# Development and Evolution of the Spider Silk Producing System

I n a u g u r a l - D i s s e r t a t i o n

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Köln, 2008

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| Tag der mündlichen Prüfung: | 1. Dezember 2008 |

# Abstract

The spider silk producing system comprises a complex set of different gland types ending in specialized appendages called spinnerets. Using these organs, all spiders produce different types of silk that they employ for a wide range of tasks, which is a unique trait in the animal kingdom. Unfortunately, the evolutionary origin of the silk producing system is currently poorly understood. Available hypotheses proposed that the different components of the system are homologous to particular other spider organs. If this is true, then it is likely that conserved features, such as parts of the genetic program that regulates their development, are shared between different elements of the silk producing system and their postulated homologs. Examining the morphological and molecular basis of silk gland and spinneret development could therefore provide evidence for their evolutionary origin. The goals of this thesis thus were to describe the embryonic and post-embryonic development of the silk glands and spinnerets in our model species *Cupiennius salei*, to localize the position of the primordia of the silk glands and to investigate the genetic patterning of the developing spinneret limb buds.

In order to facilitate these goals, the late embryonic and early post-embryonic staging system of *C. salei* was reassessed and modified. The silk glands of the first free foraging stage were described in detail and were found to be already remarkably similar to the adult glands, including a system that allows for the production of silk during molting. It was confirmed that the silk glands first appear inside the spinnerets, supporting their supposed epidermal origin. Detailed investigation of the epidermis of the spinneret limb buds showed invaginations that possibly are the earliest morphological indications of the primordia of the silk glands, and provide a lead to investigating which molecular factors are involved in their early developmental origin. Finally, the study of expression patterns of leg patterning genes in the spinneret limb buds provided a way of comparing these structures with other spider appendages. Taken together, this thesis provides a solid basis for further research to shed more light on the evolution of the spider silk producing system.

# Kurzzusammenfassung

Der seideproduzierende Apparat der Spinnen umfasst einen komplexen Satz verschiedener Drüsentypen, die in spezialisierten Gliedmaßen enden, den sogenannten Spinnwarzen. Mit Hilfe dieser Organe produzieren Spinnen mehrere unterschiedliche Arten von Spinnseide, die sie für eine große Anzahl verschiedener Aufgaben verwenden. Dies ist im Tierreich eine einzigartige Fähigkeit. Bedauerlicherweise sind über den evolutionären Ursprung des Spinnapparats bis heute sehr wenige gesicherte Fakten bekannt. Vorhandene Hypothesen schlagen vor, dass die verschiedenen Komponenten des Spinnapparates zu anderen Organen der Spinnen homolog sind. Also sollten die Komponenten des Spinnapparates konservierte Eigenschaften, wie z. B. ähnliche genetische Netzwerke während ihrer Entwicklung, mit den vorgeschlagenen Homologen aufweisen. Die Untersuchung der morphologischen und molekularen Grundlagen der Spinndrüsen- und Spinnwarzenentwicklung sollte demzufolge Hinweise auf ihren evolutionären Ursprung liefern. Das Ziel der vorliegenden Arbeit war es daher, die embryonale und postembryonale Entwicklung des Spinnapparates in dem Modellorganismus *Cupiennius salei* zu beschreiben, die Lage der Spinndrüsenprimordien zu bestimmen und die genetische Musterbildung während der Entwicklung der Spinnwarzenanlagen zu untersuchen.

Um dies zu bewerkstelligen, wurde zunächst die Einteilung der späten Embryonal- und frühen Postembryonalstadien von *C. salei* zu überdacht und neu zu definiert. Der Spinnapparat des ersten sich selbstständig ernährenden Stadiums wurde im Detail beschrieben und erwies sich als erstaunlich differenziert, einschließlich eines Mechanismus der das Seidenspinnen während der Häutung erlaubt. Es wurde bestätigt, dass die Spinndrüsen in den Spinnwarzenknospen angelegt werden. Eine detaillierte Untersuchung der Oberfläche der Spinnwarzenanlagen zeigte Invaginationen, die vermutlich die frühesten morphologischen Indikatoren der Spinndrüsenanlagen sind. Dies bietet daher einen Anhaltspunkt zur Erforschung der molekularen Faktoren ihres ontogenetischen Ursprungs. Darüber hinaus hat die Analyse von Expressionsmustern an der Musterbildung in Beinanlagen beteiligter Gene eine Basis zum Vergleich der Spinnwarzenanlagen mit anderen Gliedmaßenknospen des Spinnenembryos ergeben. Diese Arbeit bildet somit eine solide Grundlage für weitergehende Forschung, welche die Frage nach der Evolution des Spinnapparates aufklären könnte.

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# Chapter 1

# General Introduction

## 1.1 Arthropod silk

Most people know silk from fine and expensive fabrics and do not often see the connection between their new peignoir and the spider webs they remove from the corners of their ceilings. In scientific terms however, silks can be defined as fibrous proteins made up of repetitive amino acid sequences spun or pulled from an animal under tension (Craig, 2003), and thus include the secretions of the caterpillars of the moth *Bombyx mori* that are used to weave silken fabrics, but also the threads spun by spiders. And even though spider silk has been much more difficult to produce in large quantities, a lot of investigation is been done to exploit the superb materialistic properties of spider silk for high tech applications, such as surgery of the human nervous system (Allmeling *et al.*, 2007; Kluge *et al.*, 2008). Obviously, the creatures producing silk do not do that in order to provide humans with superb materials, but silk rather evolved in many animal lineages over fast periods of time, which opened up new ways for these animals to interact with their environment. Spiders are remarkable in this respect, because they make use of silk in a tremendous amount of different ways, which is unsurpassed in the animal kingdom. This thesis is about our present knowledge of the evolutionary processes that gave rise to the spider silk producing system and that likely made spiders one of the most successful groups of animals in terrestrial ecosystems and in particular about what the development of spiders can teach us about these processes.

### 1.1.1 The use of silk by arthropods other than spiders

Present day arthropods include insects, crustaceans (lobsters, crabs and their relatives), myriapods (millipedes and their relatives) and chelicerates (spiders and their relatives, figure 1.1). Among the insects the use of silk is very diverse. For

example, caterpillars of many moth species spin themselves into a protective silken cocoon before going into the delicate process of metamorphosis. Less known is that many other insects also produce silk (reviewed by Vollrath & Knight, 2001). Examples are the fibers that bees and wasps embed into the wax of their combs to provide strength, or the sticky proteins used by caddisfly larvae to bind debris into their underwater shelters.

Remarkably, crustaceans, which are mostly aquatic and are the arthropods most closely related to the insects (Giribet *et al.*, 2001), do not seem to produce silk. An exception might be the viscous secretions produced by terrestrial crustaceans, such as the woodlous *Porcellio scaber*, that produce threads of sticky fluids that come from glands attached to the two extrusions on the back of their body (Lereboullet, 1853). It has been hypothesized that the fluid is involved in defense from predators and evolved as an adaptation to terrestrial life (Weirich & Ziegler, 1997). The molecular structure of these secretions has not been studied.

Some myriapods produce fibrous proteins that could be called silk. According to Craig (2003), "Male myriapods secrete fibrous proteins from their accessory glands that they use to produce sperm webs, sperm stalks and mating threads. Females ... also produce fibrous proteins that they use for molting, egg cocoons, defense and communication". For example, it has been shown that a sticky fluid is produced by the coiling millipede *Glomeris marginata* associated with toxic chemicals. Another example is the chilopod centipede *Orphnaeus brasilianus* that also produces a sticky and toxic secretion (Eisner, 2003). Little is known about these secretions either.

Chelicerates include the marine animals in the class Xiphosura (horseshoe crabs) and the all-terrestrial class Arachnida. Silk is produced in the well known orders of Araneae (true spiders, including tarantulas, wolf spiders and web building spiders) and Acari (mites and ticks), but also in the much less species-rich and also less known orders Pseudoscorpiones (false scorpions) (Wood & Gabbutt, 1979a,b) and arguably also in the Amblypygi (tailless whip scorpions or whip spiders). Animals in other arachnid orders, for example Scorpionida (true scorpions) and Opiliones (daddy longlegs) do not produce silk.

All arachnids share, to a certain extend, a similar body plan. Their bodies can be divided into a prosoma, which is the front part of the body bearing the brains, feeding appendages and the walking legs, and an opisthosoma, which is the hind part of the animal that includes the genitals and breathing organs, and typically bears no appendages, as discussed below. In the orders of pseudoscorpions and mites, and also in some spider species, silk is primarily made in the prosoma. In addition, in most species of all silk producing arachnid orders some sort of fibrous secretion is produced in association with both male and female genitals on the opisthosoma. These secretions are used by males of many species for making stalks on their spermatophores (sperm capsules), whereas females often produce

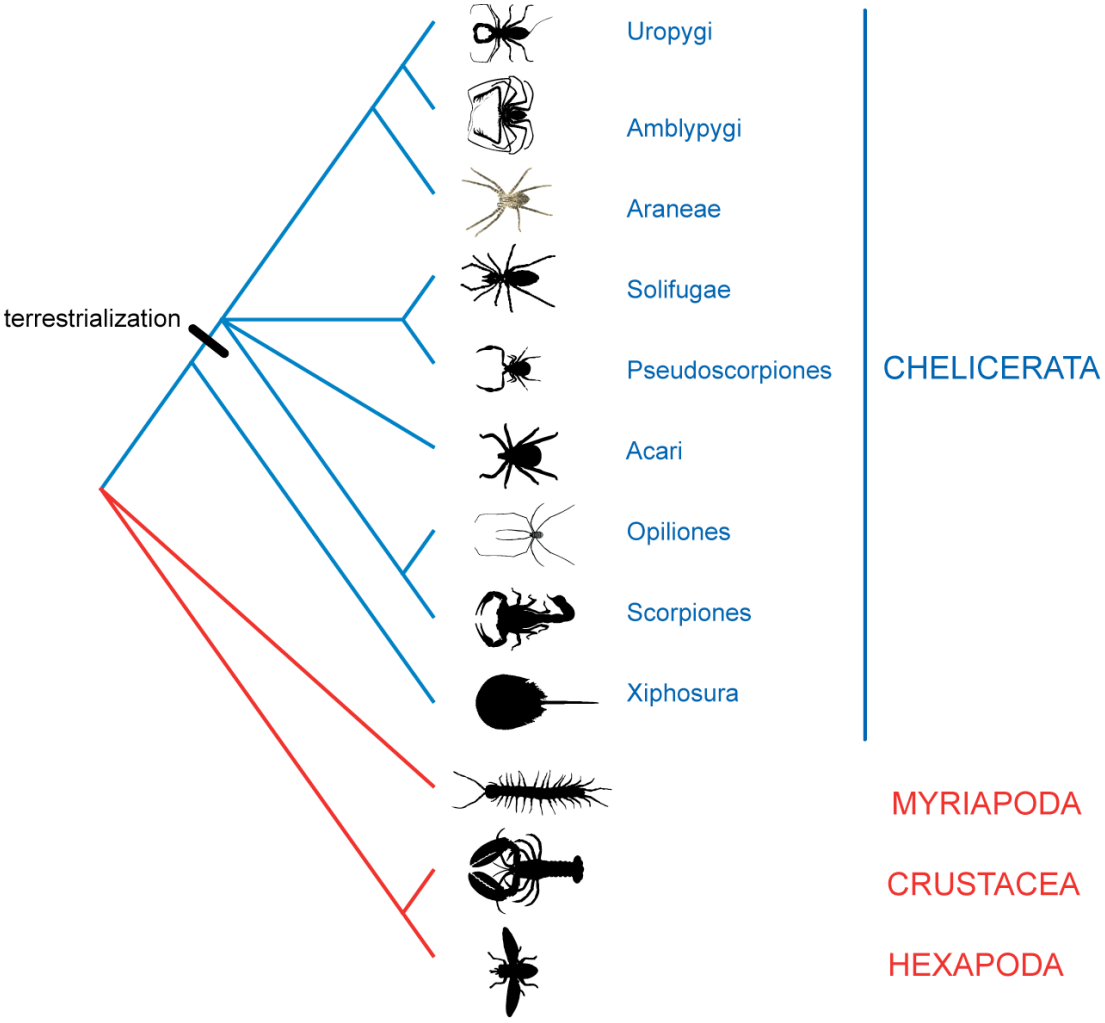



**Figure 1.1:** Cladogram of the Arthropoda, with a focus on present day Chelicerata. All terrestrial Chelicerata jointly form the class Arachnida and are the sister group to the aquatic Xiphosura (horseshoe crabs). Higher-order relationships of the arachnid orders are not well supported, but the Uropygi (Vinegaroons) and Amblypygi (whipspiders) are likely the sistergroup to the Araneae (spiders) and together form the Tetrapulmonata, named after the two pairs of book lungs present on the second and third opisthosomal segment (Shultz, 2007). Numbers of book lungs vary in the other orders, the Scorpiones (scorpions) most notably carry four pairs of book lungs on opisthosomal segments four to seven (Selden, 1998). The number of terrestrialization events is debated but book lung morphology supports a single event (Scholtz & Kamenz, 2006). Acari, ticks and mites; Crustacea, crustaceans; Hexapoda, insects; Myriapoda, millepedes and centipedes; Opiliones, daddy longlegs; Pseudoscorpiones, pseudoscorpions; Solifugae, camel spiders.

a secretion to encapsulate their eggs. Both uses seem to be adaptations to the terrestrial environment.

### 1.1.2 The use of silk by spiders

In spiders, the use of silk reached an unsurpassed complexity. They are the only arachnids that spin silk from glands in their opisthosoma that open on specialized opisthosomal protrusions, the spinnerets. Also, all spiders are capable of producing more than one silk type for which they use different glands, although some glands are capable of producing more than one type of silk as well (Craig, 2003). This intricate way of producing silk allows spiders to occupy a wide range of habitats. For example, many ground dwelling spiders live in burrows, probably as an adaption to life on land, because it reduces temperature minima and maxima (Humphreys, 1987). To provide support to their burrows, they line them with silk (Coyle, 1986). A very derived form of using silk to influence the microclimate is seen in the water spider *Argyroneta aquatica*. These animals build a diving bell from silk to breath under water (Levi, 1967).

Silk is used by many spiders to disperse as well. A very common way for young spiders to fly away from their place of birth is by making a small line of silk which gets picked up by the wind, a process called "ballooning" (Bonte *et al.*, 2007; Decae, 1987). Many spiders also use a so-called "drag line" of silk to facilitate foraging and dispersal (Barth *et al.*, 1991). This line can be clearly seen when one finds a spider at home that lowers itself from the ceiling.

Another use of spider silk is predation. Well-known are the beautiful orb webs of orb weaving spiders, but many spiders make webs that appear chaotic and nevertheless are very effective in catching prey (Benjamin & Zschokke, 2003; Morewood *et al.*, 2003; Zschokke & Vollrath, 1995).

A further use of spider silk that should be mentioned is communication. In many species, including the wandering spider *Cupiennius salei*, which is the organism used in the experiments described in this thesis, silk is used to leave behind pheromones (Papke, 2000). In species that build orb webs, conspecific male and female spiders often communicate via fibrations. Finally, it is thought that communication via silk played a prominent role in the evolution of spider social behavior (e.g. Agnarsson & Kuntner, 2005; Jantschke & Nentwig, 2001).

Together all these uses of spider silk not only influence the life of the spiders themselves, but also have a substantial impact on the ecosystem, because spiders are very important in catching a lot of herbivorous insects (Nyffeler, 2000). Also, an indirect influence was found in that the silk draglines that spiders leave as they move through the vegetation reduce the foraging of herbivorous insects (Hlivko & Rypstra, 2003).

All these examples make clear that, even though spiders are not unique in

producing silk, the number of ways they put them to use is unsurpassed by any other animal group.

## 1.2 Diversity of the spider silk producing system

The ecological complexity of the spider silk producing system is reflected in the morphology of the spinnerets and in the histochemistry, morphology and number of different glands present in a single spider. Although spinnerets and glands are tightly integrated functionally, the diversity of both organs is introduced separately for comprehensibility.

### 1.2.1 Spinneret diversity

Spinnerets are opisthosomal protrusions unique to spiders. They are muscular, flexible and highly controlled (Peters, 1967; Whitehead & Rempel, 1959). Depending on the species, the spinnerets are covered more or less densely with sensory hairs and cuticular nozzles, which are the openings of the underlying silk glands. The spinnerets of a spider, together with the surrounding cuticle that can also bear nozzles, form its "spinning field". The diversity of spinning fields is large and many characters used for constructing spider phylogenies are based upon the morphology of the spinnerets (Coddington & Levi, 1991). The importance of the spinnerets for spider taxonomy is reflected in the names of the two suborders that present-day spiders are divided in: the Mesothelae and the Opisthothelae (Platnick & Gertsch, 1976), where Meso- (middle, intermediate) and Opistho- (behind, posterior) refer to the position of the spinnerets on the anterior-posterior axis of the opisthosoma (see figure 1.2).

Figure 1.2 shows a simplified phylogram of the current ideas on the evolution of the major spider lineages (Platnick & Gertsch, 1976), together with a schematic drawing of the most frequent spinneret topologies in those groups (Marples, 1967). Branching-off at the base of the Araneae are the Mesothelae, or "segmented spiders"; a small number of species that represent a whole suborder and that have been called "plesiomorphic" (i.e. retaining ancestral characters, Coddington *et al.*, 2004). The name Mesothelae refers to the position of their spinnerets, which are located around the middle of their opisthosoma (meso-: Greek for middle, intermediate), which consists of twelve segments in total (Yoshikura, 1955). Mesothelid spiders from the genus *Liphistius* have two pairs of spinnerets on opisthosomal segment four (O4) and two pairs on opisthosomal segment five (O5). Each of these pairs develops from one limb bud only. During development, the buds divide in a dorsal and ventral portion, which become separate spinnerets.

Therefore, the anterior-most spinnerets have been called anterior lateral (*al*) and anterior medial (*am*) and the posterior-most spinnerets posterior lateral (*pl*) and posterior medial (*pm*, figure 1.2). Notably, although the (*am*) are well developed structures, they are not associated with silk glands in any of the present day Mesothelae (Marples, 1967).

A second suborder, the Opisthothelae, includes all other present day spiders. As the name suggests, in this clade the spinnerets are placed at the posterior end of the opisthosoma (opistho-: Greek for posterior). Nevertheless, the spinnerets in this group also originate from O4 and O5 and their posterior placement is the result of broadening of the ventral portion of the segments anterior to the spinnerets and reduction of the segments posterior to the spinnerets during development. So even though the outer appearance of the adult spinnerets of both suborders is different, they are considered homologous structures.

The Opisthothelae are further subdivided in the infraorders Mygalomorphae and Araneomorphae. The Mygalomorphae (tarantulas, trapdoor spiders and their kin) are mostly large, ground dwelling spiders (Hedin & Bond, 2006). Numbers of spinnerets in this group are reduced in comparison to the Mesothelae (Marples, 1967) and no Mygalomorph spider is known with four pairs of spinnerets. Most mygalomorph species have only two pairs, both situated on O5. These have been called *pl* and *pm*, in concordance with the nomenclature of the Mesothelae (Marples, 1967). Some Mygalomorphae however have three pairs of spinnerets, with two on O5 and one on O4 (e.g. Montgomery, 1909; Yoshikura, 1958). The single pair on O4 is thought to be homologous to the *al* of mesothelae and named accordingly (Marples, 1967).

The most species-rich and morphologically diverse group of spiders are the Araneomorphae. Some species in this group build very complex silk structures, including for example the webs of orb- and cobweb spiders (Benjamin & Zschokke, 2003; Zschokke & Vollrath, 1995). Spinneret numbers in this group are also reduced; no araneomorph spider is known with four pairs of spinnerets. Instead, the great majority of araneomorph spiders, including *C. salei*, has three pairs of spinnerets, one pair on O4 and two pairs on O5. In this thesis, the homology of araneomorph spinnerets is subject of investigation (see chapter 5) and therefore the suggestive terms *al*, *pl* and *pm* (Marples, 1967)) are avoided. Instead, the names anterior spinnerets (ASp) on O4, and posterior (PSp) and medial spinnerets (MSp) on O5 are employed (figure 1.2). In most araneomorphae, as it is the case in *C. salei*, medially and slightly anteriorly to the anterior spinnerets lies a small unpaired cuticular protrusion called the colulus (figure 1.2). The function of this protrusion is not known.

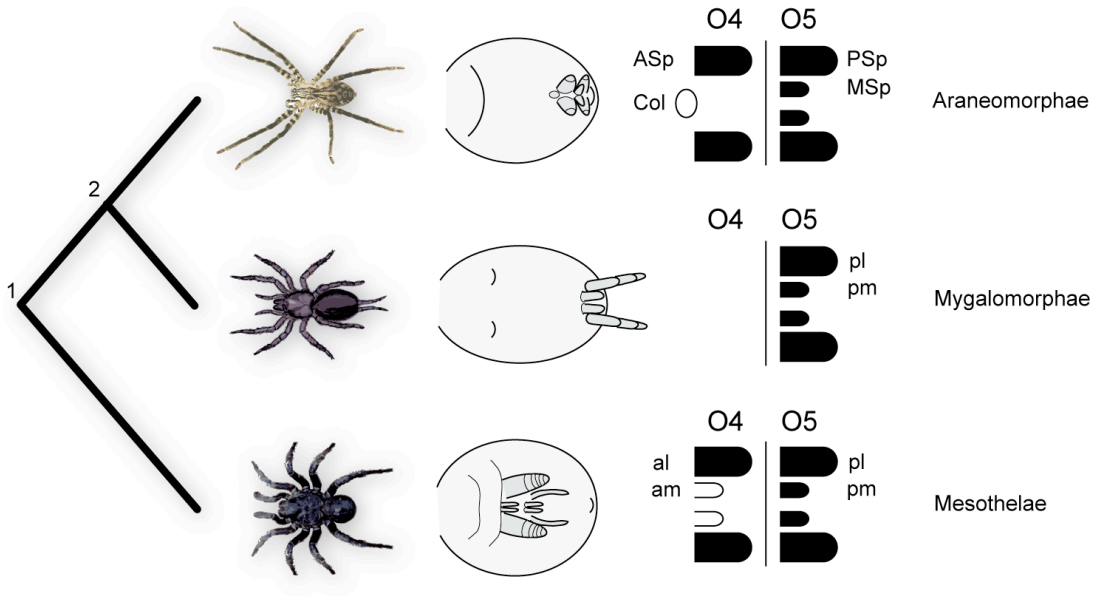



**Figure 1.2:** Cladogram showing the three major present day spider lineages. The Mesothelae (segmented spiders) are considered the sister group to all other spiders and are though to retain many plesiomorphic characters (Coddington *et al.*, 2004). The Araneomorphae (true spiders) and Mygalomorphae (tarantulas, trapdoors spiders and their kin) together form the Opisthothelae. Synapomorphic characters of the Opisthothelae include the loss of anterior medial spinnerets, a reduction in size of the segments posterior to the spinnerets resulting in a posterior shift of O4 and O5 including their associated spinnerets and a reduction of neuromeres in the subesophageal ganglia (Platnick & Gertsch, 1976). The left column shows a dorsal view of representatives of the lineages, *Liphistius sp.*, *Euagrus sp.* and *C. salei* from bottom to top. The middle column shows drawings of the ventral aspect of their opisthosoma, including the spinnerets. The right column shows a schematic representation of their spinnerets (modified from (Marples, 1967)). Black structures bear silk glands, open structures do not, the vertical line represents the border between O4 and O5. **1**, last common ancestor to all spiders; **2**, last common ancestor to the Opisthothelae; **al**, anterior lateral spinneret; **am**, anterior medial spinneret; **ASp**, anterior spinneret; **Col**, colulus; **MSp**, medial spinneret; **O4, O5**, opisthosomal segments 4 and 5; **pl**, posterior lateral spinneret; **pm**, posterior medial spinneret; **PSp**, posterior spinneret.

### 1.2.2 Silk gland diversity

The diversity of spider silk glands is vast and has been subject of many studies (e.g. Apstein, 1889; Craig, 2003; Kovoor, 1977a, 1987, and references therein). In general terms, gland diversity ranges from a comparatively undifferentiated set in mesothelid and mygalomorph spiders, to a highly complex set of up to seven different gland types in orb weaving araneomorph spiders, all specialized to the production of particular types of silk (Kovoor, 1986).

These specialized gland types not only differ in the silk they produce, but also in their shape and biochemistry. Even though there is morphological variation in the glands of the mesothelid and mygalolomorph spiders as well (Glatz, 1973; Haupt & Kovoor, 1993; Küchler, 1987), silk glands in these groups are mostly aciniform (grape shaped), with short ducts. In the araneomorph spiders however, much larger glands evolved additionally, with longer ducts, which probably has to do with the length of the duct necessary to pull the silk proteins into the right molecular configuration (Craig, 2003; Kovoor & Zylberberg, 1972). Based on similarities in shape, biochemistry, number of glands of a particular type and silk spinning behavior of spiders, attempts have been made to homologize the different silk gland types found in different groups of spiders. Further evidence for the homology of different silk gland types comes from recent studies on the molecular evolution of the genes that code for the different silk proteins (e.g. Garb, 2007; Gatesy *et al.*, 2001). It appears that all silk coding genes are related, which suggests that all different spider silk glands share a common origin (Vollrath & Porter, 2006). Furthermore, molecular phylogenies of silk proteins support the postulated silk gland type homologies (Gatesy *et al.*, 2001).

Figure 1.3 shows a phylogeny of selected spider genera. On the tree, positions are indicated where a selected set of different specialized silk gland types likely evolved. Probably the first large specialized glands to evolve at the base of all araneomorph spiders were the ampullate glands. Using ampullate glands, araneomorph spiders produce very strong fibers, including dragline silk. For example, haplogyne spiders, such as the tube web spider *Segestria*, have ampullate glands in addition to smaller grape-like glands (Glatz, 1972; Millot, 1949). They probably use this ampullate silk for making "trip-wires" that radiate from their tube web and alert the spider when prey passes by. The remaining araneomorph spiders in figure 1.3, including *C. salei* , are all "higher entelegynes" and share the presence of tubuliform glands (Coddington & Levi, 1991). These glands are only present in adult females and are used for making specialized silk to wrap the egg sac, or "cocoon". Cob web spiders, such as *Achaearanea sp.* and orb web spiders, represented by *Nephila sp.* in the figure, use specialized glands for building their predatory webs. These are flagelliform glands for making capture threads and aggregate glands for making droplets on the web that stick to prey.

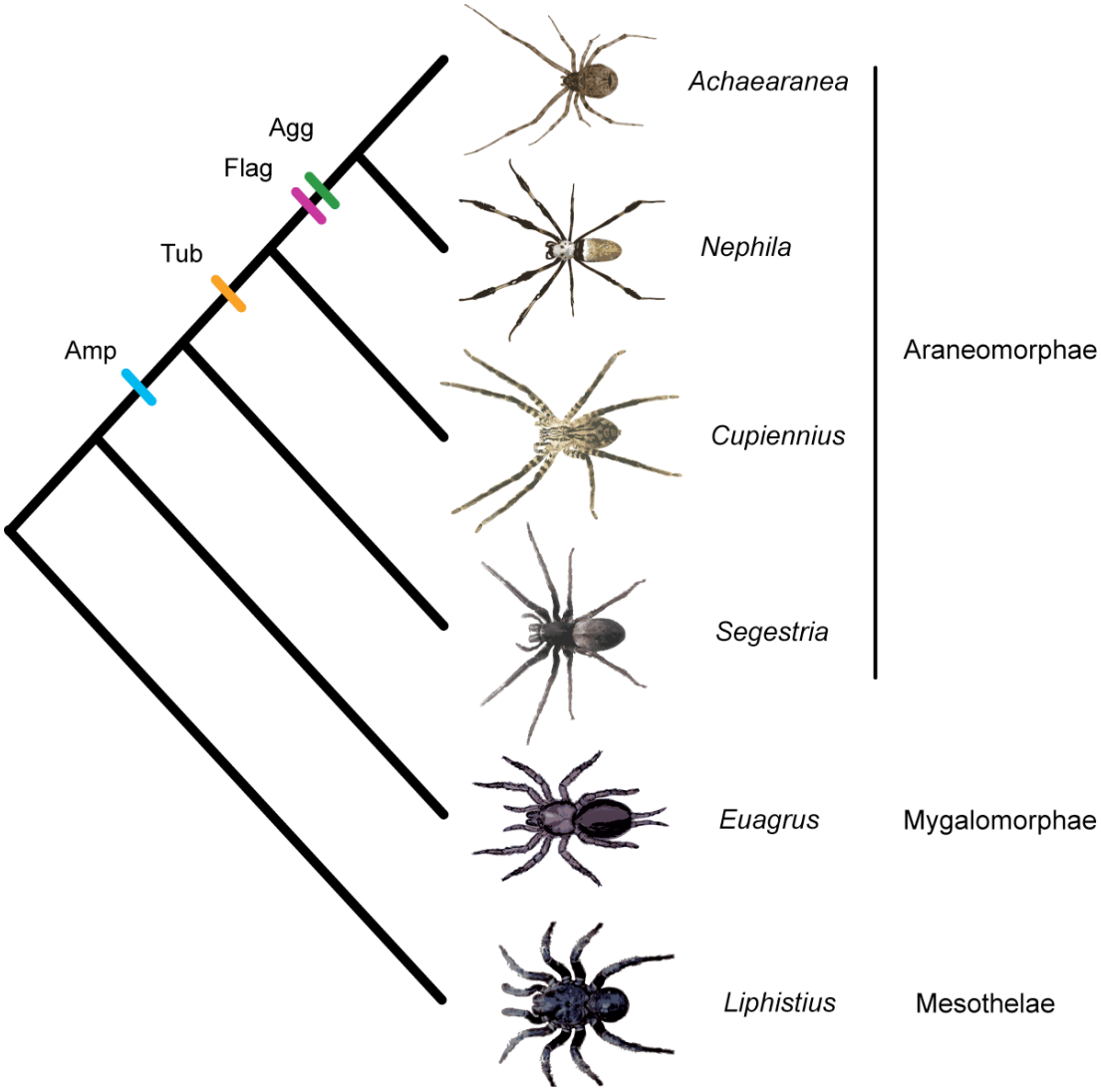



**Figure 1.3:** Cladogram of selected spider genera, showing the postulated evolutionary origin of several silk gland types in the Araneomorphae. Ampullate glands (**Amp**) are used for making strong fibers and are know from all Araneomorph spiders, including haplogyne spiders represented by the genus *Segestria* (Glatz, 1972). Tubuliform (**Tub**) glands are used for wrapping the eggs and are a synapomorphy of the higher entelegynes, which also share a modification of the female genitals (Coddington & Levi, 1991). The genus *Cupiennius* represents the "RTA clade" that includes many spider species that do not build predatory webs. Flagelliform glands (**Flag**) and aggregate glands (**Agg**) are used for web building and are only found in the Orbiculariae, such as the orb web spider genus *Nephila* and the cob web spider genus *Achaearanea*. Topology after Coddington & Levi (1991); Platnick & Gertsch (1976).

To illustrate the morphological diversity of silk producing systems of araneomorph spiders, the glands of an adult female of both *C. salei* and *Achaearanea tepidariorum* are compared schematically in figure 1.4. In *C. salei*, four different silk gland types are found (see also chapter 3) and six different types in an adult female *A. tepidariorum*. Two gland types in figure 1.4 that are not discussed above are the smaller piriform and aciniform glands. Piriform silk is very sticky and is used to attach the products of other gland types to the substrate or to each other. Aciniform silk is used for making the cocoon, but also for wrapping prey in the case of *A. tepidariorum* (Vasanthavada *et al.*, 2007). It should be noted that not only there are differences in gland types, but also in gland numbers. These differences are correlated with body size, but also with differences in ecology. For example, the apomorphic occurrence of four pairs of ampullate glands in adult *C. salei* females is thought to facilitate brood care (see chapter 3). Furthermore, particular gland types are restricted to particular spinnerets, which is a characteristic that is conserved between different spider groups.

The hitherto discussed glands types open on the spinnerets, but this is not the case for all types of glands. A further type of gland is not associated with any spinnerets, but instead with a structure called the cribellum. A cribellum occurs in some araneomorph species and consists of a transverse cuticular plate, lying anterior to the spinnerets, covered with hundreds up to to several thousands of tiny openings to cribellate glands (Kovoor, 1977a,b). The cribellum thus takes up the position of the colulus of other spider groups. The very fine silk threads emerging from cribellate glands are bundled by the spider in fluffy threads to capture prey (Eberhard & Pereira, 1993). Even though these threads are not viscous, they entangle the fine bristles on the cuticle of insect prey and are adhesive via Van der Waals forces (Vollrath, 2006). Again demonstrating the significance of the silk producing system for the current ideas on spider phylogeny, the cribellum has formerly been used as an important morphological character to distinguish between clades of "cribellate" and "ecribellate" araneomorph spiders. Later however, phylogenetic studies collapsed these groups because many closely related groups were found with both cribellata and ecribellate species, indicating that the cribellum has evolved or was lost several times independently and the character should not be used as a higher order synapomorphy (Coddington & Levi, 1991).

A final type of silk gland, that are also not associate with spinnerets, are the epiandrous, or epigastric, glands. These are small glands resembling piriform glands and are found closely to the genital openings of male spiders (Marples, 1967; Peters & Kovoor, 1991). Male spiders do not fertilize females directly, but instead deposit their sperm on a small "sperm web". Next, they take up their own sperm with specialized organs on their pedipalps and fertilize the female with their pedipalps. The epiandrous glands are involved in building these sperm

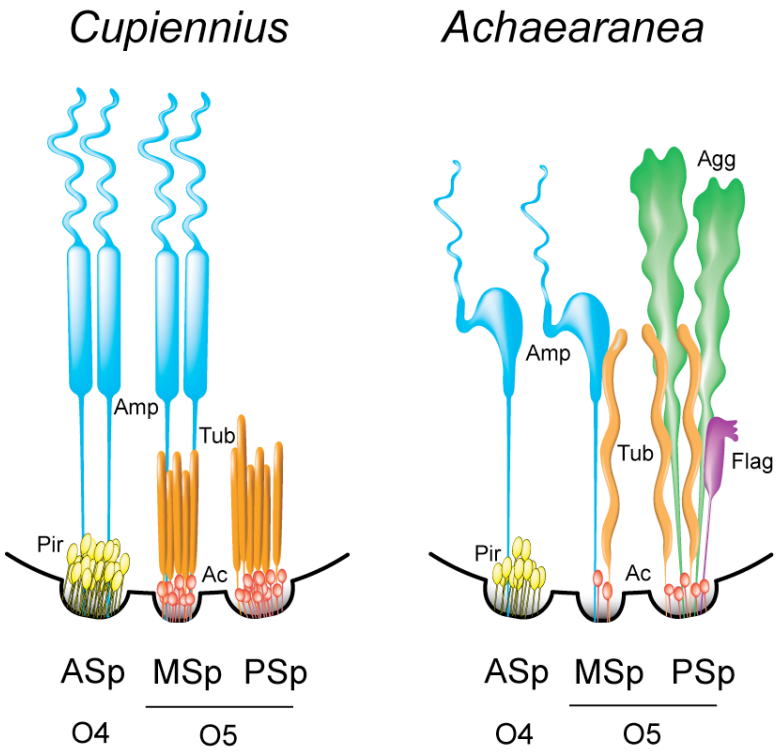



**Figure 1.4:** Schematic comparison of *Cupiennius* and *Achaearanea* adult female silk glands based on own observations and literature (Melchers, 1963; Moon & An, 2006), illustrating both the complexity of and the differences between the silk producing system of both genera. The glands and spinnerets of one body half only are depicted. See text for the postulated functions of the different gland types. **Ac**, aciniform glands; **Agg**, aggregate glands; **Amp**, ampullate glands; **ASp**, anterior spinneret; **Flag**, Flagelliform glands; **MSp**, medial spinneret; **O4, O5**, opisthosomal segments 4 and 5; **Pir**, piriform glands; **PSp**, posterior spinneret; **Tub**, tubuliform glands.

webs and are found in many spider lineages.

### 1.2.3 Other components of the silk producing system

Components of the silk producing system discussed until now are the spinnerets and the opisthosomal silk glands. However, other body parts of spiders also show modifications that are related to the use of silk. The tips of the legs for example bear claws and tufts of hairs (Dunlop, 1995a), that in many spiders are modified for handling silk (Foelix, 1970; Gorb & Barth, 1994). Modification of the claws of web building spiders made them dependent on their silken structures, which is

clearly visible when such an animal is placed on a smooth surface, because they can hardly walk outside of their webs. Another modification is seen in spiders with a cribellum, that invariantly possess a comb like structure called a callamistrum on their legs that they use to make hackled bands of cribellate silk (Opell, 2001; Opell *et al.*, 2000). Moreover, the relative proportions and slenderness of the legs of orb weaving spiders are adapted to building webs (Reed *et al.*, 1965). Finally, the complex behavioral aspects of web building (Zschokke & Vollrath, 1995) suggest that the nervous system of these spiders has adapted to living in a web as well.

## 1.3 Ideas about the evolution of the spider silk producing system

The oldest fossil known of a spinneret, including silk gland nozzles, is from the Devonian, about 380-385 million years old (Shear *et al.*, 1989). And since the silk producing system is a defining character of spiders, this is considered the oldest spider fossil Selden *et al.* (1991). The spinneret of this fossil is very similar to some of the present day spiders and although this is very interesting on its own, its information on how the silk producing system evolved is limited. Therefore, little is known about the evolutionary origin of the spider silk producing system. Nevertheless, as Shultz (1987) formulated: "The great importance of the spinning apparatus to the evolutionary success of spiders has inspired several workers to compose historical narratives in an attempt to explain the evolutionary origin of this unique feature. These notions usually rely heavily on conjecture". In other words, several authors have fantasized about possible ways the system might have evolved, but did not provided any solid evidence for their ideas. Yet, some of these hypotheses can serve as a starting point for more thorough investigation of living animals and deserve to be mentioned.

### 1.3.1 Spinneret evolution

An old idea is that the spinnerets are derived from ancestral walking legs of the opisthosoma that lost their locomotory function (e.g Jaworowski, 1896). One morphological characteristic that spinnerets share with the prosomal appendages is that they are segmented. For example, the ASp and PSp of araneomorph spiders consist of two segments that can be moved relatively to another via intrinsic muscles. Attempts have been made to homologize these muscles with the muscles of the coxa of prosomal appendages (Brown, 1945; Whitehead & Rempel, 1959). Some of these authors used this hypothesis also to explain the apparent bifurcated nature of the spinnerets, which are separated in a medial and lateral appendage

on each of the two spinneret-bearing opisthosomal segments of the Mesothelae and on opisthosomal segment 5 of the other spider groups (see figure 1.2). Their proposal was that this separation derives from a biramous ancestral appendage, homologous to crustacean pleopods. Accordingly, the lateral spinnerets are exopods and the medial spinnerets endopods (Jaworowski, 1896; Machado, 1944; Wallstabe, 1908).

Already in the 19th century however, several authors proposed that instead the spinnerets evolved *de novo*, after the disappearing of appendages on the opisthosoma of the ancestors of spiders (see Kovoor (1977a) and references therein). The latter hypothesis is supported by outgroup analysis, because no appendages are known from opisthosomal segments four and five in any of the other arachnid groups (figure 1.1) (Shultz, 1987). As Marples (1967) hypothised: "early in arachnid history the division of the body into prosoma and opisthosoma became established and the opisthosomatic appendages were suppressed in the adult though present in the early embryonic stages. In the ancestral spiders the ventral glands of the fourth and fifth opisthosomatic segments became specialized as spinnerets. ...by a process of paedomorphosis the limb-buds were retained into postembryonic life and in due course became the lateral spinnerets. Their jointed leg-like form might be secondary and not the normal jointing of a limb".

Nevertheless, there is general consensus that spinnerets are serially homologous to the prosomal appendages and to the breathing structures on the opisthosoma, because all of these organs derive from limb buds that have a very similar appearance in embryonic stages (see chapter 2). For example, Popadíc *et al.* (1998) write after detecting the expression of Distal-less (Dll) protein in the spinneret limb buds of a spider embryo: "We found that Dll is expressed in these structures, providing additional evidence for their appendicular origin and suggesting that they incorporate the distal portions of the ancestral limb". The question therefore is not *if*, but *as what* the spinnerets are serially homologous to the other appendages. Interestingly, based on further molecular evidence, Damen *et al.* (2002) postulated that the spinnerets might be homologous to the book gills of present day aquatic chelicerates, such as xiphosurans (horse-shoe crabs, see figure 1.1). Surely however, none of the terrestrial arachnids possesses gills, making the theory of Marples (1967) more likely.

Homology theories have also been postulated for the more derived features of spinnerets and on this point there appears to be consensus. It is thought that the ancestor of all spiders (node 1 in figure 1.2) looked similar to present day Mesothelae, but instead had silk glands opening on the *am* as well, and thus possessed four pairs of functional spinnerets. Moreover it is thought that, first, the PSp and MSp of Araneomorpae are homologous to the *pl* and *pm* of Mesothelae and Mygalomorpha, second, that the ASp is homologous to the *al* of Mesothelae, and third, that the proposed functional *am* of the last common

ancestor of spiders was modified into a functional cribellum and subsequently into a non-functional colulus in the Araneomorphae (Kovoor, 1977a; Marples, 1967; Platnick & Gertsch, 1976).

The third homology statement is supported by the observation that both the cribellum and the colulus are associated with muscles (Kovoor, 1977a; Whitehead & Rempel, 1959). Further support comes from the observation that in some spiders the cribellum is paired, which is considered a primitive character (Lehtinen, 1967). As Coddington & Levi (1991) formulate it: "plesiomorphic araneomorphs are unique in retaining the cribellum, a functional homolog of the anterior median spinnerets that produces extremely sticky silk. It is tempting to speculate that the diversity of araneomorph spiders is related to this important innovation".

### 1.3.2 Silk gland evolution

Several theories have been forwarded to explain the evolution of the spider silk glands. One of them, the "coxal gland hypothesis" Shultz (1987), is in line with the idea that silk might have evolved from waste products (Savory, 1960). Coxal glands have a function in excreting waste products and in ion and water balace (Butt & Taylor, 1991; Seitz, 1987). They consist of a part where excretions are accumulated and a cuticular duct that opens on the coxa of some appendages. They are segmentally iterated and although in spiders they are only found associated with the first and third walking legs, in some arachnids they also occur on more segments, including the opisthosoma (Millot, 1949). Embryologically, the interior portions of the glands derive from the coelom and the ducts develop from ectodermal invaginations (Millot, 1949). The ducts are associated with muscles (Whitehead & Rempel, 1959). The coxal gland hypothesis states that coxal glands, associated with opisthosomal appendages, evolved into silk glands. The main argument of Shultz (1987) against this theory is that coxal glands have a coelomic and thus mesodermic origin, whereas according to (Foelix, 1982), spider silk glands are of ectodermic origin.

