UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

DOTTORATO DI RICERCA IN BIOLOGIA EVOLUZIONISTICA

XXI CICLO

Non-systemic metamorphosis: millipede gonopods as a model system

Metamorfosi non sistemica: i gonopodi dei millepiedi come sistema modello

Coordinatore: Prof. Giorgio Casadoro

Supervisore: Prof. Alessandro Minelli

Dottorando: Leandro Drago

DATA CONSEGNA TESI

2 febbraio 2009

alla mia famiglia

to my family

CONTENTS

RIASSUNTO

Di norma gli artropodi, nella loro vita postembrionale, affrontano periodicamente cicli di muta per potersi accrescere, essendo dotati di un esoscheletro rigido che racchiude il corpo dell'animale. Quando la muta coinvolge improvvisi e vistosi cambiamenti nella struttura dell'animale si parla di metamorfosi, un fenomeno caratterizzato da numerosi eventi di apoptosi e proliferazione cellulare. Proprio per questa ragione, lo studio dei processi coinvolti nella metamorfosi è diventato un tema ricorrente ed importante per la biologia evoluzionistica dello sviluppo (evo-devo) degli artropodi. Il principale limite di queste ricerche, tuttavia, riguarda il fatto che sono limitate ad un ristretto numero di specie e trarre conclusioni generali basandosi unicamente sui dati raccolti da pochi sistemi modello può rivelarsi rischioso.

Gli studi più recenti sulla metamorfosi sono stati svolti su specie caratterizzate per la maggior parte da una metamorfosi di tipo sistemico. Si tratta principalmente di insetti olometaboli, nei quali il passaggio allo stadio adulto è costellato di cambiamenti che coinvolgono tutto il corpo dell'animale, eventualmente con gradi di intensità diversi. Tuttavia nella maggior parte degli altri artropodi i cambiamenti postembrionali sono notevolmente meno estesi, come avviene, ad esempio, negli insetti emimetaboli. Una situazione sicuramente meno frequente è quella in cui ad essere investite da un brusco cambiamento nello sviluppo siano uno o pochi segmenti non terminali, inizialmente uguali ai moduli che li precedono e che li seguono lungo l'asse principale del corpo. Questo tipo di trasformazione postembrionale, confinata in un distretto del corpo ben circoscritto, è definita, in questa tesi, metamorfosi non sistemica e si può trovare nello sviluppo postembrionale dei maschi dei millepiedi elmintomorfi.

Nei maschi dei millepiedi elmintomorfi, durante il processo di crescita postembrionale, avviene un brusco cambiamento che coinvolge solo uno o due paia di appendici non terminali, inizialmente non diverse da tutte quelle che le precedono e le seguono, nel mezzo di una lunga serie omonoma. Negli adulti, infatti, queste appendici, localizzate nel settimo anello del tronco, sono modificate in gonopodi, i quali sono usati come organi di presa o veicoli per il trasferimento dello sperma durante la copula. Osservando nel dettaglio lo sviluppo del settimo anello possiamo notare che: durante i primi stadi di sviluppo è identico agli altri e porta due paia di zampe convenzionali; in un qualche stadio successivo le zampe vengono sostituite da strutture estremamente ridotte (appendici squamiformi) che, successivamente, diverranno gonopodi.

Lo scopo di questa tesi è quello di indagare la metamorfosi dell'ottavo e del nono paio di zampe, corrispondenti al settimo anello del tronco (unità morfologica generalmente equivalente a due segmenti convenzionali) dei seguenti diplopodi: due blaniulidi *Nopoiulus kochii* (Gervais, 1847) e *Blaniulus guttulatus* (Bosc, 1792), uno julide nemasomatide *Nemasoma varicorne* C.L. Koch, 1847 ed un polidesmide *Oxidus gracilis* (C.L. Koch, 1847). In particolare, ho analizzato l'ultimo stadio in cui i maschi presentano l'ottavo (ed eventualmente il nono) paio di appendici locomotorie, gli stadi in cui queste sono sostituite da appendici squamiformi e il primo stadio che presenta i gonopodi.

Gli studi effettuati hanno previsto principalmente indagini di morfologia esterna, basate sulla microscopia ottica e sulla microscopia elettronica a scansione, mentre l'anatomia

interna è stata descritta principalmente attraverso l'utilizzo di protocolli istologici. Le strutture endoscheletriche associate ai gonopodi sono state studiate attraverso l'uso del microscopio confocale a scansione laser, sfruttando l'autofluorescenza della cuticola.

Dai risultati ottenuti emerge che le modificazioni esterne dovute alla metamorfosi non sistemica nei diplopodi sono associate a un notevole riarrangiamento dell'anatomia interna, documentato per la prima volta con questa tesi. Il grado di intensità dei cambiamenti interni è diverso nelle quattro specie: i due blaniulidi risentono maggiormente dell'impatto che i gonopodi hanno nell'architettura generale del tronco, in particolare a causa delle voluminose strutture endoscheletriche. Al contrario in *N. varicorne*, ed in particolare in *O. gracilis*, l'anatomia del tronco viene modificata in modo meno significativo. Nel polidesmide è stato anche possibile effettuare alcune osservazioni durante l'ultimo periodo di muta, durante il quale avvengono le maggiori modificazioni trasformando le appendici squamiformi in gonopodi.

Viene inoltre presentata un'ipotesi sui meccanismi di base che caratterizzano la formazione dei gonopodi nei millepiedi, secondo la quale i cambiamenti del settimo anello dipendono dalla presenza di un marcatore segmentale prodotto durante lo sviluppo embrionale, che viene attivato solo molto più tardi, quando iniziano i cambiamenti dovuti alla metamorfosi non sistemica.

ABSTRACT

Arthropod post-embryonic life is characterized by periodical moult cycles. These events are essential during individual growth, given that a rigid exoskeleton wraps them. When a moult event corresponds with a relatively sudden and conspicuous change in animal's structure, is called metamorphosis; this phenomenon is characterized by a relatively high level of processes like apoptosis and cellular proliferation. For this reason, the study of the processes involved during metamorphosis has become a very important subject in evolutionary developmental biology (evo-devo) of arthropods. The principal limit of these researches, nevertheless, is that they focus on a small number of species and to draw general conclusions based uniquely on data collected from a few model systems can be risky.

Recent studies about metamorphosis underpin on species characterized in most of the cases by a systemic metamorphosis. These are mainly holometabolous insects, in which the transition to the adult is rich of changes, affecting the whole body, although often with different degrees of intensity. Most of the arthropods, exhibits minor post-embryonic changes. Less usual is a situation in which a sudden developmental change affects one or a few non terminal segments, that initially are identical to those that precede and follow them along the body. This kind of post-embryonic transformation, confined in a well defined region of the body, is defined in this thesis with the term non-systemic metamorphosis. It can be observed during the post-embryonic development of males of helminthomoph millipedes.

In males helminthomorph millipedes, during post-embryonic life, a sudden changes occurs that involves only one or two pair of non terminal appendages, initially not different from the others and part of a long homonomous series. In the adults these appendages, localized in the seventh ring, are modified into gonopods, that are used as claspers or to transfer sperm during the copula. During the first stages of development the seventh ring is identical to the others and it brings two pair of conventional walking legs; in one of the following stage the legs are substituted by extremely reduced structures (called squamiform appendages), that subsequently turn into gonopods.

In this thesis I investigated the metamorphosis of the eighth and ninth pair of legs, that correspond to the seventh ring of the body (a ring is a morphological unit that generally is considered as two conventional segments) in two blanulids (*Nopoiulus kochii* (Gervais, 1847) and *Blaniulus guttulatus* (Bosc, 1792)), in one nemasomatid (*Nemasoma varicorne* C.L. Koch, 1847) and in one polydesmid (*Oxidus gracilis* (C.L. Koch, 1847)) millipedes. Special attention was paid to the last stage in which males maintain the eighth (and eventually the ninth) pair of locomotory appendages, the stages in which these are substituted by squamiform appendages and the first stage with gonopods.

I performed investigations on the external morphology, using optical and scanning electron microscopy. Internal anatomy was described through paraffin sections. The endoskeletal structures associated with gonopods, was studied using confocal laser scanning microscopy taking advantage of the autofluorescence of the cuticle.

As documented here for the first time the external modifications caused by the non-systemic metamorphosis of diplopods are associated with a huge rearrangement of

internal anatomy. The degree of intensity of internal changes is different in the four species. In the two blaniulids, the consequences that gonopods have in the general trunk architecture are bigger than in the others, in particular because of the voluminous endoskeletal structures. In *N. varicorne* and in *O. gracilis* the anatomy of the trunk is less affected. In *O. gracilis* it was also possible to perform some observations during the last moulting period: it is during this step that squamiform appendages become gonopods undergoing larger modifications.

I also present an hypothesis on the basic mechanisms that characterize gonopod formation in millipedes, according to which the changes in the seventh ring depend from the presence of a segmental marker produced during embryonic development, that is activated only much later, at the beginnig of non-systemic metamorphosis.

INTRODUZIONE

LA BIOLOGIA EVOLUZIONISTICA DELLO SVILUPPO (EVO-DEVO) NEGLI ARTROPODI

Gli artropodi sono il phylum animale più ricco di specie, i cui rappresentanti sono distribuiti pressoché in ogni ambiente abitabile, dalle profondità dei mari ai deserti, comprendendo inoltre forme parassite, le quali possono mostrare modificazioni morfologiche straordinarie. Con un tale scenario ricco di forme, lo sviluppo degli artropodi è inevitabilmente diventato uno dei temi più affascinanti per i biologi, e continua a ricevere crescente attenzione. Inoltre, il recente incontro della biologia dello sviluppo con la biologia evoluzionistica nel nuovo campo dell'"evolutionary developmental biology" (evo-devo) ha portato ulteriore interesse a questo argomento, fornendo nuove chiavi di interpretazione per problematiche che rimanevano irrisolte con i classici approcci nei rispettivi campi (per esempio Minelli, 2003, Arthur, 2004, Carroll, 2005, Müller, 2008).

Per quanto riguarda gli studi sullo sviluppo degli artropodi, questi generalmente possono essere divisi in due filoni principali, che si riferiscono rispettivamente al periodo di vita embrionale o al periodo postembrionale. Negli ultimi decenni, gli studi sul periodo embrionale sono stati molto intensi. Inoltre, molto spesso è possibile applicare queste tecniche – che forniscono informazioni che vanno dalle funzioni di determinati geni alla loro regolazione, alle aree in cui vengono espressi – a embrioni di specie diverse (per esempio, Brena et al., 2006; Brena et al., 2005; Grbic, 2003; Janssen et al., 2004; Hatini et al., 2005; Stollewerk, 2000; Stollewerk et al., 2001). Tuttavia questo approccio è solitamente limitato agli stadi embrionali precoci o, meno spesso, tardivi. La schiusa molto spesso corrisponde al momento limite, dopo il quale le conoscenze fini dei processi di sviluppo sono limitate solamente a poche specie modello. Il fatto poi che questi organismi modello, sicuramente adatti da un punto di vista pratico, siano spesso animali molto derivati, e quindi poco rappresentativi dei gruppi di cui fanno parte, rischia di portare a conclusioni errate quando ci si trova ad interpretare l'evoluzione dei processi di sviluppo (ad esempio la quasi simultanea segmentazione dell'asse principale del corpo in *Drosophila* è tutt'altro che tipica degli insetti o degli artropodi in generale). Sembra quindi necessario estendere le conoscenze sullo sviluppo degli artropodi ad un numero sempre maggiore di specie, per avvicinarsi ad una corretta interpretazione dell'evoluzione in questo phylum. Questa necessità sembra essere stata recepita nell'ambiente scientifico, e negli ultimi anni il numero di specie studiate sta aumentando (ad esempio, Abzhanov et al., 1999; Harzsch et al., 2006; Liu and Kaufman, 2005; Olesnicky et al., 2006). Questi lavori offrono informazioni molto dettagliate ma la conoscenza dello sviluppo di questi animali rimane tuttavia frammentaria, poiché raramente viene completamente coinvolta la fase postembrionale. Proprio in quest'ottica è nato il presente lavoro, con l'intento di documentare alcuni passaggi della vita postembrionale dei millepiedi, durante i quali sono coinvolte significative trasformazioni nella morfologia del corpo dell'animale; i millepiedi vengono quindi

proposti come gruppo modello per lo studio di un particolare caso di metamorfosi, qui definito (si veda Drago et al., 2008) come metamorfosi non sistemica.

