Akam, 2008). However, no functional studies have been conducted on this system to date. Not only do most arthropod taxa pattern their segments sequentially, but dynamic gene expression in conjunction with Notch signalling and also involving Wnt signalling seems to be common in many arthropods and may represent an ancestral mechanism of posterior patterning (McGregor et al., 2009). In addition, studies of segmentation processes in the annelids *Platynereis dumerilii* and *Helobdella robusta* have revealed notable commonalities between segmentation in annelids and that in arthropods, including segment polarity gene expression, pair-rule gene expression and the involvement of Notch signalling in segment generation (Prud’homme et al., 2003, de Rosa et al., 2005, Rivera & Weisblat, 2009). Whether the similarities of segmentation processes in these disparate taxa are indicative of convergent evolution or of a common origin of segmentation remains a matter of considerable debate (Patel, 2003, Tautz, 2004, Chipman, 2010).

### 6.1.3 A functional approach to *Strigamia* development

It seems highly likely that Wnt signalling is involved in (i) segment generation via an oscillatory mechanism of some sort and (ii) posterior development in arthropods.
Indeed, it seems likely that these two processes are not easily separable. A number of issues require further investigation. What role does Wnt signalling play in (i) establishing posterior identity, (ii) posterior growth and development, (iii) the establishment of a wavefront of determination for a clock and wavefront segment generation mechanism and (iv) control of oscillating gene expression in segment generation? Additionally, how are these various processes linked? In order to best answer these questions, it is necessary not only to examine the expression of Wnt genes in Strigamia in relation to these processes, but also to develop functional approaches to perturb Wnt signalling. The methods to examine the former are readily available, as in situ hybridisation techniques previously developed can be used to examine Wnt expression. Developing functional techniques in this system is more difficult, however. The most common functional approach to such problems is to use RNAi techniques. However, the thick chorion of Strigamia eggs, coupled with the pressurised material within, seem to make such an approach impractical, if not impossible (personal observation, based on tests of microinjection in Strigamia). Accordingly, the approach taken here is to make use of lithium chloride, which acts as an uncompetitive inhibitor of GSK3β, thereby mimicking the activation of the canonical Wnt pathway (Klein & Melton, 1996, Hedgepeth et al., 1997, Clement-Lacroix et al., 2005). The small size of this compound allows it to diffuse through the chorion of embryos, allowing it to be applied by simply adding it to the embryo culturing medium. This compound has been used to perturb Wnt signalling in a wide range of taxa, including Drosophila melanogaster, Lytechinus variegatus (a sea urchin) and Xenopus laevis, with concentrations used varying from 30 mM to 300 mM (Cooke & Smith, 1988, Sherwood & McClay, 1997, Berger et al., 2005). The LiCl-treated embryos will be submitted to a number of analyses, both examining LiCl-mediated phenotypic changes and LiCl-
mediated changes in expression of a number of marker genes.

6.2 Materials and Methods

6.2.1 Embryo sampling and preparation, *in situ* hybridisation and microscopy

Performed as described previously in Chapter 2.

6.2.2 Cryo-sectioning

In order to allow high-resolution analysis of the expression of some genes, sectioning of embryos already subjected to *in situ* hybridisation was performed. The samples were dehydrated into MeOH, then transferred stepwise into 20% sucrose as follows: 75% MeOH/25% PBS, 75% MeOH/25% PBS, 50% MeOH/50% PBS, 25% MeOH/75% PBS, 25% MeOH/75% PBS, 1X PBS, 1X PBS, 10% w/v sucrose/0.5X PBS, 10% w/v sucrose/0.5X PBS, 20% w/v sucrose, 20% w/v sucrose. Samples were then transferred to moulds containing OCT compound (VWR catalogue number 361603E) and frozen, in 2-methy-butane, cooled with liquid N₂. Sections were taken with an MEV SLEE cryotome, at a thickness of 15-20μM and moved onto gelatin-chromium coated slides. These were then stained with 1% toluidine blue in 70% EtOH, rinsed in ddH₂O and allowed to dry. Samples were mounted in glycerol for imaging.

6.2.3 Lithium chloride treatment

In order to distinguish between the activities of Wnt signalling in different developmental processes, lithium treatment was performed at different developmental stages: treatment during germ-band formation allows for analysis of the roles of Wnt signalling in establishment of posterior identity, while treatment during segmentation and germ-band growth allows for analysis of Wnt signalling in the later developmental
events of segmentation and posterior growth. The embryonic development of Strigamia maritima, at a culture temperature of 13°C, lasts for ~47 days from early cleavage until hatching (Brena & Akam, 2012). Lithium chloride treatment was performed by means of the replacement of some or all of the 150 mM NaCl in the normal embryo medium (LES) with LiCl. Having first performed preliminary experiments to establish suitable concentrations for treatment, two different treatment media were prepared, one with 50 mM LiCl and one with 150 mM LiCl.

Lithium treatment was performed by the simple expedient of placing the eggs in the LiCl medium, sandwiched between filter paper, and allowing lithium to pass into the egg via diffusion. The period of interest in this study stretched from the formation of the blastoderm (stage 2.1) to the end of the main period of segmentation (stage 5). When embryos are cultured at 13°C, this period lasts for 13 days (Brena & Akam, 2012). Embryos were treated either for five days at the start of the experimental period (early treatment), for the last five days of the period (late treatment), or for the entire 13-day period (constant treatment). Treatment concentrations used were either 50 mM or 150 mM LiCl, with an additional control group cultured without LiCl. After the experimental period, all embryos were fixed for 24h in 4% PFA (see Chapter 2). To determine the developmental stage of embryos, clutches collected from the field site were cultured in 1X LES under filter paper and one embryo from each clutch was examined under a stereomicroscope to ascertain its approximate stage. In situations where the examined egg was not yet at approximately stage 2.1, the clutch was allowed to develop further before being examined again. In situations where the clutch examined seemed close to stage 2.1, one egg was removed from the clutch, fixed for 24h in 4% PFA, stained with DAPI and examined under UV light to more precisely determine its
current developmental stage. Having thus confirmed the exact developmental stage of
the egg in question, the clutch from which it was taken, based on the close synchrony of
*Strigamia* clutch development (Brena & Akam, 2012), can be considered to be very
close in developmental stage to the single sampled egg. In this manner, a high degree of
confidence that all embryos were treated at the appropriate periods could be established.

6.2.4 Examination of embryonic phenotypes

The embryos from LiCl treatment experiments, after fixation, were cleared and treated
with DAPI (as described in Chapter 2). For each embryo, it was first noted whether it
had survived and formed a germ-band during the experimental period. The phenotypes
present in the surviving embryos were then examined. On the basis of the focus of the
present study being on posterior identity/development and on segmentation, four criteria
were chosen for analysis. The first three of these related to posterior identity and
development: (a) the degree of truncation of the germ-band, (b) the curving of the germ-
band and (c) the size increase of posterior structures. A fourth criterion examined was
(d) defects in segmentation. For each embryo, a score from 0 (normal wild-type
phenotype) to 4 (most extreme phenotype) was assigned. The criteria used for each
category of phenotypic change are summarised in Table 6.1.
Table 6.1: Criteria for phenotypic categorisation of treated embryos

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Severity</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>germ-band truncation</td>
<td>0</td>
<td>germ-band length normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>germ-band length decreased 25%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>germ-band length halved</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>germ-band length decreased 75%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>germ-band length &lt;500µm</td>
</tr>
<tr>
<td>germ-band curve</td>
<td>0</td>
<td>germ-band entirely straight</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>slight irregularity in germ-band shape</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>germ-band bends over 30°</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>germ-band bends over 60°</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>germ-band entirely torqued</td>
</tr>
<tr>
<td>posterior size</td>
<td>0</td>
<td>posterior normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>posterior size increased slightly</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>posterior region size increased by half</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>posterior region size doubled</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>posterior region size increased four-fold</td>
</tr>
<tr>
<td>segmentation</td>
<td>0</td>
<td>segmentation normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>mild dis ordering of some segmental boundaries</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>some segmental boundaries show branching patterns</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>many segmental boundaries misformed</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>no normal segments</td>
</tr>
</tbody>
</table>

6.2.5 Statistical analysis

The results of the phenotypic scoring were subjected to statistical analysis. As the phenotypic scores represent categorical data Chi-squared contingency table tests were employed, including both four-way and pairwise comparisons of the relevant samples. All statistical analyses were performed with the Minitab 15 software package.
6.2.6 Expression of marker genes

The effects on downstream gene expression were also examined in three marker genes. The first of these, Abdominal-B (Abd-B), is a Hox gene, expressed at the extreme posterior of the Strigamia embryo (Brena et al., 2006). It is expected that LiCl-mediated changes in anteroposterior identity will be reflected by changes in the Abd-B expression domain. The second marker gene to be examined was Delta, encoding the ligand for the Notch receptor, making it a vital component in the Notch signalling system that may be important in Strigamia segment patterning (Artavanis-Tsakonas et al., 1999). Delta is expressed as dynamic waves travelling out from the proctodeum (Chipman & Akam, 2008). So, if this Delta expression is perturbed by LiCl treatment, it would indicate Wnt activity in the putative segmentation clock of Strigamia. The third marker gene to be examined was engrailed (en), which is expressed as a segment polarity gene in a wide range of arthropods and related taxa (Davis & Patel, 1999, Damen, 2002, Eriksson et al., 2009). The expression of this gene in Strigamia has already been examined, and has been shown to take the form of single stripes at the posterior of developing segments (Kettle et al., 2003), as in other arthropod taxa. If the hypothesis of conserved functions of segment polarity genes is correct (Eibner, 2010) and engrailed plays a causative role in segment generation, its expression should still match segment boundaries after LiCl treatment.