Other hypotheses suggest a link between the gonads of the spider ancestor and the evolution of silk. One of them, the "amblypygid egg sac hypothesis" (Shultz, 1987), is based on the presence of accessory glands associated with the gonads of amblypygi, which probably are the sister group of spiders (figure 1.1). These glands are used to make egg sacs and the theory states that spider silk glands might derive from them. However, it was shown that these glands are also of mesodermic origin (Weygoldt *et al.*, 1972). Another gonad related theory is inspired on the occurrence of spermatophores (see paragraph 1.1.1) in many arachnids, and the "spermatophore- sperm web hypothesis" states that "the glands and secretions involved in construction of the spermatophore in the spider ancestor were modified for production of silken threads for use in the con-

struction of sperm webs" (Shultz, 1987). The hypothesis further includes the idea that the first selective force to give rise to the spinnerets was the need to manipulate genital products, similar to tiny appendages called "gonopods" that are found near the genital openings of amblypygids.

These hypotheses focussed on the glands and the appendages, but do not discuss the origin of the cuticular nozzles through which silk is spun. Nozzles are very important for the proper spinning of silk threads (Craig, 1997) and an hypothesis for the evolution of the silk producing system should include them. Palmer (1990) proposed that, first, silk glands are derived from "dermal glands", and second, that nozzles evolved from sensory hairs. Bond (1994) investigated this hypothesis by looking at the first nozzles that appear during the development of a mygalomorph spider and interpreted the similar morphology of the first sensory hairs and nozzles as evidence for a shared evolutionary origin of both structures.

## 1.4 Objectives of this thesis

As discussed, the evolutionary origin of the silk producing system is still very much debated and proposed theories are at the moment little more than historical narratives. What many of these theories have in common however is that it is thought that the components of the system are derived from structures that are still found in present day spiders and other arachnids. With modern methods, it is possible to make a much more detailed comparison between these structures and the different components of the silk producing system to test many of the predictions of the theories of their origin. Especially the comparison of the molecular characteristics of both the developed and the embryonic origin of the various organs could provide new insights in proposed homologies. But even though the tools are theoretically available, practically a lot of work remains to be done to establish these tools in spiders and other chelicerates. Moreover, a lot of description is lacking, especially of late spider development. For example, it is frequently assumed that spider silk glands a) develop from ectodermal invaginations, and b) that all silk glands develop in the same manner, as stated in all recent reviews on the subject. But descriptions of the early development of spider silk glands are very scarce, almost entirely date back to the early twentieth and the ninetieth century and authors of these descriptions came to mixed conclusions. Also, the embryonic origin of the medial spinneret has been discussed in numerous publications, but to date no proper description has been made of the morphogenesis of the opisthosomal limb buds at the cellular level.

The goal of this thesis therefore is to fill some of these gaps in our knowledge of spider development. The experimental work has been done in the American wandering spider *Cupiennius salei* for several reasons. One reason is that the

behaviour, physiology and general biology has been very well studied over the last decades (Barth, 2001). Also, several molecular methods have been established in this spider in recent years, including techniques to localize where genes are expressed (*in situ* hybridisation, Prpic *et al.*, 2008d) and to specifically disable the function of those genes in order to learn more about their function (RNAi, Prpic *et al.*, 2008c). A further benefit of this spider species is that its eggs are rather large, yet still available in large numbers, which facilitates their handling and observation.

In **chapter 2**, I present a new staging system for the so-called inversion of *C. salei*; a particular period of embryonic development when many morphological changes to the silk producing system take place. Also, a systematical review of the nomenclature of the early post-embryonic stages of spiders is given. As discussed, there is much more known about the silk producing system of adult spiders than of its embryonic origin, and I will use this cline in knowledge as a logical structure to build up the remainder of the thesis, working from descriptions of the late development towards studies of the embryonic origin of the glands and the spinnerets. In **chapter 3**, I describe both the outer and the inner morphology of the silk producing system of *C. salei*, starting with adults and ending with early post-embryonic stages. In chapter **4** the early embryonic differentiation of the spinneret limb buds is described using several imaging methods and hitherto un-described invaginations are uncovered that possibly represent early silk gland primordia. In **chapter 5**, the expression in the spinneret limb buds is described of several candidate genes that are thought to be involved in spider appendage development. These expression patterns are used to define the axes of the developing spinnerets in order to allow a comparison with other appendages. Also, the possible function in the spinnerets of these leg patterning genes is discussed and a proposal is made for the homology relationship of the spinnerets with the appendages of the prosoma. Finally, in **chapter 6**, future directions of the study of the spider silk producing system are discussed.

# Chapter 2

# General overview and a revised staging of the development of *Cupiennius salei*

## 2.1 Abstract

This chapter gives an overview of the embryonic and post-embryonic development of *Cupiennius salei*. Emphasis is placed on developmental phases that are of particular importance to the study of the silk producing system, including the process of inversion and the early post-embryonic stages. Moreover, a new staging system is proposed for inversion. The proposed system, contrary to an existing staging system for *Cupiennius salei*, does not depend on time after egg laying but is based on morphological characters. Although not complete, the proposed system is compatible with a staging system designed earlier to describe the development of the spider *Achaearanea tepidariorum*, and allows for future expansion of the system to include younger embryonic stages as well. Finally, the terminology of spider post-embryonic staging is reviewed, and a simple terminology is adopted for *Cupiennius salei*.

## 2.2 Introduction

There has been a recent increase in the interest in the development of spiders, driven by the advent of molecular tools. Two model species have emerged, *Cupiennius salei* and *Achaearanea tepidariorum*, that complement each other on several aspects but especially with regard to their ecology and the way they use their silk (McGregor *et al.*, 2008a). The basis of embryonic studies of *C. salei* has been the work by Seitz (1966), who described and defined the development

of this species, starting with the fertilized egg and ending with a stage he named *Jungspinne*. Seitz (1966) named the intermediate stages in German, and also provided approximated timing of these stages in hours after egg laying (hAEL). Earlier, Melchers (1963) had described external aspects of the development of *C. salei* from hatching to adulthood, thus complementing the mostly embryonic description by Seitz (1966).

Both of these descriptions form a strong backbone for developmental studies of *C. salei*, but show some shortcomings. One problem comes from the fact that more recent publications only referred to the hAEL naming, possibly to make their papers more palatable for international readers and readers that are not acquainted with details of spider development (e.g. Prpic *et al.*, 2003; Stollewerk *et al.*, 2001).

This practice is unfortunate, because the natural variation in the development of different embryos grown for the same period at the same temperature is a very interesting phenomenon on its own. For example, recent studies in *Drosophila melanogaster* have shown that there is variation within one species in the expression of a highly conserved gene involved in the patterning of the early embryo (Jaekel, 2007), variation upon which natural selection could act. To start understanding these kinds of variation in *C. salei* it would be of great benefit if hAEL would be reserved for precise measurements of developmental timing.

Also, for experiments that do not depend on precise timing, but are focussed on particular stages, measuring hAEL is not practical because almost invariantly *C. salei* females lay their eggs at night and it is very difficult to determine the exact time of oviposition. Besides, there is quite some variation in the developmental timing within one cocoon (own observations and pers. comm. Niko Prpic), making hours AEL a time unit that is to precise. Moreover, the system depends on a particular temperature, in this case 25°C. Some experiments depend on living material (e.g. injections, phalloidin stainings) and it proves to be sometimes practical to keep embryos at 20°C to slow down development and have a longer time-window of access to living embryos of particular stages.

To conclude, although the hAEL system of Seitz (1966) provides the advantage of giving direct information on the timing and order of the different stages, it is not practical. In fact, it seems that in recent *C. salei* publications the time of development was not measured, but that the stagings are based on morphological comparisons with the stages described by Seitz (1966). A way to solve this problem could be to adopt simply numbered stages, analogous to the system recently devised for *A. tepidariorum*, which starts with 1 at oviposition and reaches stage 10 when "the extending limbs become segmented and the germ band splits to shift laterally" (Akiyama-Oda & Oda, 2003; Yamazaki *et al.*, 2005).

A second shortcoming of the *C. salei* developmental description of Seitz (1966) is that the embryonic period called "inversion" is not illustrated in great detail.

These illustrations are needed because during inversion some of the main components of the silk producing system appear (see chapter 4). A final shortcoming of the existing systems for describing *C. salei* development is that the terms used for the post-embryonic stages by Melchers (1963) and Seitz (1966) do not correspond to each other, and do also not correspond to a more recent tentative to standardize the nomenclature of post-embryonic stages of spiders in different lineages (Downes, 1987). It is important to clear up this terminology, because much of the development of the silk glands occurs during post-embryonic development.

To improve this situation, in this chapter inversion is described and illustrated in more detail than by Seitz (1966). Moreover, a staging system for inversion is proposed that is compatible with the numbered stages of *A. tepidariorum*, and the terminology for the post-embryonic stages is revised. To place this information into context, and to provide an introduction to the subsequent chapters, other phases of *C. salei* development are described in lesser detail as well, starting with the fertilized egg.

## 2.3 Embryonic development

Embryonic development is defined here, in accordance with Downes (1987), as the period that starts with the fertilized zygote and concludes at hatching, i.e. the shedding of the chorion, vitelline membrane plus the embryonic cuticle including the egg tooth (see figure 2.3 1-13 days).

### 2.3.1 Early embryonic development

Early development of spiders is increasingly subject of research in recent years (see McGregor *et al.*, 2008a, and references therein). In the early embryo the body axes are set up, the germ band develops and the prosomal segments and mesoderm form. The segmented germ band elongates by sequential addition of the opisthosomal segments from anterior to posterior while the prosomal limb buds appear. In *C. salei* these processes take about 160 hours, or 7 days, at 25°C (Seitz, 1966).

### 2.3.2 Formation of the opisthosomal limb buds

About 7-8 days after egg laying (Seitz, 1966), at the onset of inversion, the final segments are added to the opisthosoma to a total of twelve, the twelfth is called the telson (Anderson, 1973). Meanwhile, limb buds appear on the opisthosomal segments 2 to 5 (O2-O5, see figures 2.1 a-c). O1 will later constrict to become -part of- the separation of prosoma and opisthosoma (see below), and remains

without limb buds. Interestingly, Yoshikura (1955) does make note of a small protrusion on O1 of the embryo of a mesothelid spider, possibly indicating a vestige of an ancestral appendage on this segment. It is generally assumed that O6-O12 remain without buds, but in fact small protrusions do appear on O6 and possibly also on O7, as is evident from expression studies of the gene *extradenticle* (see figure 5.3). These buds do not seem to develop into structures that can be distinguished in the adult, but possibly represent vestiges of ancestral appendages. Yoshikura (1955) describes protrusions on O6 of a mesothelid spider as well, without further comments on their nature.

### 2.3.3 Inversion

Soon after the opisthosomal limb buds develop inversion takes place, a process that takes until about 220 hours or 9 days after egg laying (Seitz, 1966). Figures 2.1 d-o give an overview of this period in *C. salei*, showing the external morphological changes that the posterior part of the body of an embryo goes through.

#### 2.3.3.1 Staging of *C. salei* inversion

Based on outer morphological changes of the embryo, the following stages are proposed here.

- Stage 9. The prosomal limb buds start extending distally. This stage is defined equally to stage 9 of *A. tepidariorum* (Yamazaki *et al.*, 2005).
- Stage 9 late (9l). Limb buds appear on opisthosomal segments 2 to 5.
- Stage 10 early (10e). The extending limb buds become segmented and the germ band splits to shift laterally. This stage is defined equally to stage 10 of *A. tepidariorum* (Yamazaki *et al.*, 2005).
- Stage 10 middle (10m). The dorsal edges of the two germ band halves form an angle of 180°.
- Stage 10 late (10l). The dorsal edges of the two germ band halves form an angle smaller than 180°.
- Stage 11 early (11e). Dorsal closure. Although I do not know of a formal publication, dorsal closure also seems to mark the start of stage 11 of *A. tepidariorum* in a video published publicly online (Oda & Akiyama-Oda, 2007).

- Stage 11 middle (11m). Start of constriction of the petiolus. This stage is difficult to define on gross morphology, but during stage 11m the distinct round appearance of the embryo changes into the appearance of two separate tagma.
- Stage 11 late (11l). The ventral midlines of both body halves start approaching each other.
- Stage 12. Ventral closure. Stage 12 of *A. tepidariorum* also appears around ventral closure in the video mentioned above (Oda & Akiyama-Oda, 2007).

#### 2.3.3.2 Opisthosomal changes during inversion

Early during inversion, mesoderm grows into the opisthosomal limb buds giving them a double layered aspect, and they start to differentiate (Seitz, 1966, and own observations). The buds on O2 become the book lungs (figures 2.1 b, m and 2.2 a and b). Their development progresses with the successive addition of pulmonary furrows. Purcell (1909) describes this process in the jumping spider *Sitticus floricola*, and uses the numbers of furrows to stage the development of this period. At the time of dorsal closure (figure 2.1 k), four furrows have formed in addition to the pulmonary sac (figures 2.2 a and b).

The buds on O3 develop into tubular tracheae (see figures 2.1 b, m and 2.2 a and b). These are thought to be derived of ancestral book lungs on O3 (Purcell, 1910) and in *C. salei* appear to develop as a single invagination (arrow in 2.2 b).

The most posterior opisthosomal buds, on O4 and O5 will become the spinnerets (see chapter 1). The buds on O4, marked with red arrowheads in figures 2.1 b-k, develop into the anterior spinnerets (ASp, figures 2.1 l-p and 2.2 a). The buds on O5, marked with green arrowheads in figures 2.1 b-k first appear as single buds, but over the course of inversion elongate dorso-ventrally and split into two domains; the later medial and posterior spinnerets (MSp and PSp, see figures2.1 l-p and 2.2 a). This dividing process is discussed in more detail in chapter 4.

During ventral closure, the book lungs develop more pulmonary furrows and sink into the body, as do the protrusions of the tubular trachaea (figure 2.1 n). The book lungs appear to migrate more anteriorly on the opisthosoma due to broadening of the ventral portion of O3, whereas the tubular trachaea and spinnerets move posteriorly (figures 2.1 n-o). After ventral closure, the spinnerets from both body halves lie close together at the posterior end of the body (figure 2.1 p), together forming the spinning field (SP). The SP is closely associated with a structure that many authors call the anal tubercle (AT) from this point in development on, composed of the ventral portion of the segments posterior to O5, the anus and the telson (e.g. Yoshikura, 1955).

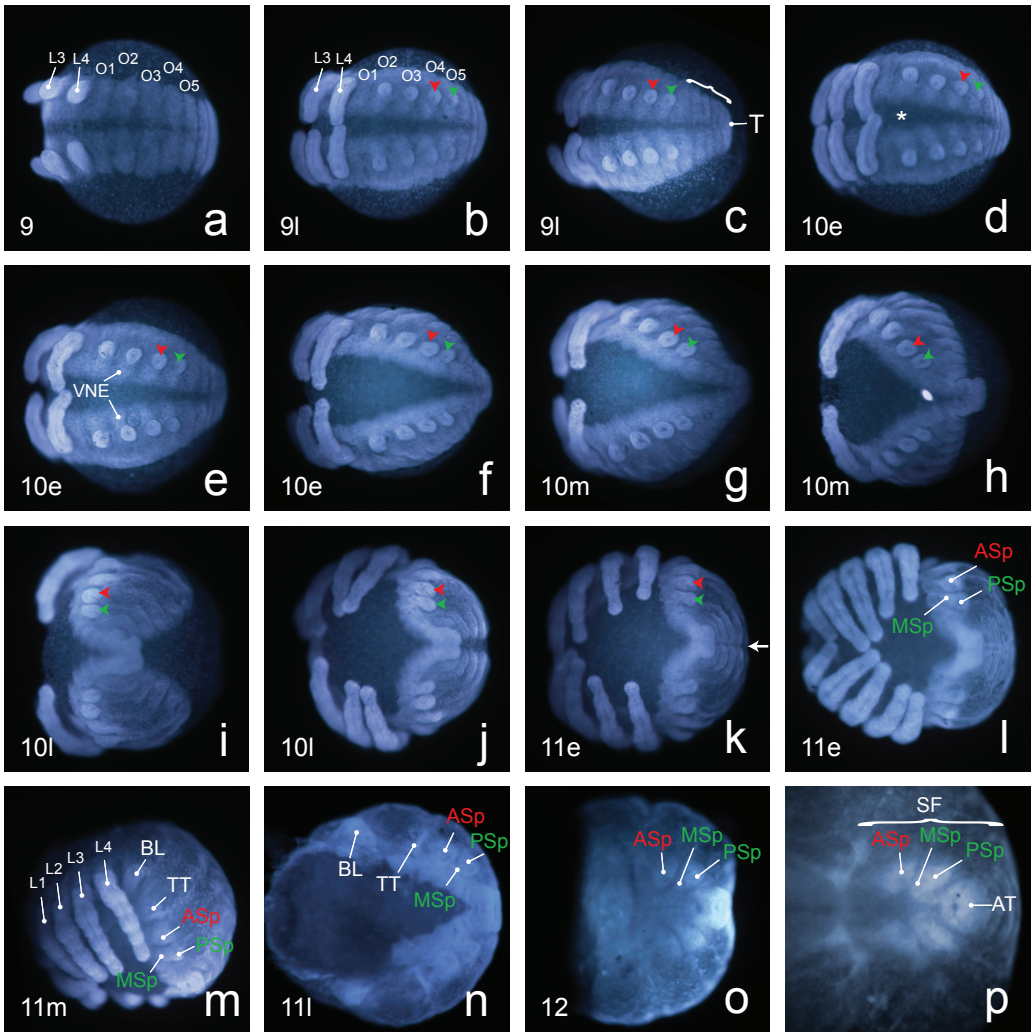



**Figure 2.1:** Developmental series of the opisthsoma of *Cupiennius salei* during inversion, stained with a fluorescent nuclear marker (dapi). At the start of inversion (**d**, stage 10e) the germ band opens up ventrally in between the ventral neuroectoderm of both body halves (marked with an asterisk). The two halves of the germ band start to move laterally, while they stay attached to one another at the head (not visible) and the telson, until they reach each other on the dorsal side (**e-k**, stages 10e-11e). Next, the ventral side closes again, marking the end of inversion (**o**, stage 12). All images are ventral views, apart from **h** and **i** which are posterior views and **m** which shows a lateral view. **a-m** show entire embryos, **n-o** close-ups of the ventral posterior part of the opisthosoma, with the prosomal part of the embryo removed. See text for stages and further explanation. The curley bracket in **c** indicates forming segments. **ASp**, anterior spinneret; **AT**, anal tubercle; **BL**, book lungs; **L1-L4**, walking legs 1 till 4; **MSp**, medial spinneret; **O1-O5**, opisthosomal segments 1 till 5; **PSp**, posterior spinneret; **SF**, spinning field; **T**, telson; **TT**, tubular trachaea; **VNE**, ventral neuroectoderm.

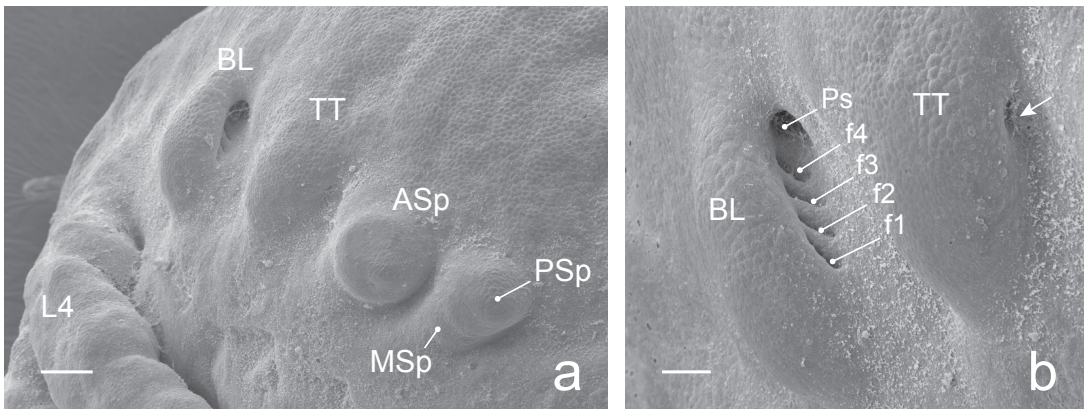



**Figure 2.2:** SEM scan of the opisthosomal extremities of *C. salei* at stage 11e. **a:**Posterior-lateral view of the opisthosomal extremities on the left side of an embryo. Scale bar 40 $\mu$m **b:**Posterior view of the developing book lungs and tubular trachaea of the left side of another embryo of the same stage. The arrow indicates the invagination site of the tubular trachaea, Dorsal is up. Scale bar 20 $\mu$m. **ASp**, anterior spinneret; **BL**, book lungs; **f1-4**, pulmonary furrows 1 till 4; **L4**, walking leg 4; **MSp**, medial spinneret; **Ps**, pulmonary sac; **PSp**, posterior spinneret; **TT**, tubular trachaea.

### 2.3.4 Embryonic development after inversion

Vachon (1957) argues that the end of inversion is the end of the embryonic period (see table 2.1). This idea is also followed by Melchers (1963), who, after Vachon (1957), calls the following stage "first prelarva", but not by Seitz (1966). Downes (1987), in an attempt to end the "plethora of terms" describing late embryonic spider development, discards the terminology of Vachon (1957) and includes the development between inversion and hatching in the embryonic period, even though not all spiders hatch at the same ontogenetic stage (Holm, 1940).

Towards the end of inversion the embryo begins to constrict slightly around O1 and this process continues after inversion. This constriction, called the petiolus, divides the embryo into two tagma; the prosoma and opisthosoma. Between about day 9 and 12 these two tagma lie perpendicular to each other, giving the embryo a "crooked" appearance, called the "abgeknickter Embryo" by Seitz (1966). During this stage septa, which are though to be of mesodermic origin (Rempel, 1957), start penetrating the yolk in a dorsoventral fashion, and divide the interior of the opisthosoma into yolk sacs. These have a structure that seems to correspond with the germ band segments, as discussed by (Holm, 1940), although Seitz (1966) is not in concordance with this theory. The septa are probably important for the

development of internal organs (see chapter 4). The constriction of the petiolus continues after day 12 and the embryo starts to stretch (see figure 2.3 13 days). After about 13-14 days at 25°C the embryo ruptures the vitelline membrane and chorion using its egg teeth, which are embryonic cuticular structures at the base of the pedipalps. Afterwards, the embryo sheds these layers together with the embryonic cuticle, which probably consists of little more than the egg teeth, as extrapolated from observations by Holm (1940) of the closely related families Pisauridae and Lycosidae (see chapter 1). Hatching of *C. salei* is beautifully illustrated by Melchers (1963).

## 2.4 Post-embryonic development

### 2.4.1 The postembryo

*C. salei* hatches at a stage that has been called "first postembryonic stage" by Seitz (1966), "second prelarva" by Melchers (1963) and "stage 1 larva" by Stollewerk & Seyfarth (2008) (see table 2.1). This stage has its walking legs bent down underneath the prosoma, like the embryonic stages after inversion (see figure 2.3 16 days). It does not move and does not have sensory hairs or pigmentation (Stollewerk & Seyfarth, 2008). Downes (1987) argues that one should not call this stage first instar, because the embryonic cuticle that is shed is not considered a true molt, which is defined as "a molt with limbs" (see Holm (1940) for a review of the embryonic cuticle). Instead, this stage is called "postembryo", defined as: "the stage starting with hatching: the shedding, but not necessarily the discarding, of the chorion" (Downes, 1987).

Stollewerk & Seyfarth (2008) report that in about 50 % of the cases *C. salei* sheds the cuticle of the postembryo together with the chorion, vitelline membrane and embryonic cuticle. If this would be true, it would imply that in many cases the embryo hatches as first instar, but this has never been confirmed by own observations and is here discarded as a common phenomenon in *C. salei*. However, Holm (1940) writes that spiders of the genus *Zora* (that is also included in the family Ctenidae together with *C. salei*) do hatch as first instar. Similar to *C. salei*, spiders of related families Pisauridae and Lycosidae hatch as postembryo.

### 2.4.2 The first instar

After the first true molt, the first instar emerges: "The first instar starts with the shedding, but not necessarily the discarding, of the first integument that has legs" (Downes, 1987). Seitz (1966) calls this *C. salei* stage *Jungspinne*, Melchers (1963) the "third incomplete stage" and Stollewerk & Seyfarth (2008) "stage 3

**Table 2.1:** Nomenclature of embryonic and post-embryonic developmental stages according to different authors. Authors referring to *Cupiennius salei*: Melchers (1963); Seitz (1966); Stollewerk & Seyfarth (2008). Authors referring to other spider species or spider development in general: Akiyama-Oda & Oda (2003); Downes (1987); Holm (1940); Kovoor & Muños-Cuevas (1995); Oda & Akiyama-Oda (2007); Vachon (1957); Yamazaki *et al.* (2005)

| **Definition and short description of stage** | **Holm (1940)** | **Vachon (1957)** | **Melchers (1963)** | **Seitz (1966)** | **Downes (1987)** | **Kovoor & Muños-Cuevas (1995)** | **Oda & Oda(2003); Yamazaki *et al.* (2005)** | **Stollewerk & Seyfarth (2008)** | **other names** | **this thesis** |
|---|---|---|---|---|---|---|---|---|---|---|
| The developmental stage that concludes at hatching (Downes, 1987). Several authors divide the embryo in a stage before and after inversion. | A | embryo / 1st prelarva | embryo / 1st prelarva | embryo | embryo | | see text | | pullus | see text |
| hatching from vitelline membrane and chorion plus molt of embryonic cuticle (including egg tooth) | | | | | | | | | | |
| The developmental stage between hatching and the discarding of the integument of the first true molt (Downes, 1987). Postembryos are motionless and do not react to mechanical stimuli (Stollewerk & Seyfarth, 2008). | B | 2nd prelarva | 2nd prelarva / 2nd incomplete stage | 1. Post-embryonal-stadium | postembryo | first instar | | stage 1 larva | postpullus, deutovum, quiescent stage | postembryo |
| first true integument molt | | | | | | | | | | |
| The developmental stage resulting from the discarding of the integument of the first true molt (Downes, 1987). The first hairs are visible on the cuticle of the first instar. Spiders of this stage do not walk. The late first instar starts to spin silk. | C | larva / prenymph | 3rd incomplete stage | Jungspinne | first instar | second instar | | stage 2 larva | | first instar |
| second molt | | | | | | | | | | |
| Second instarsno longer feed on yolk. Eyes are well developed. The proportions of the the pro- and opisthosoma, as well as of the legs and palps resemble those of the adult. This is the stage that emerges from the cocoon. | | first nymph | first nymph | | second instar | third instar | | stage 3 larva | | second instar |
| (typically) 10 molts | | | | | | | | | | |
| The last sexually immature stage. | | | 11th nymph | | penultimate instar | | | | | penultimate instar |
| last molt | | | | | | | | | | |
| Sexually mature stage. It takes about 220 days in total to reach adulthood (Melchers, 1963). | | | imago | | adult | | | | | adult |

embryo" (see table 2.1).

The first instar does not walk, although it does react to mechanical stimuli by wiggling its legs in an uncontrolled fashion. The legs are stretched and the eyes are evident (see figure 2.3). It takes somewhat less than two weeks at room temperature to molt into the second instar (Melchers (1963) and own observations). According to Seitz (1966) this stage takes about twenty hours only (360-380 hAEL), which raises the suspicion that he either did not observe this stage with great care, or that he only refers to the first twenty hours of development. The cuticle of the first instar is transparent, and the cuticle of the next instar can be seen underneath. The pigmentation of the latter cuticle increases during these twelve days, showing a typical pattern (figure 2.3, 19 days until 28 days). Not many other outer changes can be seen, but a couple of days before the next molt very fine silk threads start to be produced from the spinnerets, indicating that -some of- the silk glands are already functional.

Much of the internal development of all other major organs occurs in the first instar, while it feeds on large amounts of yolk still present in both the posterior part of the prosoma and throughout the opisthosoma. *C. salei* first instars quite frequently feed on unfertilized eggs, in addition to their own yolk, indicating that at least parts of the digestive tract start to be functional. This behavior of *C. salei* has not been described before, but egg feeding is a known phenomenon in other spider species (Valerio, 1974). It is unknown what effect egg feeding has on the development of *C. salei*. Unfortunately, very few publications describe this very interesting period of organogenesis and organ differention in the first instar of spiders (but see for example Kovoor & Muños-Cuevas, 1995; Rempel, 1957).

### 2.4.3 The second and subsequent instars

The second true molt of *C. salei* takes place after about 25-30 days at 25°C. The resulting second instar leaves the cocoon after the mother has opened it and resembles later instars in many aspects; the proportions of the different body parts are very similar (see figure 2.3, 31 days), it is very agile, it no longer feeds from its yolk reserves and starts hunting. Moreover, it uses silk to wander around.

During the first days, most second instar spiders stay around the cocoon in a chaotic web that is spun by all emerging second instars jointly. The function of this web does not primarily seem to to catch prey because the silk is not very sticky, although a predatory function can not be excluded, and *Drosophila* flies get trapped by it (own observations). Another possibility is that the animals stay around the cocoon for a short period while some parts of their internal development comes to an end. *C. salei* second instars do not climb on the back of their mother, as is the case with for example many wolf spider species. At some point, the young spiders start exploring the surroundings and make use of

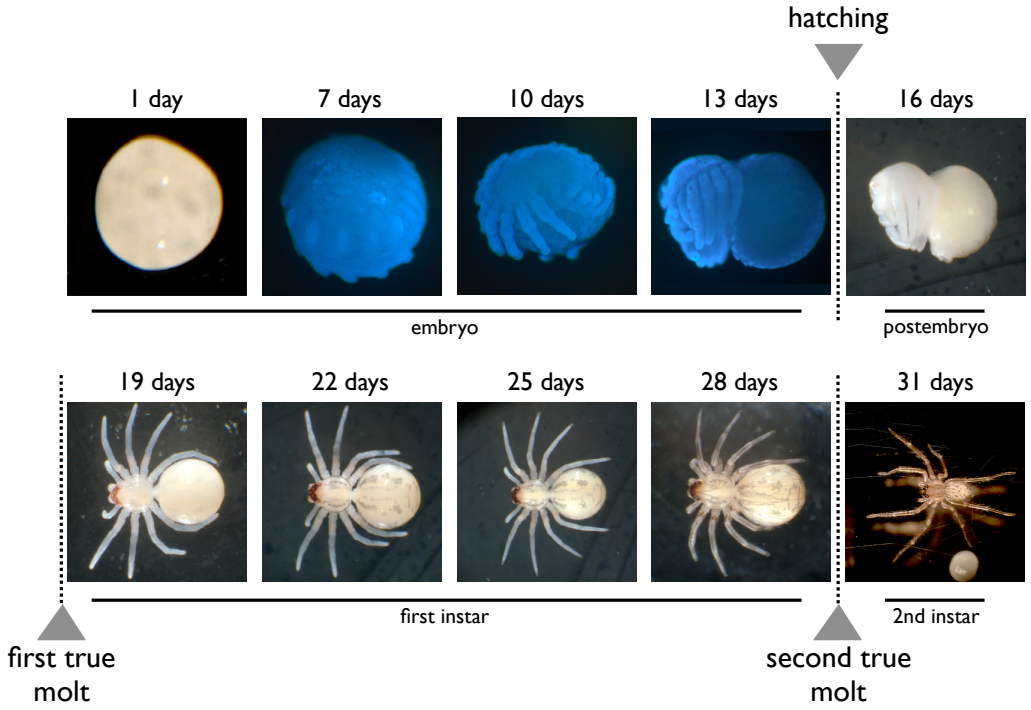



**Figure 2.3:** Overview of the development of *Cupiennius salei* at 25°C. **Day 1:** A fertilized egg, about 1.2-1.4 mm in diameter. **Days 7-13:** Stainings with a fluorescent nuclear marker (dapi) of embryonic stages (see text). **Days 16-28:** The development that *C. salei* goes through inside the cocoon after hatching from the egg. The cuticles of the postembryo and first instar lack pigmentation and are transparent, showing the underlying cuticle of the next instar. The pigmentation of the latter increases during the last ten days of the first instar. **Day 31:** After about a month at this temperature a fully pigmented second instar emerges after the second molt. Note that the proportion of the opisthosoma to the rest of the body of the second instar is much smaller than in preceding stages because the yolk has been consumed. At this stage the spider leaves the cocoon, starts hunting and produces silk as can be seen in this picture. The round structure next to the second instar is a *C. salei* egg.

their silk to disperse. *C. salei* second instars do not "balloon" i.e. do not spin a short line of silk to be taken by the wind, but perform what has been called "drop and swing dispersal behavior" (Barth *et al.*, 1991).

The second instar, as well as the third and the fourth instar are grey-brown with the pattern on pro- and opisthosoma that is visible already in the second instar. Apart from the smaller size, one of the most conspicuous differences with older instars is that it is very difficult if not impossible to tell male and females apart based on outer characters and pigmentation, and it is not clear to what extent -internal- sexual differences might have already developed. Melchers (1963) describes a transitional period between the fourth and eighth instar where darker and lighter individuals become apparent and where these color differences become more and more correlated with the gender of these animals. From the eighth molt on, sexual differences are clear from the color and color pattern. Typically, in total eleven sexually immature instars develop, the last being called the penultimate instar that molts into the twelfth and adult instar. This is true for both males and females. When underfed, sometimes spiders become adult in less molts (Melchers, 1963). During these subadult molts, *C. salei* spiders increase dramatically in size and in captivity seem to gradually change their behavior to being more calm (although feeding behavior remains explosive (Hergenröder & Barth, 1982; Melchers, 1967). No detailed study has been published on the life history changes of the use of silk by *C. salei*, but some work has been done in the not to distantly related wolf spider *Pardosa amentata* (Richter (1970), see chapter 3).

### 2.4.4 The adult

The adult molt of *C. salei* (see figure 2.4) is the final one. That is, unlike some mygalomorph and liphistiomorph species (Fujii, 2001), adults of *C. salei* do no longer molt, which means for example that after a female has successfully mated, she will continue to produce offspring without the need of further matings. Female spiders of species that continue molting after they mated become virgin again because the internal lining of their gonads gets shed together with the cuticle. Adult *C. salei* spiders show pronounced differences in behavior, with the males being more active than the females (Schmitt *et al.*, 1990). There are also many differences in the use of silk. One of the most prominent differences between males and females is the use of a special silk type by the female for constructing cocoons (see figure 2.4 a). Other uses of silk are described in more detailed in chapter 3. In captivity males live in total for about 500 days, females for about 600 days (Melchers, 1963), which could be related to their differences in acticity.

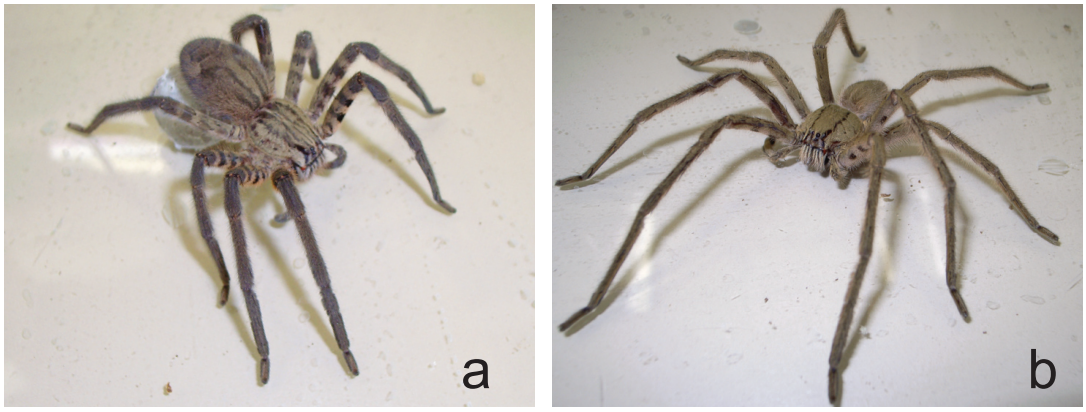



**Figure 2.4:** *Cupiennius salei* adults. **a:** Female carrying a cocoon. **b:** Male.

## 2.5 Methods

### 2.5.1 Dapi stainings and imaging

Embryos were collected at different stages of development, and fixed according to standard methods (Prpic *et al.*, 2008a). After rehydrating the embryos into PBS supplemented with 0.1% Tween 20, the embryos were incubated with about 1 ng/ml of the fluorescent nuclear marker dapi ( 4',6-diamidino-2-phenylindole, Roche) for 1 hour. After several washes they were photographed under a dissecting microscope using an UV light source. In Adobe Photoshop, the saturation of the colors was reduced.

### 2.5.2 SEM imaging

Fresh embryos were fixed for four hours on a wheel in Dubosq-Brasil fixative (consisting of a freshly made working solution of 14 parts stock solution and 1 part glacial acetic acid; Stock solution 60% ethanol, 8% formaldehyde and 0.5% picric acid) and washed several times in 70% ethanol. After an ethanol dehydration series embryos were critical point dryed (BALTEC CPD 030), mounted on stubs and sputter-coated with gold (BALTEC SCD 005). Images were made on a LEO 1450 VP SEM microscope with help of C. Wolff.

# 2. GENERAL OVERVIEW AND A REVISED STAGING OF THE DEVELOPMENT OF *CUPIENNIUS SALEI*

# Chapter 3

# Post-embryonic development of the silk producing system of *Cupiennius salei*

## 3.1 Abstract

This chapter provides a description of the post-embryonic development of the silk producing system of *Cupiennius salei*. The adult and late post-embryonic silk glands were found to be similar in organization to the glands described for wolf spiders and lynx spiders. A complex set of ampullate glands was found in the second instar, which probably represents a system able to produce silk during molting and comparable to systems earlier described for orb weaving spiders and wolf spiders. A detailed 3D reconstruction of the major ampullate glands in *C. salei* allowed for the correlation of these glands with the position of their respective cuticular openings on the anterior spinnerets, as shown by SEM scans of the spinning field. Sections of a first instar and postembryo demonstrated that the silk glands originate from the spinnerets at the posterior of the body. The ampullate glands grow through the opisthosma in the first instar towards their final position anteriorly. Cues for the direction of this growth might be provided by the septa of the yolk sacs and/or by the ventral muscles.

## 3.2 Introduction

The post-embryonic development of the silk producing system of spiders has been studied by few authors. Most of this work has been done on the spinnerets and their cuticular structures (see Yu & Coddington, 1990, and references therein). These cuticular structures include nozzles, which are microscopic funnel shaped

openings of the underlying silk glands. Because the nozzles and glands form a functional unit, the studies of the former indirectly tell us something about the latter. Direct studies of the post-embryonic development of silk glands are even more scarce, although the importance of the post-embryonic development for the final differentiation of the silk glands has been recognized for a long time. As Rempel (1957) pointed out: "Gland formation ... at the time of hatching is still quite incomplete. In spiders the great development and differentiation of the spinning apparatus is a postembryonic phenomenon".

Some authors studied the silk producing sytem of later post-embryonic stages, i.e. from the third instar up till the adult (see chapter 2). For example, Richter (1970) found many interesting changes of silk gland size and number taking place during the life history of the wolf spider *Pardosa amentata*, which he used as a basis to postulate functions of the different kinds of glands and silk. A later series of studies on the late post-embryonic development of orb weaving spiders and wolf spiders explained the significance of cuticular structure called "'tartipores" and "nubbins", which are related to the nozzles, and revealed a system for the use of ampullate silk during molting (Townley & Tillinghast, 2003; Townley *et al.*, 1993; Townley, 1991).

Others studied the silk producing system of early post-embryonic stages, including the postembryo and first and second instar (see chapter 2). There are descriptions of the early development of silk glands in wolf spiders (Jaworowski, 1896) (see also chapter 4) and different aspects of organogenesis including the silk glands of a cob-web spider (Rempel, 1957) and lynx spiders (Kovoor & Muños-Cuevas, 1995). Unfortunately, Jaworowski (1896) and Rempel (1957) do not correlate their findings with later stages, making it difficult to follow how the post-embyronic development of the silk glands comes about. In a unique publication, Kovoor & Muños-Cuevas (1995) recognized three different silk gland types in the first instar that can be related to later silk gland types.

Thus far, no direct study of the sub-adult or the adult silk glands of *C. salei* has been published, but Melchers (1963) gives a broad overview of numbers of nozzles of different sizes in adult *C. salei* males and females on the different spinnerets. Using these numbers, ideas on the possible numbers and nature of *C. salei* silk glands can be deduced from studies of related spider lineages (Griswold, 1993), such as the work on wolf spiders by Richter (1970). A comparison of both studies is provided in table 3.1.

In *P. amentata* there are four different types of glands. Ampullate glands, which are the main silk glands concerned with the production of the drag-line; piriform glands, which produce the attachment discs and play a role in cocoon construction; aciniform glands, which might play a role in molting and tubuliform glands (adult females only), which are used for cocoon construction (Richter, 1970). These glands connect in a typical pattern to three pairs of appendages, the

spinnerets. There are anterior spinnerets (Asp), part of the fourth opisthosomal segment, medial (MSp) and posterior (PSp) spinnerets, that both develop from the fifth segment (see chapters 1 and 2).

**Table 3.1:** The nozzles of adult male and female *Cupiennius salei* spiders (Melchers, 1963) and the silk glands of the wolf spider *Pardosa amentata* (Richter, 1970). **amp**, Ampullate glands; **pir**, Piriform glands; **ac**, Aciniform glands; **tub**, Tubuliform glands.

| stage | spinneret | Number of nozzles in *Cupiennius salei* | | | Number of silk glands in *Pardosa amentata* | | | |
|---|---|---|---|---|---|---|---|---|
| | | spigots (4-6 µm) | fusules (1 µm) | fusules (1.5 µm) | amp | pir | ac | tub |
| adult female | ASp | 2 | 300 | - | 2 | 26 | - | |
| | MSp | 2 | - | 60-90 | 2 | - | 13 | 20 |
| | PSp | - | - | 120-130 | - | - | 18 | 23 |
| adult male | ASp | 1 | 300 | - | 1 | 15 | - | - |
| | MSp | 1 | - | 27 | 1 | - | 9 | - |
| | PSp | - | - | 60 | - | - | 14 | - |
| sub-adult | ASp | | | | 2 | 13-16 | - | - |
| | MSp | | | | 2 | - | 7-8 | - |
| | PSp | | | | - | - | 12-16 | - |

In many spider species, the nozzles of the ampullate glands are called "spigots" and are larger than those of other glands, called "fusules". Since Melchers (1963) distinguishes between nozzles of different sizes her counts provide some insights in the types of glands attached, see table 3.1. Comparing the findings of Melchers (1963) and Richter (1970) shows that, in all likelihood, *C. salei* adult spiders have a very similar set of silk glands when compared to *P. amentata*. Females most likely have two functioning ampullate glands on each of their anterior (ASp) and medial spinnerets (MSp), whereas males have only one such gland on these spinnerets, and both sexes are very similar with regard to the about 300 piriform glands on their ASp. Aciniform glands are present in both sexes on the MSp

and posterior spinneret (PSp), and females in addition bear tubuliform glands on their MSp and PSp.