LO SVILUPPO POSTEMBRIONALE DEGLI ARTROPODI: MUTE E METAMORFOSI

Lo sviluppo postembrionale degli artropodi mostra un sorprendente livello di complessità. Una delle peculiarità più immediate da evidenziare in questo gruppo di animali è che, data la presenza di un esoscheletro rigido che racchiude l'animale, il suo aumento di dimensioni viene limitato da questo rivestimento. Questo problema viene risolto attraverso la muta ed il successivo ripristino dell'esoscheletro chitinoso. Il ciclo di muta consiste infatti in una elaborata sequenza di eventi atti a costruire una nuova cuticola, più grande della precedente, all'interno di quella vecchia (Nijhout, 1994). Gli eventi di muta che si susseguono durante il ciclo vitale variano in numero a seconda della specie; spesso possono esserci differenze anche a livello individuale. Quando un evento di muta è caratterizzato da un vistoso e relativamente brusco cambiamento nella struttura dell'animale, si parla di metamorfosi. Il riarrangiamento morfologico ed anatomico può avere gradi di intensità diversi, ma tipicamente prevede la perdita di strutture larvali più o meno specializzate e la formazione delle strutture caratteristiche dell'adulto. La metamorfosi rappresenta perciò il momento in cui maggiormente vengono coinvolti fenomeni di apoptosi e di proliferazione cellulare a livello postembrionale e proprio per questa ragione è diventata uno dei temi principali della biologia dello sviluppo di questi animali.

Negli ultimi decenni, intanto, la revisione del concetto di metamorfosi in un'ottica evo-devo ha prodotto interessanti interpretazioni, anche al di fuori degli artropodi, soprattutto riguardo la classificazione dei modi di sviluppo nei metazoi (Cameron et al., 1998; Davidson, 1991; Peterson et al., 1997; Peterson and Davidson, 2000), su cui Davidson (2001) ha fondato uno schema concettuale e comparativo per l'interpretazione dell'origine del piano organizzativo dei bilateri, quale conseguenza dell'evoluzione del network genetico che lo controlla.

Tornado agli artropodi, gli studi attuali sulla metamorfosi sono svolti su specie caratterizzate, per la maggior parte dei casi, da una metamorfosi di tipo sistemico. Si tratta principalmente di insetti olometaboli nei quali il passaggio allo stadio adulto è caratterizzato da una serie di cambiamenti che coinvolgono tutto il corpo dell'animale, eventualmente con gradi di intensità diversi. Tra gli insetti questo tipo di metamorfosi sembra rappresentare una condizione derivata (per esempio Brusca & Brusca 2003) e quindi di limitato interesse ai fini dello studio dell'evoluzione degli artropodi nel loro complesso. Va messo in evidenza che nella maggior parte degli insetti i cambiamenti postembrionali sono notevolmente meno estesi (meno "sistemici"). Negli insetti emimetaboli, ad esempio, le trasformazioni più cospicue che portano dallo stato ninfale all'adulto sono localizzate: un caso interessante di queste modificazioni postembrionali è fornito dalle cicale, le cui zampe anteriori sono sviluppate nell'adulto come un tipico paio di appendici locomotorie, mentre nella ninfa sono vistosamente modificate ed adattate per lo scavo nel suolo, rendendo così implicito che la muta ad adulto sia accompagnata da una sorta di "metamorfosi regionale". Il resto del piano corporeo, infatti, subisce solo minimi cambiamenti, sicuramente non comparabili al globale cambiamento che avviene in un olometabolo. Similmente, negli stadi ninfali delle libellule, il labbro è modificato in una maschera molto mobile, usata per catturare le prede, una specializzazione che va perduta con la muta finale, dalla quale emerge l'adulto che presenta pezzi boccali di tipo convenzionale. In entrambi i casi, i cambiamenti morfologici nella forma delle appendici sono ovviamente accompagnati da

cambiamenti nella muscolatura ed è ragionevole ipotizzare, sebbene non sia documentato, che allo stesso modo debbano esserci cambiamenti nella geometria dell'innervazione. In termini di evoluzione della progressione di sviluppo, la condizione che si ritrova nell'adulto, sia nelle cicale che nelle libellule, deve essere considerata come più primitiva rispetto a quella ninfale. Per certi versi, questa situazione è analoga a quella degli insetti olometaboli, la cui larva rappresenta una innovazione evolutiva rispetto alla condizione più conservata dell'adulto (Minelli 2003). Una situazione che fino ad ora è stata scarsamente investigata è quella in cui ad essere investite da un brusco cambiamento nello sviluppo sono esclusivamente uno o pochi segmenti non terminali, inizialmente uguali ai moduli che li precedono e che li seguono lungo l'asse principale del corpo. È proprio per il fatto che questo tipo di trasformazione postembrionale rimane confinato in un distretto del corpo ben circoscritto, che viene definita metamorfosi non sistemica in uno dei lavori in cui si articola questa tesi (Drago et al., 2008). Un esempio di questa metamorfosi si riscontra durante lo sviluppo dei maschi dei millepiedi elmintomorfi.

DIPLOPODA

I millepiedi (Diplopoda) sono un gruppo di artropodi riferibili al subphylum Myriapoda, che comprende circa 12000 specie descritte e 80000 specie stimate (Hoffman et al. 2002). Essi rappresentano un gruppo di animali terrestri molto antico: il più antico animale terrestre descritto, considerato capace di respirare ossigeno atmosferico, è *Pseumodesmus newmani*, un millepiedi del Siluriano (Wilson and Anderson, 2004). Tra i Myriapoda è possibile separare facilmente i Diplopoda da Chilopoda, Symphyla e Pauropoda, grazie a quella che probabilmente è la loro più nota peculiarità, ossia la presenza di diplosegmenti: i moduli del corpo dei millepiedi sono infatti confrontabili con coppie di segmenti fusi tra loro a due a due. La maggior parte delle specie conosciute è detritivora e si ciba principalmente di foglie e legno in decomposizione. I millepiedi assumono così un importante ruolo, dal punto di vista ecologico, contribuendo in modo significativo alla decomposizione dei vegetali e alla mobilitazione di vari elementi chimici (Cárcamo et al. 2000), in particolare il calcio (Seastedt & Tate 1981), componente importante della cuticola della maggior parte delle specie. Alcune specie si cibano invece di tessuti vegetali vivi costituendo talvolta un problema dal punto di vista economico (per esempio, Blower, 1985).

Nonostante queste caratteristiche che porterebbero ad ipotizzare un notevole interesse nei loro confronti, molti aspetti della biologia, anatomia e filogenesi di questi animali sono ancora lontani dall'essere compresi. Mancando un consenso sulla filogenesi degli artropodi (si veda Giribet et al. 2005 per una revisione ed una estesa analisi filogenetica), anche la collocazione dei miriapodi al loro interno non è risolta. Le relazioni fra le classi di miriapodi sono ancora in discussione e un'ipotesi supportata da convincenti analisi morfologiche è quella che associa diplopodi e pauropodi nel gruppo Dignatha (Dohle, 1980; 1988; 1997); viene riconosciuto anche il legame tra Symphyla e Dignatha, considerati sister groups (Sierwald and Bond, 2007). Inoltre anche all'interno dei diplopodi persistono problemi ad ogni livello tassonomico e filogenetico. Una revisione dello stato attuale delle conoscenze di tassonomia e filogenesi dei diplopodi comprensiva di un'analisi dei vari ordini dei millepiedi effettuata sia a livello morfologico che molecolare è stata presentata nel 2007 da Sierwald & Bond. Enghoff (1981) ha fornito una dettagliata analisi cladistica dell'ordine Julida utilizzando un approccio morfologico, rivista successivamente nel 1991. Restano tuttavia ancora irrisolti molti aspetti della filogenesi dei diplopodi.

Per quanto riguarda la loro morfologia, la caratteristica più evidente è, come già sottolineato, la diplosegmentazione, particolarità che peraltro da il nome a questo gruppo di animali. Per descrivere questo tipo di organizzazione è necessario far riferimento all'organizzazione generale del corpo degli artropodi: la cuticola che riveste il corpo forma un esoscheletro costituito da piastre di rivestimento, gli scleriti, i quali sono relativamente rigidi e separati tra loro da zone di cuticola più flessibile (membrana artrodiale). Gli scleriti dorsali sono chiamati tergiti, quelli ventrali sterniti e quelli laterali pleuriti. In genere ogni segmento è composto da un tergite, uno sternite e due pleuriti laterali e normalmente si riscontra una stretta corrispondenza tra i segmenti corporei e le appendici. Se queste ultime sono presenti, ciascun segmento ne porta un paio. Nei diplopodi la situazione è diversa: la prima unità del tronco, il collum, è senza appendici ed è seguita (di regola) da tre segmenti con un paio di zampe ognuno, seguiti a loro volta da una serie di unità, ciascuna con due paia di zampe, stigmi ed altre strutture duplicate (Minelli, 2004). Il termine segmento può generare ambiguità parlando di millepiedi: Enghoff et al. (1993)[1] adottano una terminologia diversa per evitare questo problema. Nei diplopodi degli ordini basali come i Penicillata, i Pentazonia ed alcuni Colobognatha (Polyzoniida e Siphonophorida) parlano di tergiti dato che gli scleriti sono indipendenti e connessi dalle membrane artrodiali. Nei Colobognatha Platydesmida e nei Nematophora usano il termine pleurotergiti dato che pleuriti e tergiti sono fusi assieme, mentre gli sterniti restano liberi. Utilizzano infine il termine anelli per i Polydesmida e gli Juliformia, nei quali, dopo il quarto tergite, i quattro scleriti di ogni unità segmentale sono tutti fusi assieme.

Un'ulteriore complicazione è data dal fatto che la seconda, la terza e la quarta unità del corpo assomiglino a segmenti convenzionali. Studi effettuati su polidesmidi e juliformi hanno evidenziato un maggiore grado di complessità nell'apparato scheletro-muscolare di questi che spesso vengono chiamati segmenti toracici rispetto ai diplosegmenti post toracici. Di conseguenza, alcuni autori (Kraus, 1990; Manton, 1961; Demange, 1967) hanno ipotizzato che i tre segmenti toracici derivino da diplosegmenti, in seguito alla perdita di alcune strutture, compreso un paio di zampe. Tuttavia, i segmenti toracici non presentano duplicazione del ganglio ventrale (Miley 1930) né degli ostii o dei muscoli alari (Seifert 1932); inoltre gli studi di Dohle (1974) su embrioni di *Glomeris marginata* non trovano alcuna evidenza a sostegno dell'ipotesi della soppressione del secondo paio di appendici. Il confronto tra queste due ipotesi è ben riassunto nel lavoro di Wilson (2002), il quale, oltre a fornire una dettagliata descrizione della muscolatura del torace di *Phyllogonostreptus nigrolabiatus*, dà anche una plausibile spiegazione riguardo la complessità dell'apparato muscolare di questi segmenti. Egli associa il maggior grado di complessità al fatto che gli sterniti dei segmenti toracici siano liberi, mentre nei segmenti post toracici, completamente fusi a formare un anello, si assiste ad una riduzione della muscolatura. Questo è confermato osservando la muscolatura dei millepiedi degli ordini basali: infatti, quando tutti gli sterniti del corpo sono liberi, il livello di complessità elevato permane in tutto l'animale. La seconda, la terza e la quarta unità non deriverebbero quindi da diplosegmenti semplificati ma, al contrario, rappresenterebbero la condizione di partenza.