6.3 Results

6.3.1 Early Wnt expression in embryo’s posterior

The expression of all 11 Wnt genes present in Strigamia was examined in relation to the establishment of posterior identity. One Wnt gene, Wnt11, was implicated in this, due to
its early posterior expression. In the blastoderm (at stage 2.1), this gene is expressed as a broad domain in the presumptive future posterior (Figure 6.1). This expression zone will later retreat into a smaller region surrounding the proctodeum as the germ-band forms. As this expression of \textit{Wnt11} is present in the future posterior of the embryo before any anteroposterior polarity is visible, the expression of this gene is a likely candidate for the establishment of initial anteroposterior polarity. It is also noteworthy that its early expression bears a clear similarity to the early expression of \textit{Wnt1}, \textit{Wnt8} and \textit{WntA} in \textit{Tribolium} (Bolognesi et al., 2008a) and of \textit{Wnt8} in \textit{Parasteatoda} (McGregor et al., 2008). Considering that \textit{Wnt8} seems to be absent from the genome of \textit{Strigamia} (see Chapter 3), it seems possible that the posterior identity establishment functions of \textit{Wnt8} may have been assumed by \textit{Wnt11} in \textit{Strigamia}.

\textbf{Figure 6.1:} \textit{Wnt11} expression in future posterior of \textit{Strigamia} during early development (stage 2.1).
6.3.2 Posterior Wnt expression during germ-band growth

All 11 Wnt genes present in Strigamia were examined in relation to possible roles in posterior growth and development. Nine Wnt genes were expressed at the posterior of the embryo during germ-band growth. Three of these, Wnt2, Wnt9 and Wnt16, only display very low transcript levels near the proctodeum, making them unlikely candidates for important roles in posterior development. Four Wnt genes, Wnt1, Wnt6, Wnt10 and Wnt11, are expressed in a small domain surrounding the proctodeum during stages 4-5 (Figure 6.2 A-D). The small size of this expression zone does not suggest control of posterior growth, as the region of growth during the period is much larger than this small periproctodeal region. Two Wnt genes, Wnt5 and WntA, are expressed in much larger posterior domains (Figure 6.2 E-F). Accordingly, these two genes are the best candidates for roles in posterior growth. Additionally, as the edge of the expression domains of both of these genes correspond to the segment-forming region (Chipman & Akam, 2008), these genes are also suitable candidates for roles in defining a wavefront for the putative segmentation mechanism of Strigamia. When examined in flat-mounted embryos, these two genes appear to be almost completely co-expressed. However, there are subtle differences between them, with the boundary of expression of WntA being more clearly defined than that of Wnt5. Moreover, comparison of the expression domains of the two genes in sagittal sections (Figure 6.2 G-H) reveals that WntA expression is more restricted in this region, seemingly present in the ectoderm only, while Wnt5 expression seems to extend into the mesoderm. Of additional interest is the fact that WntA is the only gene that seems to show the presence of dynamic waves of gene expression, which are present as rings surrounding the embryo (Figure 6.3). However, this expression is quite transient, having faded away by stage 5. This expression may point towards a role for WntA in control of the putative oscillator.
Figure 6.2: Expression of Wnt1 (A), Wnt6 (B), Wnt10 (C), Wnt11 (D), Wnt5 (E, G) and WntA (F, H) in flat-mounted (A-F) and sagitally sectioned (G-H) Strigamia embryos.
6.3.3 Common LiCl-mediated phenotypic changes

LiCl treatment of embryos produced noticeable changes in morphology from wild-type forms (described in Chapter 4). Even before embryos were assessed via phenotypic scoring and the results analysed statistically, clear differences were visible between the different treatment groups, albeit with a certain degree of phenotypic variation within each group. Readily apparent differences were seen between late-treated embryos and early or constantly treated embryos, but the effect of LiCl concentration seemed to be minimal. Late-treated embryos (treated for a 5-day period corresponding to the primary period of segmentation and germ-band growth) displayed the smallest difference from wild-type morphology. These characteristics included mild to moderate defects in segmentation, with the usually straight segmental boundaries becoming undulating and branching (Figure 6.4 C). The germ-band was also slightly truncated, usually by ~25% (Figure 6.4 D). In addition, the germ-band was slightly curved (Figure 6.4E) and the proctodeum and the surrounding posterior region grew in size (Figure 6.4B). In embryos subjected to either early treatment (a 5-day period from germ-band formation onwards)
or constant treatment (a 13-day period from germ-band formation onward) the phenotypes produced were generally similar. In the more strongly affected embryos, typical features included severe shortening of the germ-band to a fraction of its normal length (Figure 6.5 A-F), with the embryo curving in on itself and enclosing a small portion of the egg yolk, instead of the whole egg, as is normal (Figure 6.5 D-E). The posterior region, including the proctodeum, was also noticeably increased in size (Figure 6.5 B-C). As with late-treated embryos, those treated with 150 mM LiCl showed slightly more severe defects than those treated with 50 mM LiCl. The trends towards truncation of the germ-band, lateral curvature of the germ-band and increase in posterior size all point to a role for \textit{Wnt} gene activity in posterior development and identity, while the segmental defects point to a role for \textit{Wnt} gene activity in segment generation. Some less frequent phenotypic effects were also present in a minority of embryos, with the most striking of these being the presence of two proctodeums in a small number of embryos (Figure 6.6). This LiCl-mediated posterior doubling provides additional support for the hypothesis of \textit{Wnt}-mediated conferring of posterior identity in \textit{Strigamia}. 
Figure 6.4: Embryonic morphology in DAPI-stained flat-mounted (A-C) and intact (D-G) *Strigamia* embryos treated with LiCl during the late treatment period.
Figure 6.5: Embryonic morphology in DAPI-stained flat-mounted (A-C) and intact (D-F) Strigamia embryos treated with LiCl during early treatment period.
6.3.4 Effects of LiCl treatment on morphological measures

In order to better quantify the effects of LiCl treatment, all embryos that survived the treatment period were categorised and scored according to the phenotypic scoring scheme devised for this purpose and described in section 6.2.3. However, due to the relatively small surviving sample size for constant and early LiCl treatment (32 and 35, respectively), the 50 mM and 150 mM LiCl concentration samples were pooled. Due to the equal distribution of 50 mM and 150 mM in each of the treatment-period categories and the small effect of LiCl concentration, this pooling should not introduce any biases. Examining the relative proportions of embryos falling into each of the phenotypic categories reveals a number of patterns, illustrated in Figure 6.7.
Figure 6.7: Histograms of phenotypic scoring of embryos treated with LiCl for different periods (early, late or constant), scored in relation to segmentation defects, germ-band truncation, germ-band curvature/polarity and posterior size. Negative control group scored “0” for each category for every embryo and is not shown.

Most prominent among the patterns illustrated here is the difference between the late-treated embryos and the early-treated and constant-treated groups in their distributions of phenotypic scoring categories. The latter two groups, in keeping with the remarks made previously, show a large degree of similarity in each of the scoring categories.
Also of interest is the lesser degree of difference between the late-treated group and the early-treated and constant-treated embryos in the category of segmentation defects than in the other three categories, all of which relate more directly to posterior identity and development. This is consistent with the establishment of posterior identity and much of posterior development occurring before the main phase of segmentation, as might be expected based on analysis of *Strigamia* development (Brena & Akam, 2012).

The scores obtained from this work were then analysed statistically for significance. First, the scores were pooled, with scores from 0-1 and from 2-4 being combined, creating a binary distinction between unaffected or nearly unaffected embryos and moderately or severely affected embryos for each of the four categories of phenotypic change mentioned previously. These scores were then analysed by means of Chi-square contingency table tests. First, a four-way test was performed to establish the presence of significant differences between the four test groups (early-treated, late-treated, constant-treated and negative control). Then, a series of pairwise comparisons was performed, as shown in Table 6.2. All four categories of phenotypic change measured showed the same pattern, with a highly significant result for all comparisons except for the comparison of early-treated and constant-treated embryos. This is in keeping with previously described trends, with highly significant differences between the late-treated group and both early-treated and constant-treated groups, with all three groups also being highly significantly different from the negative control group (Table 6.2).
Table 6.2: Summary of the results of Chi-square tests (Seg, segmentation defects; Tru, truncation defects; Cur, curvature/polarity defects; Pos, posterior size defects).

<table>
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<th>d.f</th>
<th>P</th>
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<td>79.352</td>
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<td>Seg-Early, Seg-Late</td>
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</tr>
<tr>
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<td>0.008</td>
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</table>
6.3.5 Effects of LiCl treatment on Abd-B expression

*Abd-B*, as the most posterior-expressed of the *Hox* genes, has an expression domain that is restricted to the post-segmental region of wild-type *Strigamia* embryos (Brena et al., 2006). However, in embryos that have been treated with LiCl, this region of expression is significantly extended, being expressed in a number of the posterior-most leg-bearing segments (Figure 6.8 B). This transformation is only seen, however, in embryos treated from early development. As LiCl treatment mimics universally activated Wnt signalling, this extension of the *Abd-B* expression domain provides strong evidence that Wnt signalling confers posterior identity.

**Figure 6.8:** Expression of *Abd-B* in wild-type (A) and LiCl-treated (B-D) flat-mounted *Strigamia* embryos.
6.3.6 Effects of LiCl treatment on Delta expression

The examination of Delta expression in LiCl-treated embryos was chosen as a suitable method to examine the putative Notch-signalling based segmentation clock in Strigamia maritima. Considering the previously discussed segmentation defects in LiCl-treated embryos, if segmentation proceeds via a Notch-based oscillator, embryos with segmental defects would be expected to also display associated changes in Delta expression. Delta expression is indeed altered in LiCl-treated embryos with segmentation defects. The waves of expression are lost at the posterior of the embryo or, where present, are disorderly in form (Figure 6.9 B). The loss of posterior waves of Delta bears some similarity to a phenotype observed in Parasteatoda, where Wnt8 RNAi (perturbing normal Wnt signalling) causes the failure of Delta transcripts to “clear” from the posterior region of the spider embryo (McGregor et al., 2008). Together with dynamic gene expression in both taxa (Damen et al., 2007, Chipman & Akam, 2008), this points towards commonality of posterior segmentation mechanisms in spiders and centipedes.