To build on this knowledge, in order get a better understanding of the post-embryonic development of spider silk glands and spinnerets in general, and of the silk producing system of *C. salei* in particular, I studied the silk glands in adults, second and first instar, as well as in the postembryo of this species. Questions to be addressed were what the adult silk glands and spinnerets of *C. salei* look like, whether there is also a system in place to allow for the production of ampullate silk during molting, and if so, when during ontogeny this system comes about.

Moreover, it was of particular interest to find out when and where the different types of glands develop, because this knowledge is very important to test hypotheses about the evolutionary origin of the spider silk producing system (see chapter 1). According to some of these theories, silk glands evolved from structures of mesodermic origin, such as the coxal glands still found in many arachnids (Shultz, 1987). Others proposed that the glands are derived from structures that developed from the epidermis (Bond, 1994; Shultz, 1987). Although the current consensus is that spider silk glands have an epidermal origin (e.g. Craig, 1997), no convincing evidence has ever been shown for this notion (see chapter 4). Tracing the developmental origins of the silk glands of *C. salei* can provide support for either group of theories. Finally, knowledge of the development of these glands allows for understanding better which organs and structures the developing silk glands might interact with to regulate their growth and differentiation, as is also a topic of the research of the development of the silk glands of the silkworm *Bombys mori* (Julien *et al.*, 2004).

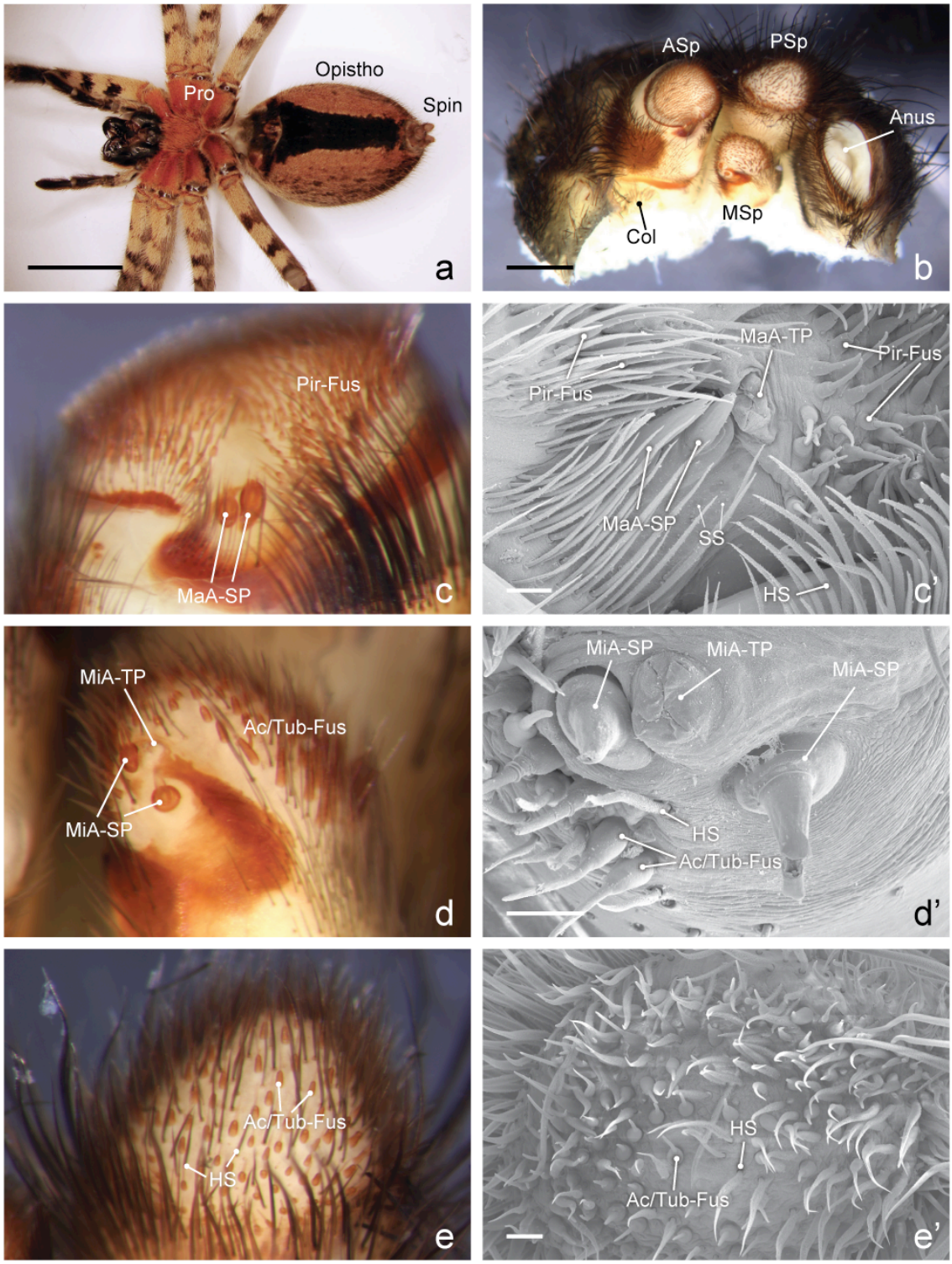
## 3.3 Results

### 3.3.1 The adult spinnerets

In order to complement the observations of Melchers (1963), the spinning field, which is the cuticular region that comprises the spinnerets and surrounding structures, was dissected from adult male and female spiders and inspected with a scanning electron microscope (SEM, see figure 3.1). The spinnerets of only one body half were inspected, and all further text in this paragraph relates to one body half only.

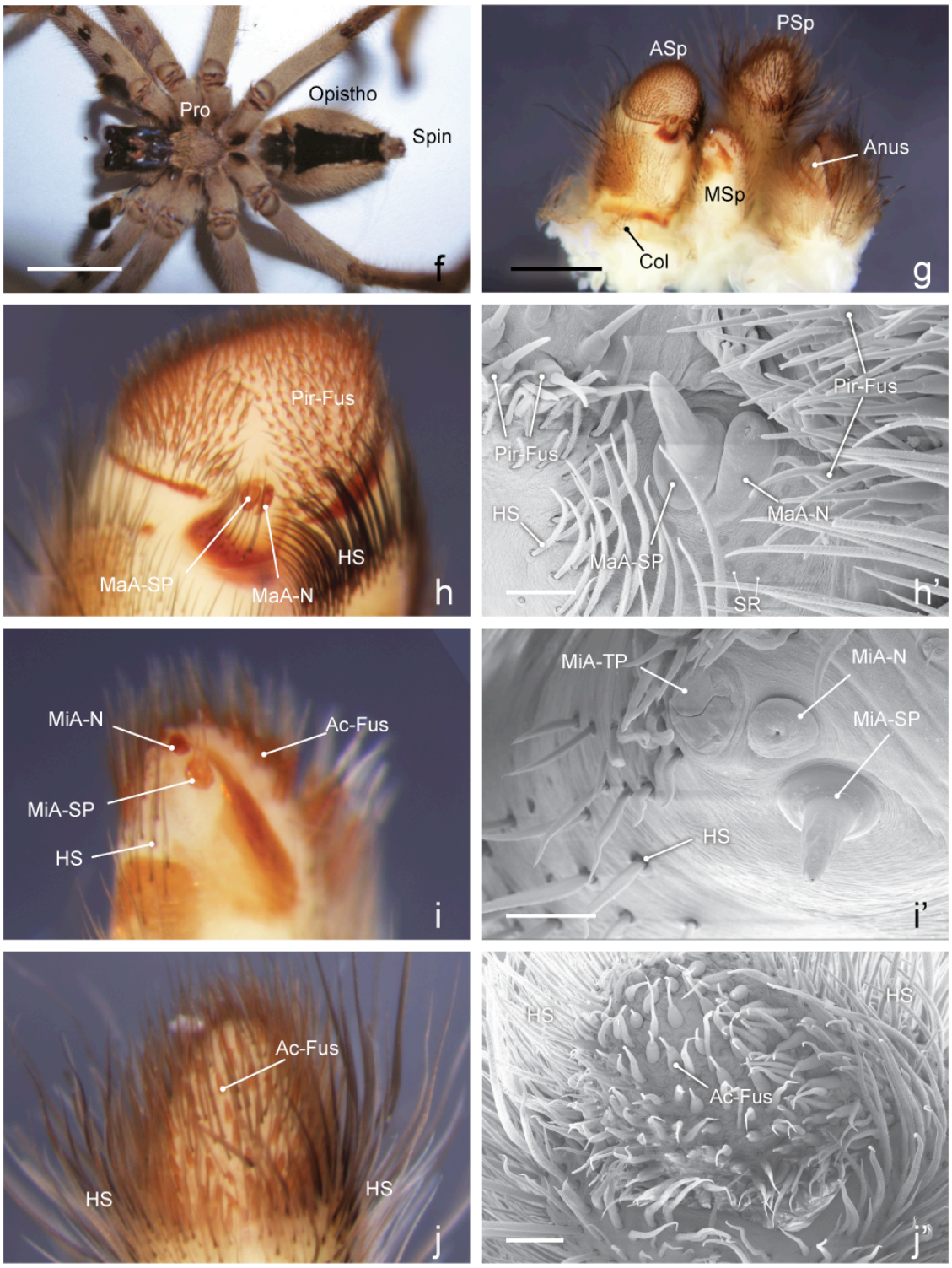
It was found that the ASp and PSp are similar in size. The PSp however bairs a "crown" of long sensory hairs that is not present as such on the ASp. Interestingly, even though the MSp is clearly smaller than the ASp and PSp, its general appearance is similar to the ASp. In between the ASp's there is a small cuticular structure, called the "colulus". No precise counts were made of the

**Figure 3.1:** *Cupiennius* adult spinnerets. **a, f:** Ventral view of an adult *C. salei* female and male respectively, showing the spinnerets at the posterior part of the opisthosoma. Scale bars 1 cm. **b, g:** Dissected spinnerets of the left body half of male and female resp. Scale bars 1 mm. **c, h:** Detail of the tip of the anterior spinneret of male and female resp. **c', h':** SEM scan of the region around the ampullate spigots of the anterior spinneret of a male and female resp. Scale bars 50 $\mu$m. **d, i:** Detail of the tip of the medial spinneret of a male and female resp. **d', i':** SEM scan of the region around the ampullate spigots of the medial spinneret of a male and female resp. Scale bars 50 $\mu$m. **e, j:** Detail of the tip of the posterior spinneret of a male and female resp. **e', j':** SEM scan of the tip of the posterior spinneret of a male and female resp. Scale bars 100 $\mu$m. **Ac-Fus**, fusules of an aciniform gland; **Ac/Tub-Fus**, spigot of aciniform or tubuliform gland; **ASp**, anterior spinneret; **Col**, colulus; **HS**, hair sensilla; **MaA-N**, nubbin of a major ampullate gland spigot; **MaA-SP**, spigot of a major ampullate gland; **MaA-TP**, tartipore of a major ampullate gland; **MiA-N**, nubbin of a minor ampullate gland spigot; **MiA-SP**, spigot of a minor ampullate gland; **MiA-TP**, tartipore of a minor ampullate gland; **MSp**, medial spinneret; **Opistho**, opisthosoma; **Pir-Fus**, spigot of a piriform gland; **Pro**, prosoma; **PSp**, posterior spinneret; **Spin**, spinnerets; **SS**, spigot sensilla.




**(I)**

**Figure 3.1:** *Cupiennnius* adult spinnerets; female




**(II)**

**Figure 3.1:** *Cupiennnius* adult spinnerets; male

fusules but their numbers on the ASp, MSp and PSp seemed to be in concordance with the observations of Melchers (1963) (see table 3.1). The ampullate spigots, easily identifiable by their larger size and dark pigmentation, are located on a stretch of cuticle without sensory hairs or other nozzles on the ventral part of the tips of the ASp and MSp of both sexes, see figure 3.1 c,d,h and i). Ampullate glands that lead to the ASp have been named "major", and those leading to the MSp "minor", both sexes therefore have major and minor ampullate glands. Also verifying the observation of Melchers (1963), and possibly homologous to the situation in *P. amentata* (Richter, 1970), I observed difference between the sexes with regard to the ampullate spigots. There are two of these spigots present on both the ASp and MSp of the female, whereas the male only bears one on these spinnerets. There are no nozzles present on the colulus. Around the ampullate spigots on the ASp, tiny sensory structures can be seen, that have been named spigot sensilla, and probably serve to control the spinning of major ampullate silk more precisely (Gorb & Barth, 1996).

Close to the ampullate spigots, in both males and females, structures are visible on locations were spigots are expected that appear as scars on the cuticle, or as undeveloped spigots. These are tartipores and nubbins respectively (Townley & Tillinghast, 2003). Adult females have one ampullate tartipore on both the ASp and the MSp, and no ampullate nubbins. Males also have one tartipore, and in addition one nubbin on these spinnerets (see figure 3.2 b). Therefore, in both sexes, the total of ampullate spigots, tartipores and nubbins equals three on both the ASp and the MSp. Interestingly, nubbins possess a small protruding structure in the center (3.2 b, arrow). The significance of tartipores and nubbins is discussed in paragraph 3.4.3.1.

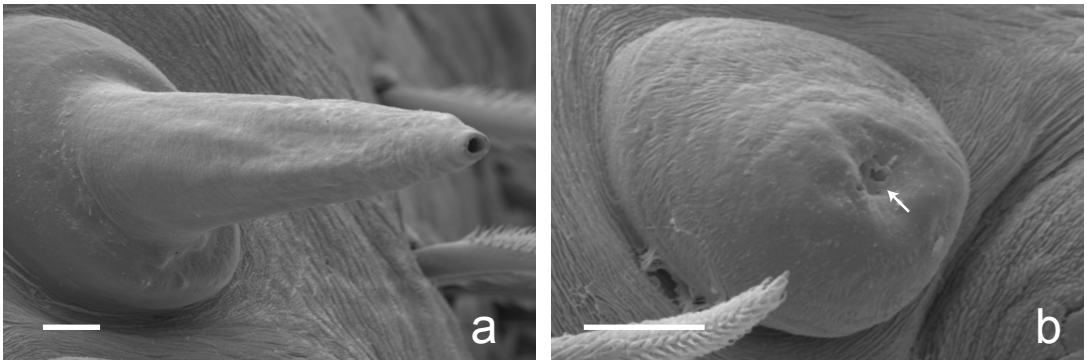



**Figure 3.2:** Detailed SEM scans of a spigot (**a**) and nubbin (**b**) from an adult male medial spinneret (see figure 3.1 i'). Scale bars 10 $\mu$m. The arrow indicates a small protrusion from the center of the nubbin, see text.

### 3.3.2 The adult silk glands

Dissection of the silk glands of an adult male and female (figure 3.3) shows that in terms of gross morphology the glands are indeed very similar to those of *P. amenata*, merely differing in number and size (figure 3.4). In both sexes, a large amount of piriform glands is present on the ASp, and a similar mass of aciniform glands is seen on the MSp and PSp. Males have one large "major" ampullate gland leading to the ASp, and one smaller "minor" ampullate gland leading to the MSp. Females have two major and two minor ampullate glands, and bear in addition a large number of tubuliform glands on their MSp and PSp. No attempt was made to count the exact number of the small and abundant aciniform and piriform glands, or of the more elongate tubuliform glands of the female, but their numbers appear very similary to the number of spigots observed by Melchers (1963) (table 3.1).

The ampullae of the major ampullate glands are located in the anterior lateral part of the opisthosoma, slightly posterior to the book lungs (see figure 3.4). The minor ampullate glands are somewhat smaller, and lie at a more medial and posterior position. The posterior division (terminology based on the morphologically similar silk glands of the moth *Bombyx mori* (Akai, 1983)) of the male ampullate glands (see 3.3 b) is larger than the posterior division of the female ampullate glands (3.3 a). The ducts of all ampullate glands, in both females and males are very long and slender, tapering towards the spinnerets. Leaving the ampullae, they lead almost to the spinnerets, turn 180 degrees and lead back to the ampullae only to turn yet another time 180 degrees and then lead all the way to the spigot on one of the spinnerets. This zig zag pattern makes the ducts very long, which is probably crucial for the proper formation of the silk protein (Craig, 1997).

### 3.3.3 The spinnerets of the second instar

The second instar is the first free-living instar, that leaves the cocoon (see chapter 2). There are no outer sexual differences visible, for this reason no distinction between sexes is made in the following analyses. To study the cuticle of the silk producing system in this stage an SEM scan was made of the spinning field (see 3.5). All three spinnerets are well developed, and all of these bear sensory hairs, as well as nozzles. Nozzle numbers are much reduced in comparison to the adult stages. On the ASp, there are six fusules present, most likely belonging to piriform glands. Five fusules on the MSp and eight on the PSp are probably associated with aciniform glands. On both the ASp and MSp there are two larger spigots present as well as one tartipore. Comparing the spigots of the ASp with the silk glands (see §3.3.4) it is clear that both spigots connect to functional

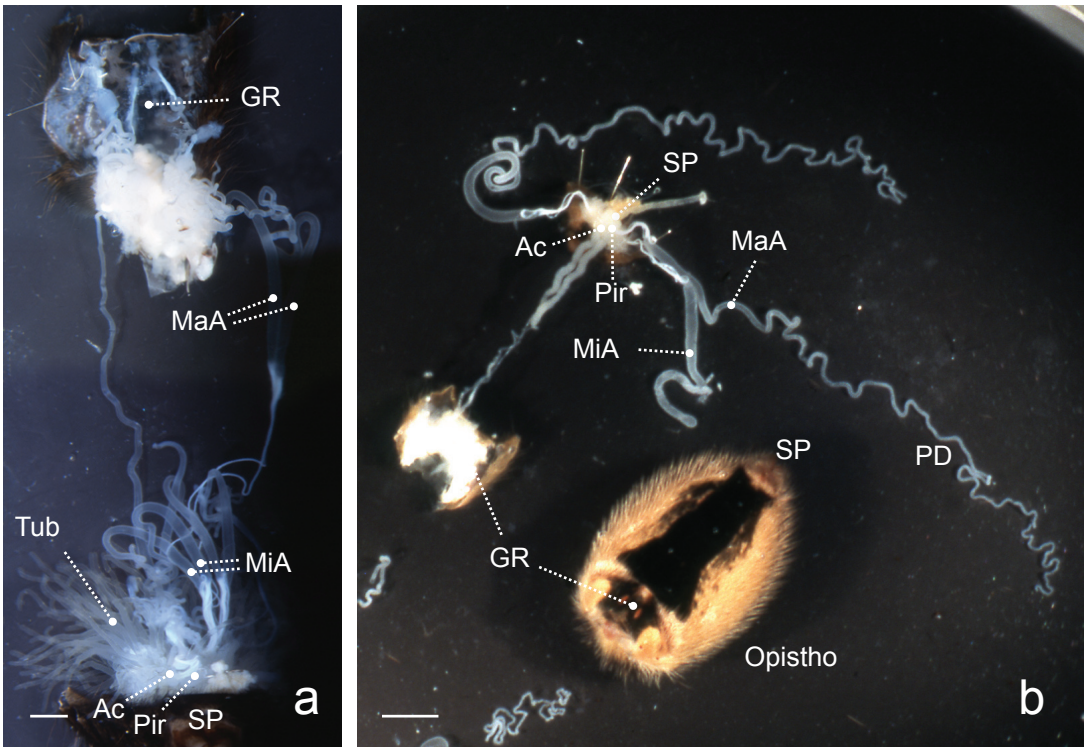



**Figure 3.3:** Dissections of adult *Cupiennius salei* silk glands. Scale bars 5 mm. **a:** Female. All organs and most of cuticle of the opisthosoma are cut away, apart from a piece of cuticle and associated tissue around the genital region, pinned down to facilitate dissection. Two of the major ampullate glands are pulled anteriorly, opening up the zig-zag shaped ducts. Anterior is up. This dissection was performed by Kristen A. Panfilio. **b:** Male. An undissected opisthosma (seen ventrally) is shown next to a dissected one. The latter is treated in a similar way as the female opisthosoma in **a:**. The spinnerets are pinned down and not visible. It is not known what the structure between the spinnerets and the piece of cuticle around the genital opening is, possibly a spermatophore. Posterior is up. **Ac**, aciniform glands; **GR**, gential region; **MaA**, major ampullate glands; **MiA**, minor ampullate glands; **Opistho**, opisthosoma; **PD**, posterior division of the gland; **Pir**, piriform glands; **Pro**, prosoma; **SP**, spinnerets; **Tub**, tubuliform glands.

**Figure 3.4:** The silk glands of adult *Cupiennius salei*. Schematic drawings of the silk glands of one body half only are shown, other opisthosomal organs are left out. Drawings of the prosoma in **a** and **f** were modified with permission from AustralianMuseumOnline (2002). **a:** female, overview of all glands. **b-e:** female, single gland types. **f:** male, overview of all glands. **g-i:** male, single gland types. **Ac**, aciniform glands; **MaA**, major ampullate glands; **MiA**, minor ampullate glands; **Opistho**, opisthosoma; **Pir**, piriform glands; **Pro**, prosoma; **Tub**, tubuliform glands.

glands, the more anterior spigot to the 1° and the more posterior spigot to the 2° major ampullate gland.

### 3.3.4 The silk glands of the second instar

The opisthosoma of a second instar *C. salei* is about 1 mm in length (see figure 3.6 f), and not well-suited for dissection. Therefore, near-to-complete stacks of transversal and coronal sections were made in order to make a reconstruction of the internal organs in three dimensions (see figure 3.6 i and j). Three types of glandular structures were found, and these correspond almost certainly to piriform, aciniform and ampullate silk glands (compare with Kovoor & Muños-Cuevas, 1995). Based on the aspect of the content of their lumen after fixation and haematoxylin/eosin (HE) staining, these glands are all actively producing silk protein (see fig 3.6 a' and c'). The piriform glands have a dark brown appearance after this treatment, and are situated ventrally close to the spinnerets (see fig 3.6 c, c' and g). In total, twelve piriform glands were counted. Putative aciniform glands colored pink, are situated slightly dorsal to the piriform glands and show a distinct globular shape (fig 3.6 c' and g ). Due to the compact organisation of the aciniform glands, and to the occasional loss or reduced quality of single sections in the stacks, it was impossible to determine their exact number; about twenty to thirty were estimated.

In total, twelve ampullate glands were found; six on each body half. The schematic drawing in figure 3.6 h shows their organization in one body half, and the remaining text in this paragraph refers to one body half only. Two of the larger ampullate glands could be traced via long ducts to two large spigots on the ASp, and are therefore considered the major ampullate glands (MaA-GL's, drawn in blue and red). these glands lie laterally in the anterior ventral portion of the opisthosoma (see figures 3.6 i and j), close to the book lungs (figure 3.6 g). The ampul of the MaA-GL depicted in blue is large and folds back on itself, the ampul of the MaA-GL drawn in red is slightly smaller. Based on these size differences, I named them 1° MaA-GL and open 2° MaA-GL respectively, in accordance with the terminology of Townley & Tillinghast (2003). The ampullae of the 1° and open 2° MaA-GL's appear very similar in cross section, showing many large granules (figure 3.6 a'), and so does the content of their lumen, even though in this particular section the content of the 1° MaA-GL was lost on both sides (but see figure 3.6 g). The active ampullate glands are not completely developed yet in this stage, because the posterior division of the gland (see figure 3.3) is completely absent.

The ducts of both of these MaA-GL's have a zig-zag shape, very similar to the ducts of ampullate glands in adult animals (see above). They are slightly tapering and are not surrounded by epithelium in the two first loops, named 1

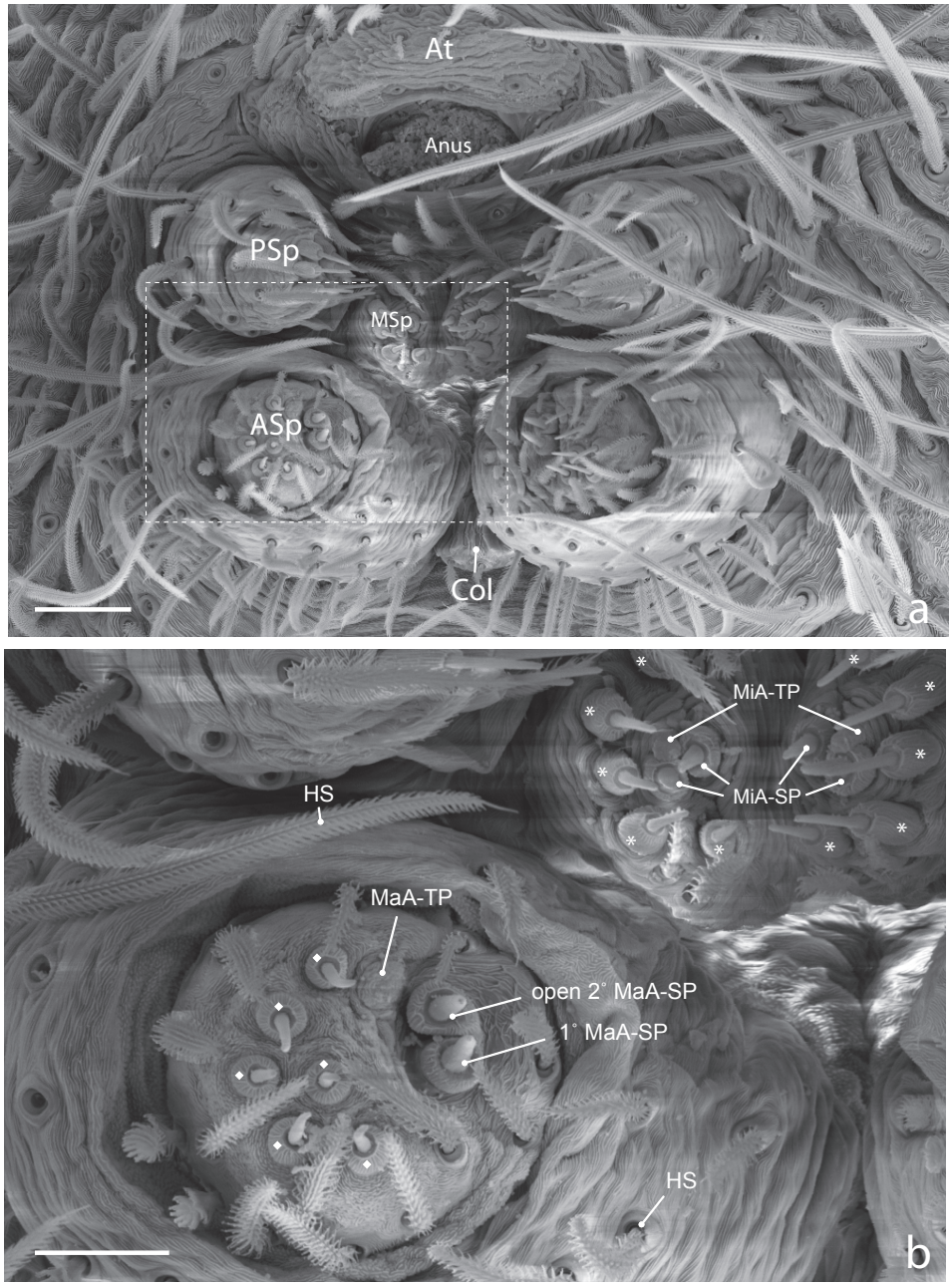



**Figure 3.5:** SEM scans of the spinnerets of the second instar of *C. salei*. **a:** Overview of the spinning field. Posterior is to the top. The dashed box indicates the area shown in b. Scale bar 40 $\mu$m. **b:** Higher magnification of the left ASp and MSp. Asterisks indicates aciniform, diamonds piriform fusules. Scale bar 20 $\mu$m. **1° MaA-SP**, primary major ampullate spigot; **At**, anal tubercle; **ASp**, anterior spinneret; **Col**, colulus; **HS**, hair sensilla. **MaA-TP**, major ampullate tartipore; **MiA-SP**, minor ampullate spigot; **MiA-TP**, minor ampullate tartipore; **MSp**, medial spinneret; **open 2° MaA-SP**, open secondary major ampullate spigot. **PSp**, posterior spinneret.

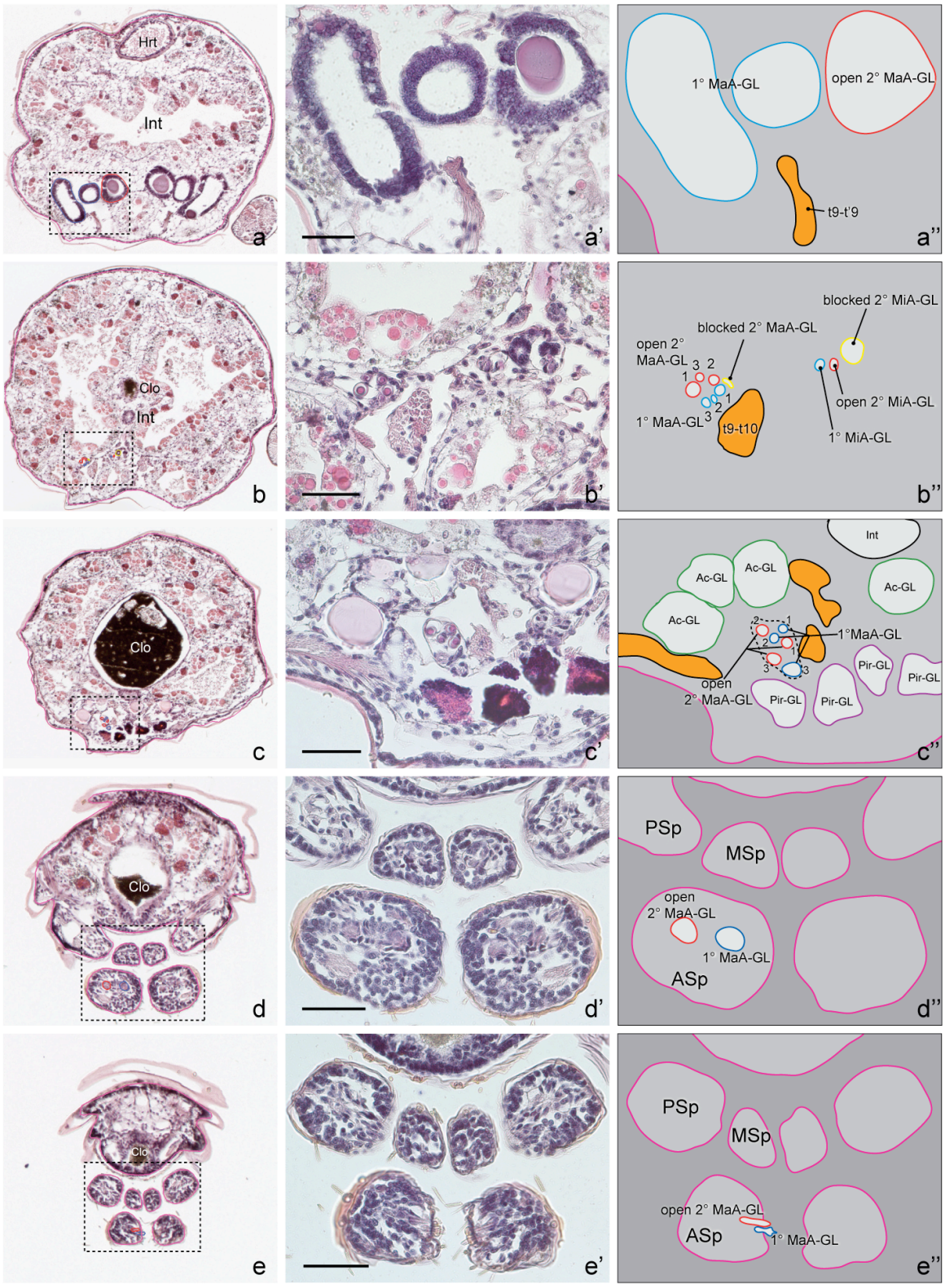
and 2 (figures 3.6 b', c' and h). Close to the spigot, towards the more posterior part of loop 3, surrounding nuclei can be seen (see figure 3.6 d'), that are possibly part of a sphincter muscle. The duct of the 1° MaA-GL leads to a spigot more ventrally on the ASp than then spigot from the open 2° MaA-GL (see figure 3.6 e') and it was possible to make the connection between the 3D model of the glands and the SEM scan of the ASp (figure 3.5 b). Closely connected to the 2° MaA-GL, there is a third glandular structure visible in these sections, drawn in yellow (figures 3.6 b', i and j). This is probably a blocked 2° MaA-GL (see discussion). It was not possible to follow the duct of this gland all the way to the spinnerets, and it is not clear if this gland makes such a connection in this stage (represented by a grey dashed line in figure 3.6 h). No silk was found in the ampul of the blocked 2° MaA-GL.

The smaller ampullate glands, which are very likely the minor ampullate glands (MiA-GL's), are smaller than the MaA-GL's and are located medially and more posteriorly. Apart from these differences, their organization appears very similar. On each body half there are three MiA-GL's. The ampullae of the two larger ones of these are filled with silk protein, whereas the third and much smaller one is not. One of the two active MiA-GL's is larger than the other and folded back on itself. Based on these similarities to the MaA-GL's, the MiA-GL's glands were termed 1° MiA-GL (blue), open 2° MiA-GL (red) and blocked 2° MiA-GL (yellow). The ducts of none of these glands individually could be traced to the spinnerets, but a bundle of tracts could be followed until very close to the MSp (indicated by grey dashed lines in figure 3.6 h).

### 3.3.5 The spinnerets of the first instar

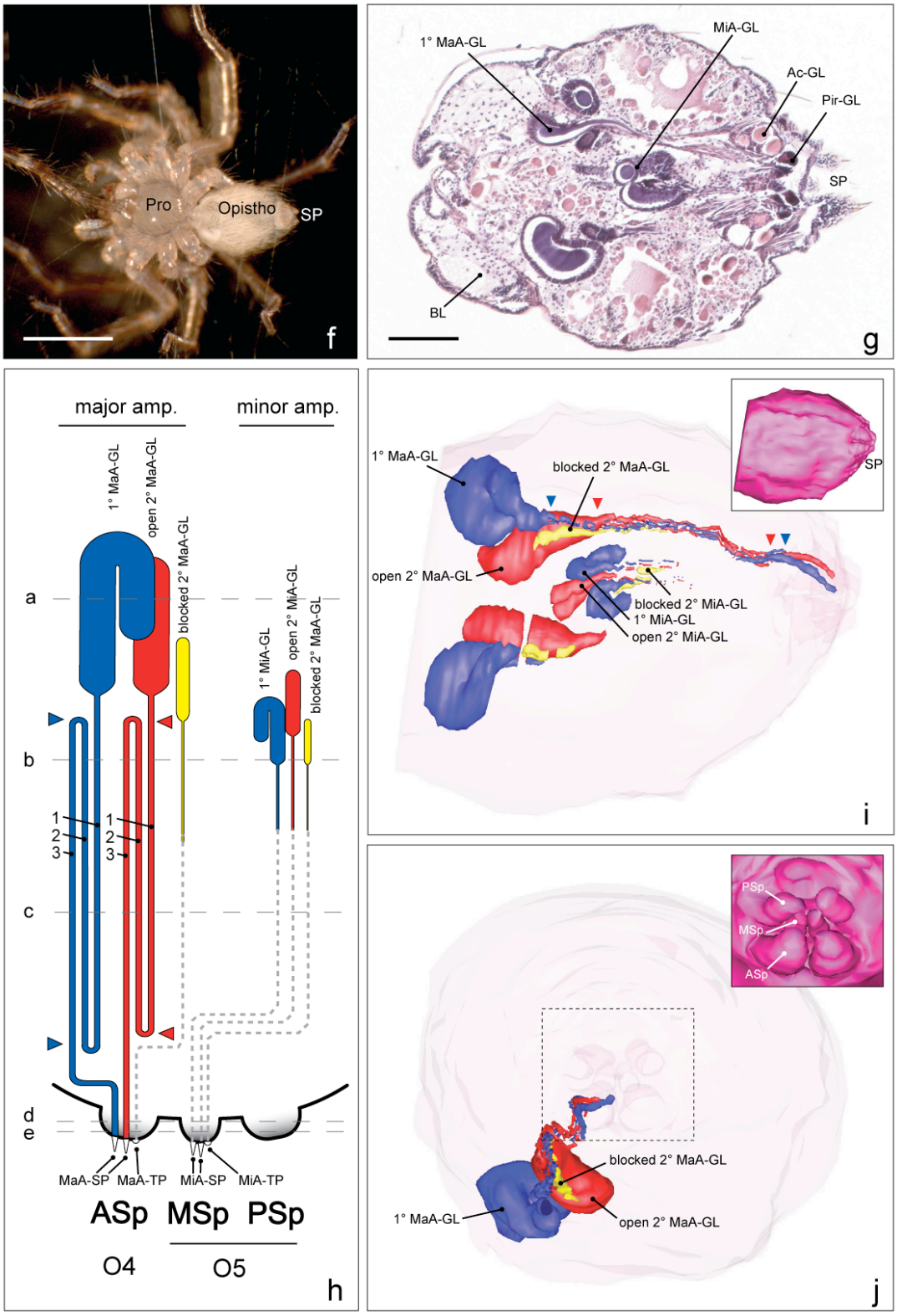
The first instar takes about two weeks. Animals are capable of movement, but normally lie on their back (when taken from the cocoon) and do not walk. All remaining yolk of the embryonic stages is used up in this instar, and many organs probably undergo their final differentiation. This is also true for the silk glands, because towards the end of the first instar, very fine silk starts to be spun from the spinnerets (see chapter 2). To study the silk producing system in this stage, again the first step was to inspect a SEM scan of the spinning field (see figure 3.7). Exteriorly, all three types of spinnerets, as well as the colulus are well developed. They are easily visible, because in comparison to the second instar the cuticle of the first instar is covered with very few sensory hairs, as mentioned by Stollewerk & Seyfarth (2008). A small number of nozzles are present on the tips of all three spinneret types. There appear to be three nozzles on each of the PSp's, four on the MSp's and at least two on each of the ASp's (3.7 a). These two spigots on the ASp have the position on the spinneret and the appearance of ampullate spigots (see figures 3.7 b and 3.2 b), and in this particular specimen one of the spinnerets

**Figure 3.6:** Silk glands of the second instar of *C. salei*. **a-e:** Transversal paraffin sections, haematoxylin and eosin stained. The positions of the sections in relation to the ampullate silk glands is shown schematically in **h**. **a'-e':** Magnifications of dashed boxes in **a-e**. Scale bars 50 $\mu$m. **a"-e":** Schematic drawings of selected elements from **a'-e'**. **f:** Ventral view of a living second instar. Scale bar 1 mm. **g:** Coronal paraffin section of the ventral part of the opisthosoma of a second instar, haematoxylin and eosin stained. Scale bar 200 $\mu$m **h:** Schematic drawing of the ampullate glands and spinnerets of one body half. Horizontal dashed lines mark the location of the sections shown in **a-e**. Blue and red arrowheads indicate the positions of the loops of the ducts of the 1° MaA-GL and open 2° MaA-GL respectively, see **i**. Gray dashed lines indicated likely connections that were not verified by direct observation (see text). **i:** 3D model of the ampullate glands of a second instar, ventral view. The secretory portions of all ampullate glands for both body halves are shown, but ducts only for the major ampullate glands of the left body half. The cuticle is shown translucently. The insert shows the solid outline of the cuticle in smaller magnification. **j:** Same model, view from posterior. The region in the dashed box is repeated in the insert, which shows the solid outline of the cuticle of the spinning field (compare to fig 3.5 a). **1° MaA-GL**, primary major ampullate gland; **1° MiA-GL**, primary minor ampullate gland; **Ac-GL**, acinifom gland; **BL**, book lungs; **blocked 2° MaA-GL**, blocked secondary major ampullate gland; **blocked 2° MiA-GL**, blocked secondary minor ampullate gland; **Clo**, cloaca; **Hrt**, heart; **Int**, Intestinal tract; **MaA-SP**, spigot of a major ampullate gland; **MaA-TP**, tartipore of a major ampullate gland; **MSp**, medial spinneret; **O4**, (part of) opisthosomal segment 4; **O5**, (part of) opisthosomal segment 5; **open 2° MaA-GL**, open secondary major ampullate gland; **open 2° MiA-GL**, open secondary minor ampullate gland; **Opistho**, opisthosoma; **Pir-GL**, piriform gland; **Pro**, prosoma; **PSp**, posterior spinneret; **SP**, spinnerets; **t9-t9'**, muscle between entochondrite t9 and t9'; **t9-t10**, muscle between entochondrite t9 and t10.




**(I)**

**Figure 3.6:** Silk glands of the second instar of *C. salei*; sections.




**(II)**

**Figure 3.6:** Silk glands of the second instar of *C. salei*; model.

was fixed while spinning silk. As indicated by a diamond, there are structures present that might be piriform fusules or undeveloped sensory hairs.

### 3.3.6 The silk glands of the first instar

Sectioning the opisthosoma of the first instar is problematic. On the one side, there is a well developed cuticle present, that makes it difficult to get good penetration with many types of fixatives. On the other side, a large proportion of the volume of the opisthosoma is made up of yolk, which is difficult to fix. Many first attempts to cut paraffin sections were unsuccessful, due to brittleness of the fixed specimen. Unfortunately, frozen sections of the first instar were also problematic and did not adhere to the slide very well. Different epoxy embedding resins were tried, and reasonable results were obtained with a protocol described by Pernstich *et al.* (2003). Preliminary results (not shown) with a protocol modified from the method described by Anderson (1964) showed that in principle it is possible to obtain high quality paraffin sections of this stage. Kovoor & Muños-Cuevas (1995) also used a modification of this protocol and produced reasonably paraffin sections, but unfortunately it was not possible to obtain their protocol. Optimization of the protocol will hopefully allow for staining of rehydrated sections in the near future, like for example in situ hybridization or antibody staining.

Sagittal sections were cut of the opisthosoma of a twenty-four day old first instar (see figure 3.8). The (lobes of) large yolk sacs can be seen in all presented sections, separated by septa. They are drawn as light grey areas with a bit of space in between for clarity, but in reality the septa are in close contact to one another (Rempel, 1957). Developing organs are seen in between the yolk sacs, for example the midgut, including the cloaca and laterally associated Malpighian tubules, slightly anterior to the PSp. A lot of the musculature also lies in between yolk sacs, such as the t9-t'9 muscles that cross the opisthosoma dorso-ventrally (figures 3.8 a, a', b and b') and the ventral muscle nodes (entochondrites) t8 and t9 (Peters, 1967) (figures 3.8 c and c').