Per quanto riguarda la segmentazione, in quasi tutti i miriapodi progoneati esiste una discrepanza tra strutture segmentali ventrali e dorsali: i diplopodi e molti pauropodi hanno meno tergiti che sterniti, mentre i sinfili, al contrario, hanno più tergiti che sterniti. Una dissociazione comparabile è stata riscontrata in crostacei notostraci (Linder 1952) e in alcune forme fossili (Chen et al. 1995; Ramsköld et al. 1996). Questi

---

[1] Enghoff et al. 1993 fanno riferimento alla filogenesi riassunta in Fig. 1

esempi suggeriscono che i meccanismi di segmentazione possono essere differenti tra i lati dorsale e ventrale del corpo, cosa che è stata dimostrata in *G. marginata* (Janssen et al., 2004). Questo induce a rivedere i concetti di aplo- e diplosegmento: in seguito a studi sull'espressione dei geni della segmentazione, risulta che i confini di un tergite non corrispondono direttamente ai confini (ventrali o dorsali) di unità segmentali embrionali e che l'ectoderma ventrale deve essere considerato separatamente da quello dorsale. I tergiti in *G. marginata* si estendono dalla metà di una unità segmentale embrionale alla metà dell'unità successiva. Dunque, se i diplosegmenti sono stati originariamente considerati come un set di due segmenti coperti da un diplotergite e gli aplosegmenti come singoli segmenti coperti da un aplotergite, tali termini non possono essere più applicati nel senso originale, ma possono essere mantenuti solo a fini descrittivi.

I diplopodi sono caratterizzati da uno sviluppo postembrionale di tipo anamorfico. In questi artropodi, infatti, il giovane sguscia dall'uovo con un corpo breve, formato da pochi segmenti e generalmente provvisto di tre sole paia di zampe (ad esempio Fig 2), e va incontro ad una serie di mute attraverso le quali il numero dei segmenti e delle paia di zampe cresce progressivamente (per esempio Blower (1985)). I segmenti vengono aggiunti nella parte posteriore del corpo, subito prima dell'ultimo segmento o anello. Esistono tre differenti modalità di anamorfosi (Enghoff et al., 1993). Quando ad ogni evento di muta corrisponde l'aggiunta di nuovi segmenti e tale processo prosegue per tutta la vita dell'animale si parla di euanamorfosi, tipica di Julida e Colobognatha. Se le mute continuano per tutta la vita dell'animale, ma l'aggiunta di segmenti procede solo fino ad un determinato stadio di sviluppo, si parla di emianamorfosi, caratteristica di Polyxenida e Glomerida. Se invece ad un certo stadio di sviluppo, tipicamente quello della maturità sessuale, le mute cessano, si parla di teloanamorfosi, situazione che si ritrova in Chordeumatida e Polydesmida. Il periodo che intercorre tra una muta e la successiva viene definito stadio di sviluppo postembrionale o stadium: il numero di questi stadi varia da specie a specie ed in taluni casi, in particolare tra i millepiedi euanamorfici, si può riscontrare una notevole variabilità a livello individuale.

In letteratura, la maggior parte degli studi concernenti lo sviluppo dei millepiedi si riferisce agli stadi embrionali, dal classico approccio descrittivo (ad esempio, Anderson, 1973; Dohle, 1974; Johannsen and Butt, 1941)) fino a lavori più recenti nel campo della genetica dello sviluppo (Dove and Stollewerk, 2003; Janssen et al., 2004; Prpic et al., 2005). Per quanto riguarda lo sviluppo postembrionale dei millepiedi, invece, sono disponibili vecchi studi, ancora molto utili, di Verhoeff (riassunti in Verhoeff 1926-32), seguiti da diversi altri contributi (riassunti in Enghoff et al. (1993) e in Hopkin & Read (1992)).

Nel contesto dello sviluppo postembrionale, indipendentemente dal tipo di anamorfosi, è possibile ritrovare una peculiarità molto diffusa: nei maschi dei millepiedi elmintomorfi – tutti i diplopodi esclusi Penicillata e Pentazonia –, durante lo sviluppo anamorfico avviene un brusco cambiamento che coinvolge solo uno o due paia di appendici non terminali, inizialmente non diverse da tutte quelle che le precedono e le seguono, nel mezzo di una lunga serie omonoma. Negli adulti, infatti, queste appendici, localizzate nel settimo diplosegmento, sono modificate in gonopodi, i quali sono usati come organi di presa o veicoli per il trasferimento dello sperma durante la copula (per esempio, Hopkin and Read, 1992). È presente un solo paio di gonopodi nei millepiedi polidesmidi (ottavo paio di zampe modificato), mentre ce ne sono due paia nei gruppi rimanenti (ottavo e nono paio di zampe negli julidi). Una particolarità dei millepiedi è che le aperture genitali non sono situate nello stesso segmento dove sono presenti i gonopodi: i gonopori sono infatti situati nella terza unità del tronco, anteriormente ai gonopodi. Prima della copula, i maschi devono quindi far arrivare gli spermi ai gonopodi,

operazione che avviene ripiegando il corpo e avvicinando così il terzo ed il settimo anello.

I gonopodi hanno sempre ricevuto molta attenzione da parte dei miriapodologi, principalmente per il loro valore tassonomico (per esempio, Brölemann, 1923; Enghoff, 1985; Mesibov, 2003; Schubart, 1947; Sierwald et al., 2003). La loro complessa forma e la grande varietà morfologica esistente fanno inoltre ipotizzare un loro ruolo nella competizione sessuale che va oltre al semplice trasferimento di spermi: nello spirosteptide *Orthoporus pyrhocephalus* è stato documentato l'utilizzo dei gonopodi per la rimozione degli eventuali spermi di altri maschi dai genitali femminili (Barnett et al., 1993). Restano però limitate le conoscenze sulla morfologia funzionale dei gonopodi (Tadler, 1993, 1996); il ruolo che hanno le singole componenti di queste appendici, durante la copula, il più delle volte è stato soltanto ipotizzato e non verificato con osservazioni. Molto frammentaria è, in aggiunta, la comprensione della formazione dei gonopodi, argomento che non ha mai ricevuto particolare attenzione in passato. Se si osserva il settimo anello di un millepiedi maschio durante lo sviluppo postembrionale, si può riconoscere una sequenza di eventi comune a tutte le specie. Durante i primi stadi di sviluppo, il settimo anello del tronco è identico agli altri possedendo due paia (l'ottavo ed il nono) di **zampe** normali. In un qualche stadio successivo le zampe vengono sostituite da strutture estremamente ridotte, le **appendici squamiformi**, le quali in seguito vengono modificate nei **gonopodi**. In generale il processo di formazione dei gonopodi ha ricevuto scarsa attenzione in passato e la maggior parte dei lavori riguardanti queste appendici è puramente descrittiva e si limita a rappresentare la loro struttura esterna "finita" per poterli utilizzare come carattere tassonomico. Nemmeno nei lavori di Sahli (Sahli, 1983, 1985, 1990a, b), relativi a quegli julidi in cui è presente in fenomeno della periodomorfosi[2] (Verhoeff, 1923), vengono considerate le modificazioni morfologiche dei gonopodi.

Osservazioni sulla sequenza di cambiamenti di queste appendici sono state effettuate da Miley (1925), Petit (1973, 1976) e Filka and Shelley (1980) in alcuni polidesmidi e da Berns (1968) in uno spirobolide. In ogni caso, tutti questi lavori danno un interessante quadro del fenomeno, ma esclusivamente da un punto di vista della morfologia esterna. Uno dei pochi studi di anatomia interna che consideri anche i gonopodi è stato effettuato da West (1953), sempre in un polidesmide; sono tuttavia assenti informazioni riguardo gli stadi precedenti alla comparsa dei gonopodi.

Complessivamente, fino ad ora non ci si è occupati delle modificazioni ontogenetiche a carico di questa regione e dei meccanismi che le regolano; sono inoltre limitate le conoscenze istologiche e anatomiche riguardo i gonopodi ed il settimo anello del tronco, e praticamente assenti per quanto riguarda le appendici squamiformi.

SCOPO DELLA TESI

Lo scopo del presente lavoro è stato quello di indagare la metamorfosi dell'ottavo e del nono paio di zampe, corrispondenti al settimo anello del tronco, in quattro specie di diplopodi: due blaniulidi *Nopoiulus kochii* (Gervais, 1847) e *Blaniulus guttulatus* (Bosc, 1792), un nemasomatide *Nemasoma varicorne* C.L. Koch, 1847 ed un paradoxosomatide *Oxidus gracilis* (C.L. Koch, 1847). Le prime tre specie rientrano in due famiglie dell'ordine Julida, mentre la quarta fa parte dell'ordine Polydesmida (Fig. 1). In

---

[2] in alcune specie il differenziamento dei gonopodi è reversibile: ad uno stadio che presenta gonopodi ne possono seguire uno o più con maschi "intercalari" nei quali i gonopodi sono regrediti; solo in seguito ad un'altra muta i gonopodi si presentano nuovamente differenziati.

questa ricerca si è cercato di descrivere nel dettaglio i cambiamenti morfo-anatomici associati alla metamorfosi non sistemica nei diplopodi, inserendo queste osservazioni in un'ottica evo-devo. Nello specifico, ho svolto un'analisi comparativa della morfologia esterna e dell'anatomia interna del settimo anello e delle relative appendici in questi millepiedi durante gli stadi di sviluppo coinvolti nella formazione dei gonopodi; in particolare sono stati analizzati l'ultimo stadio in cui i maschi presentano l'ottavo (ed eventualmente il nono) paio di appendici locomotorie, gli stadi in cui queste sono sostituite da appendici squamiformi e il primo stadio che presenta i gonopodi. I risultati ottenuti, fornendo un quadro completo delle modificazioni a carico di questa precisa regione, possono fungere da punto di partenza per studi sulle dinamiche cellulari – processi di apoptosi e di proliferazione – coinvolte nella formazione dei gonopodi. Inoltre le conoscenze morfo-anatomiche che emergono da questa tesi diventano essenziali per studi sui pattern di espressione postembrionale di ipotetici geni implicati a monte di questo tipo di metamorfosi.

METODI

Gli studi effettuati sulle specie sopra menzionate hanno previsto principalmente indagini di morfologia esterna, basate sulla microscopia ottica e sulla microscopia elettronica a scansione. L'anatomia interna è stata descritta attraverso l'osservazione di raccolte seriali di sezioni in paraffina, colorate con ematossilina ed eosina o, più frequentemente, con la colorazione tricromica di Mallory. Per la comprensione delle strutture endoscheletriche associate ai gonopodi è stato necessario utilizzare un moderno protocollo, basato sull'uso del microscopio confocale a scansione laser; tale tecnica, sfruttando l'autofluorescenza della cuticola, permette di rilevare queste strutture senza dover ricorrere a dissezioni, le quali sono estremamente complicate in specie di dimensioni ridotte, in quanto potebbero provocare danni alle parti da esaminare.

La tesi si articola nelle tre parti qui sotto descritte.