Figure 6.9: Expression of Delta in wild-type (A) and LiCl-treated (B) flat-mounted Strigamia embryos.
6.3.7 Effects of LiCl treatment on *engrailed* expression

The segmental marker gene chosen, *engrailed*, is expressed at the posterior of developing segments in a wide range of arthropods. It has been used as a marker of segmentation in previous functional studies of arthropod development, where its expression still conforms to formed segmental boundaries in developmentally perturbed embryos with altered segmentation patterns (Ober & Jockusch, 2006, Bolognesi et al., 2009, Pechmann et al., 2011). Accordingly, it would be expected that the expression of *engrailed* in LiCl-treated embryos will follow the same pattern. In some treated embryos, this seems to be the case, with *engrailed* transcripts present at the posterior border of the segments, even when the segment boundaries are malformed (Figure 6.10F). However, important exceptions to this pattern are present, taking a number of forms. Firstly, some LiCl-treated embryos have many segments without any detectable *en* expression. This is clearly visible in Figure 6.10 B-C, where the posterior segments are fully formed yet lack *engrailed* transcripts. This demonstrates that segmental boundaries can form in an *en*-independent fashion in *Strigamia*. Secondly, some LiCl-treated embryos display *en* expression in regions of developing segments that do not lie along the posterior boundary (Figure 6.10 D-E). Therefore, it seems to be the case that *en* expression is neither required nor sufficient for segment boundary formation.
Figure 6.10: Expression of *en* in wild-type (A) and LiCl-treated (B, D, F) flat-mounted *Strigamia* embryos. B and D are seen counter-stained with DAPI in C and E, respectively.
6.4 Discussion

6.4.1 Wnt gene activity in posterior identity, growth and development

The evidence presented here, together with expression data from other studies (Chipman & Akam, 2008), can be used to build a tentative model for Wnt activity in the linked processes of posterior development and segmentation. Firstly, Wnt signalling is involved in the initial establishment of posterior identity required to allow germ-band development. Functional evidence for this derives from the effects of LiCl treatment during early germ-band formation, causing disruption of the establishment of anteroposterior identity, with concurrent defects in anteroposterior polarity. Additionally, early Wnt11 expression in the future posterior is consistent with this model and indicates that Wnt11 is the Wnt gene involved in this process. After this initial establishment of the anteroposterior axis, Wnt signalling is also important in posterior growth and development during germ-band extension. Though there are many Wnt genes with small zones of expression near the proctodeum, two Wnt genes, Wnt5 and WntA, are strong candidates for posterior growth and development roles. The results of LiCl treatment during the developmental period under study here, including truncation of the germ-band, duplication of the proctodeum and the change in Abd-B expression domain, provide functional support for such an interpretation. This proposal, with the activity of multiple Wnt genes in the posterior development of Strigamia, bears a resemblance to Wnt gene activity in Danio and Tribolium (Shimizu et al., 2005, Bolognesi et al., 2008b).

6.4.2 Wnt gene activity in a Notch-based segmentation clock in Strigamia

When considered in the context of previous work describing oscillatory gene expression in Strigamia (Chipman & Akam, 2008), the data presented here provide strong evidence
for a clock and wavefront based segment generation mechanism in *Strigamia maritima*. Furthermore, this mechanism seems to be regulated by Wnt signalling, as evidenced by the effects of LiCl treatment on segment formation and the LiCl-mediated disruption of wave-like *Delta* expression. The functions of Wnt signalling in the putative clock and wavefront mechanism could be mediated (a) by controlling the wavefront of determination or (b) by acting to control the oscillation of gene expression. The expression pattern of *Wnt5* and *WntA* at the posterior of the embryo is consistent with the former. Additionally, the LiCl-mediated increase in size of the post-segmental region of the germ-band can best be interpreted as waves of gene expression travelling further from the posterior due to ectopic Wnt pathway activation, again supporting activity via the former mechanism. However, the disruption of *Delta* expression after LiCl treatment and the early expression of *WntA* in a wave-like pattern are more consistent with the former. It is, therefore, possible that Wnt signalling is involved in both processes.

### 6.4.3 Segmentation in *Strigamia* seems to be *en grailed*-independent

The evidence of *Wnt* gene activity in both posterior identity and development and in segment generation suggests commonalities between the development of *Strigamia* and that of other arthropods. However, the finding that segment boundary formation in *Strigamia* seems to occur without *en* expression is novel and this has not previously been described in any other arthropod. Despite the operation of the segment polarity genes being characterised as a highly conservative developmental module (Oppenheimer *et al.*, 1999, Von Dassow *et al.*, 2000), this is an example of one of its most well-known elements seemingly varying from its canonical function. As no second *en grailed* gene has been found in the *Strigamia* genome, it is unlikely that this function has been assumed by a second *en grailed* gene. This may be due to *en grailed* expression
not being necessary to activate the hedgehog signalling pathway which leads to maintenance of Wnt1 expression in the canonical segment polarity system. As engrailed is still expressed in the canonical position, it may be the case that engrailed acts in its canonical fashion, but has a less important role in Strigamia than in other systems, perhaps merely playing a minor role in stabilising existing patterns. This also serves as an example of the danger of extrapolating too readily from conservation of expression to conservation of function.

6.4.4 Concluding remarks
The data presented here provide the basis for a possible model in which Wnt signalling plays multiple roles in the linked processes of posterior development and segmentation. First, localised Wnt11 expression helps to establish posterior identity. Wnt5 and WntA then operate in posterior growth and development, while also playing roles in the control of the wavefront of determination for a Notch-based segmental oscillator. WntA is also seen to operate in the oscillation system. However, the molecular basis of the segmental boundaries produced in this fashion is unclear, as their initial formation appears to be en-independent. In order to further elucidate the role of Wnt signalling in Strigamia development, attention should be paid to the expression of other developmental genes in LiCl-treated embryos. Possibly informative target genes include caudal, which is expressed both at the posterior and in a pair-rule fashion in Strigamia (Chipman & Akam, 2008) and operates in posterior patterning in Drosophila and Danio, in the latter of which it operates downstream of Wnt (Moreno & Morata, 1999, Shimizu et al., 2005). Attention to putative segment polarity genes other than engrailed could also reveal which are of the greatest importance in establishing segmental boundaries.
Chapter 7:
General discussion

Scutigera coleoptrata embryo
7.1 Theoretical Background

The advancement of science is an inherently iterative process, starting from simple initial conceptions and progressing to more complex theoretical constructions via multiple rounds of hypothesis-testing using empirical data, followed by improvement in, and increasing the complexity of, the theoretical model concerned. Accordingly, scientific progress in the field of evolutionary developmental biology requires that the inherent complexity of biological systems is engaged with properly and that understudied and neglected taxa, like Chilopoda, are not assumed to be simple organisms that can act as a substitute for the ancestral arthropod form. In the case of Chilopoda, such misconceptions were based on their superficially basic morphology, with the homonomy of chilopod segments used to equate the centipedes with a hypothesised arthropod ancestor sharing this trait (Grenier et al., 1997). This conception falls down on a number of grounds. First, the fact that the Chilopoda may be primitive in one respect is not sufficient to allow assumptions to be made about other traits. Second, modern phylogenetic evidence places the Myriapoda at internal nodes within the Arthropoda (Edgecombe, 2010). Third, the Scutigeromorpha, despite being an ancient group containing the oldest fossil centipedes (Anderson & Trewin, 2003), have an abnormal body morphology with unequal dorsal and ventral segmentation, providing evidence against attempts to equate chilopod segments with those of an ancestral arthropod. Fourth, the novel leg-derived forcipules of centipedes are further evidence against any attempts to equate them with an ancestral and simple ur-arthropod (Dugon & Arthur, 2012a).

Thus, we are forced to look at the Chilopoda as we look at other taxa: a product of complex evolutionary and developmental histories. When considering the Wnt
signalling system, two naïve views would be either (a) to expect the presence of few \textit{Wnt} genes in the centipedes, on the basis of the supposed simplicity of the Chilopoda, or (b) to expect the conservation of the complete ancestral system, with no genes lost and no genes involved in novel functions, based on the centipedes supposedly being a “primitive taxon”. However, the recognition that chilopods are as evolved as other taxa necessitates dispensing with such assumptions. A more appropriate approach would instead place emphasis on comparing chilopods with other taxa in an explicit phylogenetic framework, as has been done throughout this thesis.

7.2 Diverse Findings

This thesis has examined the developmental systems of centipedes in detail, with emphasis placed on Wnt signalling in \textit{Strigamia maritima}. In particular, the following themes were addressed: combinatorial action and co-expression of \textit{Wnt} genes (Chapter 3), comparative segmentation and limb development (Chapter 4), forcipular development (Chapter 5) and posterior development and segment generation (Chapter 6). These investigations have produced multiple notable findings. These include implicating \textit{Wnt} genes in segment boundary formation, limb development, forcipular identity and development, establishment of posterior identity, posterior growth, establishment of a wavefront of determination for a putative clock and wavefront based segment generation system, control of posterior oscillatory gene expression, head development and the development of segmentally reiterated structures. Each of these findings is interesting in its own way and has been examined already in the context of the questions addressed in each chapter. However, the use of a broader perspective, examining the totality of the results obtained here for overarching trends, yields valuable insight into how the Wnt system as a whole operates and how it evolves.
It is difficult to extract general results from investigation of such complex and multi-faceted systems as this without succumbing to the temptation to remove additional complexities from consideration or to force results into a pre-determined framework. However, two important points arise from a consideration of \textit{Wnt} gene activity in centipedes as a whole, along with a final wide-ranging finding. These are discussed in turn as follows.

\textbf{7.3 \textit{Wnt} Genes Have Both Conserved and Novel Functions}

Of the large number of expression patterns and putative functions of \textit{Wnt} genes identified here, some, including posterior development functions, segment-polarity type expression, expression in limb development and possible functions in controlling a clock and wavefront segment generation mechanism, indicate the maintenance of ancient and conserved functions in centipedes. However, other findings are indicative of novel functions for \textit{Wnt} genes within the centipede group. Forcipular \textit{Wnt} expression is the clearest example of this, but \textit{Wnt} expression in the pre-antennal region and in some segmental patterns also fall within this category. It is noteworthy that genes with highly conserved expression in one region may seemingly be operating in a novel way in another part of the embryo. \textit{Wnt7} fits this pattern, with the segmental expression of this gene in \textit{Strigamia} matching that in \textit{Parasteatoda} and \textit{Glomeris} (Janssen et al., 2010), but with forcipular expression of this gene as a novel element. An alternative situation is seen in the case of \textit{Wnt8}, which is missing from the \textit{Strigamia} genome, but whose canonical roles seem to have been assumed by other \textit{Wnt} genes (Chapter 6). Such patterns, with \textit{Wnt} genes operating in a mosaic of conserved and novel contexts, is consistent with the previously described conception of these genes, whereby they act as
part of a “genetic toolkit” and can be repurposed in many ways to serve a multitude of functions, while different functions can be looked at as relatively separable and can evolve independently.