The ampullae of both the developing major (figures 3.8 b and b') and minor (figures 3.8 d and d') ampullate glands lie side by side with the ventral muscles. There are two major and two minor ampullae visible on each body half. In the first instar of this age, both of these gland types are located slightly posterior to the position they take up in the second instar (see above). Present in the sections between those depicted in figure 3.8 (not shown), are the ducts that connect the ampullae to the spinnerets. It is not clear whether these have already developed their typical zig-zag shape (see figure 3.6). Clearly developed aciniform and/or piriform glands are not visible. However, there are are a lot of cells visible inside the ASp, MSp and PSp and although their nature could not be deduced from these sections, it is likely that a large fraction of this tissue, apart from muscles

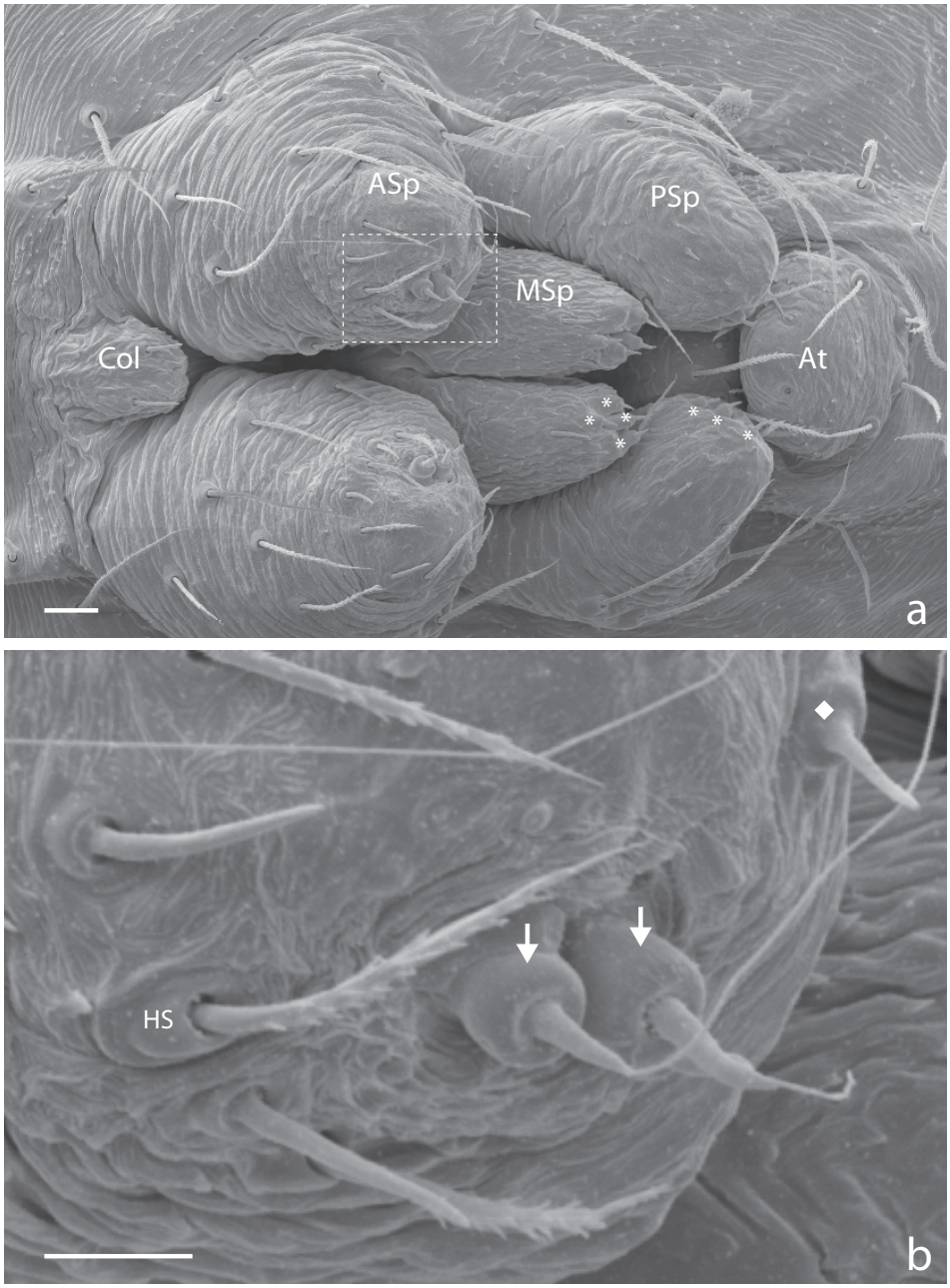



**Figure 3.7:** SEM scan of the spinnerets of a first instar of *C. salei*. **a:** Overview of the spinning field. Acifiniform fusules on the spinnerets of the right body half are marked with asterisks. The dashed box indicates the area shown in b. Scale bar 20 $\mu$m. **b:** Higher magnification of the tip of the left ASp. Arrows indicate two ampullate spigots excreting silk. The diamond indicates a possible third nozzle (see text). Scale bar 10 $\mu$m. **ASp**, anterior spinneret; **At**, anal tubercle; **Col**, colulus; **HS**, hair sensilla. **MSp**, medial spinneret; **PSp**, posterior spinneret.

**Figure 3.8:** Internal morphology of the opisthosoma of a first instar *C. salei*. **a-d:** Sagittal sections of one body half of the opisthosoma of a *C. salei* first instar, from relatively lateral (**a**) to medial (**d**). **a'-d':** Interpretations of the sections shown in **a-d**. Yolk sacs are shown as light grey pockets. Muscle structures are depicted with orange and their nomenclature follows Peters (1967). Scale bars 200 $\mu$m. **ASp**, anterior spinneret; **At**, anal tubercle; **BL**, book lung; **Clo**, cloaca; **Col**, colulus; **Hrt**, heart; **MaA-GL**, major ampullate gland; **Malp**, Malpighian tubule; **MiA-GL**, minor ampullate gland; **PSp**, posterior spinneret; **t8-t9**, muscle between entochondrite t8 and t9; **t9, t9'**, entochondrites t9 and t9'; **t9-t9'**, muscle between entochondrite t9 and t9'; **t9-t10**, muscle between entochondrite t9 and t10; **tsp**, tracheal spiracle.

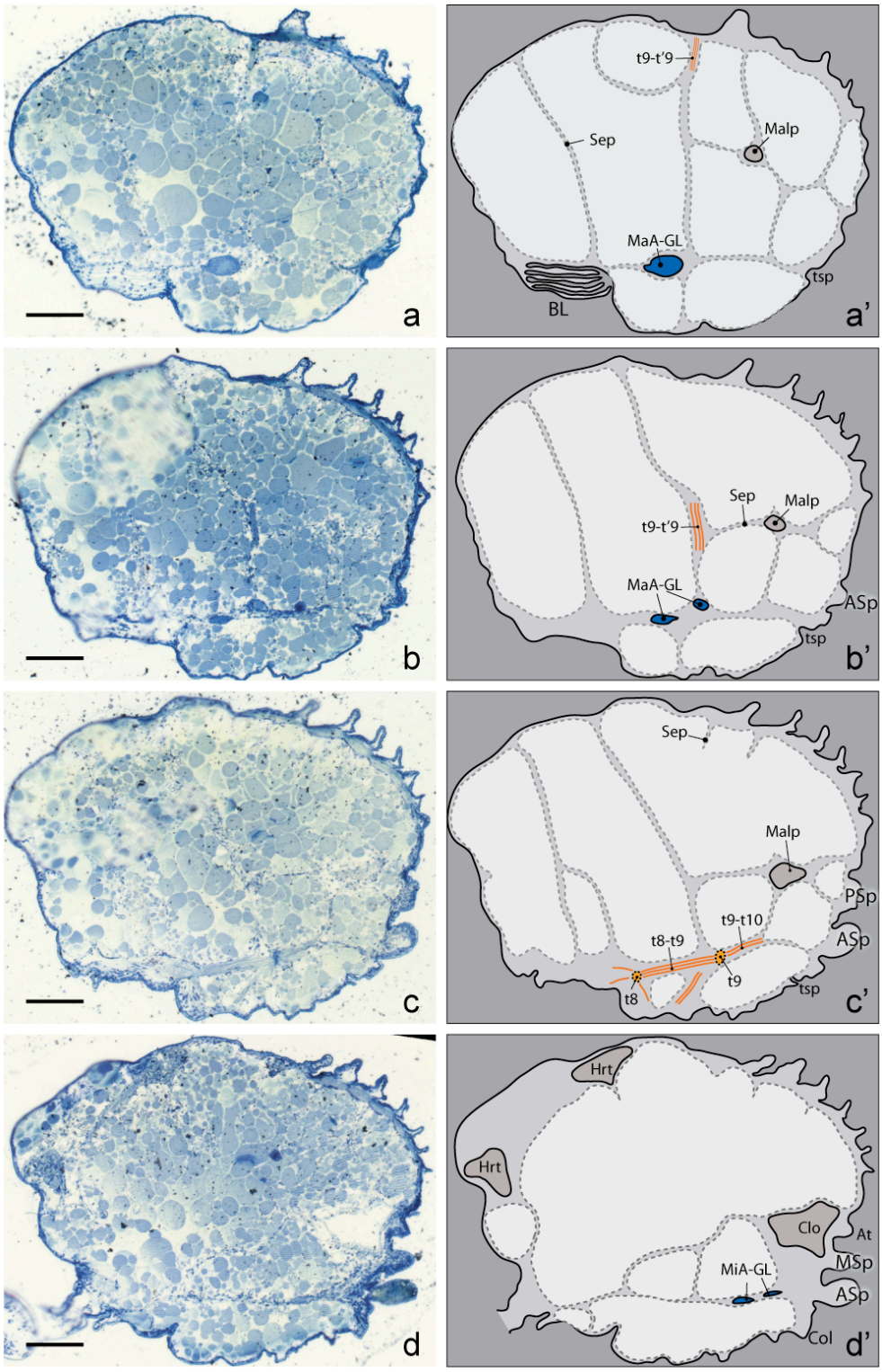



**Figure 3.8:** Internal morphology of the opisthosoma of a first instar *C. salei*

and nervous system, consists of (developing) silk glands.

### 3.3.7 The silk glands and spinnerets of the postembryo

The postembryo is the first stage after hatching, and takes up till three days before the first true molt gives rise to the first instar. Its general appearance is very similar to the late embryo in the egg, which its legs bend underneath the body. Postembryos do not move and do not react to mechanial stimuli (see chapter 2). The spinnerets and silk glands of this stage were studied by SEM and sections. The cuticle covering the spinnerets of the postembryo is free of sensory hairs and nozzles. From the cuticle it is not possible to distinguish individual spinnerets; the spinning field appears rather as a set of transversal grooves (see figure 3.9 a and b).

Sectioning of the opisthosoma of the postembryo is technically as challenging as in the first instar, because of the large proportion of yolk. Therefore epoxy embedded sections where the only method available for investigation the inner morphology (see figures 3.9 c - h). The epidermis of the spinnerets has a more complex shape than the overlying cuticle, consisting of the ASp, MSp and PSp as well as the other structures surrounding the spinning field. Inside each of these spinnerets an accumulation of nuclei is present. These nuclei are confined to close proximity of the PSp and MSp, but those associated with the ASp extend further into the opisthosoma. Although no silk gland specific marker is currently available, it is very likely that these structures consist of presumptive silk glands, in concordance with the conclusions of Jaworowski (1896) and Kovoor & Muños-Cuevas (1995). Especially the cells projecting from the ASp show a clear sac-like structure, consisting of an epithelium and a lumen. Therefore, on the ASp two glands were distinguised based on these sections (see figures 3.9 d'-g' in yellow), although it can not be said with certainty that no other glands are present. Two -smaller- glands were also distinguised on the PSp, and one on the MSp (see figures 3.9 g' and h', red and blue respectively). Interestingly, the glands associated with the ASp lie close to a septum of a yolk sac, and their leading front lies very close the entochondrite t10. Again, without a gland specific marker it is speculative to assign identity in these sections, but the location of the latter glands and their size suggests that these are the early major ampullate glands.

Other structures visible in sections of the postembryo are the tubular trachaea, which invaginate anteriorly to the spinnerets, and components of the midgut, such as the Malphigian tubules and the cloaca, which are thought to derive from yolk cells and not from invagination ectoderm (Rempel (1957) and references therein).

## 3.4 Discussion

Table 3.2 shows and overview of the silk glands and nozzles of both sexes of the adult spider and of the first three post-embryonic stages of *C. salei*. Looking at the increase in numbers of the different glands, a general distinction can be made between the piriform and aciniform glands on one side, and the ampullate glands on the other side. All of these seem to be present already in the first instar, but where the former group continues to increase in number with successive molts, the number of ampullate glands does not reach more than three. The tubuliform glands comprise a third general group, because these develop in the female only, and are not present in the first and second instar. The tubuliform glands were not the focus of this study, and will therefore be left out of the following discussion.

### 3.4.1 The piriform glands and their fusules

In *C. salei* adults, piriform glands were found in large numbers connected to the ASp's (see table 3.2) and in the ventral posterior part of the opisthosoma of the second instar (see figure 3.6). In the latter stage, twelve piriform glands were found, which corresponds exactly to the six smaller spigots present on each of the ASp's. This observation is in line with a comment of Peters (1955), that "in all likelihood, each of the mentioned glands (including piriform glands, MH) possesses its own nozzle". It is not clear, if at all, how many piriform glands are present in the first instar. Apart from two ampullate spigots, there do seem to be one or two smaller nozzles present on the ASp of this stage, but these structures might also be underdeveloped sensory hairs (see figure 3.5). No direct evidence for the presence of piriform glands in the first instar and postembryo could be obtained from sections.

The piriform glands of for example the wolf spider *P. amentata* and the orb weaving spider *Araneus diadematus* are used for making the sticky attachment discs to connect the ampullate silk threads to one another or to the substrate (Kovoor & Zylberberg, 1980; Richter, 1970), and their silk can be considered the "glue" of the constructions that these spiders build. In paraffin sections of the second instar of *C. salei* (see figure 3.6), the piriform glands show a granular appearance, very similar to the merocrine piriform glands of *A. diadematicus* (Kovoor & Zylberberg, 1980). It can therefore be speculated that the piriform glands of *C. salei* also produce attachment-disc silk. This is further supported by the observation of Melchers (1963) that silk from the ASp's of *C. salei* is used to attach the ampullate silk to the substrate during building of the silken cell prior to cocoon building. The cocoon itself is made exclusively from silk spun from the MSp's and PSp's (Melchers, 1963).

**Figure 3.9:** The silk producing system of the postembryo. **a:** SEM image of a postembryo, seen ventrally. Scale bar 200 $\mu$m. Image courtesy by Carsten Wolff. **b:** Detail of **a** showing the cuticle overlying the developing spinnerets and, with dashed lines, the estimated location of sections **d-h**. Distances between **a** and **b**, 20 $\mu$m; **b** and **c**, 10 $\mu$m; **c** and **d**, 36 $\mu$m; **d** and **e**, 70 $\mu$m. Scale bar 40 $\mu$m. **c:** 2 $\mu$m thick sagittal section of a *C. salei* postembryo. Scale bars 500 $\mu$m. The region shown in the dashed box is magnified in **e**. **d- h:** 2 $\mu$m thick sagittal sections of the posterior end of the opisthosoma of a *C. salei* postembryo. Scale bars 100 $\mu$m. **d'-h':** Interpretations of the sections shown in **d-h**. The yolk sacs are shown in light grey and are lined by septa. The blue dashed line depicts the ectoderm, underlying the cuticle shown in black. Muscle fibres are drawn as orange lines. **h:** Lower magnification of the section shown in **b** (dashed frame). Scale bar 500 $\mu$m. **at**, anal tubercle; **ASp**, anterior spinneret; **chl**, chelicera; **clo**, cloaca; **hr t**, heart; **malp**, Malpighian tubule; **MSp**, medial pinneret; **Opistho**, opisthosoma; **Pro**, prosoma; **proct**, proctodeum; **PSp**, posterior spinneret; **SP**, spinnerets; **spt**, septa of the yolk sacs; **sgl**, silk gland; **t10**, entochondrite t10; **tsp**, tracheal spiracle; **tt**, tubular trachaea.

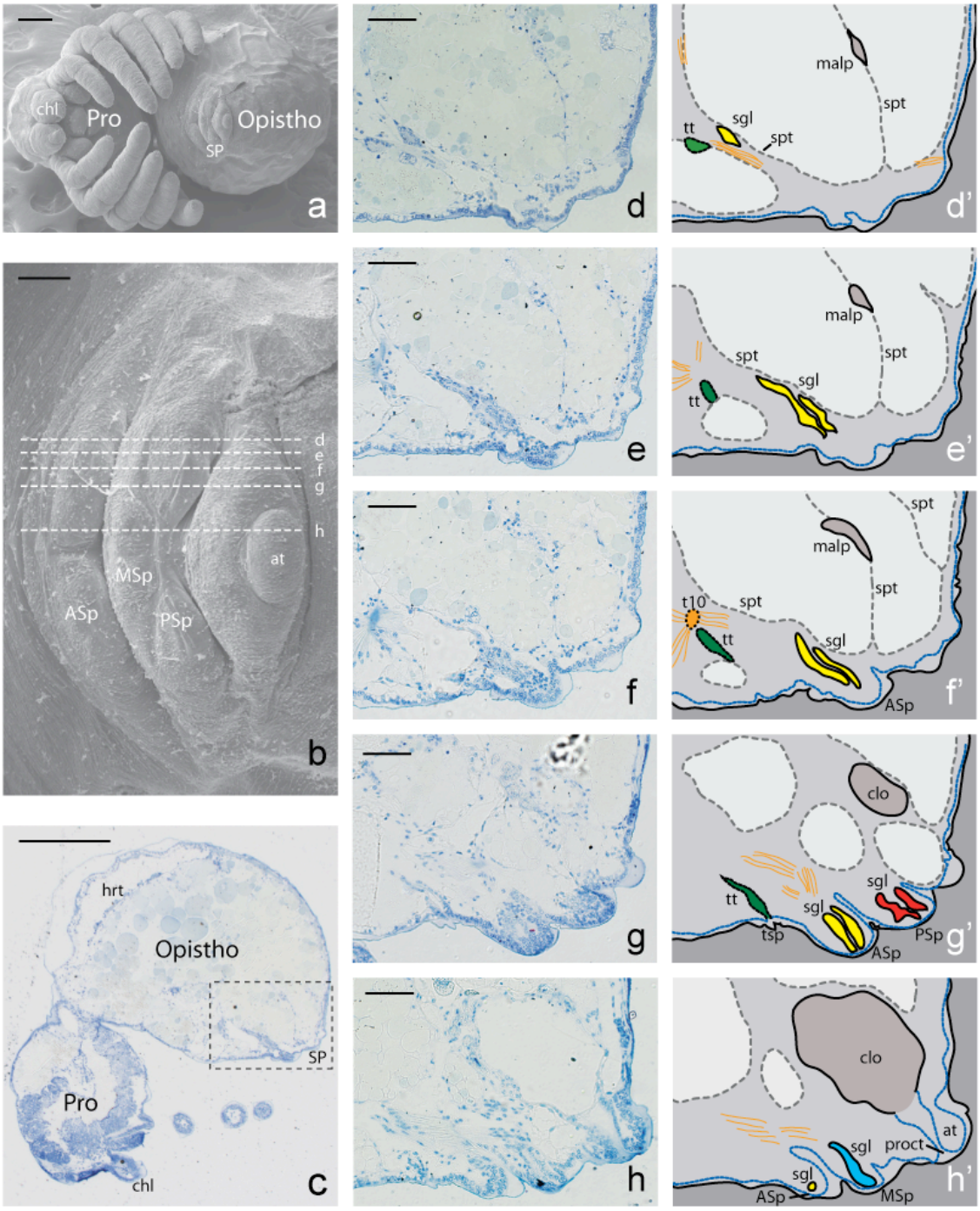



**Figure 3.9:** The silk producing system of the postembryo.

### 3.4.2 The aciniform glands and their fusules

A large amount of aciniform glands were observed in adult *C. salei* female and males, present on both the MSp and PSp (see table 3.2). Aciniform glands are also well developed in the second instar; about twenty to thirty were present, which roughly correlates with the twenty-six smaller spigots counted on both MSp's and PSp's of the cuticle of this stage (table 3.2 and figure 3.5). On these same spinnerets on the cuticle of the first instar ten nozzles were found, but, as with piriform glands, no direct evidence of the presence of aciniform glands in the first instar could be obtained from sections. However, glandular structures were observed inside the PSp of the postembryo, indicating that aciniform glands are probably already developing at that stage.

The involvement of aciniform silk in the production of the small diameter threads of the cocoon of the cob-web spider *Latrodectus hesperus* is strongly supported (Vasanthavada *et al.*, 2007). *C. salei* females build their cocoons with their MSp's and PSp's (Melchers, 1963), which could indicate the use of either aciniform or tubuliform silk, or of both types of silk at the same time. However, the presence of aciniform glands in the males and second instars (table 3.2) suggests that this silk can have other functions too. *C. salei* males for example use them for building a sperm web to take up their sperm in their pedipalps (Melchers, 1963). But this does not explain the presence of aciniform glands in sub-adult instars. On cross-section in the second instar these show a lumen well-filled with product (figure 3.6 c). Interestingly, Richter (1970) shows that the aciniform glands of *P. amentata* grow before and regress after molting, which suggests their involvement in this process, possibly for attaching the old cuticle to the surrounding substrate which allows the spider to pull itself out of the old cuticle.

### 3.4.3 The ampullate glands and their spigots

Ampullate glands were found in adults, first and second instars and probably also in the postembryo of *C. salei*(table 3.2). In older stages, they are composed of a large ampul connected to a long tapering duct, that in adults have the typical zig-zag shape found in many other spiders (e.g. Peters, 1955). In adult females, two ampullate glands were found attached to each of the ASp's and MSp's. In adult males, only one ampullate gland was observed on each of these spinnerets. These morphologies are different again from the glands found in the second instar, with three ampullate glands per ASp and MSp. Two of these clearly connect to a spigot on the spinneret; it is not clear where the duct of the third "inactive" gland leads to. These ontogenetic changes in ampullate gland numbers have not been described before in *C. salei*. This phenomenon can be understood by looking at

**Table 3.2:** Overview of the numbers of silk glands and nozzles during the life history of *Cupiennius salei*. The numbers of piriform, aciniform and tubuliform nozzles are based on (Melchers, 1963), other numbers are own observations. Numbers reflect the glands and nozzles per body halve. **ASp**, anterior spinneret; **gl**, number of glands; **MSp**, medial spinneret; **N**, a large number; **nz**, number of nozzles; **n**, a number below 10; **PSp**, posterior spinneret.

| **stage** | **spinnerets** | **ampullate** | | **piriform** | | **aciniform** | | **tubuliform** | |
|---|---|---|---|---|---|---|---|---|---|
| | | gl | nz | gl | nz | gl | nz | gl | nz |
| adult female | ASp | 2 | 2 | N | 300 | - | | - | - |
| | MSp | 2 | 2 | - | | N | 30 | N | 30-60 |
| | PSp | - | - | - | | N | 60 | N | 60-70 |
| adult male | ASp | 1 | 1 | N | 300 | - | | - | - |
| | MSp | 1 | 1 | - | - | N | 27 | - | - |
| | PSp | - | - | - | - | N | 60 | - | - |
| second instar | ASp | 3 | 2 | 6 | 6 | - | | - | - |
| | MSp | 3 | 2 | - | - | n | 5 | - | - |
| | PSp | - | - | - | - | n | 8 | - | - |
| late first instar | ASp | 2-3 | 2 | ? | ≤2 | - | | - | - |
| | MSp | 2-3 | 2 | - | - | ? | 2 | - | - |
| | PSp | - | | - | - | ? | 3 | - | - |
| Postembryo | ASp | 2? | - | ? | - | - | - | - | - |
| | MSp | 1? | - | - | - | ? | - | - | - |
| | PSp | - | - | - | - | ? | - | - | - |

the cuticle that bears, apart from ampullate spigots, tartipores and nubbins.

#### 3.4.3.1 Tartipores, nubbins and blocked ampullate glands

The presence of tartipores in adult spiders (figure 3.1) is an indication of the silk glands of the penultimate instar because, like in all araneamorphae, sexually mature spiders of this species do no longer molt. The adult cuticle forms directly underneath the cuticle of the penultimate instar, and since the penultimate instar continues to produce silk up till the final molt, the ducts of its glands necessarily have to go through the forming adult cuticle to reach the outer cuticle. Therefore, it is expected that, unless the cuticular structures on the mature molt completely replace those of the preceding molt, remainders of the ducts of the penultimate silk glands in the form of "scars" should be present on the adult cuticle. These scars have been termed "tartipores", and indicate a very interesting developmental system of the ampullate glands, that is in place in many different spiders (Townley & Tillinghast, 2003; Townley *et al.*, 1993). In these species, in any given free living sub-adult stage, two active ampullate silk glands lead to "open" spigots on both the ASp and MSp. In addition, there is a third gland present, in association with each of these two spinnerets, that is "blocked", i.e. without an open spigot. In a given instar, the ampullate gland that is blocked connects to the new cuticle. During molting, one of the two open ampullate glands continues to produce silk, but its spigot falls off with the old cuticle, and the glands gets blocked in the new instar. The newly blocked glands leaves behind a tartipore in the new cuticle. The second open glands remains open, but ceases its function temporarily while the newly formed spigot underneath the old spigot becomes functional in the new molt. All in all, this system allows the duct of at least one ampullate gland to remain attached to a spigot of the old cuticle (thus staying functional) during molting (Townley *et al.*, 1993).

The fact that this system is present in many different species from different lineages indicates that its evolutionary origin is probably ancient. Because of its complexity, it is not easily imaginable that the system developed several times independently (although this can not be excluded). A similar argument can be made for the presence of this system on both the ASp and the MSp - two spinnerets that have their origin on two different segments (see chapter 2). If it can be shown that the ampullate glands on these two spinnerets share, apart from their similar appearence, a genetic and ontogentic origin, this might indicate a shared evolutionary origin as well. Obviously, experimental evidence for this theory is lacking at present (but see chapter 5).

Although this system explains the numbers of ampullate glands in the sub-adult instars, it does not explain why in many spiders species, including *C. salei*, two ampullate glands on each spinneret are preserved in adult females and only

one in adult males, whereas in many other species the second pair of glands is lost in both sexes. Townley & Tillinghast (2003) give a detailed description of this problem, and show many example of spiders species where the female carries the cocoon attached toher 1° and 2° major ampullate spigots. *C. salei* females also carry their cocoon solely attached to their spinnerets (figure 3.3). Although the silken attachments to the cocoon were not studied in detail in this study, it can be assumed with reasonable certainty that *C. salei* females also use their 1° and 2° MaA-spigots to hold on to their cocoons.

The third structure visible, in addition to spigots and tartipores, on the ASP's and MSp's of the adult male *C. salei* are nubbins (3.2). These have been called "ontogenetic vestiges" of functional spigots. They locate the positions where functional spigots would have formed if the spider had remained a juvenile after its most recent molt (Townley & Tillinghast, 2003). Nubbins are very interesting, because the study of their development could give insights in the factors that are necessary for the correct development of spigots. Nubbins have the shape of the base of a functional spigot, but do not bear a large cone. They do however develop a very small protrusion where the cone would have formed. Bond (1994) tested the theory of Palmer (1990) that spider silk glands were derived from "dermal glands" and that nozzles were derived from sensory hairs. Bond (1994) found morphological similarities between the silk spigots and hairs of first instars of a mygalomorph spider, which: "support the hypothesis that silk spigots have evolved from setae and may suggest that silk initially functioned as a sensory mechanism". The study of the developmental mechanisms of spigots and sensory hairs could provide more data to test this evolutionary hypothesis.

Another open question is how the nozzles interact with the silk gland during their development. For example, the ampullate gland that would have attached to the nubbin is very much degenerated or completely absent in the adult male. So does the spigot needs signals from the gland to develop properly, or perhaps other way around? A third possibility is that both structures are under control of the same, yet to be identified, factor(s).

#### 3.4.3.2 Growth of the ampullate glands

From the second instar on, the ampullae of the major ampullate glands are located far anterior in the opisthsoma, close to the book lungs and far away from their spinnerets (figures 3.4 and 3.6). This distance allows the ducts of these gland to be very long, which probably is necessary for the ampullate silk proteins to obtain there configuration in the fiber (e.g. Craig, 2003). The minor ampullate glands are located more posteriorly, but are still much further placed from the spinnerets than the other gland types. This far anterior location of both types of ampullate glands is interesting, because many authors assume that the embryonic origin

of these glands lies in the spinnerets (see chapter 4). Another developmental hypothesis, analogous to for example the intestinal tract, where fore- hind- and midgut develop separately (Rempel, 1957), would be that the anterior portion of the gland develops *in situ*, and connects later to the duct (see chapter 1 for a more detailed description of this problem).

Sections made of the postembryo and the first and second instar of *C. salei* support the former hypothesis. In the postembryo no silk gland like structures were found in the anterior part of the opisthosoma, and the ampullate glands in the first instar lie slightly posterior to the position they reach in the second instar. The implies that, in order to reach their final location, the ampullate glands have to grow a long way through the opisthosoma. Most of this growth occurs in the first instar because in the postembryo silk glands are still confined to the direct vicinity of the spinnerets. This growth poses developmental questions, such as which factors direct this controlled growth, and where in the gland does the growth take place. Interestingly, a close spatial association was found between the developing ampullate glands and the ventral musculature of the opisthosoma, which seems to be already in place in the postembryo, before the main growth of the gland takes place (figure 3.9). Another possibility is that silk glands grow along the septa that line the yolk sacs (Rempel, 1957). Inspiration about where the growth of the ampullate spider silk glands takes place comes from the study of the silk glands of moth *Bombyx mori*. In this system, it was shown that cells only divide closely underneath the apex (Julien *et al.*, 2004). To answer this question dividing cells in silk glands of the first instar of *C. salei* could be labelled. A third, possibly related, question is how the zig-zag shaped ducts of the ampullate glands develop. These ducts are not connected to each other directly, but lie together in a sac like conjuntive sheat (Kovoor & Zylberberg, 1972) which could direct their growth.

This chapter provides some insights in the development of the silk producing system of *C. salei*, but also leaves many questions open. One of the most important findings is that in all likelihood all silk glands derive from the spinnerets. This means that the key for many developmental question with regard to the silk glands lies in the early development of the spinnerets. For example; why do particular glands develop on particular spinnerets only? Do the different glands share similar genetic developmental mechanisms, and which factors induce differences between them? The next chapter is about the early development of the silk glands in the spinnerets.

## 3.5 Methods

### 3.5.1 SEM imaging

Spinnerets were dissected from adult spiders and fixed in Dubosq-Brasil fixative (see 2.5.2). Opisthosomas of first and second instar spiders were dissected from their prosomas and fixed in Dubosq-Brasil. Subsequently these tissues were treated the same way as the embryos discribed in paragraph 2.5.2.

### 3.5.2 Paraffin sections and staining

Opisthosomas of second instar spiders were separated from their prosoma and fixed in Dubosq-Brasil fixative (see 2.5.2). After several ethanol washes the opisthosomas were dehydrated, moved to liquid paraffin using standard histological methods, and embedded under a dissecting microscope to orientate the opisthosoma. Sections were cut at 7 $\mu$m, mounted on slides, air dried at 37°C o/n and stained with haematoxylin and eosin according to standard histological methods.

### 3.5.3 Epoxy resin embedding, sectioning and staining

All following steps are performed on a rotating wheel at room temperature in 2 ml eppendorf tubes. 14 day old larvae were fixed for 2 days in the following fixative: 1% (w/v) picric acid (Fluka) dissolved in 1,4-dioxan (Sigma), 85 vols.; 37% formaldehyde (Sigma), 10 vols.; concentrated formic acid (Fluka), 5 vols. The larvae were washed in pure 1,4-dioxan for 8 hrs and next for 2 days, to remove excess picric acid, and then incubated overnight in a 1:1 mixture of fresh 1-4-dioxan and "SPURR" epoxy embedding resin mixed according to standard ratios ("firm") as described in the manual of the manufacturer (Serva Electophoresis, Modified SPURR Embedding Kit). The next morning the larvae were moved to pure embedding medium, and in the afternoon to an embedding mould and incubated at 70°C for 18 hrs to harden the resin. Blocks were trimmed and cut at 2 to 6 $\mu$m on a JUNG 2065 SUPERCUT microtome using a glass blade. Sections were stained with a mixture of 1% azure II (Aldrich) and 1% methylene blue (according to Ehrlich, Fluka) in a 1% aqueous borax solution (Sigma Aldrich) as described in Pernstich *et al.* (2003). The alkaline pH of this dye is important for an efficient staining of epoxy sections (Richardson *et al.*, 1960). After staining, sections were mounted in fresh SPURR resin, covered and incubated at 70°C overnight.

### 3.5.4 3D modelling

Paraffin section were fotographed under a microscope and images were aligned with the Amira (Visage Imaging) alignment function. Next stacks were imported into IMOD (Kremer *et al.*, 1996) and segmented. 3d modelling and meshing was performed in IMOD as well.

# Chapter 4

# The embryonic origin of the spinnerets and silk glands of *Cupiennius salei*

## 4.1 Abstract

Developmental studies of the silk producing system are not numerous, but date back to at least the second half of the 19th century. Most authors centered their studies on the cuticle of the spinnerets of the post-embryonic stages, and some of them also mention the development of the silk glands. However, to what extent the development of these structures is integrated is not well understood. Most authors postulate that the glands develop as invagination of the epithelium of the spinnerets. But where and how these invaginations take place is not well described or illustrated. In order to throw more light on these questions, this chapter first provides a general description of the embryonic development of the spinnerets of the model *Cupiennius salei*, and next focusses on the early silk gland development. SEM scans and confocal laser scans of nuclear and cytoskeleton markers show two pronounced invaginations. One in the center of the limb bud of opisthosomal segment four and one ventral to the bud on opisthosomal segment five. Based on their growth patterns and on the position of the invaginations on the spinnerets it is postulated that they constitute the primordia of the ampullate silk glands.

## 4.2 Introduction

The early developmental origin of the spider silk glands has been studied by several authors in the late 19th century. Jaworowski (1896) mentions the obser-

vations of a Russian arachnologist (Morin, 1888): "In a "certain stage" (of an undefined spider species, MH) a small ectodermal invagination begins at the tip of the third abdominal limb bud. It then grows, takes up a flask shape and later develops into a silk gland. A similar invagination is not seen on the fourth limb bud." Jaworowski (1896) can not confirm these observations in the wolf spider *Lycosa singoriensis*. He does not see such invaginations (figure 4.1 a), nor a cuticular membrane in the lumen of the gland, but instead postulates that the glands are derived from tissue inside the spinnerets. His drawings do not show a clear connection between the developing silk glands and the spinnerets.

Purcell (1909) however studied the jumping spider (family Salticidae) *Sitticus floricola*, and shows an invagination on the medial part of the limb bud of the anterior spinneret, at the onset of inversion (figure 4.1 b). He mentions that "the spinning glands appear at quite an early stage at the apex of the appendages", but since the focus of this publication is on the book lungs he does not discuss the further implications of this observation.

Sivickis *et al.* (1928) write that in a cob-web spider of the genus *Latrodectus*: "The primordia of the spinning glands appear in about the middle of the reversion period. They arise from the rapid multiplication of the ectodermal cells at the lower ends of the third and fourth pairs of the abdominal appendages which are modified into the spinning apparatus. Another pair of the spinning mamillae (the medial spinnerets, MH) is added after the hatching of the embryo". Unfortunately no drawings of these processes are presented.

Another publication on the development of *Latrodectus* describes the silk glands as follows: "During embryonic development the silk glands arise as a result of extensive cell proliferation of the ventral ectodermal layer of segments four and five. Each gland is a tubular structure with one layer of cells grouped around a central canal" (Rempel, 1957). The accompanying figure to this statement is not highly detailed, and the depicted embryo is of a late embryonic stage (figure4.1 c).

Several decades later, Kovoor & Muños-Cuevas (1995) describe the organogenesis of lynx spiders of the genus *Peucetia* and write about a developmental stage (that I call postembryo in this thesis, see chapter 2): "spinnerets differentiated during this instar; they did not bear any spigots, but anlagen of the large (ampullate) silk-gland ducts appeared inside them, together with the first buds of the gland sacs". The pictures in this publication are not of stunning quality, and the described stage is again older than where invaginations were observed by Sivickis *et al.* (1928) and Purcell (1909).

It seems clear that many authors have observed an association of the developing silk glands with the developing spinnerets, and my own results confirm this (see chapter 3). This is consistent with recent theories about the evolution of the silk glands, which are based upon the idea that silk glands are of ectodermic

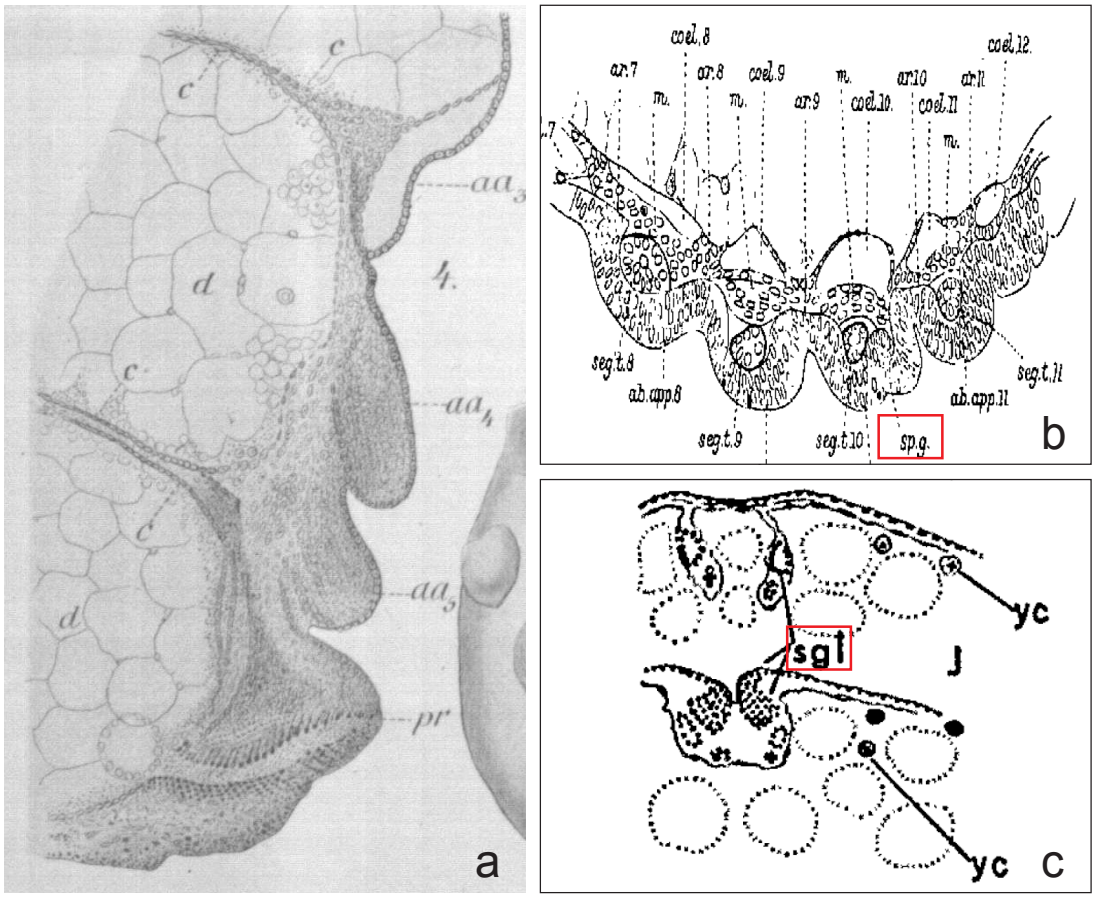



**Figure 4.1:** Illustrations of spider silk gland invaginations sites in old literature. **a:** Sagittal section through the posterior part of a late embryo (close before hatching) of the wolf spider *Trochosa singoriensis* Jaworowski (1896). **aa**$_4$, "limb bud of opisthosomal segment 4"; **aa**$_5$, "limb bud of opisthosomal segment 5" (probably medial spinneret); **c**, septum of coelomic sac; **pr**, proctodeum. **b:** Sagittal section through the medial part of the opisthosomal limb buds of the jumping spider *Sitticus floricola* at the onset of reversion (Purcell, 1909). **m**, mesoderm; **sp.g.**, (marked with a red box) spinning gland; **seg.t.10**, opisthosomal segment 4; **seg.t.11**, opisthosomal segment 5. **c:** Two parallel transversal sections through the posterior part of the opisthosoma of a late embryo (close before hatching) of the cob-web spider *Latrodectus mactans*(Rempel, 1957). **sgl**, (marked with a red box) silk gland; **yc**, yolk cell.

origin (Craig, 2003, 1997; Palmer, 1990, see chapter 1). However, even though the glands originate close to the spinnerets, the idea that they invaginate from the epidermis is based upon circumstantial evidence, because detailed descriptions of the invagination process are lacking. Also, if the glands do develop as epidermal invaginations, it is not clear where on the spinnerets and exactly when during development this process occurs. Furthermore, it is not clear whether each gland develops as a separate invagination, or branch after a shared invagination. To get insight into these questions, the spinnerets of *C. salei* during an embryonic process called inversion were investigated with several imaging techniques.

## 4.3 Results

### 4.3.1 The epithelium of the embryonic spinnerets and walking legs

In order to study the surface of the epithelium of the developing spinnerets, SEM scans were made of different stages during and after inversion, which spans the period of development where the buds of the spinnerets are present but not yet covered by any sort of cuticle, (see figure 2.1 for staging). Another incentive was that, even though it has been known for a long time that in araneamorph spiders (see chapter 1) both the medial and posterior spinnerets derive from one single limb bud on O5 (e.g. Jaworowski, 1896), to my knowledge no-one before has accurately described the morphogenesis of these limb buds with modern imaging techniques.

It was found that at stage 10e, these two limb buds have a similar globular and undifferentiated aspect (figure 4.2 a) and lie about 30 $\mu$m apart. Their surface shows individual ectodermal cells, and the bud on O4 appears to be slightly bigger and to be composed of slightly more cells than the one on O5.Their morphology soon starts to differentiate. At stage 10l both buds have grown and are almost touching each other, and their shape is no longer globular (figure 4.2 b). The bud on O4 has developed a depression (arrow) in the center, as well as a group of cells slightly protruding from the surface at its dorsal posterior side (filled circle). The bud on O5 has become somewhat elongated in a dorso-ventral fashion. One shallow groove (arrowhead), and one slightly deeper groove (double arrowhead) divide the bud in three domains (asterisks).

An embryo at stage 11e was found with a clear hole in the ectoderm at the deepest point of the depression on the bud on O4 (arrow). A hole was also observed at the same position on the bud on O4 of the other body half (not shown), indicating that this is not an artefact. The hole has a diameter of about 2 $\mu$m (see detail in figure 4.2 c') and appears to be the opening to an invagination.