MANOSCRITTO 1 – LA FORMAZIONE DEI GONOPODI NEL MILLEPIEDI *OXIDUS GRACILIS* (C.L. KOCH 1847)

In questo manoscritto viene descritto lo sviluppo postembrionale del millepiedi *Oxidus gracilis* (C.L. Koch 1847). Questa specie ha uno sviluppo di tipo teloanamorfico, in cui i giovani schiudono dall'uovo con un tronco formato da sei anelli e con sole tre paia di zampe; attraverso sette eventi di muta si arriva all'ultimo stadio (adulto) nel quale il corpo è formato da diciannove anelli. Il numero di mute è fisso ed ogni stadio è caratterizzato da un preciso assetto per quanta riguarda il numero di anelli con zampe e di anelli apodi. Una peculiarità dei maschi adulti dei polidesmidi è di possedere solo il primo paio di appendici del settimo anello modificate in gonopodi, mente il secondo paio non subisce trasformazioni. Per il primo ed il secondo stadio di sviluppo vengono confermate le osservazioni presenti in letteratura. La formazione dei gonopodi coinvolge cinque mute consecutive partendo da individui al terzo stadio di sviluppo, durante il quale l'ottavo paio di zampe è presente ed è identico alle altre paia. Il primo passo consiste nella perdita di queste zampe, che vengono sostituite da appendici squamiformi. Le tre mute che portano dal quarto al settimo stadio vedono un progressivo aumento delle dimensioni delle appendici squamiformi, accompagnato da un reciproco avvicinamento in posizione ventrale e mediana del corpo. All'interno di

11

queste strutture si evidenzia la presenza di gruppi di cellule indifferenziate che saranno poi responsabili della formazione dei gonopodi. La maggior parte dei processi morfogenetici avviene proprio a partire da queste cellule durante la muta tra il settimo e l'ottavo stadio. Durante questa muta infatti è possibile osservare l'allungamento delle appendici squamiformi e la formazione degli articoli dei gonopodi al loro interno immediatamente prima dell'ecdisi. Viene, infine presentata la situazione nel settimo anello allo stadio adulto: sono descritti i gonopodi che risultano formati da tre articoli, dei quali quello terminale presenta una certa complessità, soprattutto in riferimento ai rapporti fra le parti che lo compongono. Nello stadio adulto ci si sofferma quindi nella descrizione delle novità a livello di apparato scheletro-muscolare collegate alla presenza dei gonopodi rispetto alla situazione in cui sono presenti solo le zampe. Sono infatti presenti degli apodemi sui quali si inseriscono muscoli estrinseci responsabili dei movimenti dei gonopodi. Oltre a questi cambiamenti nell'organizzazione del tronco si assiste anche alla perdita degli stigmi associati all'ottavo paio di appendici.

MANOSCRITTO 2 – CONSEGUENZE DELLA METAMORFOSI NON SISTEMICA IN TRE MILLEPIEDI JULIDI: UN PUNTO DI VISTA ANATOMICO

Questo manoscritto descrive le modificazioni dovute alla metamorfosi non sistemica in due millepiedi blaniulidi, *Nopoiulus kochii* (Gervais, 1847) e *Blaniulus guttulatus* (Bosc, 1792), ed in un nemasomatide, *Nemasoma varicorne* C.L. Koch, 1847, fornendo un confronto tra queste tre specie. Negli julidi entrambe le paia di zampe del settimo anello vengono modificate in gonopodi. Questi animali sono caratterizzati da uno sviluppo di tipo euanamorfico: le mute continuano quindi per tutta la vita dell'animale. Nel caso di *N. kochii*, inoltre, è presente il fenomeno della periodomorfosi (vedi nota 2, pag. 10). In questo lavoro sono stati studiati l'ultimo stadio in cui sono presenti l'ottavo ed il nono paio di zampe, lo stadio successivo con appendici squamiformi, ed il primo stadio con gonopodi. Sono stati esclusi gli esemplari agli stadi successivi per poter avere un campione di specie diverse che si trovassero allo stesso punto dello sviluppo almeno per quanto riguarda le appendici copulatorie. Negli stadi in cui l'ottavo ed il nono paio di zampe sono presenti non si rilevano differenze morfo-anatomiche tra il settimo anello e le relative appendici e gli altri anelli. Le osservazioni istologiche hanno evidenziato alcune differenze tra le appendici squamiformi dei due blaniulidi e quelle del nemasomatide: nel primo caso si ritrovano gruppi di cellule indifferenziate strettamente impacchettati mentre in *N. varicorne* questi gruppi di cellule appaiono in una struttura più lassa ed sono inoltre presenti dei tessuti differenziati alla base delle appendici, più precisamente un paio di piccoli muscoli. La formazione dei gonopodi avviene in seguito ad un solo stadio intermedio con appendici squamiformi, diversamente da quanto osservato in *Oxidus gracilis* (Drago, 2009a). In tutti e tre i casi il grado di riarrangiamento anatomico è decisamente maggiore rispetto al polidesmide. In particolare si osservano dei cambiamenti più significativi nei blaniulidi, mentre nel nemasomatide la situazione può essere considerata intermedia. È presente un sacco gonopodale, una sorta di membrana cuticolare che sostituisce gli sterniti ventrali, e ad esso sono articolati i gonopodi; il sacco gonopodale permette inoltre l'alloggiamento dei gonopodi in condizione di riposo, causando una notevole riduzione del volume interno dell'anello. Le osservazioni istologiche mostrano inoltre la presenza di voluminosi apodemi a cui sono associati muscoli altrettanto imponenti: questi contribuiscono alla riduzione del volume interno, ridotto a meno della metà nei casi più estremi, causando un dislocamento di tutti gli organi in direzione dorsale. Il sistema nervoso e l'apparato digerente sono maggiormente interessati a questo effetto.

L'ampiezza del sacco gonopodale aggiunta al fatto che alcuni muscoli estrinseci dei gonopodi trovano i punti di attacco nell'anello antecedente ed in quello successivo al settimo, causano una riduzione del volume anche di questi segmenti, anche se in misura decisamente minore. La situazione è meno drammatica in *N. varicorne*, nel quale il dislocamento dorsale degli organi è molto più contenuto. I processi coinvolti nella metamorfosi non sistemica di queste tre specie sembrano quindi simili, anche se il risultato finale ha un diverso impatto sull'anatomia interna. In particolare, la presenza dello stadio con appendici squamiformi sembra essere un punto chiave dello sviluppo dei gonopodi dei millepiedi e sembra quindi rappresentare un vincolo di sviluppo imprescindibile nella loro formazione.

MANOSCRITTO 3 – METAMORFOSI NON SISTEMICA NELLE APPENDICI DEI MASCHI DI MILLEPIEDI: EFFETTO POSTICIPATO E REVERSIBILE DI UN MARCATORE DI POSIZIONE LOCALIZZATO PRECOCEMENTE?

In questo lavoro viene proposto un meccanismo che potrebbe essere alla base delle peculiarità della metamorfosi non sistemica. Questa ipotesi rappresenta una delle conclusioni più significative che si possano trarre da quanto osservato nei manoscritti 1 e 2. Si prevede che durante lo sviluppo embrionale precoce dei millepiedi venga prodotto un marcatore segmentale che rimane inespresso per tutto il periodo di sviluppo embrionale tardivo e nella prima fase dello sviluppo postembrionale. Questo marcatore verrebbe successivamente attivato da un segnale sistemico, determinando un cambiamento (o, meglio, una serie di cambiamenti) di notevole impatto ma localizzati con una impressionante precisione. Il fatto che in tutti i millepiedi elmintomorfi i gonopodi siano localizzati nel settimo anello, indipendentemente dal numero di segmenti presenti alla schiusa, fa presumere che questo marcatore venga espresso durante l'embriogenesi precoce. La natura e la posizione dei gonopodi porta a presumere che l'identità di queto marcatore sia da ricercarsi tra i geni *Hox*, nello specifico *Abdominal-B* (*Abd-B*). Questa ipotesi presuppone dunque l'esistenza di un modulo di sviluppo criptico che viene attivato soltanto mesi o addirittura anni, a seconda della specie, dopo la sua formazione nello sviluppo embrionale. In aggiunta tale modulo potrebbe essere utilizzato ripetutamente nelle specie con periodomorfosi.

BIBLIOGRAFIA

Si veda "references" a pag. 23-28.

I do not know what I may appear to the world; but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

Isaac Newton, *From Brewster, Memoirs of Newton* (1855)

INTRODUCTION

ARTHROPOD EVO-DEVO

Arthropods are the largest animal phylum and include species inhabiting almost every imaginable habitat, from sea depth to deserts. They also comprise parasite forms, sometimes with extraordinary morphological modifications. In such a scenario, so rich of different forms, arthropod development has inevitably become one of the most fascinating themes for biologists, and is still receiving growing attention. Moreover, the recent marriage of developmental biology and evolutionary biology in the new "evolutionary developmental biology" (evo-devo) is contributing to increase the interest in this subject, providing new keys to interpret questions that remained unsolved with classic approaches (e.g., Minelli, 2003, Arthur 2004, Carroll 2005, Müller 2008).

As for studies about arthropod development, these can be usually divided in two principal groups, referring to the embryonic and the post-embryonic life period respectively. In the last decades, researches about embryonic development have been very prolific, mostly because of the emergence of many new, sophisticated molecular techniques. Moreover, very often it has been possible to apply techniques (than can supply different types of information, from the function of putative genes to their regulation, to the areas where they can be expressed) to the embryos of many different species (Brena et al., 2006; Brena et al., 2005; Grbic, 2003; Janssen et al., 2004; Hatini et al., 2005; Stollewerk, 2000; Stollewerk et al., 2001).

However, this kind of approach is usually limited to embryonic stages, precocious and sometimes late. In most cases, hatching represents the limit of knowledge, after which information available about developmental processes concerns only very few organisms, usually model species. The fact that these model organisms are suitable from a practical point of view, but are often very derived and then less representative of the groups to which they belong, can lead to wrong conclusions when interpreting the evolution of developmental processes (e.g., the almost synchronous segmentation of the main body axis in *Drosophila* is not so typical of insects, or of arthropods in general). Thus, it seems necessary to try to extend our knowledge about arthropod development in a higher number of species, in order to get a correct interpretation of the evolution of development in this phylum.

This necessity has possibly been received by the scientific community and in the last years the numbers of studied species is growing (e.g., Abzhanov et al., 1999; Liu and Kaufman, 2005; Harzsch et al., 2006; Olesnicky et al., 2006). These works offer very detailed information, but the knowledge of development remains fragmentary, since the post-embryonic phase is rarely included. The present study finds its place in this scenario. Its aim is to document some phases of millipede post-embryonic life, in which important transformations in animal morphology are involved; millipedes are thus proposed as a model group for the study of a particular case of metamorphosis, here described as non-systemic metamorphosis (Drago et al., 2008).

ARTHROPOD POST-EMBRYONIC DEVELOPMENT: MOULTS AND METAMORPHOSIS

Arthropod post-embryonic development shows a surprising level of complexity. One of the most conspicuous peculiarities of this process relates to the existence of a rigid exoskeleton that wraps the individual, limiting its growth. This problem is solved through the moults and the subsequent restoring of the chitinous covering. A moult cycle, in fact, consists in an elaborated sequence of events that results (generally) in the production of a larger new cuticle inside the previews one (Nijhout, 1994).

The number of moult events during life cycle can vary between species and often also at the individual level. The term metamorphosis is used when a moult event is characterised by a conspicuous and relatively sudden change in the structure of the animal. This morphological and anatomical rearrangement can have different degrees of intensity, but typically it involves the loss of larval and less specialized structures and the formation of the characteristic structures of the adult. Thus, metamorphosis represents the moment in which apoptosis and cell proliferation processes are mostly involved during post-embryonic life and for this reason it has become one of the main themes in arthropod developmental biology. In the last decades, moreover, the revision of the metamorphosis concept from an evo-devo point of view produced interesting interpretations, even outside arthropods, even suggesting a classification of metazoan development modes (Cameron et al., 1998; Davidson, 1991; Peterson et al., 1997; Peterson and Davidson, 2000), which has established a comparative and conceptual framework for subsequent interpretations of the origin of the bilaterian body plan as a consequence of the evolution of the underlying genetic pathways (Davidson 2001).