7.4 Non-Canonical Segment Boundary Formation in Centipedes

The segment polarity and segment boundary formation system of arthropods is noted for its large degree of evolutionary conservation (von Dassow et al., 2000). This is one of the facts that have led to the segmented germ-band being described as the arthropod “phylotypic stage”, the period of development at which members of the arthropod phylum are most similar (Peel et al., 2005, Kalinka et al., 2010). The segment polarity module includes the action of *engrailed* and *Wnt1*, where the canonical and highly conserved expression of these two genes takes the form of segmental stripes of *Wnt1*, expressed anterior to stripes of *engrailed*, with the expression of these two genes being required for segment boundary formation. However, the present study has documented two cases of *en/Wnt1* activity deviating from this canonical model in centipedes. Firstly, *Wnt1* expression in *Scolopendra* is not consistent with its involvement in a canonical segment boundary formation role (see Chapter 4). Secondly, the effects of LiCl treatment on *engrailed* expression do not support an important role for this gene in segment boundary formation (see Chapter 6). In line with the cautionary remarks made above, these findings indicate that the condition present in centipedes may not mirror the primitive arthropod state. These results also cast doubt on the degree of evolutionary conservation present in the segment polarity module, suggesting that detailed studies in other arthropod taxa may reveal additional variation in this respect.
7.5 Concluding Remarks

In addition to these two issues, the present study revealed the ubiquity of Wnt expression, the conservation of a large complement of Wnt genes in centipedes and the broad effects of Wnt signal perturbation on segmentation and posterior development. Taken together, these findings point towards the developmental importance of these genes and the complexity of the Wnt signalling system. The investigations performed here have documented a wide range of Wnt gene activities in centipedes and have shed light on the development of this under-studied group. It is hoped that this work has made a useful contribution to the field of arthropod evolution and development.
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Appendices

Appendix A: Buffers and Solutions

Phosphate Buffered Saline (PBS)

1L 10X PBS:

2.56 g \( \text{NaH}_2\text{PO}_4 \)
11.94 g \( \text{Na}_2\text{HPO}_4 \)
102.2 g \( \text{NaCl} \)
800 ml \( \text{ddH}_2\text{O} \)

pH adjusted to 7.4

\( \text{ddH}_2\text{O} \) added up to 1L

Phosphate Buffered Saline with Tween-20 (PBT)

100 ml 1X PBT

10 ml 10X PBS
90 ml \( \text{ddH}_2\text{O} \)
100 \( \mu\text{l} \) Tween-20

Tris-Acetate-EDTA (TAE)

1L 50X TAE

900 ml \( \text{ddH}_2\text{O} \)
242 g \( \text{Tris} \)
57.1 ml Glacial acetic acid
18.6g \( \text{EDTA} \)

\( \text{ddH}_2\text{O} \) added up to 1L

Saline-Sodium Citrate buffer (SSC)

1L 20X SSC

175.3 g \( \text{NaCl} \)
88.2 g Sodium citrate
800 ml \( \text{ddH}_2\text{O} \)

pH adjusted to 7

\( \text{ddH}_2\text{O} \) added up to 1 L
Alkaline Phosphatase buffer (AP buffer)

100 ml AP buffer

2 ml 5M NaCl
5 ml 1M MgCl₂
10 ml 1M Tris-HCl, pH 9.5
10 μl Tween-20
83 ml ddH₂O

Hybridisation Buffer

100 ml Hybridisation buffer

25 ml 20X SSC
2.5 g Dextran sulfate
250 μl 20mg/ml yeast tRNA
250 μl 20mg/ml heparin
100 μl Tween-20
50 ml deionised formamide

ddH₂O added up to 100 ml

MOPS Electrophoresis Buffer

1 L 10X MOPS

41.8 g MOPS (3-morpholinopropanesulfonic acid)
700 ml DEPC-treated ddH₂O
pH adjusted to 7 with NaOH
20 ml 1M sodium acetate
20 ml 500 mM EDTA, pH 8

ddH₂O added up to 1 L

Locust Embryo Saline (LES)

1 L of 10X LES

87.6 g NaCl
2.23 g KCl
2.64 g CaCl₂
1.2 g MgSO₄
11.46 g TES

ddH₂O added up to 1 L
Carbonate Buffer

10 ml carbonate buffer

67.2 mg \( \text{Na}_2\text{HCO}_3 \),
127.1 mg \( \text{Na}_2\text{CO}_3 \)

ddH2O added up to 10 ml
### Appendix B: Oligonucleotide Primers

**B1: Primers used for amplification of *Strigamia Wnt* genes**

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<tr>
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<td>63</td>
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<tr>
<td>Wnt6F2</td>
<td>TCTACATTTGCTCTCTCGAAGC</td>
<td>23</td>
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<tr>
<td>Wnt6R1</td>
<td>AAGGGTGCATCCTCGACAG</td>
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<tr>
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<tr>
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<tr>
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<td>62</td>
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<tr>
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<tr>
<td>Wnt9F1</td>
<td>TCTCAGAGGGTCGGACAGTTG</td>
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<td>25</td>
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<td>GCACGCTGCAACTCCTATTCG</td>
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<td>52</td>
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<tr>
<td>Wnt10F2</td>
<td>GCATCAATATCGCTCAAACC</td>
<td>20</td>
<td>50</td>
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</tr>
<tr>
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<td>AATTTGACGTAGTAAAGTCCATGCC</td>
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<td>44</td>
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<td>43</td>
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</tr>
<tr>
<td>Wnt11F1</td>
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<td>19</td>
<td>57</td>
<td>64</td>
</tr>
<tr>
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<td>28</td>
<td>32</td>
<td>61</td>
</tr>
<tr>
<td>Wnt11R1</td>
<td>ATAAATGTTTGTAGTCAAGTTGTATCGTG</td>
<td>28</td>
<td>32</td>
<td>62</td>
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<tr>
<td>Wnt11R2</td>
<td>GTGCACCTTGTTAGGAAGAAGAC</td>
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<td>43</td>
<td>63</td>
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</table>
B1: Primers used for amplification of *Strigamia Wnt* genes (continued)

<table>
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<th>Sequence</th>
<th>length (bp)</th>
<th>% GC</th>
<th>°C Tm</th>
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<td>50</td>
<td>62</td>
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<tr>
<td>Wnt16R1</td>
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<tr>
<td>Wnt16R2</td>
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<tr>
<td>Wnt16F3</td>
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<td>47</td>
<td>60</td>
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<tr>
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<td>18</td>
<td>55</td>
<td>62</td>
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<tr>
<td>WntAF1</td>
<td>TGCCTGGGAATGCCAAGG</td>
<td>17</td>
<td>58</td>
<td>63</td>
</tr>
<tr>
<td>WntAF2</td>
<td>CCAATTACAAAACACTGACGAC</td>
<td>23</td>
<td>39</td>
<td>62</td>
</tr>
<tr>
<td>WntAR1</td>
<td>TATCACGATCGCAATCTCGC</td>
<td>20</td>
<td>50</td>
<td>63</td>
</tr>
<tr>
<td>WntAR2</td>
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<td>45</td>
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B2: Degenerate primers used for amplification of *Wnt* genes in *Scutigera* and *Scolopendra*

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<th>Primer name</th>
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<th>length (bp)</th>
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<tbody>
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<td>Wnt1DegF1</td>
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</tr>
<tr>
<td>Wnt1DegF2</td>
<td>ACNTGYTGGATGMGNYTNCC</td>
<td>20</td>
</tr>
<tr>
<td>Wnt1DegR1</td>
<td>CARCACCARTGRAANGTRCA</td>
<td>20</td>
</tr>
<tr>
<td>Wnt1DegR2</td>
<td>CARCATAADRTCRCANCCRTC</td>
<td>20</td>
</tr>
<tr>
<td>Wnt7DegW1</td>
<td>GNGARGCNGCNTTYACNTAYGC</td>
<td>22</td>
</tr>
<tr>
<td>Wnt7DegW2</td>
<td>GARGCNGCNTTYACNTAYGCNAT</td>
<td>23</td>
</tr>
<tr>
<td>Wnt7DegW3</td>
<td>GNGCGAARTGGGGNNGNTG</td>
<td>20</td>
</tr>
<tr>
<td>Wnt7DegW4</td>
<td>YTNATGAAAYTYNCAAYAYAA</td>
<td>20</td>
</tr>
<tr>
<td>Wnt7DegC1</td>
<td>TGNTRRTTRTANCCNCKNCCRCA</td>
<td>23</td>
</tr>
<tr>
<td>Wnt7DegC2</td>
<td>GTRRTRTANCCNCKNCCRCA</td>
<td>23</td>
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<tr>
<td>Wnt11degF1</td>
<td>TTRCANGCNCNSWNACNCC</td>
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<tr>
<td>Wnt11degF2</td>
<td>CANGCNCCNSWNACNCCRTG</td>
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</tr>
<tr>
<td>Wnt11degF3</td>
<td>CCNSWNACNCRTGRCAYTTRCA</td>
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<td>ACNMGNGARCGNNTTYGNTNW</td>
<td>23</td>
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<tr>
<td>Wnt11degR2</td>
<td>NGARCGNNTTYGNTWNGCC</td>
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<td>Wnt11degR3</td>
<td>GCNNTTYGNTWNGCNNTYNYS</td>
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B3: Conventional primers used for amplification of Wnt genes in *Scutigera* and *Scolopendra*