It was therefore called “invagination on the limb bud of O4”, or O4-i. Figure 4.2 d shows the buds of O4 and O5 of another stage 11e embryo from a slightly dorsal and anterior point of view. The protruding cells on the bud on O4 (filled circle) are clearly visible from this point of view, and their also appears to be a hole at the same position as found in the embryo shown in figure 4.2 c, albeit less conspicuous. The deeper groove on the bud on O5 (double arrowhead) has become more pronounced, separating the bud in two more or less distinct shapes. The groove on the dorsal part (arrowhead) is still present but has become less pronounced. A third groove appears at the ventral part of the bud. Interestingly, this groove also bears a hole (see detail in figure 4.2 d’), which was called O5-i. After dorsal closure it becomes more difficult to distinguish individual cells on the surface. Figure 4.2 e shows the buds on O3-O5 from a posterior point of view of an embryo at stage 11e. The entire bud on O4 of this stage will likely develop into the anterior spinneret (ASp). The depression on the ASp is still in place (arrow), but there is no more hole visible. The bud on O5 is now composed of two distinct domains, and these very likely become the posterior and medial spinnerets (PSp and MSp). O5-i is no longer visible on the base. As inversion progresses with ventral closure (figures 2.1 m-o and 4.2 f) the spinnerets become less pronounced from the surface, possibly elongating and flattening while migrating in a medial/posterior direction.

**Figure 4.2:** SEM scans showing a developmental series of the spinneret limb buds of opisthosomal segments 4 and 5, from just before inversion in **a** (comparable to 2.1 b and c) until just before ventral closure (comparable to 2.1 n). Scale bars in **a**, **b**, **c**, **d**, **d'** and **e**: 20 $\mu$m, in **c'**: 10 $\mu$m and in in **f**: 100 $\mu$m. See text for more details. **ASp**, anterior spinneret; **C**, chelicere; **O3-O5**, opisthosomal segments 3 to 5; **L1-L4**, prosomal walking legs 1 to 4; **MSp**, medial spinneret; **P**, pedipalp; **PSp**, posterior spinneret; **T**, telson.

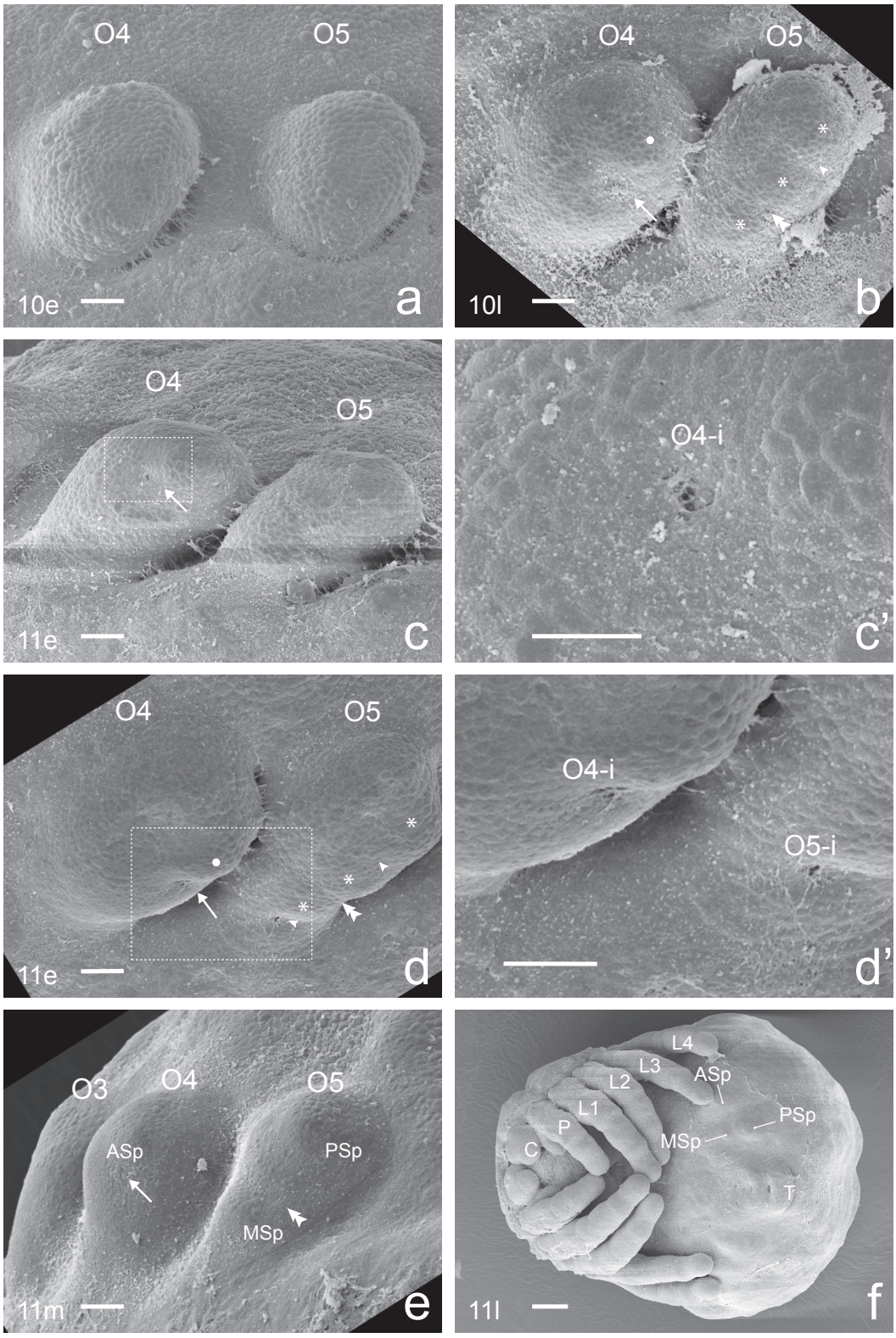



**Figure 4.2:** Developmental series of the spinneret limb buds.

# 4. THE EMBRYONIC ORIGIN OF THE SPINNERETS AND SILK GLANDS OF *CUPIENNIUS SALEI*

### 4.3.2 Invaginations of the spinnerets during inversion

To investigate the cells underneath the surface of the limb buds of O4 and O5, confocal scans were made of embryos stained with a fluorescent nuclear marker. Figure **??** shows such an embryo shortly after dorsal closure, comparable to figures 2.1 k and 4.2 d. Figure **??** b shows the orthogonal (i.e. without simulated perspective) projection of a confocal scan of the region shown in a dashed box in figure **??** a. Clearly visible are the two limb buds belonging to O4 and O5. The large bud on O4 has a globular shape whereas the bud on O5 is more elongated. Less clearly visible is the groove (double arrowhead) that divides the bud on O5 in a dorsal and a ventral portion, as seen more cleary in figures 4.2 d and e. Ventral to the two limb buds lies the ventral neuro-ectoderm. Two large invaginations were observed, one in the center of the bud on O4, and one at the ventral side of the bud on O5. In order to illustrate their size and position, their lumen was modeled in 3D and rendered yellow inside the confocal projection. Because the position of these invaginations corresponded exactly with O4-i and O5-i seen in figures 4.2 c and d, they are considered the same invaginations. O4-i grows into the tissue in an anterior direction, O5-i is smaller and its tip does not show this anterior orientation. A third, much smaller invagination was seen on the dorsal portion of the O5 limb bud, and was termed "dorsal invagination of the limb bud of O5", or O5-di.

Optical sections through the stack shown in figure **??** b show the underlying cell layers (figure **??** d and e). The positions of these sections through the stack are depicted on a perspective projection in figure **??** c. The section through the limb bud on O4 is positioned through the center of O4-i, and is slanted at an angle of about 45 ° in relation to the basal plane of the stack, in alignment with the anterior orientation of O4-i. This section clearly shows the invagination of the epithelium growing into the underlying mesoderm (figure **??** d and d'). The estimated position of the basement membrane in this stack is drawn in figure **??** d' and is strongly concave below O4-i. The section through the bud on O5 goes through the center of both O5-i and O5-di and is almost perpendicular to the basal plane (figures **??** e and e'). In this section O5-i is clearly visible, albeit somewhat smaller than O4-i, also showing a pronounced concavity of the estimated basement membrane. O5-di is markedly smaller, and the epithelial cell layer at this position does not appear concave.

### 4.3.3 The cytoskeleton of the invagination sites

It is to be expected that changes in the cytoskeleton accompany the epithelial morphogenesis of the opisthosomal limb buds shown in figure **??**. Filamentous actin, a major component of the cytoskeleton, has been stained successfully before

**Figure 4.3:** Invagination sites on the spinneret limb buds. **a**: Image under UV light of an *C. salei* embryo at stage 11e, stained with a fluorescent nuclear marker and seen from a ventral dorsal point of view. The box marks the region depicted in **b**. **b**: 3D reconstruction of a confocal stack of the limb buds of O4 and O5. The lumen of the invaginations found on these buds were modeled in 3D and these models are rendered inside the stack. Scale bar is 20 $\mu$m. **c**: Same 3D reconstruction as in **b**, additionally showing the locations of the optical sections that are shown in **d and e**. **d and e**: Optical sections through the limb buds of O4 and O5 respectively. **d', e'**: Same optical sections as in **d, e** with labeled structures drawn-in, including the 3D models of the invaginations. **BM**, basement membrane; **ecto**, ectoderm (epithelium); **L3, L4**, walking legs 3 and 4; **meso**, mesoderm; **O2-O5**, opisthosomal segments 2 to 5; **O4-i, O5-i**, major invaginations on the limb buds of O4 and O5; **O5-di**, smaller invagination on the dorsal portion of the limb bud of O5; **Te**, telson; **VNE**, ventral neuroectoderm.

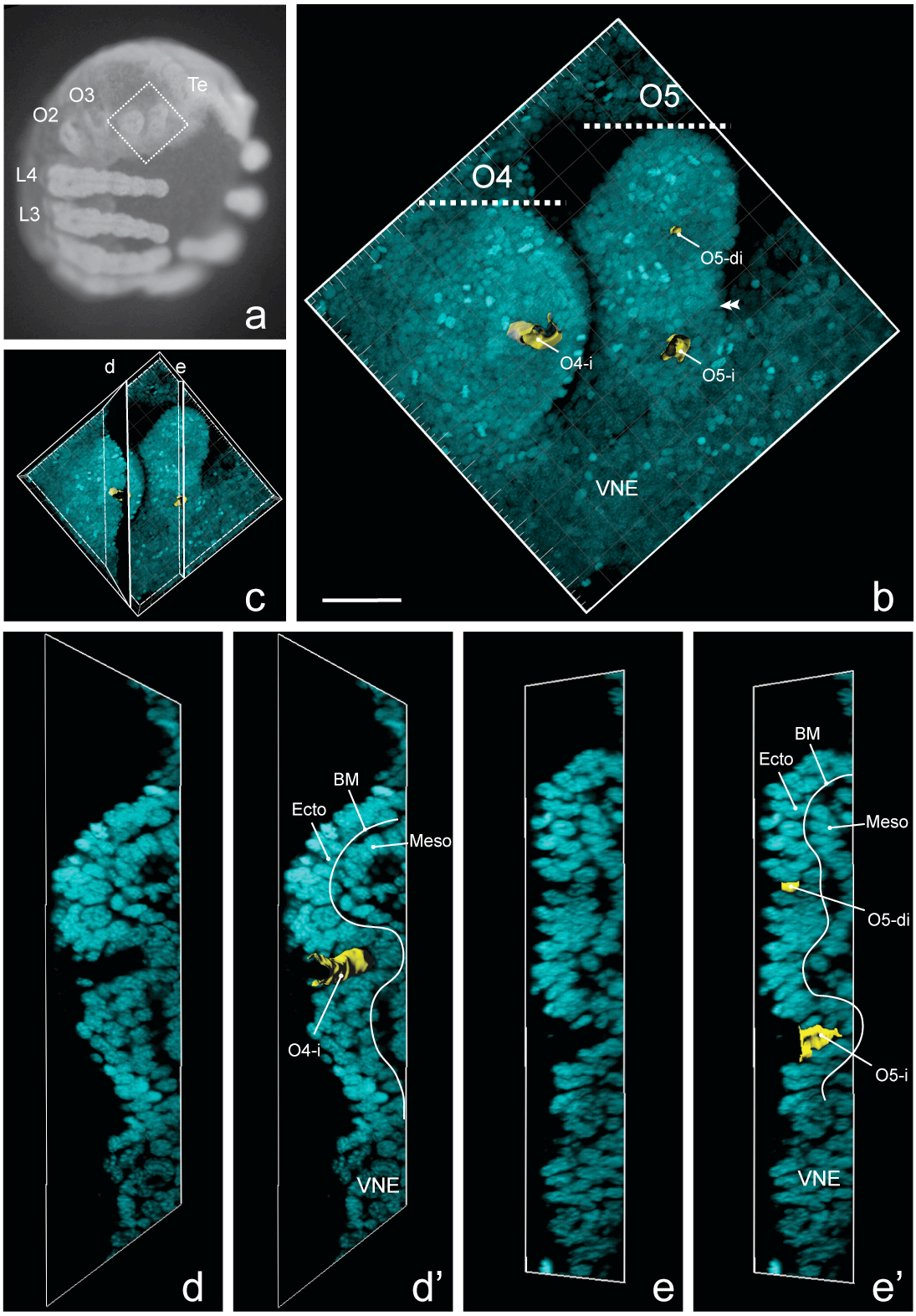


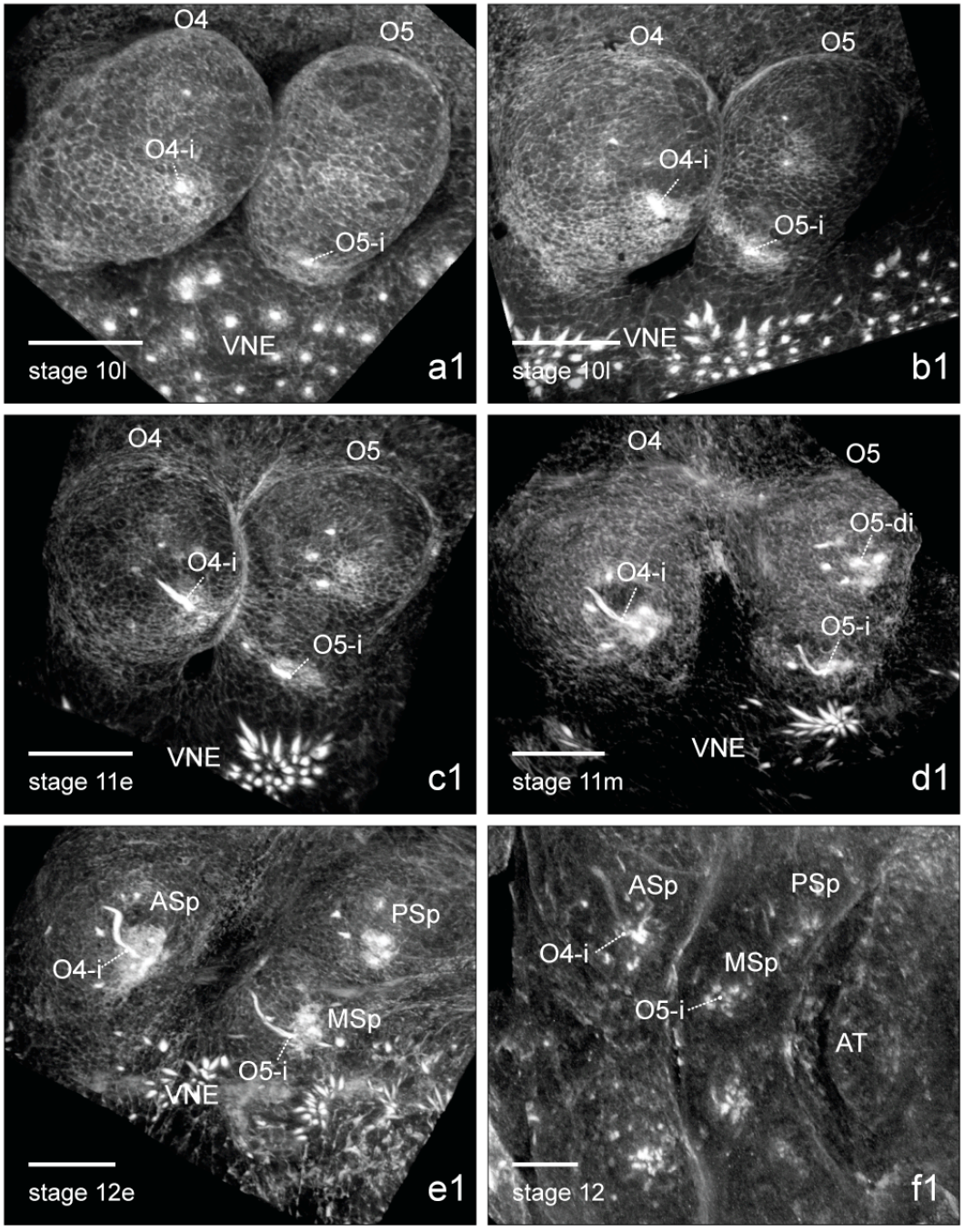

**Figure 4.3:** Invagination sites on the spinneret limb buds.

in *C. salei* in order localize cell movements of the nervous system, because it is said to "accumulate in the constricted cell processes of the sensory precursors" (Stollewerk & Seyfarth, 2008). To see whether filamentous actin accumulations can also be observed in association with the invaginations of the limb buds of O4 and O5 described above, embryos were stained with phalloidin conjugated to a fluorofore. Figures 4.4 a1 to a6 show confocal stacks of the limb buds of O4 and O5 from six successive embryonic stages. The first stage shown is when the dorsal migration of the two body halves is half way (figure 2.1 i), the last stage when the spinning field has reached its final configuration (figure 2.1 o). During this period the intercalating groups of cells of the ventral neuroectoderm can be seen as bright spots, as described by (Stollewerk *et al.*, 2001). There is also bright staining visible in the limb buds, corresponding with the location of O4-i and O5-i. Starting with a single spot on each bud in figures 4.4 a1, over the further course of inversion more spots appear and bright bundles of actin appear to be growing out of the initial spots. There is also several spots of strong phalloidin staining visible at the dorsal part of the bud on O5, one of them possibly corresponding with O5-di. From these spots no bundles grow out. The latest developmental stage analyzed (figures 4.4 f1-f4) shows a much higher level of background, probably due to the formation of the embryonic cuticle, which might hamper both staining and visualization.

The bundles of filamentous actin co-locating with O4-i were analyzed in more detail. Shown in figures 4.4 a2-f4 are orthogonal projections of rectangular blocks of tissue surrounding O4-i, that were cut-out (virtually) from the 3D rendering of each of the stacks shown in (4.4 a1-a6). Column 2 shows these block from above (same view as 1a-1f), column 3 from the long side and column 4 from the short side of the rectangle. For orientation, colors surrounding the figures correspond to the sides of the schematic block in figure 4.4 g. An arrow indicates the concentration of actin filaments that appears first (figures 4.4 a1-a4) at the surface of the epithelium and then increases in size, growing in an anterior direction at an angle of about 45 ° (figures 4.4 b-f). The serpentine shape of these bundles can clearly be seen in figures 4.4 e1-e4 . From dorsal closure on (figure 4.4 c) other spots of filamentous actin appear at the surface close to the larger bundles at O4-i, O5-i and O5-di. These do not project bundles of actin inward, apart from one spot just posteriorly to the main actin bundle on the bud of O4 (arrowhead in figures 4.4 d2-f4). At the stage when the spinning field has developed its final configuration (figures 4.4 f1-f4), and when the ASp and MSp are clearly defined, two separate bundles of actin can be seen at the tip of each of the ASp's (figures 4.4 f1-f4).

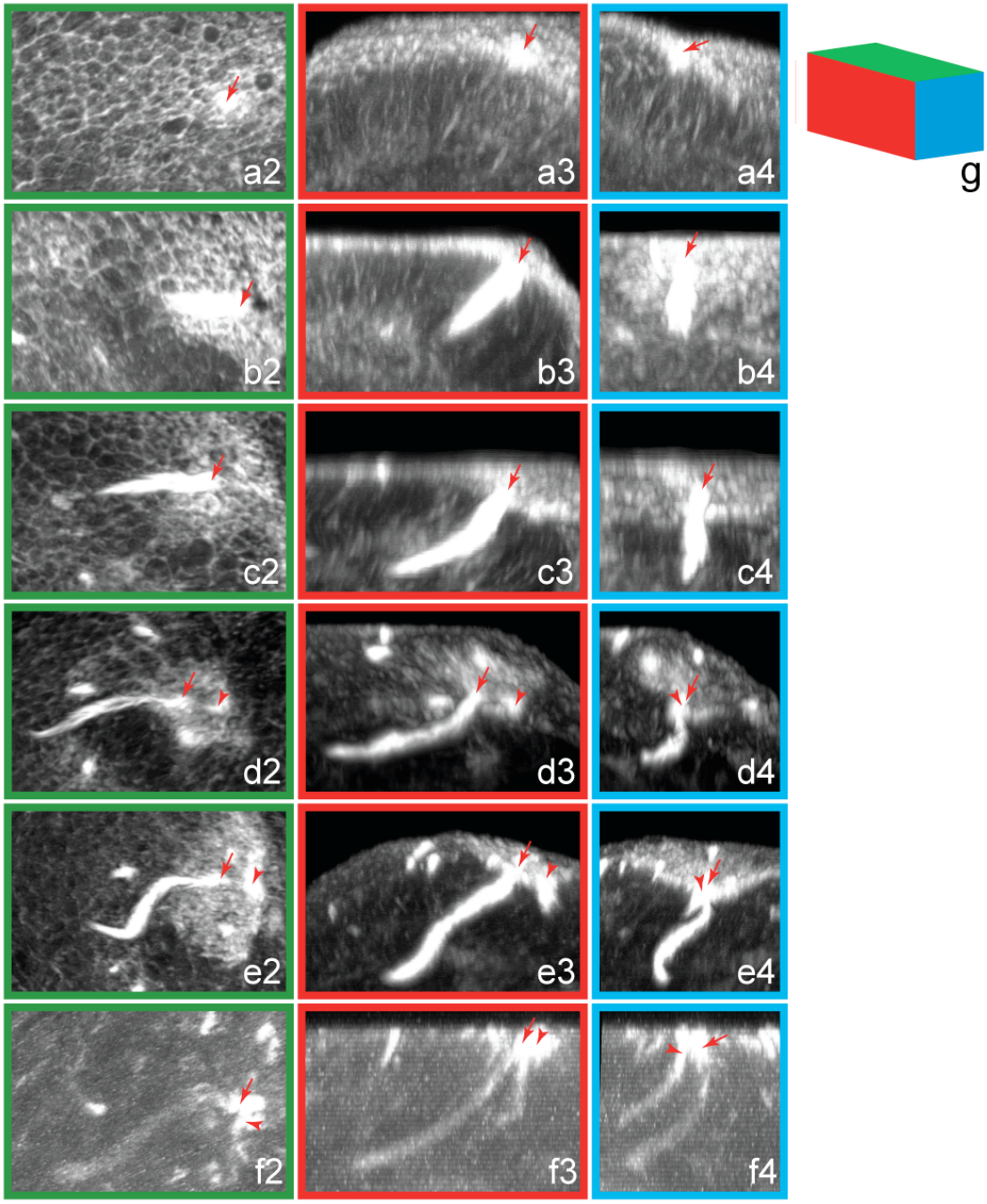
It is not clear what the significance is of the smaller spots of actin on the limb buds of both O4 and O5. These start to appear when the larger bundles at O4-i and O5-i have already gained some size (figure **??** c1). It is also not clear if they

**Figure 4.4:** Actin filaments in the developing spinneret limb buds. **a1-f1** are 3D projections of confocal scans of the spinneret limb buds of six different developmental stages during inversion, stained with phalloidin. Scale bars 50 $\mu$m. **a2-f2**, **a3-f3** and **a4-f4** show from three different angles in higher magnification the region, in 3 dimensions, surrounding O4-i in the confocal stacks in **a1-f1**. These angles are shown schematically in **g** with a color code: green is topview (same as **a1-f1**), red is sideview on the long side of the rectangle, blue is sideview on the short side of the rectangle. **a1-a4** stage 10l (corresponds to figure 2.1 i, the dorsal leading edges of the reversing embryo are approaching each other). **b1-b4** stage 10l, slightly later (corresponds to figure 2.1 j, just before dorsal closure). **c1-c4** stage 11e (corresponds to figure 2.1 k, at dorsal closure). **d1-d4** stage 11m (corresponds to figure 2.1 m, start of ventral closure). **e1-e4** stage 12e (corresponds to figure 2.1 n-o, towards the end of ventral closure). **f1-f4** stage 12 (corresponds to figure 2.1 p, shortly after ventral closure). **ASp**, anterior spinneret; **AT**, anal tubercle; **MSp**, medial spinneret; **PSp**, posterior spinneret; **O4-O5**, opisthosomal segments 4 and 5; **O4-i, O5-i**, (the location of) major invaginations on the limb buds of O4 and O5; **O5-di**, (the location of) smaller invagination on the dorsal portion of the limb bud of O5; **VNE**, ventral neuroectoderm.




**(I)**

**Figure 4.4:** Actin filaments in the developing spinneret limb buds; overview of confocal stacks.




**(II)**

**Figure 4.4:** Actin filaments in the developing spinneret limb buds; details of the anterior spinneret.

form a stereotypical pattern, that is repeated between embryos, like for example the sensory percursors in the walking legs do (Stollewerk & Seyfarth, 2008).

Based on their locations on the spinnerets, it is plausible that O4-i and O5-i are the primordia of silk glands. An alternative possibility is that these invaginations might actually be part of the developing nervous system and not of silk glands. To investigate this, the expression of *Cs-ASH2*, a spider homolog of the insect proneural gene *achaetescute* was analyzed. The gene was found expressed in the developing ventral neuroectoderm, as described before (Stollewerk *et al.*, 2001), and expression was also observed in the limb buds of O4 and O5 of developmental stages shortly after the start of inversion (figure 4.5, arrows). The expression domain on the bud of O4 is rather broad, slightly off-center to the ventral part of the bud. The domain on the bud of O5 is smaller and lies at the ventral margin of the bud.

A second gene that has been associated with the developing peripheral spider nervous system is *prospero* (Stollewerk & Seyfarth, 2008). The expression of Prospero protein in the spinneret limb buds was tested with an antibody raised against a *prospero* homolog of *C. salei* (Weller, 2002). At a stage close to ventral closure, no Prospero staining was detected in the limb buds. Unfortunately, it was not possible to obtain reliable expression data of later stages due to high levels of background.

## 4.4 Discussion

Knowledge of embryonic origin of spider silk glands is crucial for obtaining a better understanding of their evolutionary emergence. Several authors state that the silk glands originate from epidermal invaginations of the spinnerets during the embryonic process of inversion. However, descriptions of the morphogenesis of the limb buds of O4 and O5 are scarce and not very accurate (e.g. Jaworowski, 1896), nor is there sufficient empirical data available on the postulated invaginations of the silk glands. In this chapter I present three types of data supporting the presence of invagination sites on the developing spinneret limb buds of *C. salei*. SEM scans of the epithelium of the spinneret limb buds showed two pores around the time of dorsal closure, O4-i in the center of the limb bud of O4, and O5-i ventral to the bud of O5. Confocal imaging with a nuclear marker confirmed that the pores seen on the surface are invaginations of the epithelium, and phalloidin stainings showed that they are associated with high concentrations of filamentous actin, as has been shown earlier for intercalating and invaginating neuronal cells (Stollewerk & Seyfarth, 2008; Stollewerk *et al.*, 2001).

O4-i looks very similar to the invagination that was described by Purcell (1909), which he interpreted as "spinning glands" (figure 4.1 b). If this inter-

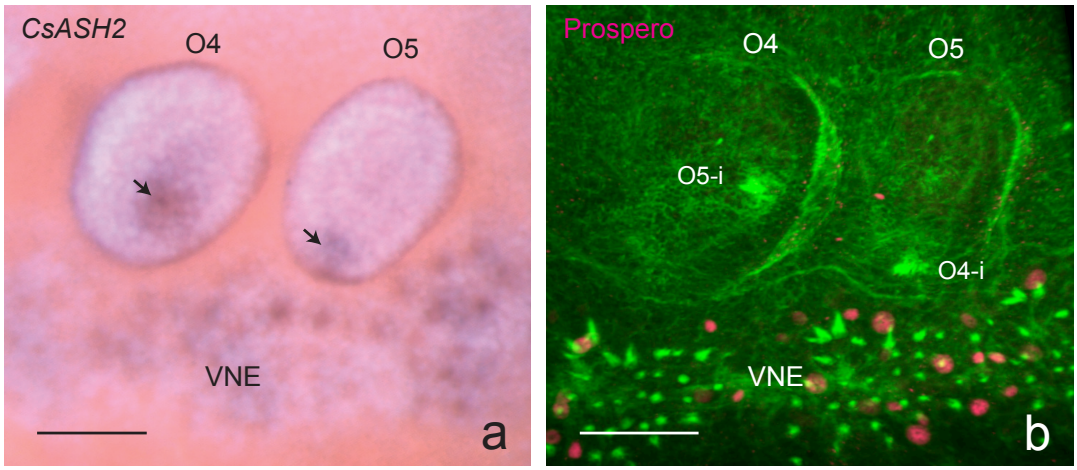



**Figure 4.5:** Expression of the proneural gene *CsASH2* and the nervous system marker Prospero in the limb buds of O4 and O5. **a:** Whole mount in situ hybridization with an RNA probe against *CsASH2*, counterstained with fluorescent nuclear marker dapi. Arrows: see text. Developmental stage shortly after ventral opening (similar to figures 2.1 f). Scale bar 50 $\mu$m. **b:** Antibody staining against *C. salei* Prospero (magenta), and phalloidin staining against filamentous actin (green). Developmental stage just before dorsal closure (similar to figures 2.1 j and 4.4 b1-b4). Scale bar 50 $\mu$m. **O4-O5**, opisthosomal segments 4 and 5; **O4-i, O5-i**, (the location of) major invaginations on the limb buds of O4 and O5; **VNE**, ventral neuroectoderm.

pretation is correct, then the question arrises if these two invaginations are the primordia of all silk glands (which are numerous, see chapter 3), or of only a subset of the glands. Since it is very likely that each silk gland possesses their own opening, or "nozzle", on the cuticle (Peters (1955) and see chapter 3), it is difficult to imagine how all glands, including their nozzles would develop from a single invagination site. It is more likely that these invaginations correspond to a subset of the glands. Because the ampullate glands have probably already developed to a length of about 100-300 $\mu$m in the postembryo (figure 3.9) and are the largest glands in later post-embryonic stages, it is expected that these originate rather early in the embryo. Therefore, it might well be that O4-i and O5-i correspond to ampullate glands. This would also be consistent with their locations on the limb buds; O4-i lies on the bud that will become the anterior spinneret, which is the spinneret that connects to the major ampullate glands in later stages, O5-i lies on the part of the bud that will develop into the medial spinneret, which is the spinneret that connects to the minor ampullate glands in

later stages.

One problem with this idea is that the total number of ampullate glands on each of the anterior and medial spinnerets is three in later stages, and at least two ampullate glands per spinneret seem to be present already in the first instar (figure 3.8), which does not seem to correspond with the single invaginations O4-i and O5-i. However, the ampullate glands on each spinneret could possibly develop one after another, which would be supported by the observation that towards the end of inversion a second bundle of filamentous actin appears close to O4-i (figure 4.4 d-f).

An alternative possibility for the nature of O4-i and O5-i is that they do not correspond to silk glands, but represent invaginating nervous cells, similar to the large sensory precursor groups described by Stollewerk & Seyfarth (2008), which are thought to give rise to internal joint receptors. Indeed, spiders display exquisite control over their spinnerets, which implies extensive innervation and the presence of mechanoreceptors. For example , a study on the neuroethology of *C. salei* describes mechanoreceptors, and their accompanying axon, around the spigots of the major ampullate glands on the adult anterior spinnerets (Gorb & Barth, 1996). It is possible that O4-i and O5-i are involved in the development of these structures.

To investigate this alternative, embryos were stained with an RNA probe agains the gene *CsASH2*. Interestingly, *CsASH2* expression was observed in the limb buds of O4 and O5, roughly correlating with the positions on the buds where shortly afterwards the first signs of O4-i and O5-i appear (figures 4.5 a and 4.4 a1-a4). This would be in concordance with a nervous system fate of O4-i and O5-i because *CsASH2* expression was also observed prior to the formation of sensory precursor groups in "the corresponding areas" on the walking legs of *C. salei* Stollewerk & Seyfarth (2008). However, although the function of *achaete-scute* has been extensively studied in *Drosophila melanogaster* (e.g. Skeath & Doe, 1996, and references therein), functional studies of the *achaete-scute* homologs in *C. salei* have not produced convincing results that *CsASH2* is indeed involved in the development of sensory precursor groups; RNAi phenotypes do not appear very reproducible and especially the large sensory precursor groups of the walking legs are affected only in a small percentage of injected embryos (Stollewerk & Seyfarth, 2008).

Embryos were also stained with an antibody against the nervous system marker Prospero (Weller, 2002). Prospero is said to be "transiently expressed in the sensory neurons and the glia, which delaminate from the epithelium to occupy a basal position, and it accumulates in the nuclei of the sheath cells that remain in the epithelium" Stollewerk & Seyfarth (2008). The protein was found to be expressed in the ventral neuroectoderm, as described earlier, but not in association with O4-i and O5-i in developmental stages close before dorsal clo-

sure (figure 4.5). Unfortunately, stainings with Prospero in later developmental stages proved to be difficult. These results speak against the sensory precursor hypothesis, although it could be that Prospero is not expressed in O4-i and O5-i until later in development, because in the large precursor groups the protein is only expressed in the basal cells Stollewerk & Seyfarth (2008).

A third hypothesis would actually incorporate both the idea that O4-i and O5-i are the precursor of silk glands as well as the expression pattern of *CsASH*. According to an evolutionary theory proposed by (Palmer, 1990), the cuticular nozzles of the silk glands (see chapter 3), are derived from sensory setae. Bond (1994) tested this theory, and found that the nozzles that appear earliest in development of a mygalomorph spider indeed look very similar to the earliest sensory hairs. Interestingly, in *C. salei* is also difficult to distinguish between the sensory hairs and the nozzles in the first instar (figure 3.7). If this is the way silk glands evolved, then it might just as well be that molecular factors associated with the development of sensory hairs are also involved in early development of silk glands and their cuticular structures.

It is clear that the discovery of invagination sites on the limb buds of O4 and O5 is not the final evidence that these are indeed the primordia of silk glands in general, or ampullate glands in particular. It does seem to be very likely however that these are the structures that were observed by Purcell (1909) as judged from his drawings (figure 4.1 b), and possibly also by Morin (1888) and Sivickis *et al.* (1928), albeit in other spider species than *C. salei*. The smaller size of O5-i and its locations at the base of the limb bud might be the reason why Morin (1888) and Purcell (1909) overlooked them.

In order to get more confidence about the nature of these invaginations, the most direct approach would be to trace these cells into later stages, either by injection of a traceable compound, and/or by sections of a fine-scaled developmental series starting at inversion. Such fine-scaled staging would also give more information about whether the other silk glands that originate in the spinnerets develop as epidermal invaginations or by some other means. But at the moment high quality sectioning of these stages is hampered by technical limitations, as discussed in chapter 3, and no techniques are available yet for injections of single cells in spiders. A more indirect approach would be to test the expression of more genes and proteins that are for example known to be associated with early nervous system development, or on the other hand are recovered from silk glands in later stages and could be used as developmental markers.

Taken together the evidence presented in the literature and the data in this chapter, it is apparent that we should be careful in assigning the developmental origin of the silk glands to ectodermal invaginations, as some theories require (Craig, 1997; Palmer, 1990). Indeed, circumstantial evidence points towards such an origin, but solid evidence is currently still lacking and should be persued.

## 4.5 Methods

### 4.5.1 SEM imaging

See paragraph 2.5.2.

### 4.5.2 Whole mount in situ hybridization

See paragraph 5.5.2.

### 4.5.3 Antibody and phalloidin stainings

Embryos for phalloidin stainings or for combined antibody and phalloidin stainings were fixed in paraformaldehyde prepared according to the standard antibody protocol of the Patel lab (Patel, 2006) for 20 minutes. Next, embryos were washed for several hours with PBS-Triton X 0.3%, blocked with 5% normal goat serum for 1 hour. Subsequently, embryos were either incubated with a 1:500 dillution of FITC-conjugated phalloid (Sigma P5282) and incubated at 4°C o/n followed by imaging (paragraph 4.5.5), or for combined antibody and phalloidin stainings, incubated with a 1:200 dillution of rat-anti-Prospero antibody (raised against *C. salei* Prospero by Weller (2002) and kindly provide by A. Stollewerk) at 4°C o/n. After intensive washing Cy5-anti-Rat secondary antibody and FITC-conjugated phalloid were added, both at a 1:500 dillution and incubated at 4°C o/n followed by imaging (paragraph 4.5.5)

### 4.5.4 Sytox staining

Embryos were fixed according to standard fixation methods (Prpic *et al.*, 2008a), washed for 1 hour in 10mM Tris Buffered Saline at pH 7.4, incubated with 5$\mu$M SYTOX green (Molecular Probes S-7020) for 2 hrs, and washing thoroughly followed by imaging (paragraph 4.5.5).

### 4.5.5 Confocal imaging and 3D analysis

Embryos stained with fluorescent antibodies, FITC-phalloidin and SYTOX were prepared for confocal imaging by dissected the germ band from the yolk followed by embedding on a slide with Prolong Gold anti fading reagent (Invitrogen) and an incubation time of at least 24hrs. Scans were made on a Leica SP2 confocal microscope. Leica stacks were directly imported in Imaris 5.7.2 (Bitplane AG) for 3D analysis. Orthogonal and perspective projections were made in the Surpass View window.

# Chapter 5

# Genetic patterning of the spinneret primordia

## 5.1 Abstract

In this chapter a detailed gene expression analysis is presented in the primordia of the spinnerets and the opisthosomal breathing structures of *C. salei*. The investigated genes have in common that they previously have been associated with the development of *C. salei* prosomal appendages. These expression patterns are compared between the different opisthosomal structures and are discussed in relation to the prosomal appendage patterns. The goal was to define the axes of the spinnerets, to better understand the morphological development of the spinnerets and to ultimately investigate the evolutionary relationship of the spinnerets with the prosomal appendages and opisthosomal breathing organs.

It was found that the primordium of the spinnerets on opisthosomal segment 5 consists of two proximo-distal axes that are patterned in different ways. The dorsal portion of the primordium (the later posterior spinneret) is patterned by the expression of *Cs-exd-2*, *Cs-dac*, *Cs-hth-2* and *Cs-Dll*, the ventral portion (the later medial spinneret) by *Cs-hth-2* only. The patterning of the spinneret on opisthosomal segment 4 (the later anterior spinneret) shows many more similarities with the posterior spinneret than with the medial spinneret. The proximo-distal patterning of the anterior and posterior spinneret primordia show many similarities with the patterning of the prosomal appendages.

In contrast, the studied genes showed a very complex patterning of the dorsoventral axes of the spinnerets, that is not univocally comparable with the prosomal appendages. Nevertheless, the gene expression analysis of the ventral portions of the spinneret primordia provide the first experimental evidence of serial homology of the colulus and medial spinnerets. Moreover, based on *Cs-pby-2*

expression the theory is raised that the ventral portions of the spinnerets are serially homologous to the gnathobase of the prosomal appendages.

Finally it was concluded that, despite the high resolution of this gene expression analysis, most of the postulated hypotheses of common patterning because of common decent are rather speculative partially because so little is known about the function of these genes in the different studied organs. Future functional genetic studies and the discovery of new and organ-specific candidate genes are crucial to gain more insights in the evolutionary origin of the spinning apparatus.

## 5.2 Introduction

Numerous studies on the molecular base of arthropod appendage development have been published in recent years, and these have uncovered many conserved aspects of gene expression and gene function (e.g. Angelini & Kaufman (2005) and references therein). Currently, most knowledge is based on the fruitfly *Drosophila*, but studies of other arthropod groups, including crustaceans, chelicerates and myriapods (see figure 1.1), give a much broader perspective on appendage evolution. The comparison of gene expression in the prosomal appendages and other parts of the body of the spider *Cupiennius salei* with other animals has been the focus of a series of recent publications (reviewed in McGregor *et al.*, 2008a). In several of these publications, mention has been made of the expression in the spinneret primordia of genes that are thought to have a function in the development of the prosomal appendages. However, in most cases the expression patterns in the spinneret primordia have not been studied in detail, and have not been discussed in relation to the expression patterns of the other appendages.

An exception is a study on the expression of the Distal-less protein (Dll) (Popadíc *et al.*, 1998): "On embryological and phylogenetic grounds, it has long been believed that the spinnerets of spiders are serially homologous to the other appendages. We found that Dll is expressed in these structures, providing additional evidence for their appendicular origin, and suggesting that they incorporate the distal portions of the ancestral limb".

The hypothesis referred to by Popadíc *et al.* (1998) has indeed been forwarded much earlier based on comparative morphology, and spinnerets and other spider appendages are often said to be serially homologous, or spinnerets are said to be "modified appendages" (reviewed in Kovoor, 1977a; Shultz, 1987). For example, Whitehead & Rempel (1959) and Brown (1945) studied the musculature of spiders and both came up with proposals to homologize the muscles of the coxa of the walking legs with muscles of the spinnerets. However, this statement of serial homology is ambiguous because it is not clear *as what* the spinnerets and other appendages might be homologous. Serial homologous characters occur when the

underlying developmental program of a structure is duplicated and expressed in a new location (Hall, 1995), but in case of the spinnerets it is unclear which program of which ancestral structure duplicated.

One way to approach this problem is to look at fossils and present day outgroups, such as other arachnids (see figure 1.1), and to try to find structures that the spinnerets might be homologous to or derived from. But the opisthosoma of all other arachnids, including fossil groups like for example the trigonotarbids (Dunlop, 1995b), appear to be free of appendages (exceptions are the scorpions and Amblypygi, because they have sensory organs called pectines on their third (Selden, 1998) and gonopods used for manipulating gential products on their second (Weygoldt *et al.*, 1972) opisthosomal segments respectively).

However, the apparent lack of opisthosomal appendages of the arachnida might be deceiving, because opisthosomal appendages, in the form of book-gills, are known from aquatic chelicerates like the Xiphosura (figure 1.1). It is thought that the book lungs and trachaeae of arachnids derive from similar ancestral gills (Scholtz & Kamenz, 2006; Selden, 1998). The apendicular origin of these breathing structures in spiders is suggested for example by the resemblance of their developmental primordia with the limb buds of the prosomal appendages, as argued similarly for the spinnerets (see figure 2.1). So the breathing structures of spiders can also be considered of apendicular origin, even though they are not protruding appendages in the developed spider.