In arthropods, the current research on metamorphosis involves species characterised by a systemic change. These are mainly holometabolous insects, in which the transition to the adult stadium is characterised by a series of changes that affects the whole body of the animal, although often with different degrees of intensity. Within the insects, this type of metamorphosis seems to be a derived condition (e.g., Brusca and Brusca, 2003) and thus of limited interest for the study of arthropod evolution. It should be noticed that in most insects post-embryonic changes are considerably less extended (less "systemic"). In the hemimetabolous insects, for example, the most conspicuous transformations involved in the transition from nymphal to adult stadium are more localised: an interesting case of this post-embryonic modification is provided by the cicadas, where fore legs in the adult are a typical pair of locomotory appendages, while in the nymph they are conspicuously modified and adapted for digging in the soil, and it is thus implicit that the moult into the adult shall be accompanied by a sort of "regional metamorphosis". The rest of the body plan goes through only a few minor changes, not comparable with the global change that affects holometabolous insect. In a similar way, in the nymphal stadia of dragonflies, the labium is modified to highly mobile mask that is used to catch the prey, a specialization that is lost with the final moult, from which emerges the adult with its "normal" mouthparts. In both cases, morphological changes in the form of appendages are obviously accompanied by changes in the musculature and it is reasonable to hypothesize, even if it is not documented, that in the same way there should be changes in the geometry of innervation. In terms of evolution of development progression, the adult condition, both in cicadas and in dragonflies, should be considered as more primitive than the nymphal one. In some way, this situation is analogous to that of holometabolous insects, where the larva represents an evolutionary innovation respect to the (primitively) more conserved condition of the adult (e.g., Minelli, 2003). A situation that until now has scarcely been investigated is when just one or few non-terminal segments of the individual, initially identical to those

that precede and follow them amidst a long homonomous series, are affected by a sudden change in development. As this type of post-embryonic transformation is confined to a well-defined region of the body, this type of metamorphosis is defined here as "non-systemic" (Drago et al., 2008). An example of this type of metamorphosis can be seen during the development of all the males of helmintomorph millipedes.

MILLIPEDES (DIPLOPODA)

Millipedes are a group of arthropods that belong to the subphylum Myriapoda. It includes about 12000 described and 80000 estimated species (Hoffman et al. 2002). Millipedes represent a very ancient group of terrestrial animals: current research indicates that the oldest myriapod known, and also the first known terrestrial oxygen-breathing organism was the millipede *Pneumodesmus newmani* from the mid Silurian (Wilson and Anderson, 2004). Whithin Myriapoda, it is easy to recognize the Diplodopa from Chilopoda, Symphyla and Pauropoda for their probably most known peculiarity, that is the presence of diplosegments: the units forming a millipede body are composed of pairs of segments fused together. Most known species are detritivores and feed predominantly on leaves and decaying wood. Millipedes, thus, play an important ecological role, contributing to the decomposition of plant matter and to the mobilisation of various chemical elements (Cárcamo et al., 2000), particularly calcium (Seastedt & Tate, 1981), an important component of the cuticle of most millipede species. Some species, otherwise, feed on fresh plant tissues and sometimes are an economic problem (Blower, 1985).

Although these characteristics may suggest that millipede elicit a high interest, many aspects of their biology, anatomy and phylogeny are very poorly known. Given the lack of a general consensus on arthropod phylogeny (see Giribet et al. (2005) for a review and an extended phylogenetic analysis), also the position of the Myriapoda is not clear. Relationships among the classes of Myriapoda are still under discussion and a well supported hypothesis, based on morphological analysis, associates Diplopoda and Pauropoda in a group Dignatha (Dohle, 1980; 1988; 1997), and also a close affinity of Symphyla and Dignatha is recognized, considering them as sister groups (Sierwald and Bond, 2007). Also within Diplopoda there are problems at every taxonomic and phylogenetic level. A review of the current knowledge of millipede taxonomy and phylogeny, including an analysis of the various order of millipedes combining both morphological and molecular data was presented in 2007 by Sierwald & Bond. Enghoff (1981; 1991) gave a detailed cladistic analysis of the order Julida, using a morphological approach. However, many aspects of Diplopoda phylogeny are still unsolved.

Concerning the morphology of these animals, as already mentioned, their most evident characteristic is diplosegmentation, a peculiarity that gives also the name of this taxon. To describe their organisation it is necessary to refer to the general organisation of arthropod body: the cuticle that coats the body forms an exoskeleton, composed by plates, sclerites, that are relatively rigid and separated each to the other by more flexible zones of cuticle, the so-called arthrodial membranes. Dorsal sclerites are called tergites, the ventral ones sternites and the lateral ones pleurites. Generally, each segment is composed by a tergite, a sternite, and two lateral pleurites. Normally there is a tight correspondence between body segments and appendages. If there are appendages, each segment carries a pair of them. In Diplopoda the situation is different: the first body unit, called the collum, does not carry appendages and is followed by three segments with a pair of legs each, followed in turn by a series of units with two pairs of legs, spiracles and other duplicated structures (Minelli, 2004). The term

"segment" is often ambiguous, when applied to millipedes: Enghoff et al. (1993)[3] adopt a different terminology to avoid this problem. In Diplopoda of basal orders, as Penicillata, Pentazonia and some Colobognatha (Polyzoniida and Siphonophorida), these authors use the term tergites, giving that sclerites are independent and connected to arthrodial membranes. In Colobognata Platydesmida and in Nematophora, instead, they use the word pleurotergites, giving that pleurites and tergites are fused together, while sternites are free. Finally, they use the term rings for Polydesmida and Juliformia, where, after the fourth body unit, the four sclerites of each units are all fused together.

A further complication is that the second, the third and the fourth body unit seem to represent conventional segments. Studies on polydesmids and juliforms showed a higher degree of complexity in the skeleto-muscular apparatus of the so-called thoracic segments, than in the post-thoracic diplosegments.

Consequently, some authors (Manton, 1961; Demange 1967, Kraus 1990) hypothesized that the three thoracic segments derive form diplosegments, after the loss of some structures, including a pair of legs. Nevertheless, the thoracic segments do not present duplication of ventral ganglia (Miley, 1930) neither of ostia nor alar muscles (Seifert, 1932); moreover, Dohle's (1974) studies on the embryo of *Glomeris marginata* did not find any evidence supporting the hypothesis of a second pair of appendages. The comparison between this two theories is well resumed by Wilson (2002), who gives a detailed description of the thorax musculature of *Phyllogonostreptus nigrolabiatus* and gives also a plausible explanation of the complexity of the muscular apparatus of these segments. He associates the higher degree of complexity with the fact that sternites of thoracic segments are free, while in post-thoracic segments, completely fused to form a ring, there is a reduction of musculature. This can be confirmed observing the musculature of basal millipede orders: when all body sternites are free, an elevate degree of complexity is still found in the whole animal. The second, the third and the fourth units, thus, are not derived from simplified diplosegments but, at the contrary, they shall be the starting condition.

As for segmentation, in almost all the progoneate myriapods there exists a discrepancy between ventral and dorsal segmental structures: Diplopoda and many Pauropoda have fewer tergites than sternites, while Symphyla, at the contrary, have more numerous tergites that sternites. A comparable dissociation was found in notostracans crustacea (Linder, 1952) and in some fossil forms (Chen et al. 1995, Ramsköld et al. 1996). These examples suggest that the mechanisms of segmentation can be different between dorsal and ventral body sides, as demonstrated for *G. marginata* (Janssen et al., 2004). These results induce to reconsider the concepts of haplo- and diplo-segment. Studies about the expression of segmentation genes indicate that the boundaries of a tergite do not correspond directly to the boundaries of the dorsal or ventral segmental embryonic units and that the ventral ectoderm behaves separately from the dorsal one. Tergites in *G. marginata* extend from the half of a segmental embryonic unit to the half of the following one. Thus, if a diplosegment was originally considered as a set of two segments covered by a diplotergite and haplosegments were considered as single segments covered by a haplotergite, these terms can not be still applied with their original sense, although they can be maintained as descriptive terms.

Diplopods are characterised by an anamorphic post-embryonic development. In these arthropods, indeed, the young hatches from the egg with a short body, composed by few segments and usually provided by only three pairs of legs, and goes through a series of moults, following which the number of segments and pair of legs progressively

---

[3] Enghoff et al. 1993 referred to the phylogeny summarized in Fig. 1.

increases (Blower, 1985). The segments are added in the posterior part of the body, immediately before the last ring. There are three different types of anamorphosis (Enghoff, 1983). The term euanamorphosis is used when to each moult event corresponds the addition of new segments and this process continues for the whole life of the animal; euanamorphosis is typical of Julida and Colobognatha. If moults continue for the whole life of the animal but the addition of segments stops after a given stadium of development, this is described as hemianamophosis, and it is characteristic of Polyxenida and Glomerida. Finally, if at a given stadium of development, typically the one when the animal reaches sexual maturity, the moults stop, this is called teloanamorphosis, a behaviour that can be found in Chordeumatida and Polydesmida. The period from a moult to the following one is called a post-embryonic developmental stadium: the number of these stadia vary from species to species and in some cases, in particular in euanamorphic millipedes, there can be a high variation within the species.

Embryonic development of millipedes was studied using the classical descriptive approach (Anderson, 1973; Dohle, 1974; Johannsen and Butt, 1941) and recently using developmental genetic approach (Dove and Stollewerk, 2003; Janssen et al., 2004; Prpic et al., 2005). The post-embryonic development was the object of old, still useful studies by Verhoeff (summarized in Verhoeff 1926-32), followed by several other contributions summarized in Enghoff et al. (1993) and Hopkin and Read (1992).

Independently from the kind of anamorphosis, in male helmintomoph millipedes – that is all millipedes, exluded Penicillata and Pentazonia – during this period a sudden change involves only one or two pairs of non-terminal appendages, initially not different from all the appendages that precede and follow them, inside a long homonomous series. In adults, indeed, these appendages, localised in the seventh diplosegment, are modified into gonopods that are used as claspers or to transfer sperm during the copula (Hopkin and Read, 1992). In male polydesmid millipedes there is only one pair of gonopods (modified eighth pair of legs), while there are two pairs in the remaining groups (eighth and ninth pair of legs in julids).

A particularity of millipedes is that the genital openings are not situated on the same segment as the gonopods: gonopores, indeed, are in the third trunk unit, anterior to the gonopods. Before copulation, males should thus let the sperm arrive to gonopods, by rolling their body and getting the third and the seventh ring closer.

Gonopods have always received attention from myriapodologists, mainly for their taxonomic value (Brölemann, 1923; Enghoff, 1985; Mesibov, 2003; Schubart, 1947; Sierwald et al., 2003). Their fascinating high complexity and their amazing diversity has suggested a role in sexual competition, more important than the mere sperm transfer: in the spirosteptid *Orthoporus pyrhocephalus*, the use of the gonopods to remove sperm from competing males from female genitalia has been documented (Barnett et al., 1993). The knowledge about functional morphology of gonopods, however, remains limited (Tadler, 1993, 1996); most of the times, the role of each component of this appendages during copulation was only supposed and not verified by observations.

Moreover, very fragmentary is our understanding of gonopod formation, a subject that never received particular attention in the past. If we observe the seventh ring of a male millipede during post-embryonic development, we can recognise a sequence of events common to all the species: during the first stages of development the seventh trunk ring is identical to the others, with two pairs (eighth and ninth) of normal walking legs; in one of the following stadia the legs are substituted with extremely reduced structures, the squamiform appendages, that are later modified into

gonopods. In the past most of the studies about gonopod were merely descriptive and just concerned with their "finished" external structure in order to use them as a taxonomical character. No special attention was paid to the morphological modifications of the gonopods, not even in Sahli's papers (Sahli, 1983, 1985, 1990a, b), devoted to the julids with periodomorphosis[4] (Verhoeff, 1923).