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<th>° C Tm</th>
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<td>55</td>
<td>62</td>
</tr>
<tr>
<td>ScolwgR1</td>
<td>AGTGAAATGTACAACTGCACC</td>
<td>21</td>
<td>42</td>
<td>61</td>
</tr>
<tr>
<td>ScolwgR2</td>
<td>TGTACAACCTGCACCTCTCG</td>
<td>19</td>
<td>52</td>
<td>62</td>
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<tr>
<td>Scutwn11F1</td>
<td>CGATTTCATAGCCTGCAG</td>
<td>19</td>
<td>47</td>
<td>60</td>
</tr>
<tr>
<td>Scutwn11F2</td>
<td>GTGCAGTTCTGGTTTACTC</td>
<td>19</td>
<td>47</td>
<td>59</td>
</tr>
<tr>
<td>Scutwn11R1</td>
<td>TGAGCGGATAAACATTTCAC</td>
<td>20</td>
<td>40</td>
<td>59</td>
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<tr>
<td>Scutwn11R2</td>
<td>CACAGGAACAGCTATGAC</td>
<td>19</td>
<td>47</td>
<td>58</td>
</tr>
<tr>
<td>Scutwn11R3</td>
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<td>Scutwn11R4</td>
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</table>

B4: Primers used for amplification from plasmid templates

<table>
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<th>% GC</th>
<th>° C Tm</th>
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<td>M13Fw</td>
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<td>M13Rev</td>
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<td>PgemTF</td>
<td>CGACGGGCAATGTAATTGTACAG</td>
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<td>50</td>
<td>66</td>
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<tr>
<td>PgemTR</td>
<td>GTGTGAATTTGTGAGCGG</td>
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<td>55</td>
<td>60</td>
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Appendix C: Standard Thermocycler Programs

C.1: Temperature gradient program for amplification of *Strigamia Wnt* genes

1: 94°C for 3 minutes  
2: 94°C for 30 seconds  
3: 48-62°C for 30 seconds  
4: 72°C for 50 seconds  
5: Return to step 2, 34 repeats  
6: 72°C for 10 minutes

C.2: Temperature gradient program for degenerate PCR

1: 94°C for 3 minutes  
2: 94°C for 30 seconds  
3: 48-62°C for 30 seconds  
4: 72°C for 50 seconds  
5: Return to step 2, 39 repeats  
6: 72°C for 10 minutes

C.3: Bacterial culture screening PCR

1: 94°C for 2 minutes  
2: 94°C for 30 seconds  
3: 55°C for 30 seconds  
4: 72°C for 40 seconds  
5: Return to step 2, 29 repeats  
6: 72°C for 10 minutes

C.4: Probe template amplification PCR

1: 94°C for 2 minutes  
2: 94°C for 30 seconds  
3: 55°C for 30 seconds  
4: 72°C for 40 seconds  
5: Return to step 2, 34 repeats  
6: 72°C for 10 minutes
Appendix D: Detailed In Situ Hybridisation Protocols

D1: Basic Strigamia in situ hybridisation protocol

Probe preparation

1μg of RNA probe was used as standard. This was first hydrolysed in 0.5X carbonate buffer, in a total volume of 30 μl, for 1h at 60°C. The reaction was stopped by the addition of 870 μl hybridisation buffer, thus creating the prepared probe.

Embryo preparation

Fixed embryos, stored at at -20°C in 100% MeOH, were rehydrated stepwise into 100% PBS as follows:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% PBS / 75% MeOH</td>
<td>5 min</td>
</tr>
<tr>
<td>50% PBS / 50% MeOH</td>
<td>5 min</td>
</tr>
<tr>
<td>75% PBS / 25% MeOH</td>
<td>5 min</td>
</tr>
<tr>
<td>100% PBS</td>
<td>5 min</td>
</tr>
<tr>
<td>100% PBS</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Embryos were dechorionated by hand with fine forceps, dissection needles and paintbrushes, after which they were moved to movable plastic baskets, allowing for convenient changes of the liquid surrounding the embryos by simply moving the basket to a fresh solution in a new well of a 24-well plate.

The treatments were as follows:

4-6h pre-hybridisation at 60°C in 1 ml hybridisation buffer

Hybridisation and post-hybridisation washes

16-20h hybridisation at 60°C with 1 μg hydrolysed probe in 900 ml probe preparation

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>60°C</td>
<td>in 1 ml hybridisation buffer</td>
</tr>
<tr>
<td>15 min</td>
<td>60°C</td>
<td>in 500 μl hybridisation buffer</td>
</tr>
<tr>
<td>15 min</td>
<td>60°C</td>
<td>in 500 μl hybridisation buffer</td>
</tr>
<tr>
<td>15 min</td>
<td>60°C</td>
<td>in 1 ml 2X SSC with 0.1% Tween-20</td>
</tr>
<tr>
<td>12 min</td>
<td>60°C</td>
<td>in 1 ml 0.2X SSC with 0.1% Tween-20</td>
</tr>
<tr>
<td>12 min</td>
<td>60°C</td>
<td>in 1 ml 0.2X SSC with 0.1% Tween-20</td>
</tr>
<tr>
<td>5 min</td>
<td>RT</td>
<td>in 1 ml PBT, shaking at 40 rpm</td>
</tr>
<tr>
<td>5 min</td>
<td>RT</td>
<td>in 1 ml PBT, shaking at 40 rpm</td>
</tr>
<tr>
<td>5 min</td>
<td>RT</td>
<td>in 1 ml PBT, shaking at 40 rpm</td>
</tr>
</tbody>
</table>

Blocking, antibody binding and post-binding washes

2-4h at RT in 1 ml 0.1X goat serum, 0.9X PBT, shaking at 40 rpm
12-18h at 4°C in 1 ml 0.1X goat serum, 0.9X PBT, shaking at 40 rpm, with added antibody (see below):
For DIG-labelled probes:
0.3 μl 150 u/μl anti-DIG-AP, Fab fragments

For Flu-labelled probes:
7.5 μl 30 u/μl anti-Flu-POD, Fab fragments

30 min at RT, shaking at 40 rpm
20 min at RT in 1 ml PBT, shaking at 40 rpm
20 min at RT in 1 ml PBT, shaking at 40 rpm
20 min at RT in 1 ml PBT, shaking at 40 rpm
20 min at RT in 1 ml PBT, shaking at 40 rpm
20 min at RT in 1 ml PBT, shaking at 40 rpm

Staining: DIG-labelled probes

5 min at RT in 1 ml AP buffer, shaking at 40 rpm
5 min at RT in 1 ml AP buffer, shaking at 40 rpm
5 min at RT in 1 ml AP buffer, shaking at 40 rpm

1h-days at RT in 1 ml BM purple, shaking at 40 rpm

Staining continued until desired colour depth attained

Reaction stopped with:

5 min at RT in 1 ml PBT, shaking at 40 rpm
5 min at RT in 1 ml PBT, shaking at 40 rpm
5 min at RT in 1 ml PBT, shaking at 40 rpm

Staining: Flu-labelled probes

10min-hours at RT in 10% diaminobenzidine solution in 90% peroxide buffer

Staining continued until desired colour depth attained

Reaction stopped with:

5 min at RT in 1 ml PBT, shaking at 40 rpm
5 min at RT in 1 ml PBT, shaking at 40 rpm
5 min at RT in 1 ml PBT, shaking at 40 rpm
D2: Basic *Scolopendra in situ* hybridisation protocol

Probe preparation

1.5 μg of RNA probe was used as standard. This was first hydrolysed in 0.5X carbonate buffer, in a total volume of 45 μl, for 1h at 60°C. The reaction was stopped by the addition of 1.3 ml hybridisation buffer, thus creating the prepared probe.

Embryo preparation

Fixed embryos, stored at at -20°C in 100% MeOH, were rehydrated stepwise into 100% PBS as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Buffer Composition</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25% PBS / 75% MeOH</td>
<td>5 min</td>
</tr>
<tr>
<td>2</td>
<td>50% PBS / 50% MeOH</td>
<td>5 min</td>
</tr>
<tr>
<td>3</td>
<td>75% PBS / 25% MeOH</td>
<td>5 min</td>
</tr>
<tr>
<td>4</td>
<td>100% PBS</td>
<td>5 min</td>
</tr>
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</table>

Embryos were dechorionated by hand with fine forceps, dissection needles and paintbrushes, after which they were moved to movable plastic baskets, allowing for convenient changes of the liquid surrounding the embryos by simply moving the basket to a fresh solution in a new well of a 24-well plate.

The treatments were as follows:

4-6 h pre-hybridisation at 65°C in 1.5 ml hybridisation buffer

Hybridisation and post-hybridisation washes

16-20 h hybridisation at 65°C with 1.5 μg hydrolysed probe in 1.3 ml probe preparation

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature</th>
<th>Buffer Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>65°C</td>
<td>in 1.5ml hybridisation buffer</td>
</tr>
<tr>
<td>15 min</td>
<td>65°C</td>
<td>in 1 ml hybridisation buffer</td>
</tr>
<tr>
<td>15 min</td>
<td>65°C</td>
<td>in 1 ml hybridisation buffer</td>
</tr>
<tr>
<td>15 min</td>
<td>65°C</td>
<td>in 1.5 ml 2X SSC with 0.1% Tween-20</td>
</tr>
<tr>
<td>12 min</td>
<td>65°C</td>
<td>in 1.5 ml 0.2X SSC with 0.1% Tween-20</td>
</tr>
<tr>
<td>12 min</td>
<td>65°C</td>
<td>in 1.5 ml 0.2X SSC with 0.1% Tween-20</td>
</tr>
<tr>
<td>12 min</td>
<td>65°C</td>
<td>in 1.5 ml 0.2X SSC with 0.1% Tween-20</td>
</tr>
<tr>
<td>5 min</td>
<td>RT</td>
<td>in 1.5 ml PBT, shaking at 40 rpm</td>
</tr>
<tr>
<td>5 min</td>
<td>RT</td>
<td>in 1.5 ml PBT, shaking at 40 rpm</td>
</tr>
<tr>
<td>5 min</td>
<td>RT</td>
<td>in 1.5 ml PBT, shaking at 40 rpm</td>
</tr>
</tbody>
</table>
Blocking, antibody binding and post-binding washes