Nevertheless, and most importantly, even if one considers the breathing structures as modified appendages as well, the homology of opisthosmomal appendages of different arachnids is largely agreed upon (Scholtz & Kamenz, 2006), and the only arachnid appendages known from opisthosomal segments four and five are spider spinnerets and scorpion book lungs. And even though the higher order relationships of the arachnids are not well resolved (Shultz, 2007), the scorpions are not likely to be the closest relatives to spiders, and might even have derived from a separate terrestrialization event (Selden, 1998). In conclusion, general consensus is that the arachnid ancestors that stood at the cradle of the first spiders did not bear developed appendages or appendage-like structures on those opisthosomal segments where spinnerets evolved.

Puzzled by this problem, and inspired by the morphological similarities of the embryological primordia of the spider breathing structures and the spinnerets, Marples (1967) proposed that spinnerets are indeed derived from ancestral breathing structures, but that they did not evolve directly, but instead via a process of "paedomorphosis". Supposedly, ancestral gills on opisthosomal segments four and five of an arachnid ancestor got reduced to vestiges, and from these vestiges later the spinnerets evolved. This theory has never been investigated systematically, but interestingly, in a later study molecular similarities were found between the spinnerets, the spider breathing structures and the book gills of the present

day aquatic chelicerate *Limulus* (Damen *et al.*, 2002), giving support to the hypothesis of Marples (1967).

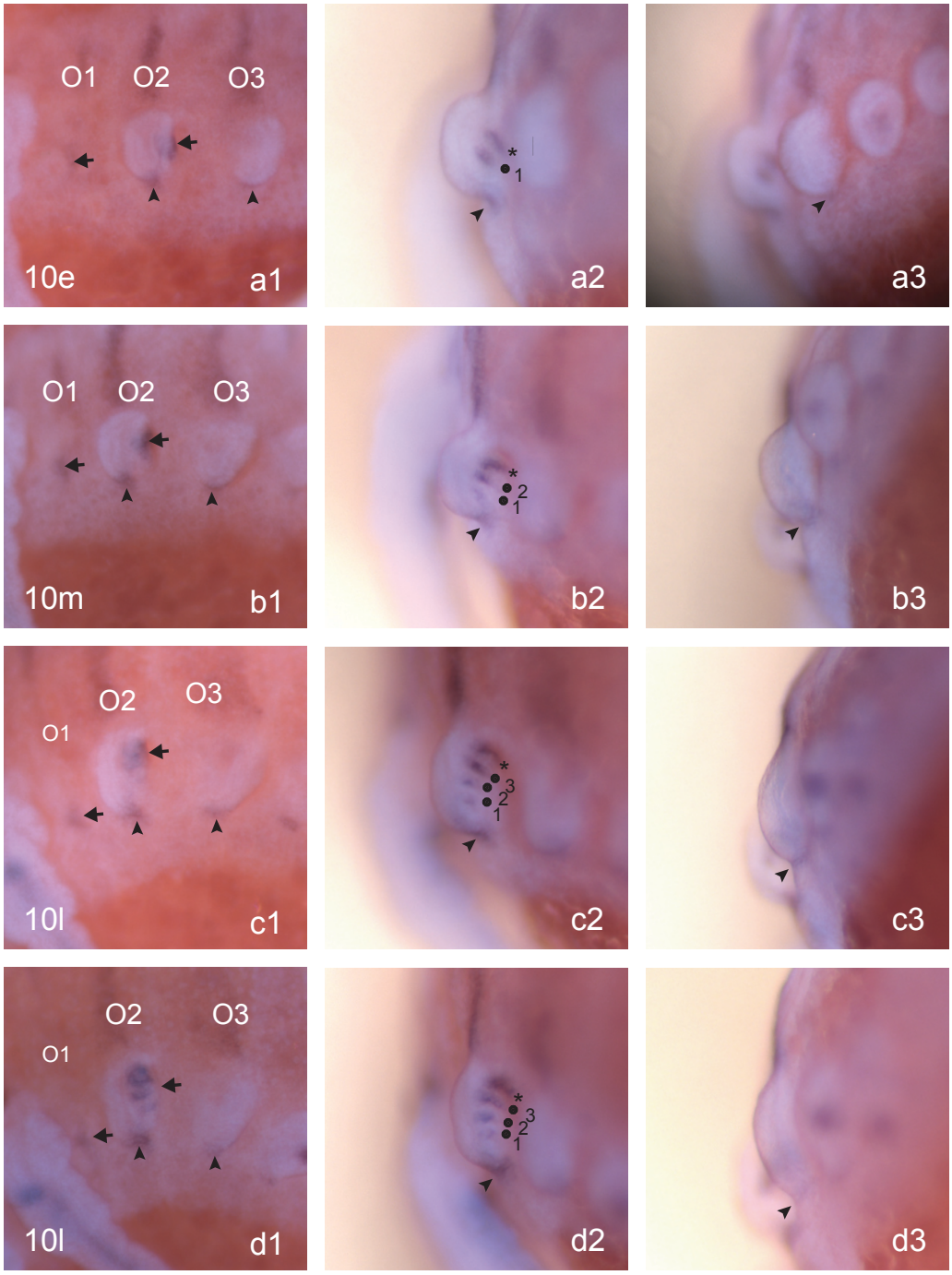
A problem with testing this theory, and with the comparison of present day organisms that diverged hundreds of millions of years ago in general, is that the evolution that took place might have left behind traces, but that subsequent evolutionary events could have also masked signals that can be used to homologize structures back to a particular level. For example, one of the reasons why the morphology of spinnerets is difficult to compare with other spider appendages is that the spinnerets consist of a medial and a lateral portion (i.e. the PSp and MSp in araneamorph spiders, see figure 1.2), whereas the prosomal appendages and the primordia of the breathing structures seem to have one proximo-distal axis only. This observation has led several authors in older publications to postulate that spinnerets derive from an ancestral biramous appendage, consisting of and exopodite and endopodite (reviewed in Kovoor, 1977a), but Marples (1967) and Shultz (1987) argued that this biramous morphology is probably derived, in concordance with the paedomorphosis theory.

Another problem with comparing the spinnerets with other appendages is that it is not clear if the spinnerets on opisthosomal segments four and five appeared separately, or that maybe one of them evolved first and its developmental program got duplicated on the other segment with subsequent modifications, as seen by the variation of spinnerets in present day spiders (figure 1.2). The latter possibility would suggest that the spinnerets on both segments share a commonly derived ground plan, whereas there is no *a priori* reason to assume developmental similarities between the different spinnerets if they evolved independently.

The great challenges to understand the origin of the spinnerets are thus to try to tease out the morphological and molecular traces that several subsequent big evolutionary events might have left behind, and to find out which aspects of the spinnerets can be related to their origin at the base of the spider clade, and which aspects are either plesiomorphic to all appendages or apomorphic of particular spinnerets. Because only if we know which modifications occurred in relation to which events we can for example start understanding which ecological circumstances might have driven these changes. A potential way to separate these traces is by making a detailed comparison of the shared characters of the developmental programs of the different appendages found in the spider, as demonstrated by the results of Damen *et al.* (2002) and Popadíc *et al.* (1998). In other words: what are the differences and similarities of the spinnerets, how do these similarities relate to the other opisthosomal limb buds and how do these similarities relate to the prosomal appendages?

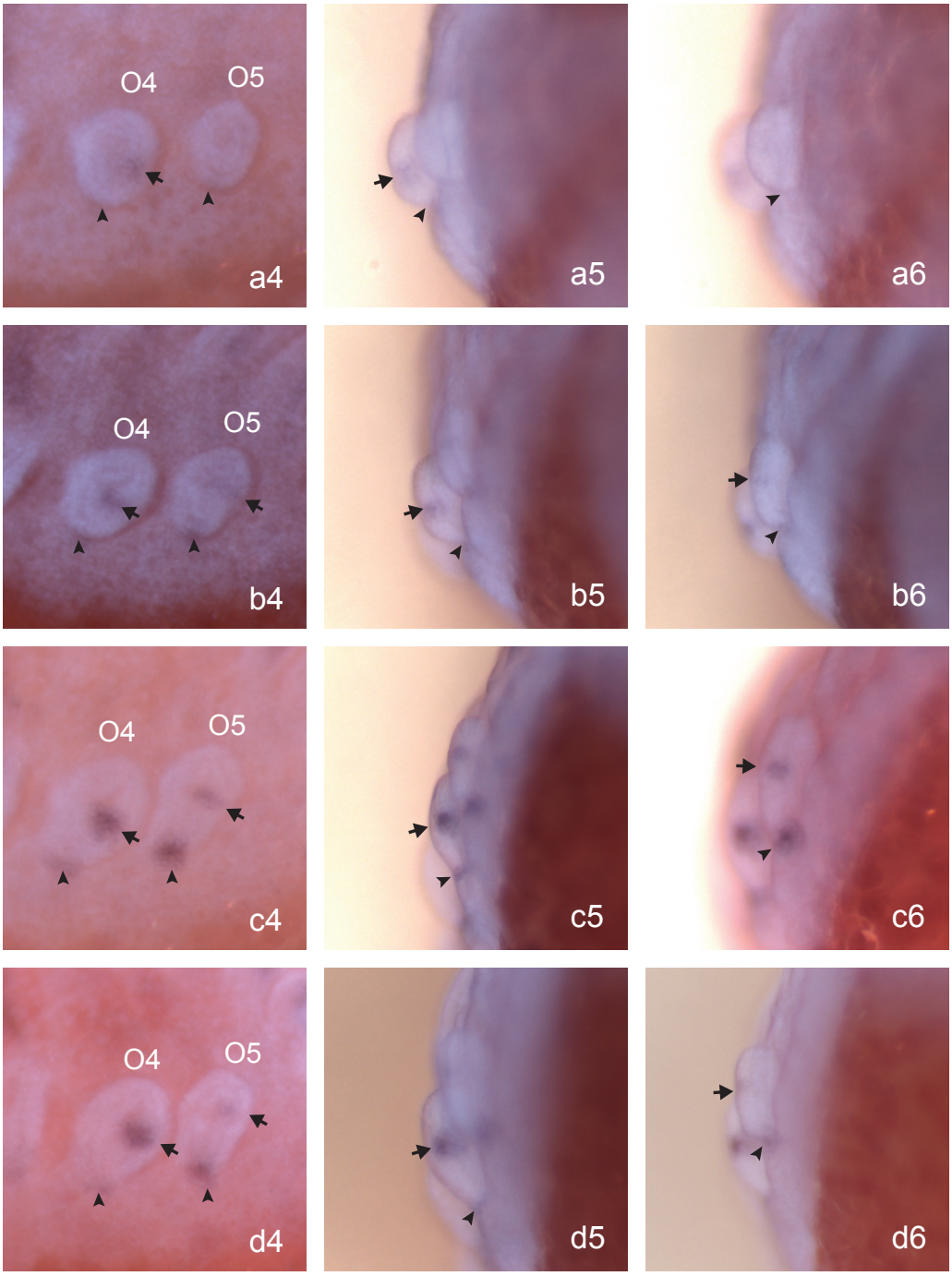
These questions imply a long research program to discover the factors that are involved in the development of the different appendages, which was beyond the scope of this project. However, an exploratory study was possible by looking

**Figure 5.1:** Expression of *Cs-wingless* in the opisthosomal limb buds. Limb buds of the left side of the embryo are shown; limb buds on O2 and O3 from stage 10e until stage 11e, buds on O4 and O5 from stage 10e until stage 12. Row **a**, Stage 10e; row **b**, stage 10m; row **c**, stage 10l; row **d**, stage 10l (slightly later); row **e**, stage 11e; row **f**, stage 11l; row **g**, stage 11l (just before ventral closure); row **h**, stage 12. Column **1**, limb bud region on O1-O3. O1 will degenerate and become part of the petiolus, the bud on O2 is the primordium of a book lung and the bud on O3 is the primordium of a tubular trachaea; column **2**, posterior view of the limb bud on O2; column **3**, posterior view of the limb bud on O3; column **4**, limb buds on O4-O5; column **5**, posterior view of the limb bud on O4; column **6**, posterior view of the limb bud on O5. **O1-O5**, opisthosomal segments 1 to 5. Dashed line in **h4** demarcates the ventral midline. Images are of the left body half and overlays of normal light and a fluorescent nuclear marker (dapi). See text for further description.



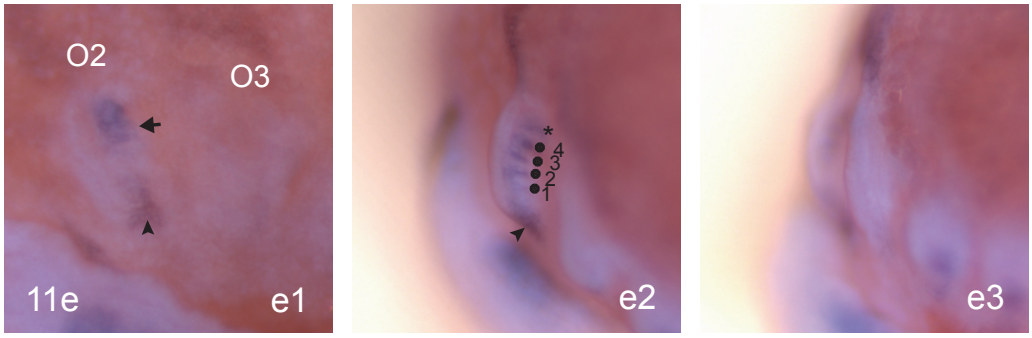

**(I)**

**Figure 5.1:** Expression of *Cs-wingless* in the opisthosomal limb buds. Legend on page 87.



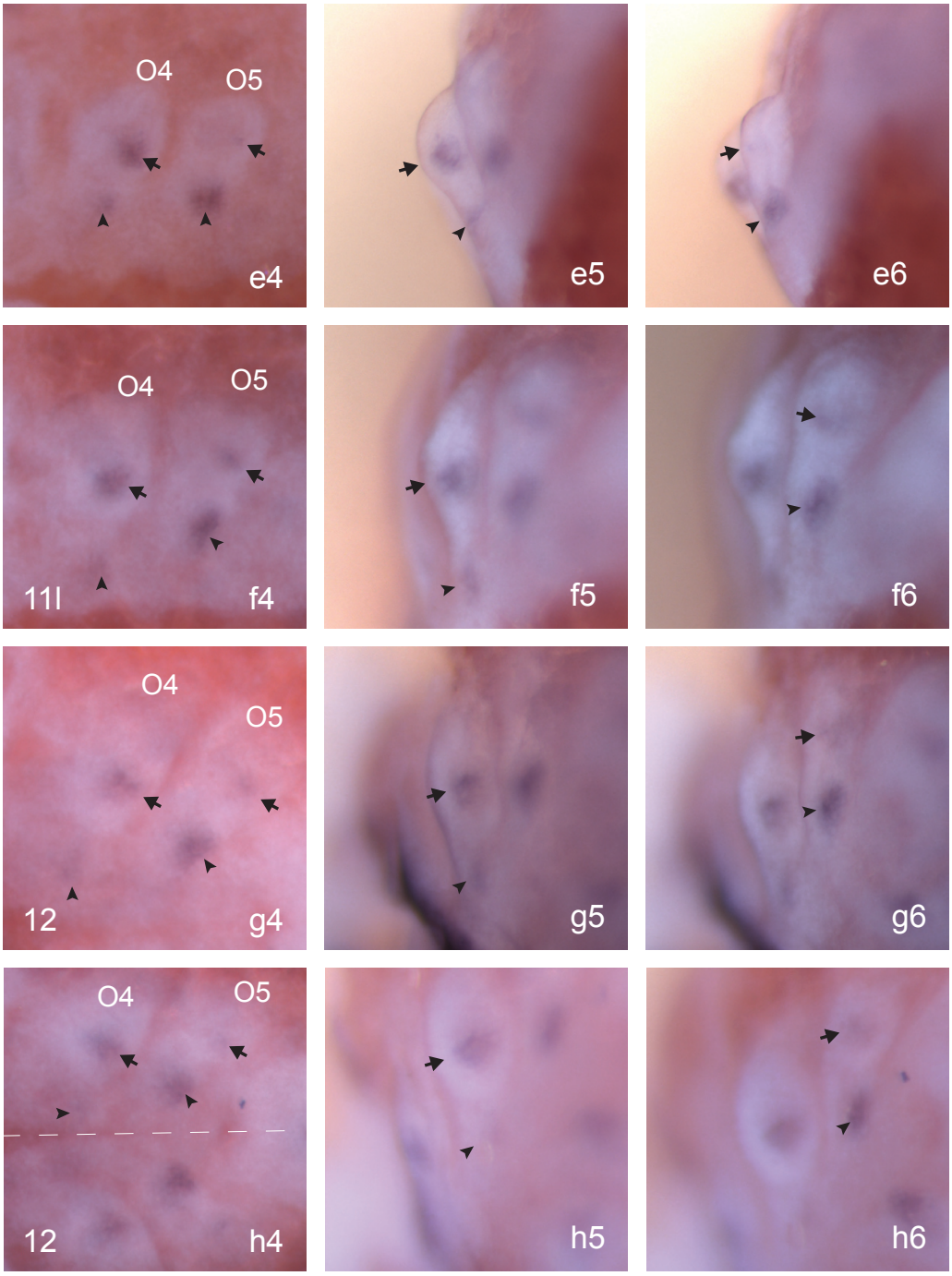

**(II)**

**Figure 5.1:** Expression of *Cs-wingless* in the opisthosomal limb buds (continued). Legend on page 87.




**(III)**

**Figure 5.1:** Expression of *Cs-wingless* in the opisthosomal limb buds (continued). Legend on page 87.




**(IV)**

**Figure 5.1:** Expression of *Cs-wingless* in the opisthosomal limb buds (continued). Legend on page 87.

in detail at the expression in the spinneret limb buds of several genes that were previously only casually reported to be expressed in the spinnerets, including: *Cs-wingless* (*Cs-wg*), *Cs-extradenticle-1* (*Cs-exd-1*), *Cs-extradenticle-2* (*Cs-exd-2*), *Cs-dachshund* (*Cs-dac*), *Cs-homothorax-1* (*Cs-hth-1*), *Cs-homothorax-2* (*Cs-hth-2*), *Cs-Distal-less* (*Cs-Dll*), *Cs-pairberry-2* (*Cs-pby-2*), *Cs-optomotor blind-2* (*Cs-omb-2*), *Cs-H15-2* and *Cs-Activator Protein-2* (*Cs-AP-2*).

In this study, using the morphology of the spinneret limb buds and the expression patterns of some of these genes, the axes of the spinnerets are defined to allow a comparison with the other appendages. Since these genes have been associated with particular functions in the prosomal appendages, there possible function in patterning the spinneret limb buds is discussed. Moreover, it is discussed to what extend the expression of these genes can be compared between the two spinneret limb buds, and between the spinneret limb buds and other appendage primordia. Also, a comparison is made between these expression patterns and morphological features on the surface of the spinneret limb buds, such as the invaginations discussed in chapter 4.

## 5.3 Results

### 5.3.1 Expression of *Cs-wingless*

A relatively small number of signaling pathways is involved in most of animal development (Pires-daSilva & Sommer, 2003). One of them is the wingless related (Wnt) pathway, and Wnt genes and other components of this pathway have been found trough-out the animal kingdom (Prud'homme *et al.*, 2002). The best studied arthropod Wnt gene is *wingless* (*wg*), the *Drosophila* homolog of the vertebrate gene *Wnt1*. *wg* codes for a secreted signaling molecule that has a role in many developmental processes, including segmentation, the diversification of cell types in the embryonic epidermis, neuroblast differentiation and control of cellular proliferation during the formation of the midgut (Siegfried & Perrimon, 1994). A further role in *Drosophila* is the function of *wg* during early patterning of the limbs. Gradients of Wingless together with another signaling molecule Decapentaplegic lie at the base of a gene cascade that pattern the proximo-distal axis of the fly appendages, similar to the segmentation cascade that organizes the germ band of the embryo (e.g. Angelini & Kaufman (2005); Estella *et al.* (2008) and references therein).

Several Wnt homologs have also been discovered in the spiders *C. salei* and *A. tepidariorum* (Damen, 2002; McGregor *et al.*, 2008b). Similar to *D. melanogaster*, *C. salei wg* (*Cs-wg*) is expressed in a parasegmental fashion during embryogenesis, which suggests a conserved role in segmentation. In inversion stage embryos,

expression was also observed on the anterior-ventral portion of the prosomal appendages (Damen, 2002), and it was proposed that *Cs-wg* also has a key role in the early patterning of the proximo-distal axis of spider appendages (Prpic, 2004; Prpic *et al.*, 2003). Expression of *C. salei* in the opisthosoma has been described just posterior to the opisthosomal limb buds of O2, O4 and O5, and as a stripe dorsally and a spot ventrally to each of the opisthosomal limb buds, but possible functions of the gene in these expression domains were not discussed (Damen, 2002).

A closer inspection of the expression of *Cs-wg* in the opisthosomal limb buds during inversion reveals complex patterns (figure 5.1). To facilitate the description of expression patterns, in the remaining text the limb buds of opisthosomal segments 2 till 5 for are abbreviated with bO2 till bO5. Spots of expression appear at stage 10e, which is the start of inversion, ventrally at the base of each of the buds (arrow heads in figures 5.1 a1-a6). However, the manner that these expression domains develop differs for each of them. The domain at the ventral base of bO2 increases in size and becomes wedge-shaped at stage 11e (left arrow head in figures 5.1 a1-e1 and arrow head in a2-e2). The domain ventral to bO3 never becomes very pronounced and disappears after dorsal closure at stage 11e (right arrow head in figures 5.1 a1-e1 and arrow head in a3-e3). The domain ventral to bO4 does not become very pronounced either, but persists after dorsal closure. Interestingly, close before ventral closure this domain migrates more ventrally until it almost reaches the ventral midline at stage 12, adjacent to the corresponding expression domain of the other body half (left arrow head in figures 5.1a4-h4 and arrow head in a5-h5). The domain at the base of bO5 is very similar to the corresponding domain on O4 during stages 10e and 10m (arrow heads in figures 5.1 a4-a6 and b4-b6), but at stage 10l becomes much more intense (arrow heads in figures 5.1 c4-c6 ), which correlates with to the appearance of a depression of the surface of the limb bud and also with the appearance of the invagination O5-i at this stage, as described in chapter 4.

The expression of *Cs-wg* posterior to bO2 was described as to "... represent the lamellae of the book lung" (Damen, 2002), but in fact *Cs-wg* is expressed in the invaginations in between the lamellae, and marks the pulmonary sac and pulmonary furrows (asterisk and filled circle respectively in figure 5.1 a2). The dorsal-most invagination is the pulmonary sac and the ventral invaginations are the furrows (Purcell, 1909). Over the course of inversion, new furrows appear in between the sac and the already existing furrows. The furrows are numbered in order of appearance in figures 5.1 a2-e2. Since the expression is stronger in the more dorsal furrows in comparison to the more ventral furrows it is apparent that expression in the furrows weakens during differentiation of the furrows. *Cs-wg* is not expressed posterior to bO3, and therefore does not mark the position where the tubular trachaea originate (figures 5.1 a3-e3 and figure 2.2 b).

**Figure 5.2:** Expression of *Cs-hth-1* and *Cs-hth-2* in the opisthosomal limb buds. **a-c**, images are overlays of normal light and a fluorescent nuclear marker (dapi). **a**, expression of *Cs-hth-1* in the limb buds of O2 to O5 at stage 10e (ventral view); **b**, expression of *Cs-hth-2* in the limb buds of O2 to O5 at stage 10l; **c**, expression of *Cs-hth-2* in the limb buds of O3 to O5 at stage 10l; **d-f**, expression of *Cs-hth-2* in the limb buds of O4 and O5 at stages 11m to 12, (normal light); **d'-f'**, staining of **d-f** with a fluorescent nuclear marker (dapi); **d"-f"**, overlay of **d-f** and **d'-f'**; **L3, L4**, walking legs 3 and 4; **O2-O5**, opisthosomal segments 1 to 5. Images are of the left body half unless otherwise stated. See text for further description.

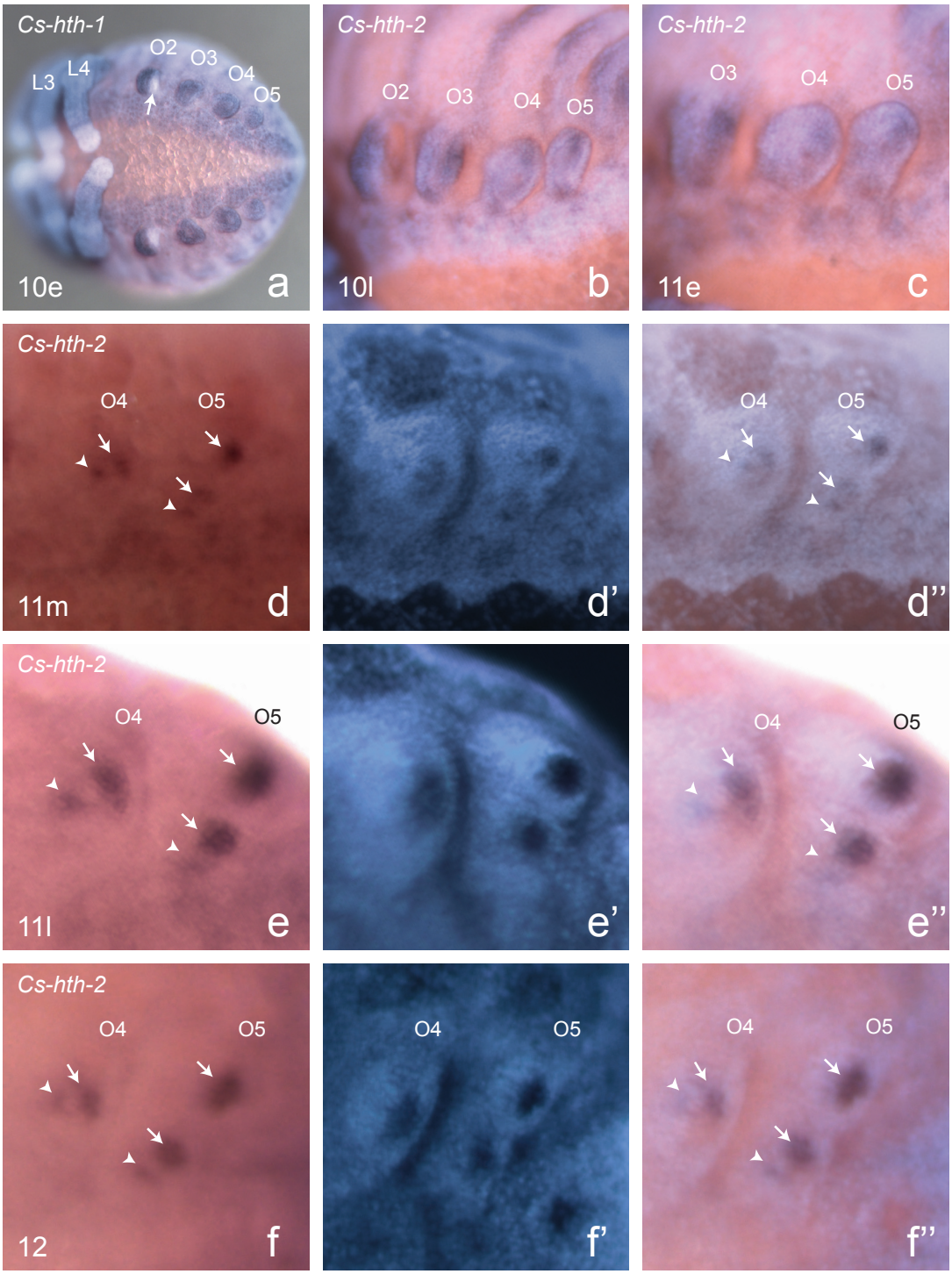



**Figure 5.2:** Expression of *Cs-hth-1* and *Cs-hth-2* in the opisthosomal limb buds. Legend on page 94.

Expression of *Cs-wg* on the dorsal portion of the spinneret limb buds starts at stage 10e with a broad domain on bO4 (arrows in figures 5.1 a4 and a5). This domain correlates with a depression of the surface of the epithelium (arrow in figure 4.2 b). During inversion this expression domain increases in intensity until ventral closure at stage 12 (figures 5.1 b4-h4, b5-h5 and figure 4.2). The dorsal expression domain on bO5 is weak at stage 10e, increases in intensity at stage 10l and then weakens again (right arrow in figures 5.1 a4-h4 and a6-h6). This domain also correlates with a depression of the surface of the bud (upper arrow heads in figures 4.2 b and d).

## 5.3.2 Expression of the leg gap genes

Homologs of *homothorax* (*hth*), *extradenticle* (*exd*), *dachshund* (*dac*) and *Distal-less* (*Dll*) are required for the proper formation of whole regions of the appendages of many insects and other arthropods Angelini & Kaufman (2005); Sewell *et al.* (2008); Tanaka & Truman (2007), and have been called leg gap genes (Rauskolb, 2001). The expression in the legs of *C. salei* homologs of these leg gap genes has been described in detail previously, and it has been proposed that they have a function in patterning spider legs too (Prpic *et al.*, 2003).

### 5.3.2.1 *Cs-homothorax-1 and 2*

Two homologs of *hth* have been discovered in *C. salei*, with remarkably different expression patterns. *Cs-hth-1* is expressed throughout the appendage save the distal tip, whereas *Cs-hth-2* is expressed in several rings of expression (Prpic *et al.*, 2003). Investigation of the expression of both homologs in the opisthosomal limb buds also shows obvious differences. From the first appearance of these buds until the end of inversion *Cs-hth-1* is uniformly expressed on all four of these (figure 5.2 a and data not shown), except from the posterior part of bO3, where the furrows of the book lungs invaginate (arrow in figure 5.2 a).

Until dorsal closure at stage 11, *Cs-hth-2* is similarly uniformly expressed in the opisthosomal limbs (figure 5.2 b and c). After this stage, the uniform expression of *Cs-hth-2* makes place for several distinct expression domains in the limb buds of O4 and O5. On the surface of bO4, a broad spot of expression appears (left-most arrows in figures 5.2 d-f and d"-f"), which corresponds to a protrusion of the surface of the bud (closed circle in figures 4.2 b and d). Slightly ventral on the bud a smaller second spot of expression appears deeper inside the tissue (left-most arrow heads in figures 5.2 d-f and d"-f"). Interestingly, a very similar set of expression domains, consisting of a domain at the surface and a smaller domain inside the tissue, is also visible on the ventral portion of bO5, corresponding to the region that will become the MSp (lower arrows and lower

arrow-heads respectively in figures 5.2 d-f and d"-f"). On the surface of the region that corresponds to the later PSp, a strong expression domain appears as well, but this domain is not accompanied by a second domain deeper inside the tissue (right-most arrow in figures 5.2 d-f and d"-f").

#### 5.3.2.2 *Cs-extradenticle-1 and 2*

The first description of the expression of the EXD protein in the developing appendages of a spider was done by means of an antibody, raised against *Drosophila* EXD, in the cobweb spider *Steatoda triangulosa* (Abzhanov & Kaufman, 2000). Later efforts to clone the *C. salei* homologous gene with degenerate primers revealed that at least two homologs are transcribed in *C. salei* (Prpic *et al.*, 2003). *Cs-exd-1* is expressed in the proximal part of the walking legs and decreases in intensity towards the more distal parts. At stage 10e, a faint medial ring appears as well, which increases in intensity. The second homolog *Cs-exd-2* is expressed in two rings around the middle of the leg, plus a domain ventrally on the coxa (Prpic *et al.*, 2003). As far as a comparison is possible based on the published data, the findings of (Abzhanov & Kaufman, 2000) and (Prpic *et al.*, 2003) are compatible.

In the opisthosomal limb buds, *Cs-exd-1* is ubiquitously expressed, from the start of inversion until ventral closure (figure 5.3 a, b and c). At stage 10e, the second homolog *Cs-exd-2* is expressed uniformly in the tissue surrounding the opisthosomal limb buds including a ring at the base of the buds, but the remainder of the buds stay free of expression (figure 5.3 d). The expression of *Cs-exd-2* directly surrounding bO4 persists over the course of inversion. This is also true for the expression around bO5, with the exception of the ventral-most part of the bud. As soon as the latter bud starts to elongate around stage 10m the expression of *Cs-exd-2* ventral to it disappears (arrow in figures 5.3 e' and f). Interestingly, *Cs-exd-2* is also expressed around groups of cells on segments O6 and O7 that appear to be vestigial limb buds on these segments (figure 5.3 e').

#### 5.3.2.3 *Cs-dachshund*

Genes homologous to *Drosophila Dachshund* are involved in the development of the appendages, eyes and different parts of the nervous system in a variety of animals, including arthropods (Angelini & Kaufman, 2005; Davis *et al.*, 1999; Loosli *et al.*, 2002; Mardon *et al.*, 1994; Sewell *et al.*, 2008). The first description of the expression of a spider *dac* homolog was made in the spider *Steatoda triangulosa*. In this spider the gene is highly expressed in a domain in the middle of the walking legs. In the opisthosomal limb buds expression was seen in the "lateral portion" of bO4, but not in bO5 (Abzhanov & Kaufman, 2000). Cloning

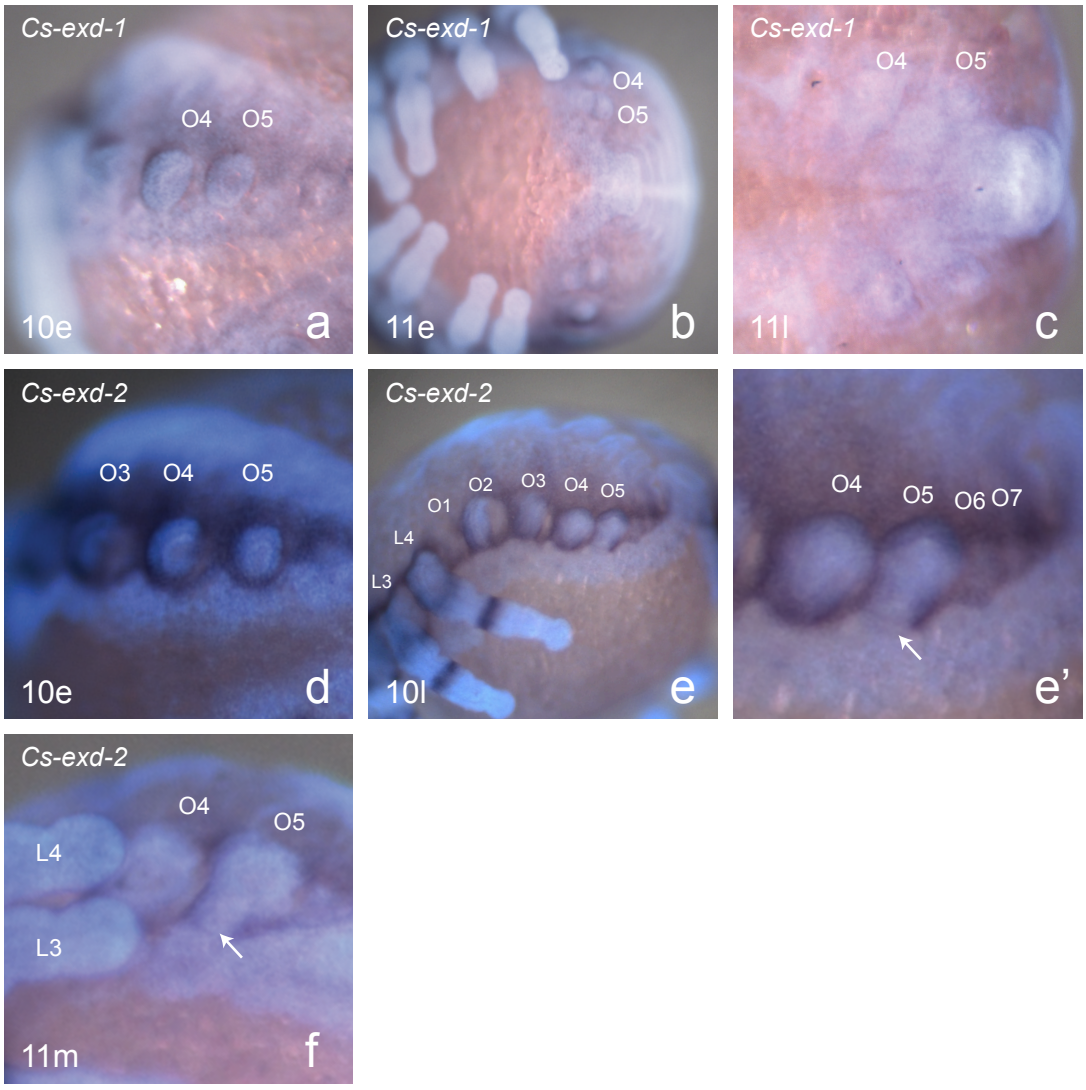



**Figure 5.3:** Expression of *Cs-exd-1* and *Cs-exd-2* in the opisthosomal limb buds. **a**, expression of *Cs-exd-1* in the limb buds of O4 and O5 at stage 10e; **b**, expression of *Cs-exd-1* at stage 11e (ventral view); **c**, expression of *Cs-exd-1* in the spinning field at stage 11e (ventral view); **d**, expression of *Cs-exd-2* in the limb buds of O3-O5 at stage 10e; **e**, expression of *Cs-exd-2* in the limb buds of O2-O5 at stage 10l; **e'**, detail of **e** showing the limb buds of O4 and O5 plus presumed vestiges of limb buds on O6 and O7: **f**, expression of *Cs-exd-2* in the limb buds of O4 and O5 at stage 11m (ventral view); **L3, L4**, walking legs 3 and 4; **O1-O7**, opisthosomal segments 1 to 7. Images are overlays of normal light and a fluorescent nuclear marker (dapi). See text for further description.

efforts in *C. salei* revealed one *dac* homolog, *Cs-dac*. *Cs-dac* is not expressed in the early prosomal limb buds, but appears first when these appendages have extended a bit. As in *Steatoda triangulosa*, *Cs-dac* is expressed in a broad domain medially on the proximo-distal axis of the walking legs. Later during inversion, weaker expression domains emerge in the dorsal part of the coxa of the legs. Further expression domains were described in a "very late stage" in a few cells at the tips of the walking legs, and might be involved in the development of peripheral nervous system. To my knowledge, expression of *Cs-dac* in the opisthosomal limb buds has not been described before.

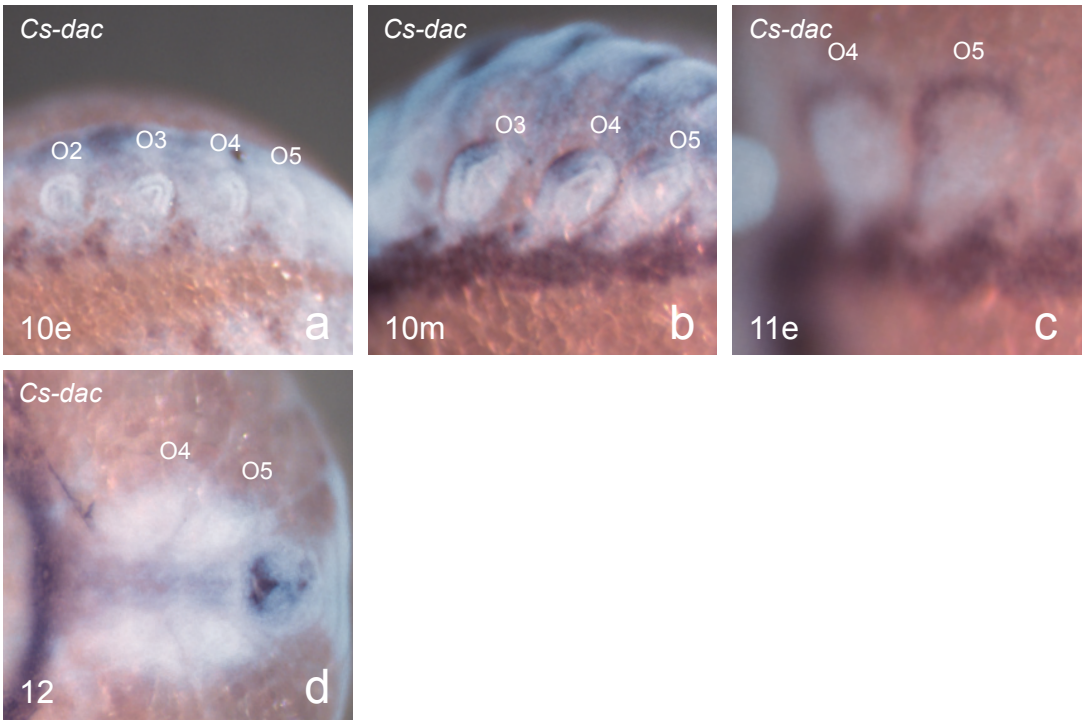



**Figure 5.4:** Expression of *Cs-dac* in the opisthosomal limb buds. **a**, the limb buds of O2 to O5 at stage 10e; **b**, the limb buds of O3 to O5 at stage 10m; **c**, the limb buds of O4 and O5 at stage 11e; **d**, the spinning field at stage 12 (ventral view); **O2-O5**, opisthosomal segments 2 to 5. Images are of the left body half unless otherwise stated and overlays of normal light and a fluorescent nuclear marker (dapi).

Investigation of the expression of *Cs-dac* in the opisthosomal limb buds shows no expression at stage 10e (figure 5.4 a). At stage 10m expression appears dorsally to the opitshosomal limb buds (figure 5.4 b). These domains are initial rather

fussy, but get more pronounced after dorsal closure in a sickle shaped domain dorsally on both of the spinneret limb buds, which is different from what Abzhanov & Kaufman (2000) observed (figure 5.4 c). At stage 12 *Cs-dac* expression in the spinnerets is absent (figure 5.4 d).

#### 5.3.2.4 *Cs-Distal-less*

*Distal-less* encodes a transcription factor that is involved in the development of distal limb structures and other forms of body outgrowths throughout the Metazoa (Angelini & Kaufman, 2005; Cohen, 1990; Panganiban, 2000; Panganiban *et al.*, 1997). The expression of *Distal-less* in a spider was first described by Popadíc *et al.* (1998), who stained an *Achaearanea tepidariorum* embryo with an antibody against the DLL homeodomain (Panganiban *et al.*, 1995).

The expression of the mRNA of the *C. salei* homolog *Cs-Dll* was described in general terms in Schoppmeier & Damen (2001) and was found to be very similar. In the prosoma of both species, expression was seen in the labrum, in the head region and in the distal portion of the appendages. In the opisthosoma, there is expression in the posterior-most end of the embryo, as well as in the spinneret limb buds. An interesting difference between both studies is that no gene expression was observed in the other opisthosomal limb buds of *C. salei*, whereas antibody staining was described in bO2 and bO4 of *A. tepidariorum*, primarily in the operculum of the developing book lungs on O2 (Panganiban *et al.*, 1995).