(Miley, 1925), (Petit, 1973, 1976) and (Filka and Shelley, 1980) observed the sequence of changes that affects these appendages in some polydesmids, while (Berns, 1968) observed them in a spirobolid. In every case, all these studies give an interesting picture of this phenomenon, but exclusively under the point of view of external morphology. One of the few studies on internal anatomy that considered also gonopods was performed by (West, 1953) in a polydesmid. However, informations about the stadia before the appearance of gonopods are still lacking.

At any rate, just nobody focussed on the ontogenetic modifications that occur in this region and on the mechanisms that regulate it; moreover, histological and anatomical knowledge about gonopods and the seventh ring are limited, and almost absent in regard to the squamiform appendages.

QUESTIONS TARGETED BY THIS THESIS

The aim of the present work was to investigate the metamorphosis of the eighth and ninth pair of legs, corresponding to the seventh trunk ring, in four diplopod species: two blaniulid species *Nopoiulus kochii* (Gervais, 1847) and *Blaniulus guttulatus* (Bosc, 1792), the nemasomatid *Nemasoma varicorne* C.L. Koch, 1847, and the paradoxosomatid *Oxidus gracilis* (C.L. Koch, 1847). The first three species belong to two families of the order Julida, while the fourth species belongs to the order Polydesmida (Fig. 1). In this research, then, I tried to describe in detail the morpho-anatomical changes associated to non-systemic metamorphosis in diplopods, and to interpret these observations from an "evo-devo" point of view. Specifically, I performed a comparative analysis of external morphology and internal anatomy of the seventh ring and its appendages in these millipedes during the stadia of development involved in gonopod formation; in particular, I analyzed the last stadium where males present the eighth (and eventually the ninth) pair of locomotory appendages, the stadia where these are substituted by squamiform appendages and the first stadium with gonopods. The results I obtained, as they represent a complete picture of modifications of this body region, can be a starting point for the study of the cellular dinamics – apoptosis and proliferation processes – involved in gonopod formation. Moreover, the morpho-anatomical information that emerges from this thesis turn into a precious basis for the study of post-embryonic expression patterns of genes hypothetically involved this kind of metamorphosis.

---

[4] In some species, the differentiation of male gonopods is reversible; that is, a mature male may undergo a moult to an 'intercalary' stage with dedifferentiated gonopods, eventually followed by another moult leading it to a second reproductive stage with re-differentiated gonopods.

METHODS

On the four species mentioned above I performed mainly investigations of the external morphology, using optical and electronic scanning microscopy. Internal anatomy was described studying serial collections of paraffin sections of individuals stained with hematoxylin-eosin or, more frequently, with trichromic Mallory staining. In order to reconstruct the endoskeletal structures associated with gonopods, it was necessary to adopt a modern protocol based on confocal laser scanning microscopy; this technique takes advantage of the autofluorescence of cuticle, and allows to detect without dissections structures that are extremely difficult to handle in species of small size because dissection could cause damage to the parts of interest.

This thesis is articulated in three parts, as described below.

MANUSCRIPT 1 - GONOPOD FORMATION IN *OXIDUS GRACILIS* (C.L. KOCH 1847)

In this manuscript, I describe post-embryonic development of the millipede *Oxidus gracilis* (C.L. Koch 1847). This species has a teloanamorphic development, where the young hatches from the egg with a trunk of six rings and only three pairs of legs; adulthood is obtained following seven moults. In the last stadium, the body is formed by nineteen rings. The number of moults is fixed and each stadium is characterised by a defined number of rings with legs and of apodous rings. In polydesmid adult males only the first pair of appendages of the seventh ring is modified into gonopods, while the second pair does not undergo transformations.

As for the first and second stadium of development I could confirm observations found in the literature. Gonopod formation involves five consecutive moults, starting from individuals at the third stadium of development where the eighth pair of legs is present and identical to the others. The three following moults that lead from the fourth to the seventh stadium show a progressive increase in size of the squamiform appendages, which progressively became closer together towards a ventral median position.

Inside these structures, the presence of clusters of undifferentiated cells can be highlighted. These cells will be later responsible for gonopod formation. Most morphogenetic processes involving these cells happen during the moult between the seventh and the eighth stadium. During this moult it is possible to observe the extension of squamiform appendages and the formation of the articles of gonopods inside them, before the ecdysis. I finally present the situation of the seventh ring in the adult stadium: the gonopods are formed by three articles, where the terminal one presents a certain degree of complexity, mostly referring to the relations with the parts that compose it. In the adult stadium, I describe the novelties in the skeleto-muscular apparatus connected to the presence of gonopods, comparing them with the situation where only legs are present. In fact, in the first case, we can find some apodemes where the extrinsic muscles involved in the movement of gonopods find insertion. In addition

these changes in trunk organisation, we can also witness to the loss of the spiracles associated with the eighth pair of appendages.

MANUSCRIPT 2- CONSEQUENCE OF NON-SYSTEMIC METAMORPHOSIS IN THREE JULIDAN MILLIPEDES: AN ANATOMICAL POINT OF VIEW

This manuscript describes the modifications caused by non-systemic metamorphosis in two blaniulid species *Nopoiulus kochii* (Gervais, 1847) and *Blaniulus guttulatus* (Bosc, 1792), and in a nemasomatid species, *Nemasoma varicorne* C.L. Koch, 1847, giving a comparison between them. In male adults of the order Julida both pairs of appendages of the seventh ring are modified into gonopods. Julidan are characterized by euamamorphic development and moults are not stopped with the adult stadium. In *N. kochii* periodomorphosis occurs (see note 4, pg. 20). In this work I studied the last stadium in which the eighth and ninth pairs of legs are still present, the following stadium in which these are changed into squamiform appendages, and the first stadium with gonopods. I excluded specimens in the following stadia for the purpose to collect a sample of comparable animals, at the same point in gonopod development. When the eighth and ninth pairs of walking legs are present, no morpho-anatomical differences were found between the seventh ring and the others. Histological observations of squamiform appendages reveal some variations between the two blaniulids and the nemasomatid. In the first two species, inside the squamiform appendages there are clusters of undifferentiated packed cells, while in the third species these cells are in a loose organization. In addition, the squamiform appendages of *N. varicorne* possess, in the basal part, some differentiated tissue, more precisely there are two tiny muscles. Gonopod formation in these three species involves only one intermediate stadium, while in *O. gracilis* four stadia are required (see Drago, 2009a). The anatomical remodeling is more considerable than in the polydesmid millipede. I observed major changes in blaniulids, while the nemasomatid presents an intermediate condition. Ventrally the sternites are replaced by a gonopodal sac, a kind of cuticular membrane, within which gonopods are articulated. When the gonopods are in a resting position, the gonopodal sac causes a remarkable reduction of the internal ring volume. Histological observations reveal bulky gonopod apodemes with voluminous muscles associated to them. These structures cause a further reduction of internal ring volume that results in a dorsal displacement of internal organs, in particular of the ventral nerve corde and of the digestive tract. The size of the gonopodal sac and the fact that some extrinsic gonopod muscles are attached in different part of the sixth or the eighth ring cause a volume reduction also in these diplosegments. The situation is definitely less dramatic in *N. varicorne*, in which the dorsal dislocation of the internal organs is minimal. Processes involved in the non-systemic metamorphosis are similar in the three species, although the final results have different influence on the trunk anatomy. The existence of a stadium characterized by squamiform appendages seem to be a key point on millipedes gonopod formation, and probably represents a developmental constraint.

MANUSCRIPT 3 - NON-SYSTEMIC METAMORPHOSIS IN MALE MILLIPEDE APPENDAGES: LONG DELAYED, REVERSIBLE EFFECT OF AN EARLY LOCALIZED POSITIONAL MARKER?

In this work a mechanism is proposed that could explain the peculiarities of non-systemic metamorphosis. This hypothesis represent one of the conclusions obtained from the data of manuscript 1 and 2. I suggest that during early embryonic development a segmental marker is produced that remains unexploited throughout late embryonic and early post-embryonic development. This marker will be activated later, by a systemic signal, causing a remarkable anatomical change, strongly localized in a precise region of the body. Independently from the number of diplosegments present at hatching, gonopods of all helmintomorph millipede species are located in the seventh ring. Because of the fixation of this rule I hypothesize that the putative positional marker must be expressed during early embryogenesis. Position and nature of the gonopods suggest that the marker could be one of the *Hox* genes, probably *Abdominal-B* (*Abd-B*). Proving the validity of this hypothesis would demonstrate the existence of a cryptic developmental module that will be activated only months, or years, depending on the species, after it has been first laid down during early development. This marker could be also used repeatedly in the species with periodomorphosis event during their development.

REFERENCES

Abzhanov A, Popadic A, Kaufman TC, 1999. Chelicerate *Hox* genes and the homology of arthropod segments. Evolution & Development 1:77-89.

Anderson DT, 1973. Embryology and phylogeny in annelids and arthropods. Oxford & New York: Pergamon Press.

Arthur W, 2004. Biased embryos and evolution. Cambridge: University Press.

Barnett M, Telford SR, De Villiers CJ, 1993. Sperm displacement in a milipede? An investigation into genital morphology of the southern African spirostreptid millipede *Orthoporus pyrhocephalus*. Journal of Zoology 231:511-522.

Berns MW, 1968. The Development of the Copulator Organs (Gonopods) of a Spiroboloid Milliped. Journal of Morphology 126:447-462.

Blower JG, 1985. Millipedes Linnean Society Synopses of the British fauna(New Series). No. 35. London: E. J. Brill/Dr W. Bachuys.

Brena C, Chipman AD, Minelli A, Akam M, 2006. Expression of trunk *Hox* genes in the centipede *Strigamia maritima*: sense and anti-sense transcripts. Evolution & Development 8:252-265.

Brena C, Liu PZ, Minelli A, Kaufman TC, 2005. *Abd-B* expression in *Porcellio scaber* Latreille, 1804 (Isopoda: Crustacea): conserved pattern versus novel roles in development and evolution. Evolution & Development 7:42-50.

Brölemann HW, 1923. Blaniulidae Myriapodes. Biospeologica - Archives de zoologie expérimentale et générale 61:99-453.

Brusca RC, Brusca GJ, 2003. Invertebrates. 2nd edition. Sunderland: Sinauer Associates.

Cameron RA, Peterson KJ, Davidson EH, 1998. Developmental gene regulation and the evolution of large animal body plans. American Zoologist 38:609-620.

Carcamo HA, Abe TA, Prescott CE, Holl FB, Chanway, CP, 2000. Influence of millipedes on litter decomposition, N mineralization, and microbial communities in a coastal forest in British Columbia, Canada. Canadian Journal of Forest Research 30 (5):817-826.

Carroll SB, 2005. Endless forms most beautiful. The new science of evo-devo. Baror International, Inc., Armonk, New York, U.S.A.

Chen J, Zhou G, Edgecombe GD, Ramsköld L, 1995. Head segmentation in Early Cambrian *Fuxianhuia*: implications for arthropod evolution. Science 268:1339-1343.

Davidson EH, 1991. Spatial mechanisms of gene regulation in metazoan embryos. Development 113:1-26.

Davidson EH, 2001. Genomic Regulatory Systems: Development and Evolution. San Diego: Academic Press.

Demange JM, 1967. Recherches sur la segmentation du tronc des Chilopodes et des Diplopodes Chilognathes (Myriapodes). Mémoires du Muséum national d'histoire naturelle, N. S., série A 44:1-188.

Dohle W, 1974. The segmentation of the germ band of diplopods compared with other classes of Arthropods. Symposia of the Zoological Society of London 32:143-161.

Dohle W, 1980. Sind die Myriapoden eine monophyletische Gruppe? Eine Diskussion der Verwandtschaftsbeziehungen der Antennaten. Abhandlungen und Verhandlungen des Naturwissenschaftlichen Vereins in Hamburg 23:45-104.

Dohle W, 1988. Myriapoda and the ancestry of insects. Polytechnic, Manchester.

Dohle W, 1997. Myriapod-insect relationships as opposed to an insect-crustacean sister group relationships. In: Fortey RA and Thomas RH eds. Arthropod relationships. London: Chapman & Hall; 305-315.