2-4 h at RT in 1 ml 0.1X goat serum, 0.9X PBT, shaking at 40 rpm
12-18 h at 4°C in 1 ml 0.1X goat serum, 0.9X PBT, shaking at 40 rpm,
with added antibody (0.3 μl 150 u/μl anti-DIG-AP, Fab fragments)

30 min at RT, shaking at 40 rpm
20 min at RT in 1.5ml PBT, shaking at 40 rpm
20 min at RT in 1.5ml PBT, shaking at 40 rpm
20 min at RT in 1.5ml PBT, shaking at 40 rpm
20 min at RT in 1.5ml PBT, shaking at 40 rpm
20 min at RT in 1.5ml PBT, shaking at 40 rpm

Staining:

5 min at RT in 1 ml AP buffer, shaking at 40 rpm
5 min at RT in 1 ml AP buffer, shaking at 40 rpm
5 min at RT in 1 ml AP buffer, shaking at 40 rpm

1+ days at RT in 1 ml BM purple, shaking at 40 rpm

Staining continued until desired colour depth attained

Reaction stopped with:

5 min at RT in 1.5ml PBT, shaking at 40 rpm
5 min at RT in 1.5ml PBT, shaking at 40 rpm
5 min at RT in 1.5ml PBT, shaking at 40 rpm
Appendix F: Partial mRNA sequences

>Wnt1 (717bp)
CTTCACGTCGCCTCGGAAATGCGCAATGCAGTGCAAGTGTCACGGAATGTCCGGTTCATGCACAGTAAAAA
CATGCTGGATGCGCCTGCCGCCGTTCAGGGTCGTCGGCGACCGACTCAAGGACCGCTTCGAGATTTGCCA
CCGGTAAAGCTTCTTGGGTTTACAGGCAGGCTTCCGGACGCGAGGTCGAGATTTGCCA

>Wnt2 (688bp)
GTAAATTGCTGATTGTTGATAAAATGTGTAATTTC
TCTAATTGTGTGGAAATGCATTGAGTTTTGTGGGT
TTTTTTGCGTTATTTTTAATGAAAAACCAGTTTTAGAAGTGGAATTTAATGTTTTATATCCTATTCCAGG
TTTCTTATGCATTATTTCTTTAACTAAGTCAAGGCTTTTTATTCTCATTTTTGTTACA

>Wnt4 (763bp)
TCTTACCGCTATATGCAGATGCCAGTTTCAGCTCTCAGCCGTGGAGTACGAGTGTCTTTTACAGAC
AATCCATGTTTGACGACATCAACCTAATTTTTGCCCAGTTTGCAAGCGTTTGCAACATTTAGAGGGTA
GGGCGGTGCTGAAAGATAAGTTTGATGGTGCCACGGAAGTTTCTCAACGGAAAAAGGATCTCGACGTGA

193
>**Wnt5** (897bp)

CTCAACCGCTTTTGACGCAAATTCGCGGTGTGTGACGCAAACACTGACCTTGGCCACACCACTTATCAAGAATCATGTGGTGCAGTTTGACAACTGAGTCCCATATGATTTTTTCTTCTTCGCTCACTCATGCGGAACAAACATTGTTCTTACGCTTTCGGTTCACTTTGCTTCGCGAGGCGCGTTTGTAACAGAAAGTCGATGGGAATGGATGGTTGCAAACTGTTGTGCTGTGGCCGAGGCTTCATACTTTTAAAGGTCACTTTGAAGGAGCGATGTGATTGCAAGTTTCATTGGTGTTGT

>**Wnt6** (833bp)

CCGTTTGGCCGATATTTGGCAAGACGCGCCGAAATCTGGAAGAAGATGACGAAAGGTTCGCAAATGGCCACCAGAGAGTGTCAATTTCAGTTTCGGAATCGTAGATGGAA

>**Wnt7** (914bp)

GGTAGCAGGGAGGCAAGTTTTTTTATACGTATTTACGCTATTTCGCGGTGTGTTGACAGTGTTGAGAGCTGAGGAGACATGCGGTGTGTTGGTGCGACGAGATGATGCAACTGAGGAGGAGATTGAGGCGACGCGATCGCTGCATGGGCGGAGGCGATAATTTTTTTCTTCTTCGCTCACTCATGCGGAACAAACATTGTTCTTACGCTTTCGGTTCACTTTGCTTCGCGAGGCGCGTTTGTAACAGAAAGTCGATGGGAATGGATGGTTGCAAACTGTTGTGCTGTGGCCGAGGCTTCATACTTTTAAAGGTCACTTTGAAGGAGCGATGTGATTGCAAGTTTCATTGGTGTTGT

194
**Wnt16** (666bp)
GATTGcATTTGACGGAGCAACACAAACAAATTTGCAATGACATCTTTCGACATGTTTTACAATTTGAGTGTTGTAACCACGTCCGCAACACAGTAGATCACAGCTATGTGGACCGTTTGAGGTTCTATTGCAATAACG
TCCTCAACTCTTCAAAATGGCCACCTCTTTCATTCTTGGCCCAAGATTTTTACGTGACTTTG
GCAACAGCTTGTGCTGCCAGCTTCTGGTTGGAAGATTCATCTGATTTTTTATATTACGGCTTTTTTGATGTTTTATGGCCCTCAAGAGCATCAACAAAAATTTCGAGAAAATCGTACTCCATACAAAATGTTGTCAGCGCACTTCTTCACAGCTTCGTTGTACGATTTTCAACATGTCTTTAATTCACATGATCCTGATACACCGTGGCAACGGCATTGCAATACGCATTAATTGAC
GCAACAGCTTGTGCTGCCAGCTTCTGGTTGGAAGATTCATCTGATTTTTTATATTACGGCTTTTTTGATGTTTTATGGCCCTCAAGAGCATCAACAAAAATTTCGAGAAAATCGTACTCCATACAAAATGTTGTCAGCGCACTTCTTCACAGCTTCGTTGTACGATTTTCAACATGTCTTTAATTCACATGATCCTGATACACCGTGG

**WntA** (885bp)
TGCCAAGCAGCTTCACCAGAGCACATTTGGAACCTCAAGGGCACAATTACAAAACACATTTGCAGCAAAATTGCAATTATAGTGAAAGGCGCAACAGCAACTGCTTGGCGAACTTGGCAGCAGCAGGAGAACTTTGCGATCTGAGCGAAAATGTGCTGAAATCGGTGGGAAACGGCGCAAAATGGGCATCGACGAGTGCCAGTATCAGTTTCGCATGTCCAGGTGGAATTGTTCCACTTTTTCCGACTCGCCGTCGGTTTTTGGCGGGATGCTCAAAATAAGGAGCAGAGAGAAGGCGTACGTGTATGCGGTGTCGGCTGCGGGGGTGGCGTACAGCATCACTCGATCGTGCAGCAAAGGCGAGATAGCGGAGTGCGACTGCGATGGATAAAGTCAGGACTAAGTCGACTAAAGGGAAATGGGAGTGGGGCGGATGCTCCGAAGACGTACGGTTTGGAATCGTTTAGTAAGGATTTCGTCGATTCGAGGGAGAATGAGAATGAACGAGGGAC
TTATGAATTTGCACAAATAACGAAGCGGGGAGACGATCTATTCGTTCGAAAATGGAACTAGTGTGCAAATGTCACGGAGTAGCCGGAAGTTGTTCAGTTAGAGTCTGTTGGCGGCGCATGGCAACCTTCCagCTAGTGGGTGACGAGCTCACCGCCAGACTAGGTGGCGCATCGTTCGTCAAGATGGTGAAAAGGAAAAAAGAAAAGACTGCGCCCTATGACATAAAAATCGCAAAAAAACCCACGAAAAAAAGTTCTCGTCTACCTAGAGGAATCACCAGACTATTGCGAAAGAAATGAAAGTGTTGGAGTATTGGGCACGCAGGAACGAACGTGTAACCGCAGTAGCTACGGTATGGACGGCTGTGGACTTTTATGTTGTGGACGAGGCTACCACACTTTAATTCA
Appendix F: Gene tree

Figure F: Gene tree of the members of the \textit{Wnt} gene family. Bootstrap values are shown at the base of clades. Wnt amino acid sequences were used from the following species: \textit{Parasteatoda tepidariorum} (At), \textit{Acyrthosiphon pisum} (Ap), \textit{Cupiennius salei} (Cs), \textit{Daphnia pulex} (Dp), \textit{Drosophila melanogaster} (Dm), \textit{Glomeris marginata} (Gm), \textit{Homo sapiens} (Hs), \textit{Ixodes scapularis} (Is), \textit{Platynereis dumerilii} (Pd), \textit{Strigamia maritima} (Sm) and \textit{Tribolium castaneum} (Tc).
Appendix G: Additional publication

Segment number, body length, and latitude in geophilomorph centipedes: a ‘converse-Bergmann’ pattern

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There is a negative relationship between trunk segment number and latitude among geophilomorph centipedes in general. A similar relationship is known to exist within the most intensively-studied geophilomorph species, Strigamia maritima, and also within several other species from this group. Previously, it was considered that this relationship did not involve body length; instead, individuals of S. maritima with more segments were considered to be more finely subdivided (not longer) than those with fewer segments. This incorrect interpretation arose from the difficulty of reliably separating post-embryonic stages and thus of making a simple and direct comparison. In the present study, we build on recent work that facilitates such comparisons; and we show conclusively that individuals with more segments are longer. Our finding means that it is now possible to connect the work on S. maritima in particular, and on geophilomorph centipedes in general, with the debate about Bergmann's 'rule': the proposal that body size increases with increasing latitude. There is a clear 'converse-Bergmann' pattern, as has been found in several other taxa. We propose an adaptive hypothesis that may explain why geophilomorphs show this pattern. © 2012 The Linnean Society of London, Biological Journal of the Linnean Society, 2012, 107, 166–174.