**Figure 5.5:** Expression of *Cs-Dll* in the opisthosomal limb buds. **a-e**, limb buds of O2-O5 (stages 9-10l); **f**, limb buds of O4 and O5 (stages 11e); **f'**, posterior view of the bud on O4 of the embryo shown in **f**; **f"**, posterior view of the bud on O5 of the embryo shown in **f**; **g-i**, limb buds of O4 and O5; **i'**, limb buds O4 and O5 of the embryo shown in **i** (view from anterior); **O2-O5**, opisthosomal segments 2 to 5. Images are of the left body half and overlays of normal light and a fluorescent nuclear marker (dapi). See text for further description.

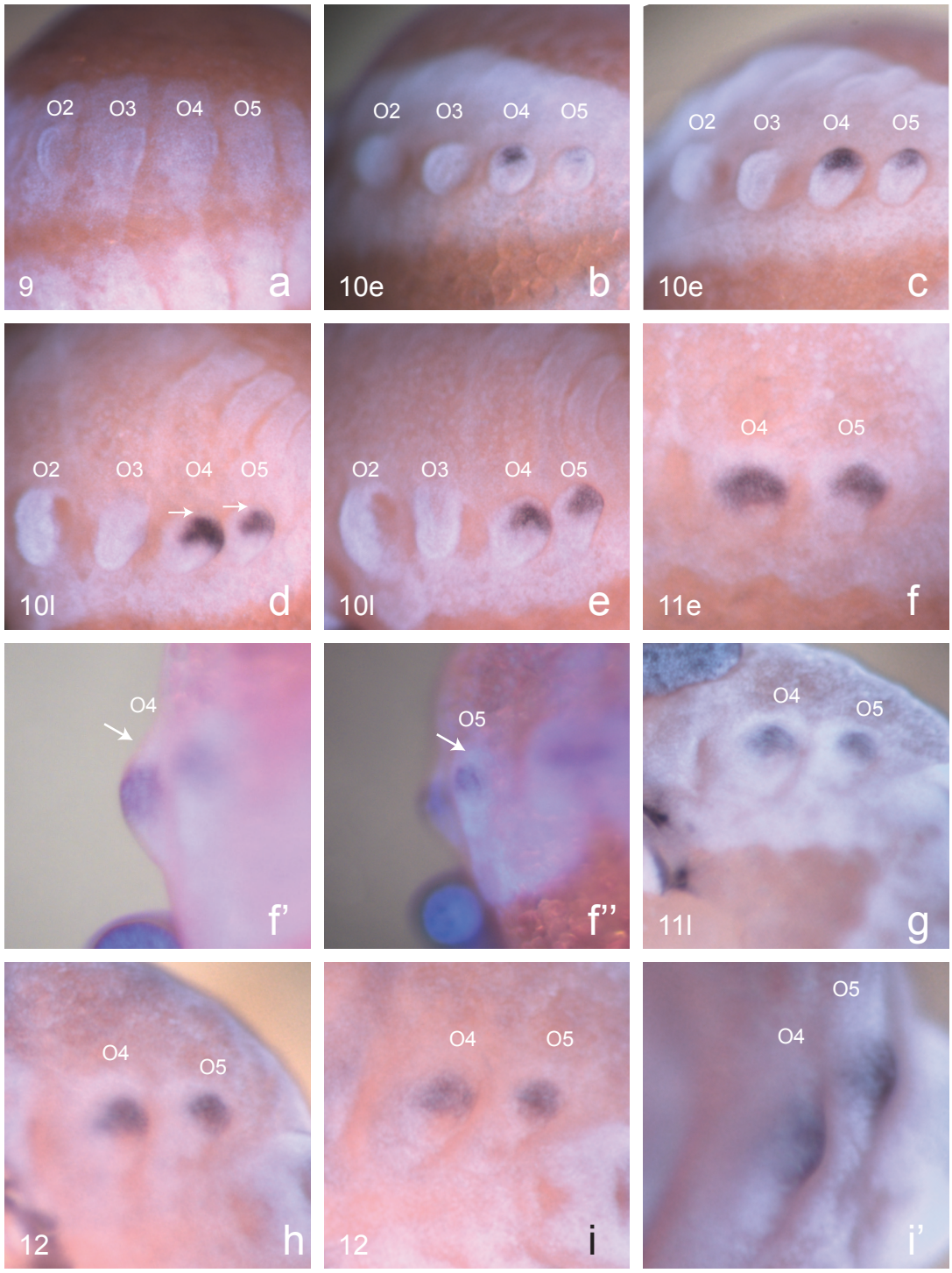



**Figure 5.5:** Expression of *Cs-Dll* in the opisthosomal limb buds. Legend on page 100.

A more detailed investigation of the expression of *Cs-Dll* on the opisthosomal limbs of *C. salei* shows that it first appears on bO4, shortly after the start of inversion (figure 5.5 a and b), directly followed by expression on bO5 (figure 5.5 c). Expression at stage 10e covers about a third of both limb buds and extends until the dorsal edge of the buds.

During the dorso-ventral elongation of bO5 the expression of *Cs-Dll* moves dorsally but does not change in size or intensity (figure 5.5 d-f). From this data it is not clear whether the *Cs-Dll* expressing cells are migrating or whether the bud proliferates at its dorsal part and *Cs-Dll* is expressed dyamically. However, whereas *Cs-Dll* at stage 10e was expressed in the dorsal most cells of both of these limb buds, at stage 11e (figure 5.5 f) tissue appears clear of expression dorsally to the *Cs-Dll* domains, plainly seen from a posterior point of view (arrows in figure 5.5 f'-f"). During ventral closure both spinneret limb buds flatten, but*Cs-Dll* expression remains strong in the most protruding parts of both buds, possibly corresponding to the distal portions of the later ASp and PSp (figure 5.5 g-i'). No expression is observed in what will become the MSp, and corroborating earlier observations (Schoppmeier & Damen, 2001), no expression was seen in the buds of O2 and O3 during the whole period of inversion.

### 5.3.3 Expression of *Cs-omb-2*

In *Drosophila*, *omb* has been shown to have a role in the development of dorsal tissue of the leg primordia (Brook & Cohen, 1996). Homologs of *omb* are known from several spider species, including *C. salei*, and it seems that the expression restricted to the dorsal parts of prosomal appendages in these species is highly conserved, suggesting a dorsalizing role in spiders as well. In *C. salei* two homologs have been found, and *Cs-omb-2* in particular shows strong dorsal expression over the course of appendage development. Moreover, dorsal expression of *Cs-omb-2* in the opisthosomal limb buds has also been demonstrated (Janssen *et al.*, 2008).

The expression of *Cs-omb-2* in the spinneret limb buds was further investigated in this study and results are shown in figure 5.6. Indeed the gene is expressed strongly at the start of inversion (stage 10e, figure 5.6) in the dorsal part of the spinneret limb buds. The ventral border of expression is not sharply demarcated and there appears to be a gradient of expression from dorsal to ventral. However, the ventral-most thirds of the spinneret limb buds are free of *Cs-omb-2* expression. This pattern does not change significantly during inversion (figure 5.6 b-e).

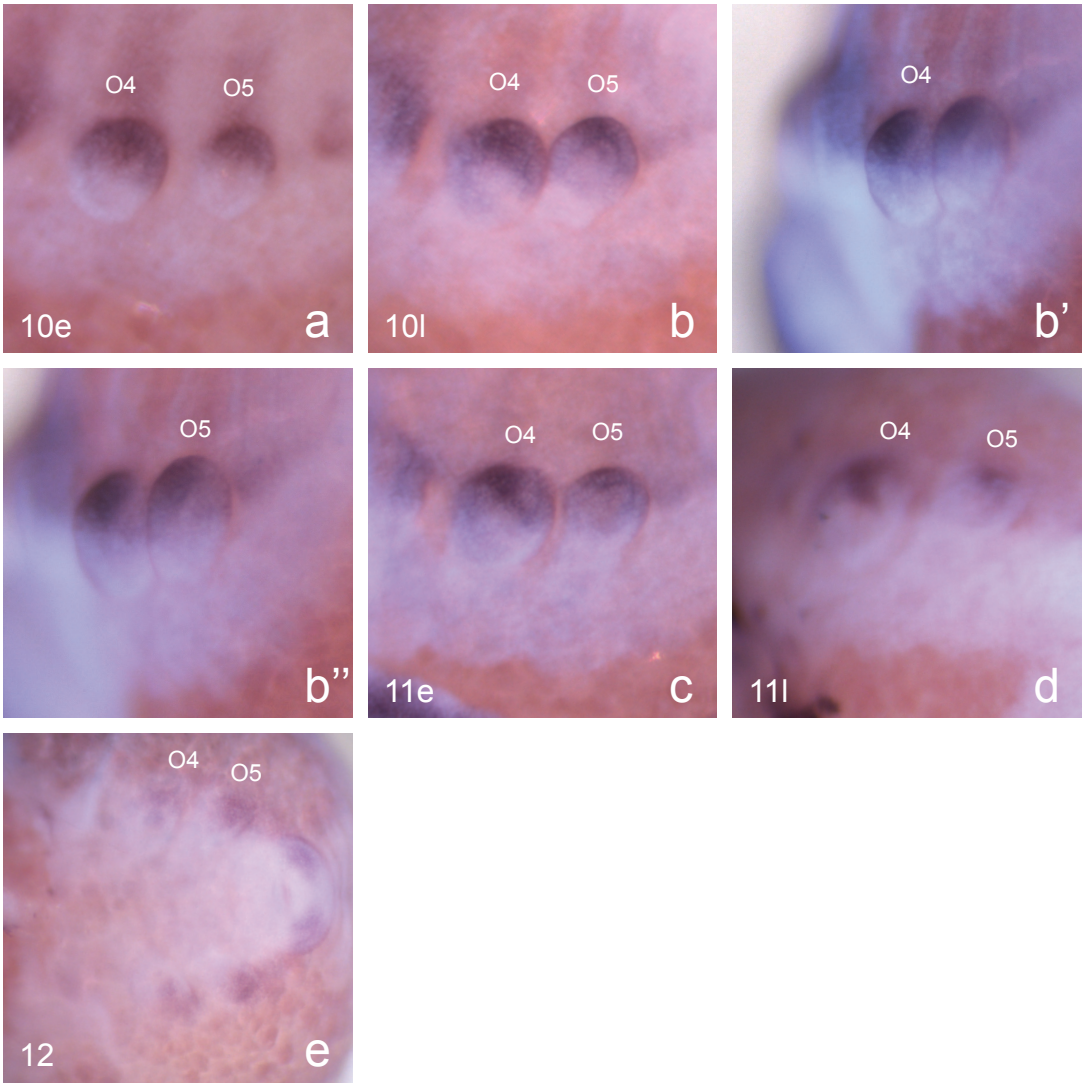



**Figure 5.6:** Expression of *Cs-omb-2* in the spinneret limb buds. **a-d**, limb buds of O4 and O5 stages 10e-11l; **e**, spinning field at stage 12 (ventral view); **O4,O5**, opisthosomal segments 4 and 5. Images are of the left body half unless otherwise stated and overlays of normal light and a fluorescent nuclear marker (dapi). See text for further description.

### 5.3.4 Expression of *Cs-H15-2*

The gene *H-15* is activated by *wg* and marks ventral tissue in the legs of *Drosophila* (Estella & Mann, 2008), which suggests a ventralizing function of the gene. In *C. salei*, two homologs of this gene have been found, *H15-1* and *H15-2*, and the presence of a third homolog is predicted from studies in other spider species (Janssen *et al.*, 2008). It has been suggested that *H15-1* and *H15-2* are involved in the development of ventral appendage tissue in *C. salei*, but also in formation of the heart, the opisthosomal neuroectoderm and nervous tissue in the tips of the walking legs (Janssen & Damen, 2008; Janssen *et al.*, 2008; Prpic *et al.*, 2003). Expression of *H15-2* was also observed in the primordia of the spinnerets (Janssen *et al.*, 2008).

The segmentally iterated expression pattern of *H15-2* in the neuroectoderm is visible before inversion starts and disappears at stage 11l. Small groups of cells are stained at the anterior part of the base of the opisthosomal limb buds (arrow heads in figure 5.7 a-g). It is not clear what these cells are, but they seem to correlate with patches of cells that are also stained by antibody DP311 raised against *Drosophila* pgIII genes (Davis *et al.*, 2005). At stage 10e, expression appears in the center of bO4 (double arrow head in figure 5.7 b and c). At stage 10l, when bO5 has started to elongate, two expression domains appear on bO5 (arrows in figure 5.7 e). Of these two domain, the more ventral one is stronger and similar in appearance to the domain on bO4 (lower arrow and double arrow head in figures 5.7 f-i). Towards stage 12 the dorsal domain on bO5 fades away (upper arrow in figure 5.7i).

### 5.3.5 Expression of *Cs-pairberry-2*

The Pax group III class (pgIII) of genes include the *Drosophila* genes *paired* and *gooseberry* (*gsb*), as well as the vertebrate genes *Pax 3* and *Pax7*. Homologs of this gene class are involved in segmentation and in the development of the central nervous system in all extant arthropod classes (Davis *et al.*, 2005). Three pgIII homologs have been found in *C. salei*; *Cs-pby-1*, *Cs-pby-2* and *Cs-pby-3*, and their joint expression resembles the expression pattern of the the pgIII genes in *Drosophila* (Schoppmeier & Damen, 2005). *Cs-pby-2* is of special interest here, because apart from being expressed in a segmentally iterated pattern in the ventral nervous system, the gene is also strongly expressed ventrally and dorsally at the base of the prosomal appendages, unlike *Cs-pby-1* and *Cs-pby-3*. Moreover, expression of *Cs-pby-2* in the opisthosomal limb buds and prosomal legs was previously reported, but not described in detail (Schoppmeier & Damen, 2005).

A closer inspection of the expression of *Cs-pby-2* in the opisthosomal limb

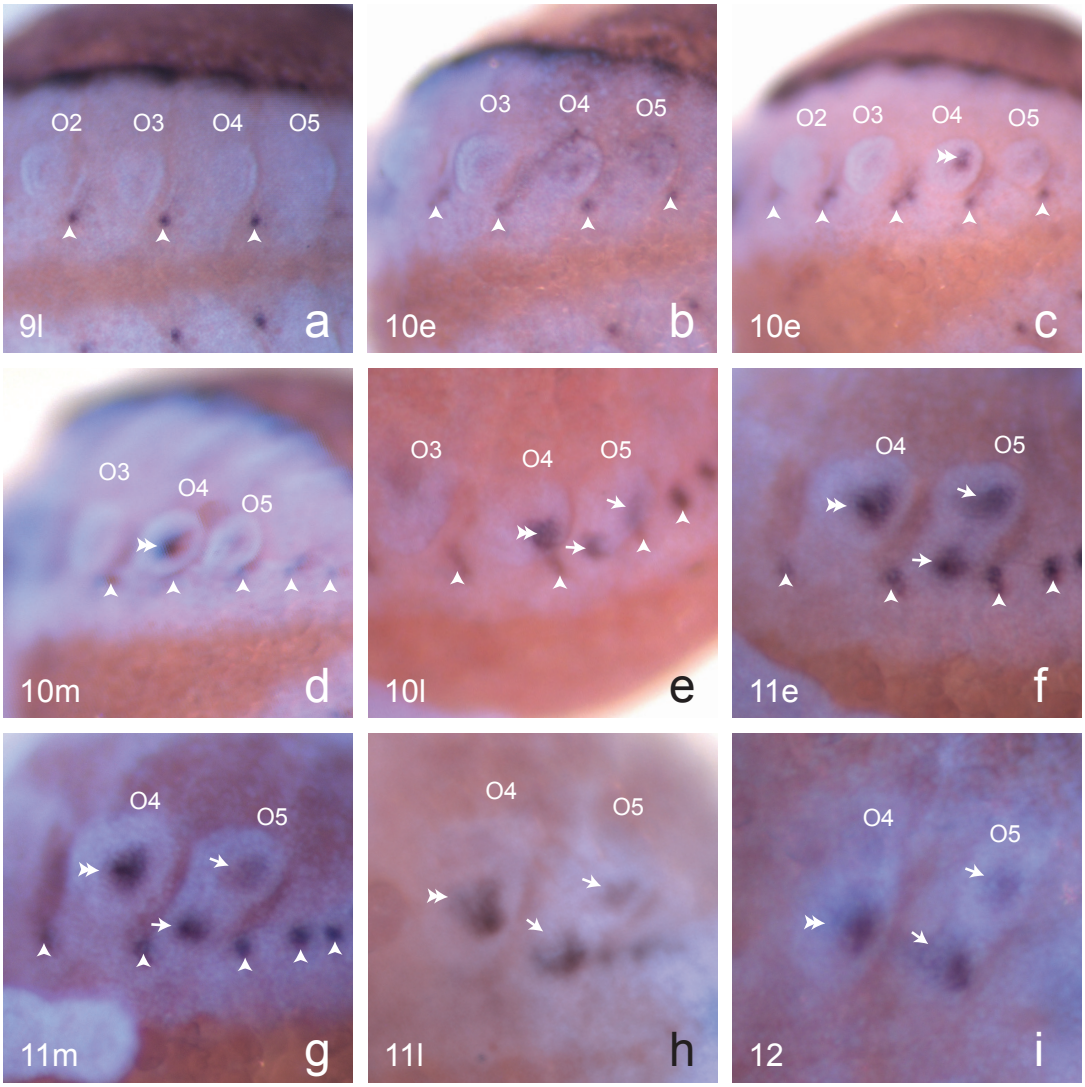



**Figure 5.7:** Expression of *Cs-H15-2* in the opisthosomal limb buds. **a-c**, limb buds of O2-O5 (stages 9l-10e); **d-i**, limb buds of O4 and O5 (stages 10m-12); **O2-O5**, opisthosomal segments 2 to 5. Images are of the left body half and overlays of normal light and a fluorescent nuclear marker (dapi). See text for further description.

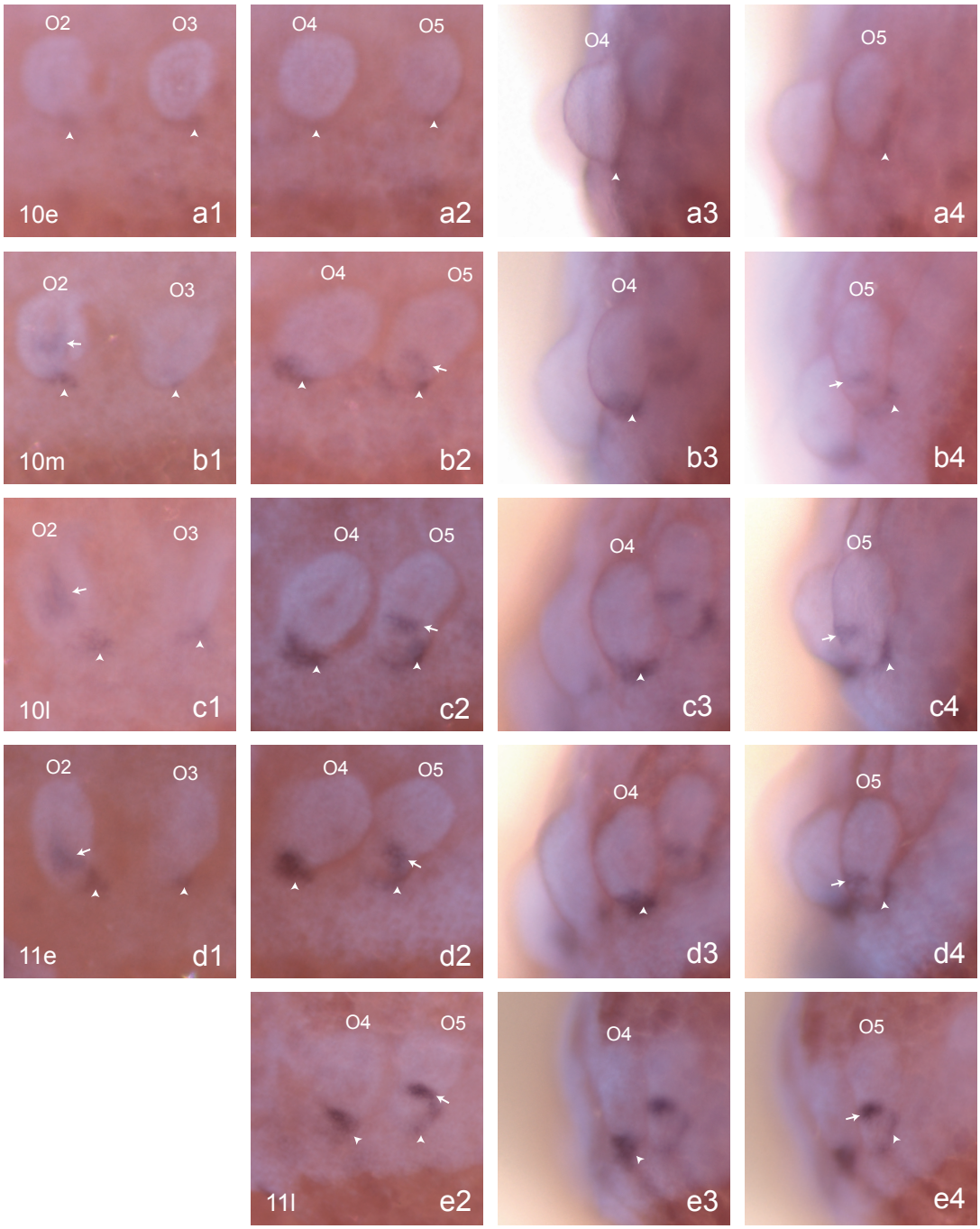
buds during inversion reveals an interesting pattern. At stage 10e faint expression appears at the posterior part of the base of each of the limb buds on O2-O5 (arrow heads in figures 5.8 a1-a4 on page 108). Soon afterwards each of these buds displays a unique pattern. The domain at the base of bO2 has broadened at stage 10m, and a second domain appears inside the bud. This pattern does not change visibly until stage 11e (figures 5.8 b1-d1). During the same period, the domain at the base of bO3 does not appear to change significantly (right-most arrow head in figures 5.8 b1-d1). The domain at the base of bO4 increases in size and intensity at stage 10m. It then continues to increase in intensity until stage 11, and stays undivided (left-most arrow head in figures 5.8 b2-e2 and arrow head in b3-e3). Remarkably, the ventral domain on bO5 does not stay undivided; at stage 10m an aditional stripe of expression appears on the limb bud, marking the later groove between the upper and lower portion of bO5 (arrow in figures 5.8 b2 and b4). The domain ventrally to bO5 elongates to a faint stripe at the base of the bud, with a stronger dot of expression at the posterior part of the base (right-most arrow head in figure 5.8 b2 and arrow in b4). This pattern on bO5 does not change significantly until stage 11l.

At the start of ventral closure, the expression of *Cs-pby-2* in the ventral neuroectoderm of segments O4 and O5 becomes more intense, presumably because the tissue of both body halves comes together in the ventral midline (asterisks in figure 5.8 f on page 109). On O4, there is still a strong domain of expression at the base of the tissue that can now clearly be distinguished as the ASp (arrow head ventral to ASp in figure 5.8 f). Interestingly, a second domain appears ventrally to this domain on the same segment, closing in a bit of tissue clear of expression (double arrow head and closed circle respectively in figure 5.8 f). The bO5 in this stage has now clearly separated in the PSp and the MSp, the latter being closed-in by dorsal, ventral and posterior expression of *Cs-pby-2* (arrow and right arrow head in figure 5.8 f).

Soon after ventral closure the embryonic spinnerets reach their final configuration. Expression of *Cs-pby-2* still marks the circumference of the MSp and the ventral border of the ASp. Although there is still tissue visible ventral to the ASp (closed circle), the expression ventral to this tissue has disappeared. No more expression of the ventral neuroectoderm can be seen (figure 5.8 g).

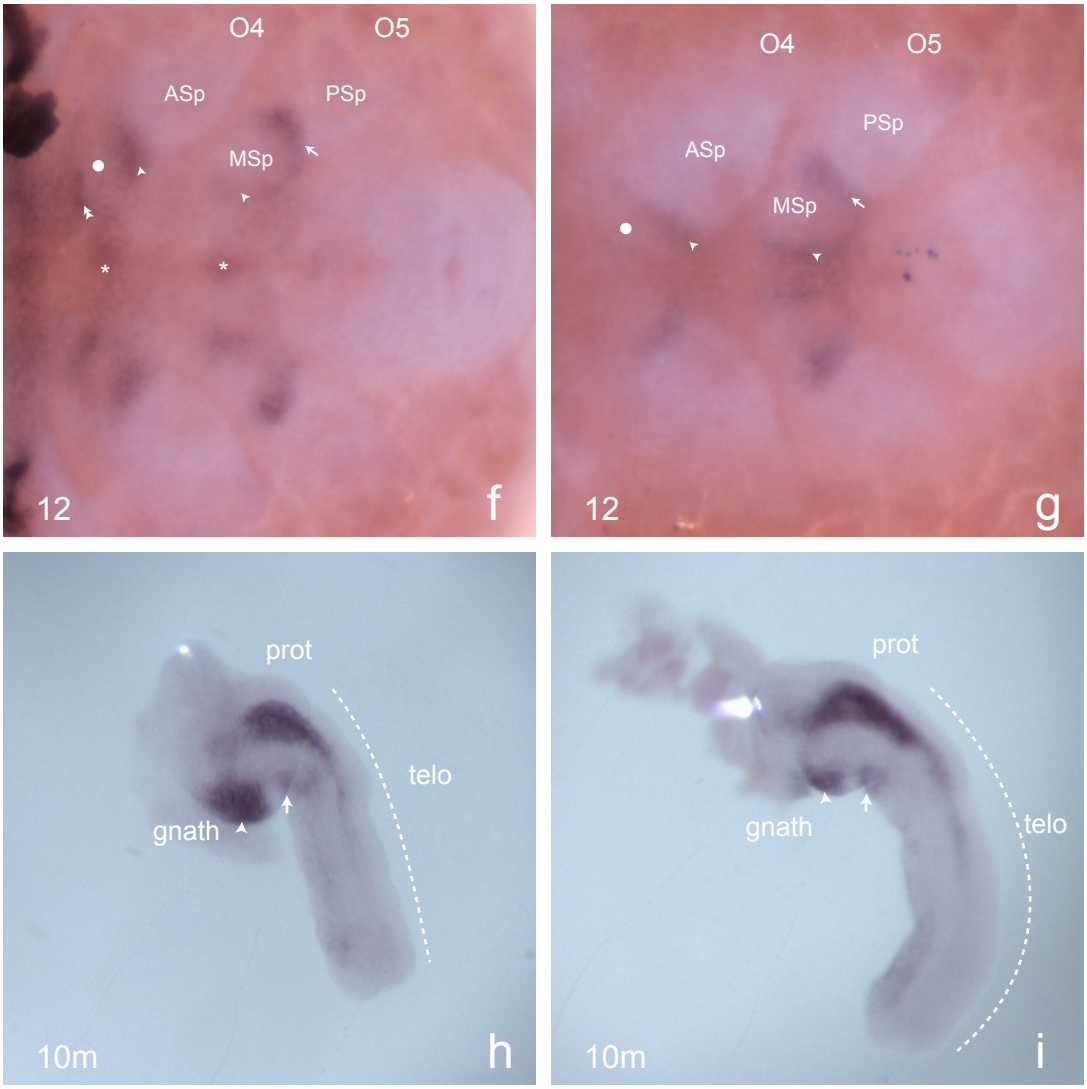
Expression of *Cs-pby-2* in the walking legs and pedipalp was also more closely investigated. Apart from strong domains of expression at the ventral (arrow heads in figure 5.8 h and i) and dorsal bases, there is a third smaller domain visible at the junction of the protruding parts (the "telopodites") and the ventral lobes (the "gnathobases") of both of these appendage types (arrows in figure 5.8 h and i).

**Figure 5.8:** Expression of *Cs-pby-2* in the opisthosomal limb buds and prosomal appendages. Row **a**, Stage 10e; row **b**, stage 10m; row **c**, stage 10l; row **d**, stage 11e; row **e**, stage 11l. Column **1**, expression of *Cs-pby-2* in the limb buds of O2 and O3; Column **2**, expression of *Cs-pby-2* in the limb buds of O4 and O5; Column **3**, expression of *Cs-pby-2* in the limb bud of O4 (posterior view); Column **4**, expression of *Cs-pby-2* in the limb bud of O5 (posterior view). **f**, expression of *Cs-pby-2* in the spinning field (ventral view, stage 12); **g**, expression of *Cs-pby-2* in the spinning field (ventral view, stage 12, slightly later); **h**, expression of *Cs-pby-2* in the pedipalp (normal light only, stage 10m); **i**, expression of *Cs-pby-2* in walking leg 3 (normal light only, stage 10m); **O2-O5**, opisthosomal segments 2 to 5; **gnath**, gnathobase; **prot**, protopodite; **telo**, telopodite (dashed line). Images are of the left body half unless otherwise stated and overlays of normal light and a fluorescent nuclear marker (dapi) unless otherwise stated. See text for further description.




**(I)**

**Figure 5.8:** Expression of *Cs-pby-2* in the opisthosomal limb buds and prosomal appendages. Legend on page 107.




**(II)**

**Figure 5.8:** Expression of *Cs-pby-2* in the opisthosomal limb buds and prosomal appendages (continued.). Legend on page 107.

### 5.3.6 Expression of *Cs-Activator Protein-2*

Homologs of the *AP-2* family of transcription factors are found in many metazoan lineages, and their function has been generalized to cell-type specific stimulation of proliferation during embryonic development (Eckert *et al.*, 2005). In *Drosophila* the homolog *dAP-2* has been associated with leg development (e.g. Ciechanska *et al.*, 2007). In *C. salei* the homolog *Cs-AP-2* is also expressed in the leg, where

it shows a dynamical pattern. Moreover, expression of *H15-2* was found in the spinnerets (Prpic, 2004).

The expression of *Cs-AP-2* in the spinneret limb buds starts at stage 10e (figures 5.9 a and b). Faint expression appears at the center of both bO4 and bO5, but whereas the domain on bO5 gets more intense (arrow in figures 5.9 b-g), the domain on bO4 disappears at stage 10l (arrow head in figures 5.9 b-c). After stage 10l *Cs-AP-2* expression on bO5 seems to be localized to the ventral part of the dorsal portion of the limb bud, i.e. the ventral part of the later PSp (figures 5.9 d-g).

### 5.3.7 Cell proliferation

As described in more detail in chapter 4, bO5 elongates over the course of inversion, and separates around stage 11e into two more or less distinct portions that give rise to the PSp and MSp. bO4 however keeps its globular shape during the same phase of development, and only starts to elongate and flatten towards the end of inversion when all ventral tissue moves towards the ventral midline (figure 4.2). To understand these morphological changes better, developing eggs were injected with BrdU labelled nucleotides at different time points during development and fixed after about 11 hours. During this incubation period, BrdU gets incorportated in all dividing cells. Cells that emerged between the time point of injection and the moment of fixation can thus be stained with an antibody against BrdU, and give an indication of the intensity of cell proliferation during the incubation time, in different parts of the embryo.

Results of the BrdU labelling experiment are shown in figure 5.10. These can be summarized as follows for the stages 10e until 10l.

- Cell proliferation in the spinneret primordia is higher than in the surrounding tissue.
- Most cell division in the spinneret primordia takes place at the margins of the buds.
- Cell proliferation seems to be higher in bO5 than in bO4.
- Most cell division in bO5 takes place at the dorsal margin, occasionally concentrations of cell division are seen at the ventral margin (arrows in figure 5.10).
- The first appearance of concentrations of cell division at the dorsal margin of bO5 is in embryos fixed at stage 10m.

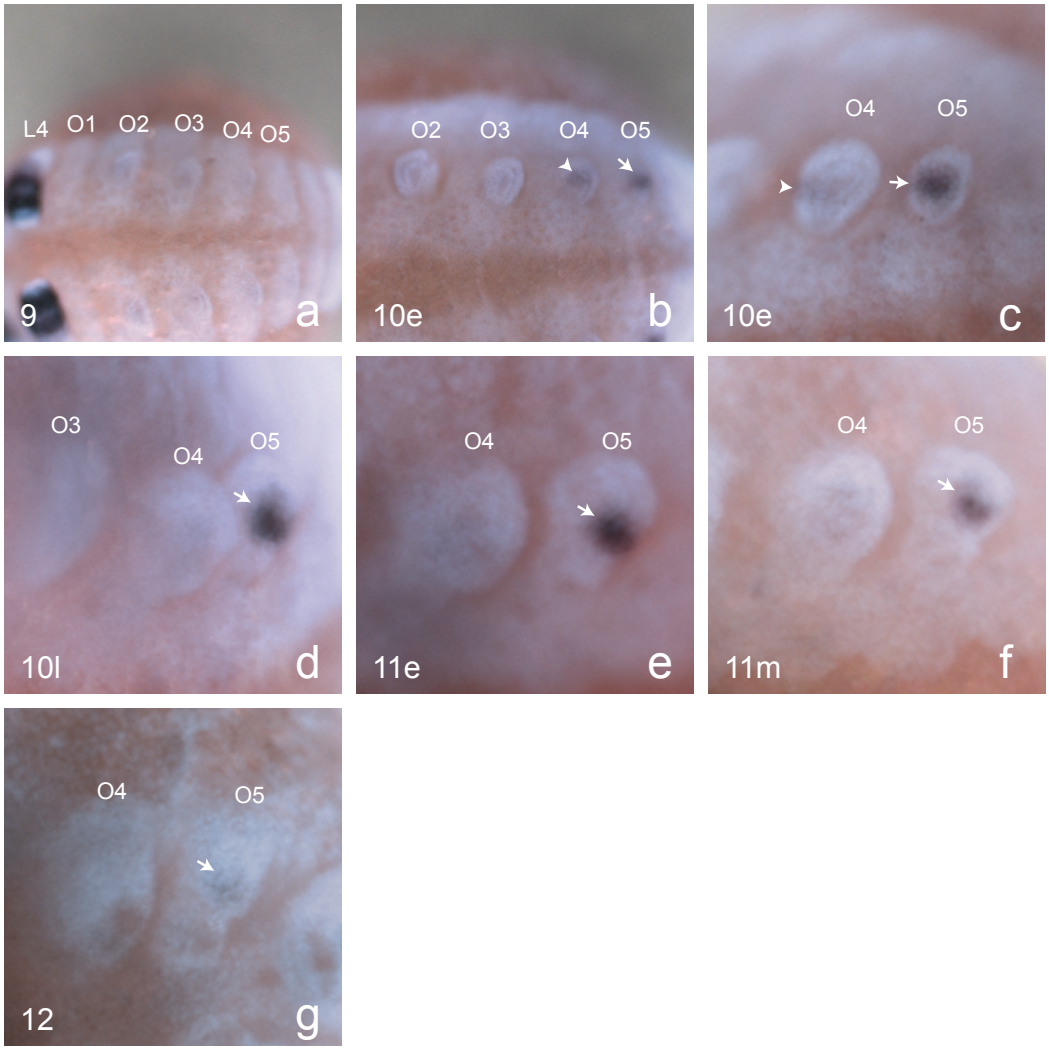



**Figure 5.9:** Expression of *Cs-AP-2* in the opisthosomal limb buds. **a, b**, limb buds of O2-O5 (stages 9, 10e); **c-g**, limb buds of O4 and O5 (stages 10e-12); **L4**, walking leg 4; **O1-O5**, opisthosomal segments 1 to 5. Images are of the left body half and overlays of normal light and a fluorescent nuclear marker (dapi). See text for further description.

## 5.4 Discussion

In this chapter the expression patterns of a selected set of genes are described in the primordia of the spinnerets on opisthosomal segments four and five, together with a study of the cell proliferation in and around them. Results are summarized in figure 5.11. In order to allow the comparison of these expression patterns between the spinneret primordia, and between these primordia and those of the limb buds of the other appendages of the spider, the first step is to define what one considers the antero-posterior (AP), the dorso-ventral (DV) and the proximo-distal (PD) axes.

### 5.4.1 The antero-posterior axis

The AP axes of the spinneret limb buds are clear. During inversion, parallel grooves can be seen on the opisthosoma that mark the segmental boundaries. Around dorsal closure bO5 becomes separated into two portions, and the axis of this fission process lies parallel to the segment boundaries. Towards ventral closure bO4 and bO5 start to become more elongated, and this process also occurs parallel to the segment boundaries. The AP axis is thus defined as the axis perpendicular to the segmental grooves.

### 5.4.2 The dorso-ventral axis

The DV axis lies perpendicular to the AP axis and can therefore also be defined based on the direction of the grooves of the segmental boundaries. The real challenge of the DV axis is not what the direction is, but whether there are one or two axes per spinneret limb bud. As already mentioned, some authors hypothesized that the medial and lateral spinnerets are homologous to the endo- and exopodite of an ancestral biramous appendage. For example Machado (1944) wrote, as reviewed by Marples (1967), that in a number of araneomorph families the adjacent medial and lateral spinnerets tend to arise on "a common membraneous base", which he regarded as the protopodite of a biramous limb.

A close investigation of the development of the medial spinneret on O5 shows that this is not the case in *C. salei* (figure 4.2). The ventral portion of bO5 likely becomes the medial spinneret, but its differentiation process can be described better as a splitting of the whole bud than in the words used by Machado (1944). Nevertheless, morphologically two DV axes develop as a result of this fission process of bO5. Confusingly however, gene expression patterns do not univocally show two axes on bO5.

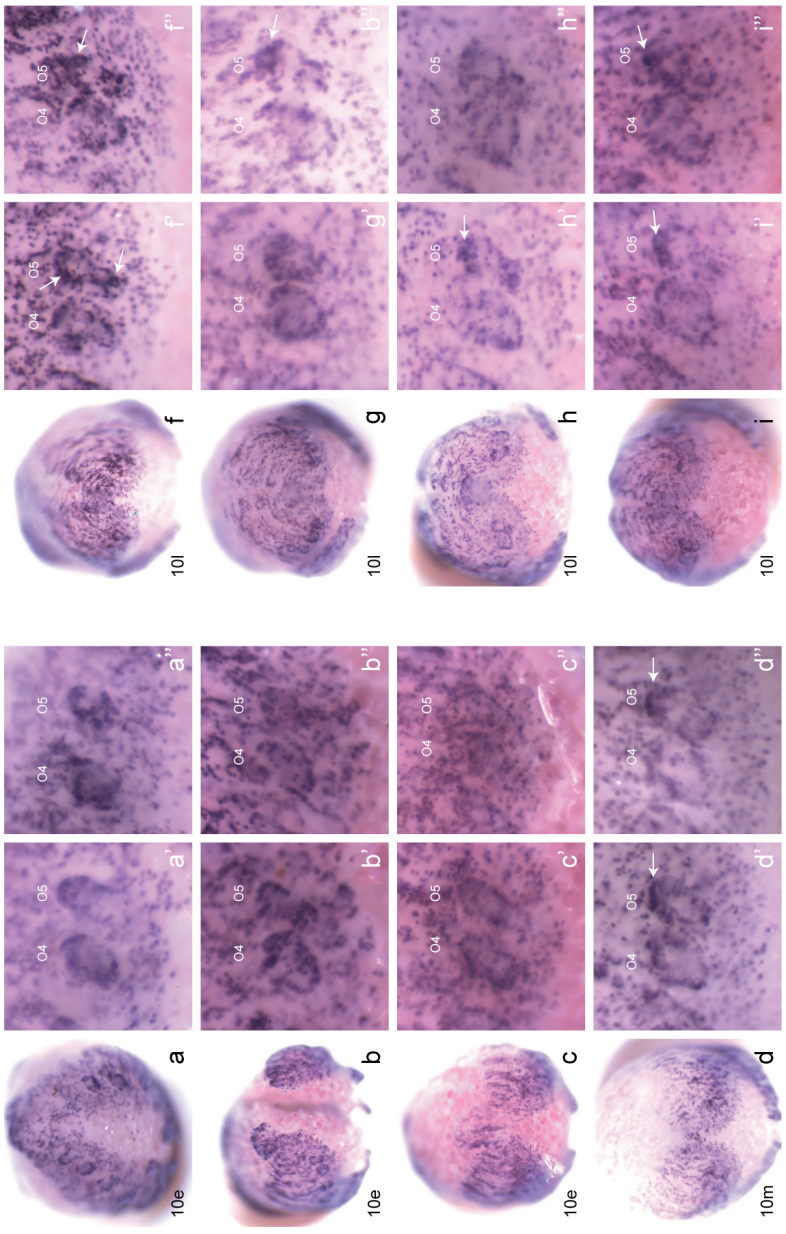



**Figure 5.10:** Cell proliferation in the opisthosomal limb buds of *C. sadei*. Developing eggs were injected with a cell division marker at different time points during development and fixed after eleven hours at stages 10e, 10m and 10l. Staining shows cells that divided during this incubation period. For stages 10e and 10l several embryos are depicted, to demonstrate the variation between embryos. **a** to **i**, posterior view of whole embryos; **a'** to **i'**, region of the limb buds of opisthosomal segments four (O4) and five (O5) from the left body half of the embryos shown in **a** to **i**; **a"** to **i"**, same region from the right body half of the embryos shown in **a** to **i**. Images **a"** to **i"** were mirrored horizontally to facilitate comparison with the other body half. Arrows indicated concentrations of dividing cells.

#### 5.4.2.1 Dorsal

A gene that clearly marks dorsal tissue in the prosomal appendages of *C. salei* (Janssen *et al.*, 2008) is *Cs-omb-2*. On the opisthosomal limb buds, it is expressed in a single domain, including bO4 and bO5. The gene marks the dorsal halves of the spinneret limb buds at stage 10e and continues to be expressed in the dorsal region of both buds while inversion proceeds. Importantly, at no time a second domain appears on bO5 (figure 5.11). In other words, the dorsal marker *Cs-omb-2* does not indicate two DV axes.

#### 5.4.2.2 Ventral and the origin of the colulus

The gene *Cs-wg*, a presumed marker of ventral appendage tissue, shows a pattern different from that of *Cs-omb-2*. *Cs-wg* is involved in the development of ventral tissue of the appendages of *Drosophila* (Brook & Cohen, 1996), and has also been associated with ventral tissue in the prosomal appendages of *C. salei* (Janssen *et al.*, 2008; Prpic *et al.*, 2003). On the spinneret limb buds in total four domains of *Cs-wg* expression can be seen, a dorsal and ventral domain on bO4 and bO5 (figure 5.11).

The two domains on bO5 could be interpreted as ventral tissue of a ventral and a dorsal DV axis on this bud. The two domains on bO4 however are more difficult to understand, because bO4 does not appear to go through a fission process and thus morphologically does not show two DV axes. However, if one follows this domain over the course of inversion, at stages 11l and 12 it migrates more ventrally, and takes up a position in the spinning field where later in the first instar the colulus (3.7) appears (see figure 1.2 and text in chapter 1). This is the first experimental evidence that the colulus is derived from cells on the early limb bud of O4 at a similar position as the cells on bO5 that will develop into the MSp, thus supporting the proposed serially homology of the colulus and MSp.