Dove H, Stollewerk A, 2003. Comparative analysis of neurogenesis in the myriapod *Glomeris marginata* (Diplopoda) suggests more similarities to chelicerates than to insects. Development 130:2161-2171.

Drago L, Fusco G, Minelli A, 2008. Non-systemic metamorphosis in male millipede appendages: long delayed, reversible effect of an early localized positional marker? Frontiers in Zoology 5:5.

Drago L, 2009a. Gonopod formation in the hothouse millipede *Oxidus gracilis* (C.L. Koch 1847). MS 1: pg. 33-51

Enghoff H, 1981. A cladistic analysis and classification of the millipede order Julida. Zeitschrift für Zoologische Systematik und Evolutionsforschung 19:285-319.

Enghoff H, 1983. Acipes - a Macaronesian genus of millipedes (Diplopoda, Julida, Blaniulidae). Steenstrupia 9:137-179.

Enghoff H, 1985. The millipede family Nemasomatidae. With the description of a new genus, and a revision of *Orinisobates* (Diplopoda: Julida). Entomologica Scandinavica 16:27-67.

Enghoff H, 1991. A revised cladistic analysis and classification of the millipede order Julida with establishment of four new families and description of a new nemasomatoid genus from Japan. Zeitschrift für Zoologische Systematik und Evolutionsforschung 29:241-263.

Enghoff H, Dohle W, Blower JG, 1993. Anamorphosis in millipedes (Diplopoda) - the present state of knowledge with some developmental and phylogenetic considerations. Zoological Journal of the Linnean Society 109:103-234.

Filka ME, Shelley RM, 1980. Structure of the gonopod primordia of the milliped *Sigmoria latior* (Brölemann) (Polidesmida: Xystodesmidae). Transaction of the American Microscopical Society 99:173-179.

Giribet G, Richter S, Edgecombe GD, Wheeler WC, 2005. The position of crustaceans within Arthropoda – Evidence from nine molecular loci and morphology. In Koenemann S & Jenner R eds. Crustacea and Arthropod Relationships. Crustacean Issues 16:305-352. Boca Raton: CRC Press.

Grbic M, 2003. Polyembryony in parasitic wasps: evolution of a novel mode of development. International Journal of Developmental Biology 47:633-642.

Harzsch S, Vilpoux K, Blackburn DC, Platchetzki D, Brown NL, Melzer R, Kempler KE, Battelle BA, 2006. Evolution of Arthropod Visual Systems: Development of the Eyes and Central Visual Pathways in the Horseshoe Crab *Limulus polyphemus* Linnaeus, 1758 (Chelicerata, Xiphosura). Developmental Dynamics 235:2641-2655.

Hatini V, Green RB, Lengyel JA, Bray SJ, DiNardo S, 2005. The Drumstick/Lines/Bowl regulatory pathway links antagonistic Hedgehog and Wingless signaling inputs to epidermal cell differentiation. Genes & Development 19:709-718.

Hoffman RL, Golovatch SI, Adis J, de Morais JW, 2002. Diplopoda. In: Adis J editor. Arachnida and Myriapoda. Pensoft, Sofia-Moscow.

Hopkin SP, Read HJ, 1992. The Biology of Millipedes. Oxford: Oxford University Press.

Janssen R, Prpic N-M, Damen WGM, 2004. Gene expression suggests decoupled dorsal and ventral segmentation in the millipede *Glomeris marginata* (Myriapoda: Diplopoda). Developmental Biology 268:89-104.

Johannsen OA, Butt FH, 1941. Embryology of Insects and Myriapods - The developmental history of insects, centipedes, and milipedes from egg deposition to hatching. New York and London: McGraw-Hill.

Kraus O, 1990. On the so-called thoracic segments in Diplopoda. In: Minelli A editor. Proceedings of the 7th International Congress of Myriapodology. Brill EJ, Leiden 480:63-68.

Linder F, 1952. Contributions to the morphology and taxonomy of the Branchiopoda Notostraca, with special reference to the north American species. Proceedings of the United States National Museum 102:1-69.

Liu PZ, Kaufman TC, 2005. *even-skipped* is not a pair-rule gene but has segmental and gap-like functions in *Oncopeltus fasciatus*, an intermediate germband insect. Development 132 (9):2081-2092.

Manton SM, 1961. The evolution of arthropodan locomotory mechanisms. Part 7. Functional requirements and body design in Colobognatha (Diplopoda),

together with a comparative account of diplopod burrowing techniques, trunk musculature and segmentation. Journal of the Linnean Society of London - Zoology 44:383-461.

Mesibov R, 2003. The millipede genus *Gasterogramma* (Diplopoda: Polydesmida: Dalodesmidae) in Tasmania, Australia, with descriptions of seven new species. Memoirs of Museum Victoria 60:207-219.

Miley HH, 1925. Development of the male gonopods and life history studies of a polydesmid millipede. Ohio: Ohio State University.

Miley HH, 1930. Internal anatomy of *Euryurus erythropygus* (Brandt). (Diplopoda). Ohio Journal of Science 30:229–255.

Minelli A, 2003. The development of animal form. Cambridge: Cambridge University Press.

Minelli A, 2004. Bits and Pieces. Science 306:1693-1694.

Müller GB, 2008. Evo-devo as a discipline. In Minelli A & Fusco G eds. Evolving Pathways:5-30. Cambridge: Cambridge University Press.

Nijhout HF, 1994. Insect hormones. Princeton, NJ: Princeton University Press.

Olesnicky EC, Brent AE, Tonnes L, Walker M, Pultz MA, Leaf D, Desplan C, 2006. A *caudal* mRNA gradient controls posterior development in the wasp *Nasonia*. Development 133:3973-3982.

Peterson KJ, Andrew CR, Davidson EH, 1997. Set-aside cells in maximal indirect development: evolutionary and developmental significance. BioEssays 19:623-631.

Peterson KJ, Davidson EH, 2000. Special Feature: Regulatory evolution and the origin of the bilaterians. PNAS 97:4430-4433.

Petit G, 1973. Étude Morphologique et Expérimentale de la Métamorphose d'un Appendice Ambulatoire en Gonopode Chez le Diplopode *Polydesmus angustus* Latz. Annales D'Embryologie et de Morphogenése 6:137-149.

Petit G, 1976. Développements comparés des appendices copulateurs (gonopodes) chez *Polydesmus angustus* Latzel et *Brachydesmus superus* Latzel (Diplopodes: Polydesmidae). International Journal of Insect Morphology and Embryology 5:261-272.

Prpic N-M, Janssen R, Damen WGM, Tautz D, 2005. Evolution of dorsal-ventral axis formation in arthropod appendages: *H15* and *optomotor-blind/bifid*-type T-box genes in the millipede *Glomeris marginata* (Myriapoda: Diplopoda). Evolution & Development 7:51-57.

Ramsköld L, Jun-Yuan C, Edgecombe GD, Gui-Qing Z, 1996. Preservational folds simulating tergite junctions in tegopeltid and naraoiid arthropods. Lethaia 29:15-20.

Sahli F, 1983. Influence de facteurs externes sur la periodomorphose chez le Diplopode *Cylindroiulus nitidus* (Verh.). Premiers resultats experimentaux. Comptes rendus des séances de l'Académie des Sciences, Serie 3 296:89-91.

Sahli F, 1985. Périodomorphose et males intercalaires des Diplopodes Julidae: une nouvelie terminologie. Bulletin Scientifique de Bourgogne 38:23-31.

Sahli F, 1990a. On post-adult moults in Julida (Myriapoda, Diplopoda). Why do periodomorphosis and intercalaries occur in males? In Minelli A. editor. Proceedings of te 7th International Congress of Myriapodology. Brill EJ.

Sahli F, 1990b. Recherches sur le cycle des mâles et la fréquence des intercalaires d'Ommatoiulus sabulosus (Myriapoda, Diplopoda, Julidae) en Bourgogne. Inversion expérimentale du nombre des intercalaires et des adultes en automne. Bulletin Scientifique de Bourgogne 43:51-59.

Seastedt TR, Tate CM, 1981. Decomposition rates and nutrient contents of arthropod remains in forest litter. Ecology 62:13-19.

Schubart O, 1947. Os Diplopoda da viagem do naturalista Antenor Leitão de Carvalho aos Rios Araguaia e Amazonas em 1939 e 1940. Boletim do Museu Nacional (Nova Serie) 82:1-74.

Seifert B, 1932. Anatomie und Biologie des Diplopoden Strongylosoma pallipes Oliv. Zeitschrift für Morphologie und Ökologie der Tiere 25 (2-3):362-507.

Sierwald P, Bond JE, 2007. Current Status of the Myriapod Class Diplopoda (Millipedes): Taxonomic Diversity and Phylogeny. Annual Review of Entomology 52:401-420.

Sierwald P, Shear WA, Shelley RM, Bond JE, 2003. Millipede phylogeny revisited in the light of the enigmatic order Siphoniulida. Journal of Zoological Systematics and Evolutionary Research 41:87-99.

Stollewerk A, 2000. Changes in cell shape in the ventral neuroectoderm of *Drosophila melanogaster* depend on the activity of the achaete-scute complex genes. Development Genes and Evolution 210:190-199.

Stollewerk A, Weller M, Tautz D, 2001. Neurogenesis in the spider *Cupiennius salei*. Development 128:2673-2688.

Tadler A, 1993. Genitalia Fitting, Mating Behaviour and Possible Hybridization in Millipedes of the Genus *Craspedosoma* (Diplopoda, Chordeumatida, Craspedosomatidae). Acta Zoologica 74:215-225.

Tadler A, 1996. Functional morphology of genitalia of four species of julidan millipedes (Diplopoda: Nemasomatidae; Julidae). Zoological Journal of The Linnean Society 118:83-97.

Verhoeff KW, 1923. Periodomorphose. 96. Diplopoden-Aufsatz (Fortsetzung). - Zoologischer Anzeiger 56 (11-13):241-254.

Verhoeff KW, 1926-32. Diplopoda. Bronn's Klassen und Ordnungen des Tier-Reichs, 5:2. Leipzig: Akademische Verlagsgesellschaft.

West WRJ, 1953. An Anatomical Study of the Male Reproductive System of a Virginia Millipede. Journal of Morphology 93:123-175.

Wilson HM, 2002. Muscular anatomy of the millipede *Phyllogonostreptus nigrolabiatus* (Diplopoda: Spirostreptida) and its bearing on the millipede "Thorax". Journal of Morphology 251:256-275.

Wilson HM, Anderson LI, 2004. Morphology and Taxonomy of Paleozoic Millipedes (DIPLOPODA: CHILOBOGNATHA: ARCHIPOLYPODA) from Scotland. Journal of Paleontology 78:169-184.