INTRODUCTION

The first case of a negative relationship between the segment number of an arthropod species and latitude was reported just over a decade ago (Kettle & Arthur, 2000). The species concerned was the geophilomorph centipede Strigamia maritima. The Geophilomorpha, consisting of approximately 1300 species, is one of a very small number of arthropod groups in which intraspecific variation in segment number (whether latitude-related or not) is the norm. Such variation is entirely absent from most arthropod groups at a comparable taxonomic level (orders). In contrast, it is commonplace in geophilomorphs, being found in all of their families except one: the basally branching family Mecistocephalidae (Bonato, Feddai & Minelli, 2003).

Geophilomorphs exhibit ‘pimorphic’ development. This means that all their segments are formed during embryogenesis/hatching, as recently confirmed by Brena & Akam (2012). Each hatching tarsus has the same number of segments as the much longer adult into which it will develop. This feature, which contrasts with the ‘anamorphic’ development of some other centipede groups and all millipedes (Engkoff, Dohle & Blower, 1993), means that the segment number distribution of a population is not directly affected by the population’s age structure. However, it may be (and is) affected by both genetic (Vedel, Brena & Arthur, 2009) and environmental (Vedel et al., 2008) factors, contrary to a previous claim that it was completely heritable (Prunescu & Capuse, 1971).

The latitudinal cline in S. maritima is almost certainly caused by temperature rather than other latitude-related factors, such as photoperiod (geophilomorphs are blind and subterranean). Segment
number has been shown to respond to temperature in laboratory experiments: higher rearing temperatures give higher mean segment number (Vedel et al., 2008). Furthermore, it has been shown that there is a particular period in early embryogenesis when temperature exerts its effect (Vedel et al., 2010).

Similar latitudinal clines exist within other geophiromorph species (Arthur & Kettle, 2001) but apparently not in all of them (Simaikis et al., 2010). Thus, it is a general, although not universal, pattern for the group. Also, the group as a whole exhibits a between-species pattern of the same sort. Across Europe (the continent for which there are most data), species with lower segment numbers are typically found at higher latitudes, as noted some time ago: 'Species of Geophiromorpha from cold and temperate regions tend to be smaller with fewer segments than those from warmer regions' (Eason, 1979). Again, this is a general, not universal, pattern. It must be emphasized: (1) that, at most latitudes, there are many species with varied mean segment numbers; and (2) that there is also a phylogenetic element to the overall pattern of segment number variation in the group, with, for example, some constituent clades (e.g. the family Himantantilidae) being characterized by species of especially high segment numbers (Foldai & Minelli, 1960).

In the present study, we investigate the relationship between segment number and body length in an intraspecific context, thus complementing the work of Minelli, Maruzzo & Fusco (2010) on the interspecific relationship. Initially, it was considered (Kettle & Arthur, 2000) that, in S. maritima, the most intensively studied species in the group, the body length distributions of samples from different segment-number classes were identical. However, we now show that this initial conclusion was wrong, and that it was probably caused by the difficulty of cleanly separating the different post-embryonic developmental stages ('stadia') within a population. We show conclusively that individuals with more segments are longer than those with fewer segments, both shortly after hatching and as adults.

Because body length is one measure of overall body size, our revised view of the latitudinal cline in segment number means that we can make a connection with Bergmann's rule; various versions of which (Blackburn, Gaston & Loder, 1999) state that, either within species or within groups of related species such as genera, families or orders, there is a positive relationship between body size and latitude. This 'rule' has been shown to be generally true of some animal groups, such as mammals (Blackburn & Hawkins, 2004), whereas others exhibit the converse pattern, or present a heterogeneous picture, such as arthropods (Blankenhorn & Demont, 2004; see also Meiri, 2011). The situation in amphibians appears to be particularly difficult to determine (Ashion, 2002; Ollera-Tarango & Rodriguez, 2007; Adams & Church, 2008). We show in the present study, in conjunction with previous work, that geophiromorph centipedes fall into the 'conversely-Bergmann' category; and we propose an adaptive hypothesis to explain why this should be the case.

MATERIAL AND METHODS

Study species

Stigmia maritima is an exclusively coastal species, living in the inter- and supratidal zones of shingle shores from Norway to France, including the coasts of Britain, Ireland, and many smaller islands. Its ecology was first studied in detail approximately half a century ago (Lewis, 1961). This early work was very thorough and provides a good basis on which to build further studies. No other geophiromorph species comes anywhere close in terms of how much is known about its ecology or its development. The main reason for this contrast is that, although S. maritima can be found at very high densities (up to approximately 1000 per m², probably as a result of the high density of macro-invertebrate prey), inland species have very sparse populations; it is usually hard to collect samples of even one individual per m².

The number of leg-bearing segments (LBS) in the order Geophiromorpha varies between 27 and 191 (Minelli & Bortolotto, 1988; Minelli et al., 2000). Apart from very occasional developmental anomalies in individual specimens (Lesniewska et al., 2009), the LBS number is always odd, probably as a result of a developmental constraint that precludes even numbers (Arthur & Farrow, 1999; Chipman, Arthur & Akam, 2004). With the exception noted earlier (family Maristostrophidae), each individual species of geophiromorph has a range of segment number variation, which can be as little as the modal number ±2 or as much as ±40 (Minelli, Pasqual & Etoni, 1984). In S. maritima, the number of LBS varies from 43 to 53. Similar to other species, there is sexual dimorphism, with females generally having two more segments than males. Thus, data on the two sexes must be analyzed separately.

Although the number of LBS does not increase during post-hatching growth, various other characters do change. One of the most notable is the number of coxal pores on the last pair of legs. This increases from one per side in the adolescents I stage to ten or more in the final (maturus senior) stage. Is between these are the adolescents II and III, plus the maturus junior. Each stage gives way to the next via a moult.

Although it would be ideal to be able to cleanly separate all these post-embryonic stages on the basis

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of coxal pore number and other characteristics, this has not yet proved possible. Only three studies have been conducted in this respect. In all of these, the adolescents I could be cleanly separated from the other stages. However, in the first two of the three studies, the later stages could be separated from each other only with difficulty (Lewis, 1961), or not at all (Vedel, 2001). This meant that length measurements could be made for the whole population (sexes separate) or for the adolescents I (again with sexes separate; this is the first stage at which the sex can be determined with confidence). Unfortunately, such measurements could not be made for any other stage on its own.

However, subsequent to the third study (Horneland & Meidell, 2009), it is now possible to cleanly separate the male maturus junior stage (the penultimate stage) from the stages before and after it (adolescents III and maturus senior). It is thus possible to compare one class of adults (maturus junior being the first reproducitively mature stage) as well as hatchings. The period between these stages is approximately 2 years.

**FIELD SAMPLING**

All samples were collected from a population in Galway Bay, western Ireland: Eare Island (Irish grid reference M316237), a small (less than 1 km across) island that is connected to the mainland by a causeway at low tides. This is a fairly dense population and allowed us to obtain mid-to-large sample sizes for measurements and counts. The sample sizes ranged from about 30–40 per segment-number category (adolescents I and maturus junior) to approximately 180 (‘general population’) (Table 1). Collections were made by hand, turning over rocks and stones and taking the centipedes from the moist substrate underneath. The term ‘general population’ refers to samples taken without regard to life-stage. These samples include the maturus junior life-stage that is also treated separately (in males). Although our taking of samples of the general population was not specifically designed to exclude the other life-stage we analysed separately (adolescents I), it can be seen from the length distributions (Figures 1, 2) that there are very few individuals of this life-stage included. This is not because they are absent but rather because of spatial segregation and/or the tendency to miss very small individuals when there is not a specific aim of focusing on them.

**DETERMINATION OF SEX**

Although this is difficult for many geophilomorph species, it is easy for *S. maritima* adults and large juveniles, in which the rearmost legs are much thicker in the males than in the females (Eason, 1964). However, at the adolescents I stage, this sexual dimorphism is not yet apparent. Instead, it is possible to sex these individuals by looking for the presence (male) or absence (female) of rudimentary gonopods (Kettle et al., 2003).

**DETERMINATION OF LIFE-STAGE**

The data were collected from three life-stage groups: a general population group, as adolescents I group and a maturus junior group. The first group consisted of individuals collected of all stages, with no staging performed. The second consisted of individuals of the first motile stadium, adolescents I. These were staged *sensu* Lewis (1961) using counts of coxal pores. The third group consisted of individuals of the first adult stage, maturus junior. These were delineated using a method derived from the work of Horneland & Meidell (2009); the number of coxal setae on the last pair of legs was counted; males with between 69 and

**Table 1. General information on the numbers of centipedes sampled and their body lengths**

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample sizes</th>
<th>Male (number of leg-bearing segments = 47)</th>
<th>Male (number of leg-bearing segments = 49)</th>
<th>Female (number of leg-bearing segments = 49)</th>
<th>Female (number of leg-bearing segments = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample sizes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adolescents I</td>
<td>32</td>
<td>32</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Maturus junior</td>
<td>42</td>
<td>28</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>General population</td>
<td>145</td>
<td>51</td>
<td>161</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>Mean lengths (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adolescents I</td>
<td>14.05</td>
<td>14.92</td>
<td>14.54</td>
<td>15.14</td>
<td></td>
</tr>
<tr>
<td>Maturus junior</td>
<td>31.47</td>
<td>33.90</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>General population</td>
<td>28.35</td>
<td>30.50</td>
<td>31.06</td>
<td>32.80</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Frequency distribution of total length in males. The three panels show the distributions for males at adolescence I and mature stages, and in the general population. LMS = age-corrected segments.
82 of these setae were considered to belong to the maturus junior stage. Females from this life-stage group were not examined because the much smaller degree of separation between the stages and the possible presence of variation between populations made staging of females potentially unreliable.