Figure 5.12 clarifies this hypothesis, by showing two schematical fate maps of the spinneret limb buds (compare to figure 1.2). The left fate map corresponds to a presumed ancestral situation. It assumes that the medial and lateral spinneret of both segments developed in a similar manner as the PSp and MSp develop on O5 of *C. salei*, i.e. by fission of the limb buds. In this ancestor, blue marks the dorsal portions of the spinneret primordia that will develop into the lateral spinnerets, and red the medial portions that will to become the medial spinnerets. To the right, a similar fate map is shown for *C. salei*, starting with early limb buds that are considered homologous to the early limb buds of the proposed ancestor. During inversion however, differences occur. The anterior limb bud does not go through a fission process, whereas the posterior bud does. Only at the ventral base of bO4 some cells separate from the limb bud, migrate ventrally

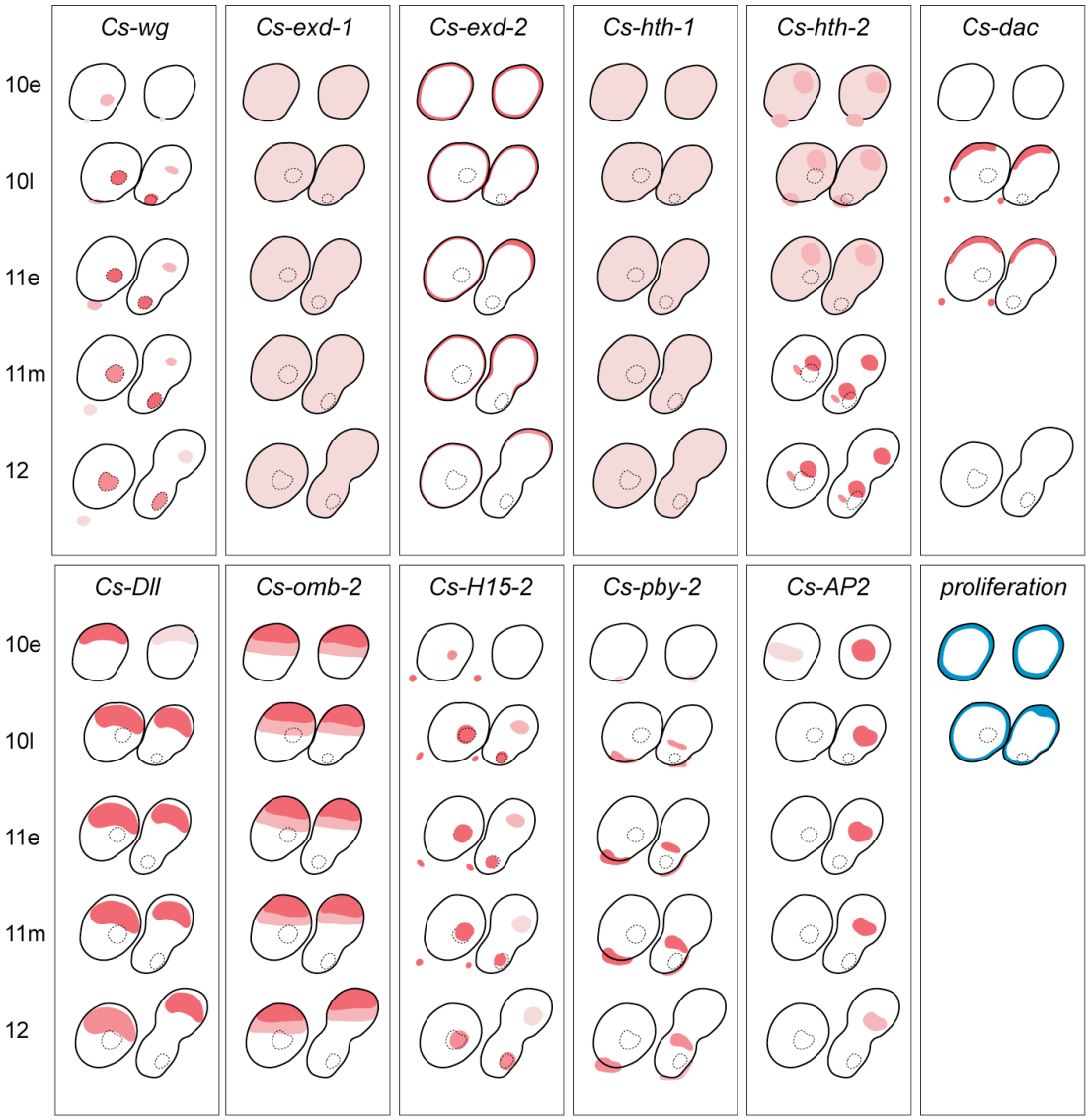



**Figure 5.11:** Schematical drawings of gene expression patterns and cell proliferation in the developing spinneret limb buds. For each depicted gene the limb buds of opisthosomal segment four (left) and five (right) are shown from stages 10e to 12. Expression domains in red. Cell proliferation in blue. Outlines of limb buds with black solid lines. Dashed domains on the limb buds from stages 10l to 12 indicated pronounced depressions of the epithelium of the buds at these stages (see figure 4.2).

and become the colulus, as seen by expression of *Cs-wg*. This postulated fate map therefore confirms the old hypothesis that the tissue of the colulus of *C. salei* is homologous to parts of the MSp. However, it does not confirm the proposed homology of the ASp with the *al*. Instead it prososes that only the dorsal part of the ASp corresponds to the ancestral *al*, and that the *am* is homologous to the colulus together with the ventral part of the ASp in araneomorph spiders such as *C. salei*.

Getting back to the question of the number of DV axes on the spinneret limb buds, according to the evolutionary scenario proposed above, *Cs-wg* indeed could be considered a ventral marker of two DV axes on each limb bud, in contrast to the conclusion based on the expression of the dorsal marker *Cs-omb-2*.

But there are other ways of interpreting the expression domains of *Cs-wg*. Some authors have questioned whether the ventralizing function of *wg* and *H15* as shown in *Drosophila* is actually conserved between other arthropods (Angelini & Kaufman, 2005). Moreover, the function of the homologs of these genes in patterning the prosomal appendages of *C. salei* has never been shown directly. Also, even if these genes have a ventralizing function in the appendages of the prosoma, they might have a different function in the opisthosoma.

The latter possibility is supported by the expression of *Cs-wg* on bO2, where the gene is expressed at the invaginations sites of the different parts of the book lungs (figure 5.1 a2-e2). Strongest expression is seen where the newly forming furrows appear, just ventral to the pulmonary sac. When the furrows mature, *Cs-wg* expression decreases. This expression dynamics thus strongly suggests a role of *Cs-wg* in the development of these furrows. No *Cs-wg* is seen on bO3, apart from the domain at the ventral base (figure 5.1 a3-e3), which is interesting because the tubular trachaea are though to be serially homologous to the pulmonary sac of the book lungs (Purcell, 1910). In *Drosophila*, *wg* is not only involved in leg patterning, but also has a function in the development of breathing structures, during tubulogenesis of the trachaea of the fly (Chihara & Hayashi, 2000; Kerman *et al.*, 2006), which supports that *Cs-wg* in *C. salei* might be involved in cell shape changes on bO2.

An indication that *Cs-wg* might be involved in cell shape changes on the spinneret limb buds, rather then being a marker of ventral tissue, is that the gene comes to expression at the positions where the invaginations O4-i, O5-i and O5-di were observed on the surface of the spinneret limb buds, as described in chapter 4. Interestingly, at these positions *H15-2* appears to be co-expressed with *Cs-wg*. Moreover, O4-i and O5-i are large invaginations, and both *Cs-wg* and *H15-2* are strongly expressed at these positions. O5-di however is much smaller, correlating with stronger expression of *Cs-wg* and *H15-2*. In conclusion, even though morphologically bO5 develops two DV axes, the genetic aspects of the DV patterning of the spinneret limb buds are not clear. The dorsal expression of

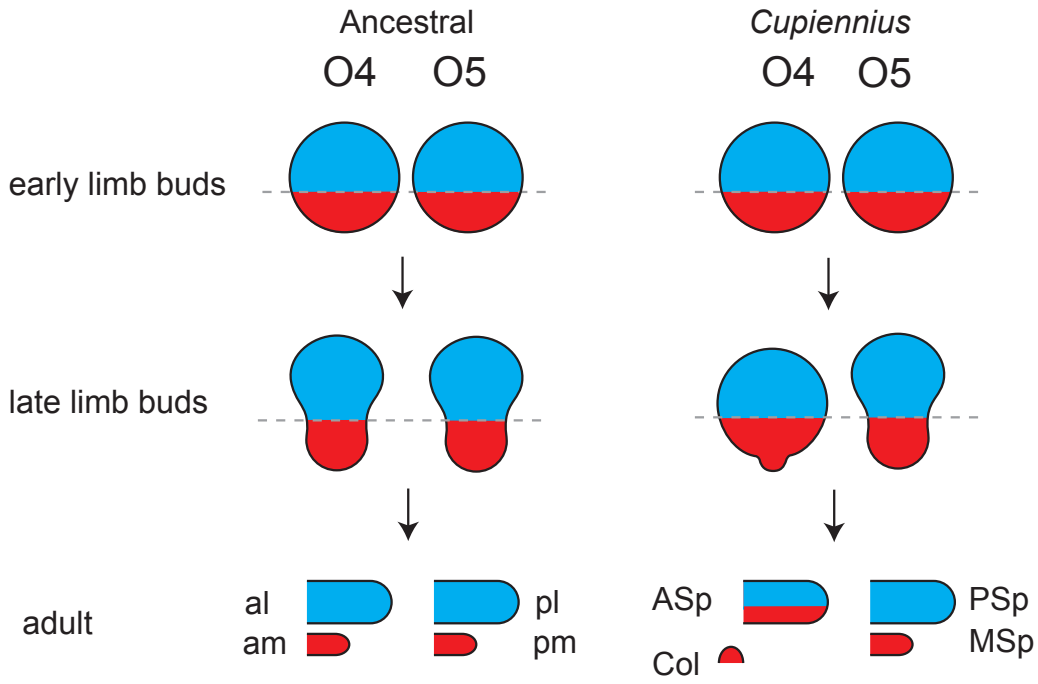



**Figure 5.12:** Schematical fate map of the spinneret limb buds of *C. salei* and a postulated fate map for the putative ancestral state. Blue marks the dorsal two-thirds of the early spinneret limb buds, that on O5 of *C. salei* develops into the PSp, and on O4 into the dorsal part of the ASp. Red marks the ventral one-thirds of the early spinneret limb buds, that on O5 of *C. salei* develop into the MSp, and on O4 into the ventral part of the ASp and the colulus. See text for a discussion of the putative ancestral state. **al**, ancestral anterior lateral spinneret; **am**, ancestral anterior medial spinneret; **ASp**, *C. salei* anterior spinneret; **Col**, *C. salei* colulus; **MSp**, *C. salei* medial spinneret; **O4,O5**, opisthosomal segments O4 and O5; **pl**, ancestral posterior lateral spinneret; **pm**, ancestral posterior medial spinneret; **PSp**, *C. salei* posterior spinneret.

*Cs-omb-2* on all limb buds of the spider suggest that this gene has a dorsalizing function on these primordia, and suggests a single DV axis of both spinneret limb buds. If *Cs-wg* and *H15-2* expression are taken as a marker of ventral tissue however, these genes suggests two DV axes on bO5, and possible also two DV axes on bO4. On the other hand, *Cs-wg* appears to have several functions on the opisthosoma, and is maybe less reliable than *Cs-omb-2* as a marker of the DV axis. Therefore, the possibility that *Cs-wg* and *H15-2* are not involved in appendage patterning on the spinneret limb buds, but instead are involved in the

development of the invagination sites of O4-i,O5-i and O5-di can not be excluded.

### 5.4.3 Proximo-distal patterning

If one postulates two DV axes on a given limb bud, then these axes necessarily also have two PD axes. The genes that pattern the PD axes could therefore also give information about the DV patterning of the spinneret primordia. The genes that are thought to pattern the PD axes of the prosomal appendages of *C. salei* are, from proximal to distal, the leg gap genes *Cs-exd-1*, *Cs-dac* and *Cs-Dll* (Prpic *et al.*, 2003).

#### 5.4.3.1 Proximal

In *Drosophila*, *exd* is thought to function only where it is co-expressed with *hth* via protein-protein interactions of the products of both genes (Rieckhof, 1997). This mutual requirement of *exd* and *hth* is probably conserved between other arthropods (Angelini & Kaufman, 2005). In *C. salei*, *Cs-exd-1* is therefore thought to pattern the proximal prosomal limbs together with one of the two *hth* homologs found in *C. salei*, or with both *Cs-hth-1* and *Cs-hth-2* (Prpic *et al.*, 2003).

In contrast to this situation in the prosomal appendages, *Cs-exd-1* was found ubiquitously expressed in all opisthosomal limb buds. Moreover, both of the presumed cofactors that are currently known in *C. salei*, *Cs-hth-1* and *Cs-hth-2* (until stage 11m), are also ubiquitously expressed. The function of *Cs-exd-1* is therefore very unlikely to be restricted to any particular region of the opisthosomal limb buds. Interestingly however, *Cs-exd-2* was found expressed as a ring around the base of the opisthosomal limb buds (figures 5.2, 5.3 and 5.11). However, if we assume that *Cs-exd-1* can interact with both *Cs-hth-1* and *Cs-hth-2*, and that *Cs-exd-2* can also interact with both *hth* homologs, then the combined function of EXD and HTH is ubiquitous in the opisthosomal limb buds.

It is therefore not clear how to interpret the ring-like expression domains of *Cs-exd-2* around the opisthosomal limb buds. One possibility is that these restricted domain of *Cs-exd-2* are not functional. Another possibility is that the function of EXD is dosage dependent, and that the combined expression of *Cs-exd-1* and *Cs-exd-2* is different from the expression of each of these genes separately. A further possibility is that *Cs-exd-2* has a greater affinity for *Cs-hth-1*, and *Cs-exd-1* a greater affinity for *Cs-hth-2*. According to the latter possibility, *Cs-exd-2* would function together with *Cs-hth-1* at the base of the opisthosomal limb buds, but not together with ubiquitous *Cs-hth-2* in the rest of the buds.

An additional indication that this might be the case is the reversed situation of the restricted expression domains of *Cs-hth-2*. Before stage 11m, *Cs-hth-2* is expressed ubiquitously, but around this stage an interesting pattern appears that

might indicate a role of this gene in patterning the PD axes of bO4, but also of both the dorsal portion and the ventral portion of bO5 (see figure 5.11). Since *Cs-hth-1* is expressed ubiquitously at that stage, *Cs-hth-2* can only have a function restricted to those domains if it either functions in a dosage dependent manner together with *Cs-hth-1*, or if it has a greater affinity for ubiquitous *Cs-exd-1*. A final possibility, that there are yet to be discovered alternative cofactors, such as other *hth* and *exd* homologs can not be excluded.

In conclusion, at present it is not clear how to interpret the basal expression domain of *Cs-exd-2*, and therefore a putative function in patterning the basal opisthosomal limb buds is uncertain. Similarly, it is also not clear how to interpret the later expression of *Cs-hth-2* in the spinneret limb buds. However, the suggested possible interactions of the different *exd* and *hth* homologs, or the existence of a dosage dependence system of these factors should be investigated further. Because if such a system is in place, both *Cs-exd-2* and *Cs-hth-2* are candidates for patterning the PD axes, as illustrated schematically in figure 5.13. Also, the existence of such a system could have implications on how to interpret the different expression domains of these genes in the other appendages of the spider (Prpic *et al.*, 2003).

#### 5.4.3.2 Medial

A gene that is likely involved in patterning the proximo-distal axis of the the prosomal limbs of *C. salei* is *Cs-dac* (Angelini & Kaufman, 2005; Prpic *et al.*, 2003). In the prosomal appendages the gene is expressed in a medial domain and in later stages also in a dorsal proximal domain. In the spinneret limb buds, *Cs-dac* is not expressed in rings, but in dorsal sickle-shaped domains (figures 5.4 and 5.11). This indicates that the gene might also be involved in the PD axes of these limb buds, albeit restricted to the dorsal part of the buds.

#### 5.4.3.3 Distal

*Cs-Dll* is expressed in protruding parts of the spinneret limb buds, of the prosomal legs and possibly also of the other opisthosomal limb buds (Schoppmeier & Damen (2001), Popadíc *et al.* (1998) and figure 5.5), which suggests a similar function of *Cs-Dll* in all appendages (Popadíc *et al.*, 1998). However, there is a difference between the prosomal appendages and the spinnerets in the way that the gene comes to expression. The earliest expression of *Cs-Dll* occupies the whole prosomal limb buds (Prpic, 2004), whereas in bO4 and bO5 the earliest expression marks only the dorsal part of the buds (figure 5.5 b and c). This suggests that the two groups of appendages develop in a different way. Nevertheless, there is also a similarity in the dynamics of the expression of *Cs-Dll* in the spinneret

limb buds and the prosomal appendages. At stage 10e, *Cs-Dll* is expressed at the upper dorsal margin of the spinneret limb buds, which could be considered the proximal part of the lim bud. Only later, around stage 10l, tissue free of *Cs-Dll* expression appears dorsal to the *Cs-Dll* expression domain (arrows in figure 5.5 d, f' and f"). So at least dorsally to the spinneret limb buds, the dynamics of *Cs-Dll* expression is similar to its dynamics in the prosomal appendages, starting with expression at the proximal base of the limb bud and with later expression restricted to the more distal parts.

A second gene that marks the distal part of developing prosomal appendages is *Cs-dpp* (Prpic *et al.*, 2003). Unfortunately, in situ hybridization with this gene did not lead to satisfying results in this study. However, published expression patterns of *Cs-dpp* show that in stage 10e *Cs-ddp* is expressed dorsally on both spinneret limb buds (Janssen & Damen, 2008). Later, in stage 11e, tissue free of *Cs-dpp* expression is visible dorsally to the *Cs-dpp* expression domain (Prpic, 2004). This aspect of *Cs-dpp* expression is thus similar to that of *Cs-Dll*, and together these genes likely mark distal parts of the spinneret limb buds. Interestingly, a further similarity between these regions of the spinneret buds of *C. salei* was suggested by staining patterns with a cross-reacting monoclonal antibody raised against a cocktail of *Drosophila* pg-III genes (Davis *et al.*, 2005). It was suggested that this antibody likely stained a spider homolog of the *Drosophila* protein Aristaless, which in *Drosophila* is involved in patterning the distal leg (Schneitz *et al.*, 1993).

In conclusion, at least dorsally on bO4 and bO5, the PD patterning of *Cs-dac* and *Cs-Dll* is reiterated between the prosomal appendages and the spinneret limb buds, and possibly *Cs-exd-2* has a role in patterning the proximal part of this axis. Figure 5.13 shows how the combined patterns of *Cs-exd-2*, *Cs-dac* and *Cs-Dll* could be interpreted to define the PD axes of the dorsal portions of bO4 and bO5. Ventrally on the spinneret limb bud on O5 however, no expression of *Cs-dac* or *Cs-Dll* was observed, and *Cs-exd-2* does also not mark the ventral-most part of bO5. Therefore there are other factors expected to pattern the PD axes on the ventral portion of bO5, such as maybe *Cs-hth-2*. A possible role of *Cs-hth-2* in PD patterning is supported by the co-expression of the gene with *Cs-Dll* on the dorsal portion of bO5 and on bO4 (figure 5.13). Finally, the PD patterning of *Cs-hth-2* on the ventral portion of bO5 is further evidence that this spinneret primordium consists of two DV axes, starting around stage 11m.

### 5.4.4 Fission of the posterior spinneret primordium

The PSp and MSp arise from the separation of bO5 into a dorsal and a ventral portion, whereas bO4 does not go through such a fission process. What are the genetic differences that underly the differences between bO4 and bO5? A

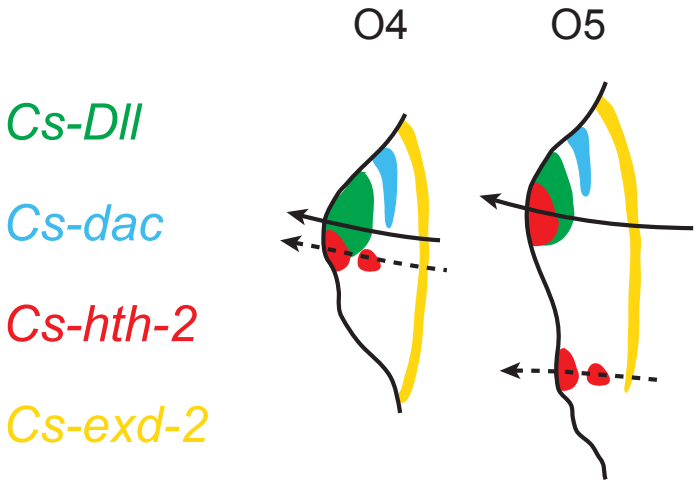



**Figure 5.13:** Schematical drawing of expression of genes proposed to be involved in proximo-distal (PD) axis formation of the spinneret limb buds. *Cs-exd-2* is expressed as a ring all around bO4 and around bO5 with the exception of the most ventral part, and is possibly involved in patterning the proximal domain. *Cs-Dll* is expressed in the protruding parts of bO4 and the dorsal portion of bO5, but not on the ventral portion of bO5. *Cs-dac* is expressed as a sickle shape domain dorsally on the spinneret limb buds, possibly patterning the cells dorsally on the bud in between *Cs-exd-2*and *Cs-Dll* expression. Solid arrows indicate the postulated PD axes marked by these genes on both spinneret limb buds. *Cs-hth-2* is expressed on the surface of all protruding parts of both limb buds, and shows separate smaller domains of expression deeper inside the tissue on bO4 and on the ventral portion of bO5. Dashed arrows indicate the postulated PD axes marked by *H15-2* on both spinneret limb buds. On bO4, the PD axes suggested by all four genes co-locate.

quick glans on the overview figure 5.11 shows that the expression of none of the investigated candidate genes, is limited to one spinneret limb bud only, apart from *Cs-AP-2*. In fact, at stage 10e the gene does also appear faintly on bO4, but that domain soon disappears and the only domain remaining in the opisthosoma lies on bO5, as noted before (Prpic, 2004).

On bO5 at stage 10e, about equal amounts of tissue lie dorsal and ventral to the expression domain of *Cs-AP-2*. However, at stage 10l more expression-free tissue appears dorsally to the expression domain. Taken this observation, together with the notion that there is tissue emerging dorsally to the *Cs-Dll* domain on bO5 as well, plus the staining of injected BrdU as proliferation marker (figure

5.10), it is clear that the bO5 grows at its dorsal margin. This seems logical, because if a large ventral portion of bO5 develops into the MSp, the primordium of the PSp has to grow to equal the ASp in size in later stages.

One could speculate about a possible function of *Cs-AP-2* in the bO5. In the prosomal appendages *Cs-AP-2* is expressed in rings. Over the course of prosomal limb development, new rings of *Cs-AP-2* emerge by fission of earlier rings. Thus, *Cs-AP-2* was proposed to have a role in cell proliferation in the opisthosomal appendages (Prpic, 2004). Such a function would make sense with the observation that *Cs-AP-2* is expressed on bO5 and not on bO4, because bO5 divides in two portions. However, no increased cell proliferation was observed on bO5 around the extimated expression domain of *Cs-AP-2* (figure 5.10). Nevertheless, the possibility of the involvement of *Cs-AP-2* in generating differences between the two spinnerets limb buds is very interesting, and deserves further investigation. For example, it would be very interesting to see if *Cs-AP-2* expression on the fourth opisthosomal limb bud of a mesothelid spider resembles the expression on bO5 of *C. salei* (see figure 1.2).

### 5.4.5 Similarities with the gnathobase

It is though that the prosomal appendages of spiders are derived from a biramous, or "split" appendage, similar to the appendages known from trilobite fossils. These trilobite appendages consisted of a protopodite, i.e. the "stem" of the appendages, and on this protopodite dorsally an exopodite and ventrally a telopodite. The idea is that in hexapods, myriapods and chelicerates (all merely terrestrial lineages, see figure 1.1) the exopodite got lost over time. The remaining uniramous appendage thus consists of a short proximal part homologous to the protopodite, and a much longer (in case of the walking legs) part homologous to the telopodite (Boxshall, 2004). At the ventral part of the protopodite of the prosomal legs of spiders a gnathobase is seen (see for example the SEM figure in Popadíc *et al.*, 1998), which is the modification of an ancestral endite; a ventral lobe of the protopodite.

To date, most studies on the patterning genes of the appendages of *C. salei* have focussed on expression domains that lie on the relatively distal part of the appendages, i.e. the telopodite (e.g. Janssen *et al.*, 2008; Prpic *et al.*, 2003). However, it is my personal impression that many genes that are associated with the patterning of the telopodite of the prosomal limbs show expression domains on the protopodite that appear to have serially homologous expression domains in the opisthosomal appendages. An example is the ring of *Cs-exd-2* around the opisthosomal appendages, that is also present around the most proximal part of the prosomal appendages (Prpic *et al.* (2003) and own observations). If the hypothesis of Marples (1967), that spinnerets derive from embryonic vestiges of

the prosoma (see introduction of this chapter) is correct, then this observation makes sense as well. Because, if all but the most proximal part of these ancestral limb buds, that later gave rise to the spinnerets, got preserved over many millions of years as an embryonic vestige, then it is likely that only those expression domains were conserved that pattern the most proximal part of the limb buds. In this sense, the groups of cells that appear to be vestigial limb buds on opisthosomal segments six and seven (see figure 5.3 e'), are very interesting, because these vestiges might give us a window back in time of what the limb buds on O4 and O5 might have looked like before spinnerets evolved at these positions. Most interestingly, *Cs-exd-2* is also expressed as rings around these vestigial limb buds.

Another example of a gene that appears to have conserved proximal domains at all prosomal and opisthosomal limb buds is *Cs-pby-2*. In the prosomal walking legs and pedipalps, this gene is expressed at the dorsal part of the protopodite, at the ventral part of the protopodite (arrow heads in figure 5.8 h and i), as well as in the region where gnathobase and telopodite come together (arrows in figure 5.8 h and i).

If the ventral-most *Cs-pby-2* expression domains of the pedipalp, walking legs and spinneret limb buds are serially homologous, and the expression domain in between the gnathobase and the telopide and the dorsal most domain of the limb bud on O5 are homologous, then this would imply that MSp could be considered a gnathobase, and the dorsal portion a protopodite. This does not mean that the ancester of spiders that evolved spinnerets must have had appendages like pedipalps or walking legs on its opisthosomal segments; this patterning of the limb bud could have been preserved in a vestigial bud.

Some of the genes discussed in this chapter do not fit with this hypothesis. *Cs-exd-2* for example is in the walking legs also expressed in the region between the gnathobase and the telopodite, but not between the dorsal and ventral portions of bO5. *Cs-Dll* is expressed at the tip of gnathobase of the pedipalp, but not at the tip of the ventral portion of bO5 (however, also not at the gnathobase of the walking legs). Other genes however do fit this hypothesis. *Cs-dac* expression could be in concordance with this theory because on the walking legs and pedipalps, apart from the broad domain medially on the telopodite, it is expressed in a small domain dorsally on the protopodite, which could be homologous to the sickle shaped domain dorsally on the spinneret limb buds. Another gene that supports this theory is *Cs-AP-2*, because apart from being expressed in rings on the prosomal appendages, it also demonstrates an expression domain at the ventral base of the telopodite (Prpic, 2004), which resemble the domain on bO5. A further gene that might fit the pattern is *Cs-hth-2*, because it shows a domain of expression on the surface of the gnathobase of walking legs (Prpic *et al.*, 2003).

### 5.4.6 Conclusions and future directions

As discussed, gene expression patterns give important clues to better understand the morphology and the morphogenesis of the spinneret limb buds. For example, the dynamics of the expression of *Cs-wg* suggests that the colulus derives from cells at the base of the primordium of the anterior spinneret. This means that the proposed evolution of the ASp from an ancestor with both an *al* and *am* could be explained by postulating that these ancestral spinnerets fused, rather than that the whole *am* got modified into a colulus or cribellum. This finding could be potentially interesting for the understanding of the diversification of the silk glands too, because glands present on this ancestral *am* could have migrated to the ASp due to this fusion.

The studied expression patterns als provided a way of defining the axes of these structures that based on morphology alone would not have been possible. It appears that the primordium of the posterior spinneret consists of two DV and PD axes, as supported by the expression of *Cs-wg*, *Cs-H15-2* and *Cs-hth-2* on the ventral portion and by *Cs-dac*, *Cs-Dll*, *Cs-dpp* and *Cs-hth-2* on the dorsal portion. These PD axes thus seem to be patterned in a different way, and because the pattern of the dorsal portion resembles the patterning of the anterior spinneret and of the principal PD axes of the prosomal appendages, it could be argued that these are serially homologous axes, and that this pattern was conserved in the structure that the spinneret limb buds evolved from. The PD patterning of the ventral axis on bO5 appears much less conserved to those of the prosomal main axes, but could instead be homologous to the gnathobase of these appendages, as is also supported by the expression of *Cs-pby-2* and *AP-2*.

However, mainly because so little is known about the function of these genes, most of the postulated hypotheses are rather speculative. For example *Cs-wg* could also be involved in the invagination of the epithelium rather than patterning the axes. Nevertheless, these functions are open for investigation. A first step should be to resolve the expression of these gene expression patterns, in the spinneret primordia but also in the other appendages, to a much finer level. It could for example be tested whether *Cs-wg* is expressed in the cells that invaginate, via confocal studies or histological sections. Alternatively, these genes could be studied via RNAi. Therefore, rather than providing definite answers, this study provides an inspiration of what kind of questions might be answered by studying the genetic base of these derived structures. Finally, because most genes investigated in this study where originally discovered while studying the prosomal appendages, it is not surprising that the expression of none of these genes is restricted to the opithisthosomal appendages only, as was shown for *C. salei apterous* and *pdm/nubbin* homologs. Possibly, many more molecular factors exist of which the expression is restricted to the opisthosomal appendages,

or to the spinneret primordia only. The candidate gene approach that led to the discovery of the the factors discussed in this chapter is not likely to identify such factors, because so little is known from the opisthosomal appendages and the spinnerets in particular that virtually no candidates are available. It seems therefore that a genomics approach to these structures is currently more appropriate. For example, it is likely that soon whole transcriptome sequencing of small amounts of tissue becomes practically and economically in reach. Because the appendage primordia of both prosoma and opisthosoma are protruding structures, there dissection from the rest of the embryo should be technically feasible as well, especially in spider species with relatively large embryos such as *C. salei*. It would be very interesting to see which and how many factors show elevated expression restricted to each of the different appendage types, and which factors are similary expressed in all limb buds.

‘

## 5.5 Methods

### 5.5.1 Sources of gene fragments

The gene fragments used in this study are described in the following sources: *Cs-wingless* (*Cs-wg*) AJ315945 (Damen, 2002); *Cs-extradenticle-1* (*Cs-exd-1*) AJ518943, *Cs-extradenticle-2* (*Cs-exd-2*) AJ518944, *Cs-dachshund* (*Cs-dac*) AJ518942, *Cs-homothorax-1* (*Cs-hth-1*) AJ518945, *Cs-homothorax-2* (*Cs-hth-2*) AJ518935 (Prpic *et al.*, 2003); *Cs-Distal-less* (*Cs-Dll*) (Schoppmeier & Damen, 2001) AJ278606; *Cs-pairberry-2* (*Cs-pby-2*) (Schoppmeier & Damen, 2005) AJ744785; *Cs-optomotor blind-2* (*Cs-omb-2*) AM774407, *Cs-H15-2* (Janssen *et al.*, 2008; Prpic, 2004) AJ518939; *Cs-Activator Protein-2* (*Cs-AP-2*) (Prpic, 2004) Courtesy of Niko Prpic.

### 5.5.2 Whole mount in situ hybridization

Embryos were fixed according to standard methods (Prpic *et al.*, 2008a). Whole mount in situ hybridization was performed according to standard methods (Prpic *et al.*, 2008d).

### 5.5.3 Detection of cell proliferation

Embryos were injected with BrdU according to standard methods (Prpic *et al.*, 2008b) and fixed according to standard methods (Prpic *et al.*, 2008a) after 11 hours of incubation at 25°C.

### 5.5.4 Acquiring and post-processing of images

Images of WMISH stained embryos were taken with an Axiocam camera on a Leica dissecting microscope equiped with a UV light source. For each embryo and orientation, bright field and UV channels were photographed separately. Post processing was done in Adobe Photoshop CS3. Images from both channels were first color and contrast enhanced using the "selective color" and "curves" algorithms respectively. Next the bright field and UV channel of each embryo and orientation were blended using the "screen" mode. This mode "looks at each channels color information and multiplies the inverse of the blend and base colors. The result color is always a lighter color. Screening with black leaves the color unchanged. Screening with white produces white. The effect is similar to projecting multiple photographic slides on top of each other" (source: Adobe Photoshop CS3 Help).

# Chapter 6

# Conclusions and future directions

The evolutionary history as well as the developmental biology of the spider silk producing system is poorly understood. For example, silk glands have been suggested to derive from ancestral organs as diverse as venom glands, accessory glands, coxal glands and dermal glands. These structures do not only differ in their function and location on the body, but are also derived from different germ layers. This demonstrates that there is not even consensus on whether the silk gland develop from the ectoderm, the mesoderm or maybe are composed of cells coming from both germ layers. The developmental and especially the evolutionary origin of the spinnerets is also puzzling. The morphological similarity of the primordia of the spinnerets with the limb buds of the prosomal is convincing that the spinnerets are of apendicular origin. However, no closely related relatives of spiders are known that bear appendages of any kind on the segments where in spiders the spinnerets develop, and the evolutionary origin of the spinnerets is thus enigmatic. Maybe the spinnerets derive from embryonic vestiges of the gills of an aquatic ancestor? If so, which parts of the development of the spinnerets reflect the development of this ancestral gill, and which parts are newly evolved? Also, how did both structures, the silk glands and the spinnerets, evolve to a tightly integrated system that has profoundly changed both the morphology and the ecology of the species in this group of arachnids? These and many other questions will not be satisfyingly answered unless there are increased research efforts describing the development of the silk producing system of different spider groups, but also of other organs of spiders and other arachnids. Such studies might uncover previously unknown similarities and differences of organs, and provide evidence in favor of and against postulated homology relationships. This thesis provides a first step into this direction, by describing the development of the silk producing system of a model species for molecular studies, the American wandering spider *Cupiennius salei*. The main findings of this work as and its consequences for future research are discussed below.

## 6.1 A new staging system of *C. salei* development

In **chapter 2** a new staging system is proposed to subdivide the period of inversion, which is an embryonic process that marks the transition from early to late embryonic development. The proposed system is based on morphological characters, rather than on time after egg laying, and is compatible with a staging system designed earlier to describe the development of the spider *Achaearanea tepidariorum*. A coherency of the nomenclature of embryonic stages of *C. salei* and *A. tepidariorum* will facilitate the discovery of differences involved in the development of the silk producing systems of these ecologically diverse species.

## 6.2 Development of the silk glands

To understand the embryonic origin of the silk glands, it was necessary to first have good knowledge of the adult morphology and of the development of the glands in early post-embryonic stages and therefore in **chapter 3** later stages are described first, followed by subsequently younger stages. The adult silk glands were found to be very similar to those of the wolf spider *P. amentata*, a species that has been studied previously, merely differing in size and numbers of glands, with four different silk gland types. One type of glands are the ampullate glands, which are of special interest because these were probably the first big and specialized glands that evolved from an ancestor with a less developed silk producing system, and might have been very important for the diversification of silk glands of Araneomorph spiders.

A developmental stage called the second instar is the first freely foraging stage of *C. salei*,, and sections of this stage showed the presence of all but one of the four adult gland types, indicating that the development of the fourth type, the tubuliform glands, is a post-embryonic process. Based on these sections, a 3D model was made that allowed for an understanding of the location and structure of the developing silk glands in the opisthosoma. For example, it showed that the ampullate glands in the second instar show a complex configuration that allows for producing silk while molting, and that their ducts already have a well developed zig-zag shape, as seen in adults.

SEM scans of the spinnerets of the preceding first instar showed that the ampullate glands are already functional in this stage. Sections of the first instar were of lesser quality, but showed the ampullate glands in a less differentiated state then in the second instar. Their ampullae were found at a more posterior position than these glands in the next instar, indicating posteroanterior growth of the ampullate glands in the first instar. This growth seems to take place along

the ventral musculature of the opisthosoma, and an interaction of both tissue types might direct the growth of the ampullate glands into the body.

Sections of the postembryo, which is the first stage to appear after hatching from the egg, showed silk gland-like structures closely associated with the spinnerets, and no silk gland like structures in the anterior part of the opisthosoma. Together with the observation of posteroanterior growth of the ampullate glands in the first instar, this strongly suggest that all silk glands originate close to the spinnerets. Glands originating from the anterior spinnerets are largest in this stage, and probably are the major ampullate glands. The tissue inside the medial and especially the posterior spinnerets was more difficult to interpret.

Unfortunately, it was not possible to obtain high quality sections of the interior of the spinnerets during late embryonic stages and therefore it was not possible to trace the post-embryonic silk glands to their first primordia unambiguously. However, it was reasoned that if the silk glands develop via epidermal invaginations, some of these invaginations might already take place before the cuticle starts forming towards the end of embryonic development. Therefore, as described in **chapter 4**, SEM scans as well as confocal laser scans of the epithelium of the spinneret limb buds during inversion were made. In total two larger and one smaller invagination sites were found. The larger ones are on the center of the limb bud of the later anterior spinneret on opisthosomal segment four (O4), and ventrally on the bud that will grow out into the medial and posterior spinnerets on opisthosomal segment five (O5). Based upon their positions on the spinnerets, it was postulated that these large invaginations sites are the primordia of the the major ampullate silk glands of the anterior spinnerets, and of the minor ampullate glands of the medial spinnerets. An alternative possibility, that they are are not the primordia of silk glands, but instead represent the early development of parts of the peripheral nervous system, can not be excluded.

## 6.3 Early development of the spinnerets

It order to understand the location and the nature of these invagination sites on the developing spinnerets better it proved to be very helpful to study expression domains on the spinneret primordia of genes that by previous workers had been cloned to study pattern formation of the prosomal appendages of *C. salei*. For many of these leg patterning genes, it had been discovered that they also have expression domains on the spinneret limb buds. Although poorly understood, this was superficially in line with old hypotheses that the spinnerets in some way are homologous to the other appendages of the spider. However, the details of these spinnerets patterns had never been studied systematically.

Therefore, in order to get more insights in the structure and morphogene-

sis of the developing spinneret primordia, but to also investigate the postulated homology relationship with the other appendages, expression patterns of various candidate genes were generated and compared, as described in **chapter 5**. It was found that the two portions that form after the posterior spinneret primordium splits during inversion are reflected by two proximo-distal axes on the underlying genetic level. Also, it was observed that, of these two axes, the dorsal one shows similarities with the patterning of the anterior spinneret limb bud and the prosomal appendages. This observation shows that not only specific gene expression is reiterated between the spinnerets and prosomal appendages, but also that aspects of the spatial interrelationship of these expression patterns is shared, providing additional evidence that the spinnerets and other appendages might share common ancestry.

## 6.4 The need for functional studies and genomics

However, many aspects of these expression patterns were also open to multiple interpretations. For example, it appeared that the expression of some presumed patterning genes was highly correlated with the structure of the surface of the spinneret limb buds, and, surprisingly, especially with the locations where the epidermal invaginations had been discovered previously. It was therefore evident that, in order to interpret these expression domains properly, future functional analyses of these genes will be necessary. These analyses might provide more insight into the evolutionary history of the spinnerets, but might also uncover genes that are involved in the development of the invaginations on the spinnerets, and thus possibly in the development of the silk glands.

Finally it was argued that the study of the molecular biology of the spinnerets, and of the silk producing system in general, could profit tremendously from genomics approaches, such as transcriptome sequencing, to discover factors that might be unique to this system. The work presented in this thesis provides a conceptual and descriptive foundation to interpret and functionally test such novel candidate factors in the future.

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# Acknowledgements

I am very grateful to Wim for his supervision and especially for giving me a lot of trust and freedom to shape this project my own way. Thanks a lot Evelyn for these great years in and outside the lab, your friendship really made a difference. Henrique: your cheerfulness was an immense suport, many thanks and tudo de bom for you and your family. Acho que vou ficar com a sua bicicleta...;-) Tobi, thank you for your friendship and endless hospitality. Without you, Cologne would have never been the same. But still.... we should have climbed down that hole at the Alter Markt...! Alistair: Thanks for cheering me up when I needed it, calling me grumpy bastard when I least needed it, and getting me drunk amongst a group of petrifying professors :-); Daniela; thanks for the great Carnivals!; Ruth: CBL's, sombreros or "freezebeeze", whatever you want, but think of "das Ei"!; Joël: looking forward to the next party!; Eva: thank you for help and all those nice cakes; Rodrigo: nao te-escesce o teu poster!; Matthias: I we're ever to drive through Heraklion together again, I swear I'll drive myself!; Naomi: next carnival I'm a wind-millsh; Isadora: obrigadão pra tudo!; Julia: zet 'em op tussen die kaaskoppen; Suma: pas op voor kaaskoppen; Maurijn: wat zijn we toch een stelletje kaaskoppen; Thanks to: Andy, Apostolis, Basti, Beatrix, Dominik, Gerti, Jeremy, Klaus, Kristen, Manuel, Nátalia; Patrick, Petra, Philipp, Ralph, Sarah K., Sarah, Till and Vera for those nice moments around the lab, in the Biergarten or on the badminton court. Erik, Ente, Gerben, Jurriaan, Jop, Mai-britt, Toon: geweldig dat we contact houden ondanks de afstand. Christiane, danke für deine Geduld! Du hast diese schwere Zeit für mich in etwas sehr schönes umgewandelt.

Papa, Mama, Rikkert, Liselot: jullie zijn fantastisch!

Many thanks to: Carsten, your input into this project was really helpful! Viktoria, Kathrin and the kitchen ladies of AG Pasparakis. Niko Prpic, Gregor Bucher, Franck Simonnet and Ralf Janssen for discussion. AG Scholtz for discussion and for a fantastic week in Berlin, Kristin Tessmar-Raible and the Arendt lab for a great and productive week at the EMBL, Alexander Haas and Thomas Kleinteich for organizing an inspiring 3D workshop, Martina Rembold and Jayan Nair for their help with the confocal.

Last but not least, lots of thanks to **Alistair**, **Carsten**, **Christiane**, **Evelyn**, **Matthias** and **Rodrigo** for commenting on the embryonic manuscript!

This document was written with LaTeX 2$_\varepsilon$ using a template that was kindly made available publicly by Harish Bhanderi. This PhD project was funded by the Marie Curie RTN ZOONET, "development and evolution of animal form: training modern comparative zoologists", contract number MRTN-CT-2004-005624.

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Teilpublikationen:

McGregor, A.P., Hilbrant, M., Pechmann, M., Schwager, E.E., Prpic, N.M. & Damen, W.G.M. (2008). *Cupiennius salei* and *Achaearanea tepidariorum*: Spider models for investigating evolution and development. *Bioessays*, **30**, 487-498.

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