MEASUREMENTS OF BODY LENGTH
The highly articulated trunk and relatively flexible exoskeleton of geophilomorph centipedes present difficulties for any attempt aiming to measure their body length. Care must be taken to ensure that their bodies are fully stretched and straightened in a repeatable manner. For the adolescents I study, a single body-length measurement was taken from each individual. For stages with longer body lengths, maximum accuracy was needed because of different degrees of curvature and stretching. Thus, for the whole-population and maturus junior studies, each individual animal was fully straightened and stretched, and then measured separately three times. A mean value of these three measurements was taken for each individual specimen. Total body lengths were measured rather than some correlated character such as head length (Lewis, 1961) because of uncertainty about the exact degree of correlation and the extent to which this might vary between populations. All measurements were made on specimens under CO₂ anaesthesia, using an Olympus SZX16 stereo-microscope equipped with an Olympus DP25 digital camera and using CELL² software. CO₂ anaesthesia was induced by having specimens resting on a porous metal plate through which CO₂ from a cylinder flowed (at a very low level to avoid mortality). Anaesthetized specimens of adolescents I were also used for measurement of metasternite length. This measurement was made from the anterior border to the posterior border of each of the selected metasternites in the ventral midline of the body.

COUNTS OF SEGMENT NUMBER
These counts were made when the specimens were under CO₂ anaesthesia. Segments were counted twice on each specimen viewed at 47 magnification under an Olympus SZX16 stereo-microscope.

STATISTICAL ANALYSIS
Because body lengths were approximately normally distributed, parametric tests (t-tests) were used. Data analysis was carried out using the statistical package MINITAB (Minitab Inc.).
RESULTS

Although individuals of *S. maritima* can have an LBS number as low as 43 or as high as 53, the fact that there is variation between populations, as well as within them, means that a sample from a particular population has a more restricted range of possible values. A typical population has two main male LBS numbers (sometimes with a further two outlier numbers) and, similarly, two main (and sometimes two outlier) female LBS numbers, with these all being shifted up by two segments from their male equivalents. The population sampled for comparison of segment number and length (Hare Island) had main LBS numbers of 47 and 49 for males and 49 and 51 for females. These four main groups made up approximately 98% of this population; consequently, outliers were ignored. The length frequency distributions of the four main groups are shown in Figure 1 (males) and Figure 2 (females).

The results of pairwise comparisons between the two main male LBS numbers in each case and (where possible) between their female equivalents are given in Table 2. The adolescents 1 life-stage and the general population both show significant differences in body length distributions when different LBS numbers are compared within each sex, as does the maturus junior life-stage in the sex (male) where we were able to confidently delineate this stage. In all cases, higher LBS number results in greater length.

Up to this point, we have been making the tacit assumption that is often made with geophilomorphs: that their body plan consists of a series of homonomous segments. That is, all are the same, unlike the situation in diptersans, for example, where not only is there marked tagmatization, but also within each tagma (e.g. the abdomen) there is often a clear (but usually not monotonic) size trend from anterior to posterior. However, geophilomorph trunk segments are not perfectly homonomous and do show an anterior–posterior pattern. Usually, this is from smaller to larger to smaller, with the largest segments being those somewhere around the centre of the trunk. Broadly similar patterns have been shown in other species (Berto, Fusco & Minelli, 1997; Fusco & Minelli, 2000), and we show it here for the adolescents 1 stage of *S. maritima* (Fig. 3). We have no reason to assume that the existence of this pattern should complicate our overall analysis, especially given that all segment number classes being compared show similar anterior–posterior patterns.

DISCUSSION

The main result of the present study is very clear: in the geophilomorph centipede species *S. maritima*, individuals with more segments are longer, on average, than those with fewer segments. This is contrary to a previous claim of no relationship between these two variables in *S. maritima* (Kettle & Arthur, 2000) but complementary to the positive relationship found in an interspecific context by Minelli et al. (2010). Because body length is one measure of ‘body size’ (other ones being body width, body volume and body mass), we can now connect the latitudinal cline that we find with ‘Bergmann’s rule’, which states that body size tends to increase with latitude. It is apparent that the within-species pattern in *S. maritima*, body length decreasing with increasing latitude, is an example of a ‘converse-Bergmann pattern’, as also has been shown in some other arthropods (Blanckenhorn & Demont, 2004). It also appears that the geophilomorphs in general show a similar between-species pattern (Eason, 1979; Minelli et al., 2010).

Many different mechanisms have been proposed to explain Bergmann’s rule, and it may well be the case that different mechanisms account for the Bergmann pattern in different animal groups in which it is found (Blackburn et al., 1999). The same may be true for animal groups that show a converse-Bergmann pattern. Thus, we do not attempt to propose a mechanism to explain the converse pattern in general. Rather, we restrict ourselves to proposing a hypothesis that might account for the converse-Bergmann pattern found in geophilomorph centipedes (a group of approximately 1300 species).

The key features of our hypothesis concern (1) the time available for embryonic development to take place in natural habitats; (2) the likelihood that embryonic stages are more vulnerable, in several respects, than post-embryonic ones; and (3) the fact that the time to complete embryogenesis is known to

<table>
<thead>
<tr>
<th>Table 2. Summary of the results of comparisons between pairs of groups</th>
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<tbody>
<tr>
<td><strong>Comparison between:</strong></td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>AIM-47, AIM-49</td>
</tr>
<tr>
<td>AIF-49, AIF-51</td>
</tr>
<tr>
<td>MJM-47, MJM-49</td>
</tr>
<tr>
<td>GPM-47, GPM-49</td>
</tr>
<tr>
<td>GPF-49, GPF-51</td>
</tr>
</tbody>
</table>

Groups are given in the first column (AI, adolescents 1; MJ, maturus junior; GP, general population; M, male; F, female). Note that, although there is a prior prediction of directionality (more segments giving greater lengths), which could argue for the use of one-tailed *P*-values, we have adopted the more conservative approach of giving two-tailed values.

be very temperature-dependent: the lower the temperature, the longer the period required to complete embryogenesis. How might these three things be inter-related?

Geophilomorph mothers coil around, or 'brood', their eggs and the immediate post-hatching stages (up to early adolescents I; Fig. 4). This is true of all species studied (and also of their sister clade, the order Scolecopendromorpha, but not of other centipede groups). The period required for brooding in *S. maritima* is approximately 6–8 weeks (usually late May until late July), with the mother leaving her brood only when her adolescents I offspring have become fully operational in terms of movement (Lewis, 1961). Although adults are considered to be top predators in the shingle-shore ecosystem, with nothing eating them (Lewis, 1961), many shore-line invertebrates, such as staphylinid beetles, are potential egg-predators; so that the eggs are more vulnerable than the adults in this respect. Also, although adults and free-living juveniles are mobile, eggs and hatchlings are not. There is always a risk of inundation by storms or high spring tides. Although a brood might survive such inundation if left intact, individual eggs, if scattered by a storm, would almost certainly not because survival appears to depend on the integrity of the brood. In this respect, there is greater vulnerability of the broods than of later stages. Finally, in an unusually cool summer, embryogenesis will be slower, which increases the risk of its noncompletion. Again, the brooded stages are more vulnerable because they cannot over-winter, whereas later stages can; *S. maritima* individuals live for up to approximately 6 years (Lewis, 1961).

One of these vulnerabilities of eggs and hatchlings, namely inundation, applies especially to *S. maritima* and those few other species of geophilomorphs with a shore-line habitat, although freshwater inundation of inland species might also occur in a prolonged brooding period. The other two vulnerabilities are potentially equally applicable to all 1300 or so geophilomorph species. Given the above points, natural selection might act within geophilomorph species as follows.

First, considering just a single population, plasticity of embryogenesis might be selected for in such a way that it reaches completion sooner than it would otherwise have occurred in cool conditions. Plasticity of segment number, and hence of body length, has been experimentally demonstrated in *S. maritima*. Specifically, lower rearing temperatures lead to fewer segments (Vedel et al., 2008). Other things being equal, this will allow embryogenesis to reach completion more rapidly than if more segments were to be made. (Trunk segments are added in anterior-posterior sequence, as has been revealed by studies of expression of the segment-polarity gene *engrailed* (Kettle et al., 2003).)

Second, if a species spreads northwards from its geographical site of origin, its more northerly populations will be subject to lower mean temperatures. Selection may then act to modify the reaction norm further, so that it becomes shifted towards lower
Figure 4. An adult female (A) lays a brood of 5–25 eggs and coils around these (B) to protect them for the whole of embryogenesis and the start of post-embryonic development. The offspring begin to lead independent lives shortly after moulting to the adolescents I stage (C). In (B), the other arthropod present, a woodlouse, has no significance; its appearance in the image was accidental.

Segment numbers and concomitantly shorter embryonic periods at all temperatures, in that the reaction norm changes so that it has lower values for all three variables (segment number, body length, and embryonic period); this trend continues the further north populations spread.

In addition to the above two levels at which selection may act within a geophilomorph species, there is another factor that might contribute to the between-species pattern. Each species will eventually reach a northern limit beyond which its range cannot extend, either because of the ultimately limited ability of selection to modify the reaction norm or for some other reason. Species with longer periods of embryogenesis would be expected to encounter this limit sooner than species with shorter periods. Other things being equal, this will mean that the species with the most segments and the longest bodies will reach their northern limit soonest, thereby producing the between-species pattern of segment number and latitude noted above (Eason, 1979). However, it is important that this pattern be considered against the background of geophilomorph phylogeny (Foddai & Minelli, 2000; Marrienne, Edgecombe & Giret, 2010). Specifically, it is crucial that it be applied at the right taxonomic level. We suggest that the appropriate level is the family. For example, it can be seen within the family Himantariidae, where no species occurring in Britain (Eason, 1964) reach the same segment number or body length as some confamilial Mediterranean species (Minelli et al., 1984). However, we should add that Mediterranean species of this family are quite varied in their modal segment numbers.

Finally, it is important to note that our hypothesis for the mechanism underlying the ‘converse-Bergmann pattern’ observed in S. maritima, and in geophilomorph centipedes in general, is just that: a hypothesis. Further work needs to be conducted to test it. With regard to the pattern itself, more work needs to be carried out to test whether the claimed exceptions to it in some species of geophilomorphs (Simaiakis et al., 2010) are real or only apparent. This will involve studying the relationships between segment number/body length and temperature, not just latitude. The way in which temperature and latitude are related is far from simple, especially in inland species with broad east–west distributions (rather than linear coastal ones), where going east might reduce the temperature by as much as or more than going a similar distance north.

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