Figure 1. Phylogenetic relationships among the millipede orders. Modified from Sierwald and Bond (2007).
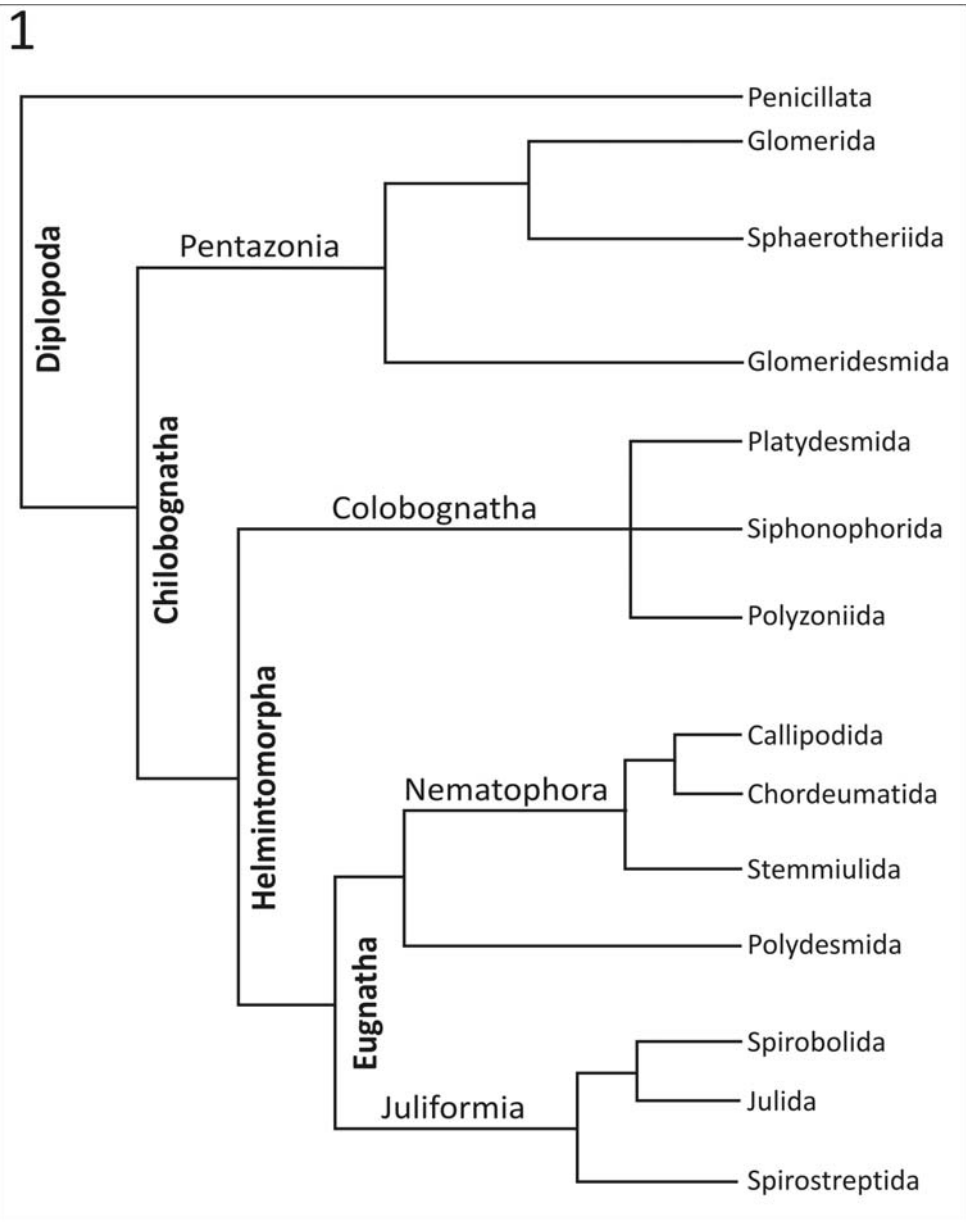
Gonopod formation in the hothouse millipede *Oxidus gracilis* (C.L. Koch 1847)

ABSTRACT

Adult males of helmintomorph millipedes possess in the seventh trunk ring specialized appendages, the gonopods, that are used for sperm transfer. These differentiate in an advanced phase of post-embryonic development. Here is described a morphological and anatomical study of seventh ring and its appendages during subsequent developmental stadia. The comparison between juvenile and adult males provides information on the mechanisms involved in the origin of gonopod. The study includes histological observations performed during the last moult event, a period characterized by the major changes in gonopod formation.

INTRODUCTION

In contrast to the still modest level of investigation hitherto obtained for the other aspects of millipede morphology, the male gonopods of helmintomorph Diplopoda have received remarkable attention for their fascinating high complexity and for their amazing diversity, which turns them into body parts of primary taxonomic value. Therefore several past authors offered us a rich number of drawing (e.g., Brölemann, 1923; Schubart, 1947), replaced in a more recent time by fascinating SEM pictures (e.g., Enghoff, 1985; Mesibov, 2003; Sierwald et al., 2003). The result of this trend is that gonopods usually received more attention than all the other parts of the animal, insomuch as for some species in literature only gonopod descriptions are available while other important information, like the life cycle and the development, is still unknown.

In contrast to the abundant description of their morphological structure and diversity only few papers have been devoted to gonopod formation. Berns (1967) and Dhaenens and VandenSpiegel (2006) described the morphological modification of copulatory appendages in two Spirobolid millipedes, and Petit has been one of the most active authors in this field, providing detailed morphological information for some species of Polydesmidae (e.g., Petit, 1973, 1976).

Gonopod formation must be considered in the context of millipede post-embryonic development, which is characterized by anamorphosis. The juvenile hatches with a short body, usually with only three pairs of legs, and undergoes a series of moults through which the number of segments and leg pairs progressively grows. Adult specimens differ from the juvenile stadia mainly in the presence of new segment and the increase in size.

Among millipedes, males of the Helmintomorpha (Diplopoda except bristly and pill millipedes), undergo a remarkable change in the aspect of their seventh trunk ring (where ring is considered as a morphological unit generally equivalent to two conventional body segments; (cf. Enghoff et al., 1993). This modification affects in particular the appendages (eighth and ninth pairs), that initially are of locomotory type, but in the adult turn into gonopods, used for indirect sperm transfer to the female gonopore.

In the Polydesmida (the largest order of millipedes in terms of number of genera and species, with a worldwide distribution; Simonsen 1990) there is only one pair of appendages modified as gonopods, the eighth one. These animals are characterized by a fixed number of moults during their life (teloanamorphosis). The males are recognizable

beginning with the fourth stadium because of the loss of the eighth pairs of legs (sexual dimorphism), that are replaced by a pair of tiny and rounded appendages called squamiform appendages or gonopod primordia. This condition persists until the adult stadium, when this structures are replaced by a couple of gonopods. The modification of the eighth pair of appendages makes the seventh ring something unique in a developmental point of view: in fact, it represents a case in which a developmental change affects, at a given time during the post-embryonic growth, only one non-terminal pair of appendages, initially identical to those that precede and follow them amidst a long homonomous series.

In this paper, I analyze the external morphology and the internal anatomy of the seventh ring and its appendages in *Oxidus gracilis* (C.L. Koch 1847) males, in different developmental stadia. This study includes observations performed during the last moult event (in particular in the pharate phase between seventh to eighth stadium), a period characterized by the major changes in gonopod formation.

Causey (1943) divided each moulting process in four periods: during the first one, animals build a moulting chamber with the substratum and organic material discharged from the anus; during the second one the animal body is detaching from the exoskeleton and increasing in length and size; the third period corresponds to the ecdydis with the animal that breaks and leaves the exuvia; the last period is necessary to the hardening of the cuticle. During the moult, the cuticle becomes soft because part of the calcium of the exoskeleton is dissolved and absorbed and the enzymes cause a partial digestion of the exoskeleton. For this reason, from the second period to the end of the process, the individual is maximally vulnerable. The pharate phase corresponds to the moulting period between apolysis and ecdysis (Nijhout, 1994): the epidermis is already detached from the old cuticle but the animal still remains inside the exoskeleton of the previous stadium. For the first time the description of millipede gonopods external morphology is accompanied by histological investigations.

MATERIALS AND METHODS

Animals

Specimens of *Oxidus gracilis* were collected in litter in Marcon di Venezia (Venice, Italy 45°33'44''N 12°17'40''E). Breeding cultures were kept at 20°C in plastic Petri dishes with an approximately 5 mm layer of 2% agar (Blower, 1974; Enghoff, 1976). Original litter material, pieces of potatoes and decaying wood (*Populus, Salix*), was used as food. Males were reared until the desired stadium of development, then they were killed using ethyl acetate vapour at 4°C, in order to prevent rolling up, and immediately fixed in a mixture of 2,5% glutaraldehyde and 2% formaldehyde in PBS. For optimal fixation, the animals were cut in two or more pieces before putting them into the fixative where they were preserved for at least 48 hours at 4°C.
In vivo observations were made using a Leica MZ125 stereomicroscope, fitted with Leica DFC 420 digital camera using Leica Application Suite software (version 2.6) and Helicon Focus software (version 3.10). The light microscope used for observation of dissected gonopods was a Leica DMRXA, fitted with an Evolution MP digital camera using ImagePro software, including the EDF-stack (Enhanced Depth of Field) algorithm.

Scanning electron microscopy

Fixed specimens were washed at least six times in PBS + 0.1% Triton®X 100, 20 minutes each; then they were dehydrated through a graded ethanol series (15%, 30%,

50%, 70%, 80%, 95%, 100% at least 1 hour for each step). For a complete dehydration the samples were immersed in hexamethyldisilazane (Nation, 1983), air dried at room temperature and finally mounted on aluminium stubs. The samples were immediately coated with gold or platinum-palladium. The investigation was made with a Cambridge Stereoscan 260, or with a Jeol JSM-6490, or with a Jeol JSM-6335-F scanning electron microscope.

To examined the endoskeletal component, the internal soft tissues of several samples were digested. Fixed specimens were washed as described above, dissected and immersed in KOH 5% at 50°C for the required time (at least 48 hours). Immersion in the KOH solution removes the soft tissues while leaving the hard cuticular exoskeleton intact. The remaining cuticle was rinsed with distilled water, dehydrated and treated as described above.

Histology

Internal anatomy was studied through serial paraffin sections. Fixed animals were initially washed in PBS + 0,1% Triton®X 100 at least six time, each time for 20 minutes, and treated overnight with chitinase from *Streptomyces griseus* (0.7 u/ml at 37 °C in HEPES buffer) to soften the exoskeleton; the samples were then washed in PBS + 0,1% Triton®X 100 and decalcified overnight in EDTA 0.5 M pH 8 at 4°C. After washing in PBS + 0,1% Triton®X 100, the specimens were dehydrated through a graded ethanol series (15%, 30%, 50%, 70%, 80%, 95%, 100% at least 1 hour for each step), then immersed in xilene, and embedded in paraffin. Sagittal, frontal and transversal sections were made at a thickness between 7 to 12 µm and stained with Mallory's triple stain (Pantin, 1964).

Slides were observed with a Leica DM5000B microscope, fitted with a Leica DFC 300 FX camera.

Pictures were elaborated using CorelPHOTO-PAINT and CorelDRAW (version 11) software.

RESULTS

The external morphology of seventh ring and its appendages in *Oxidus gracilis* was described from the first to the eighth (and last) developmental stadium, while the internal anatomy was studied only from the fourth to the eighth stadium. For each stadium specimens of different ages were used: no differences was found within each stadium. Specimens were observed also during the moulting period and the data of the pharate phase of last moult are summarized in the last paragraph of the results.

Morphology

Like all polydesmid millipedes *O. gracilis* has teloanamorphic development (Fig. 2). Juveniles emerge from the eggs (Fig. 3) usually 9-10 days after deposition (range 7 to 13 days) as described by Causey (1943). The first stadium long 18-24 hours presents a trunk of six rings with three pairs of legs (Fig. 4). During the second stadium, the animals have eight rings and six pairs of legs (Fig.5). The seventh ring is present since this stadium but it is still without appendages. These appear after the following moult. During the third stadium, the trunk of young millipedes is composed by eleven rings, with eleven pairs of legs in both sexes (Figs. 6, 7). In fact, until the following moult, males and females are identical in their external morphology. The seventh ring is

identical to the others and the eighth pair of legs are conventional locomotory appendages, composed by coxa, trochanter, praefemur, femur, postfemur, tibia and tarsus. Two pairs of spiracles open in each diplosternite, each one slightly anterior and lateral to the coxae. From the fourth stadium on, it is possible to distinguish males from females because of the absence of the eighth pair of legs. In the male, these are replaced by a pair of tiny rounded appendages (squamiform appendages or gonopod primordia) which occupy the same position of the coxae of the other legs (Fig. 8). Squamiform appendages are oriented laterally compared to the median axis of the body. The anterior pair of spiracles in this ring are now slightly deformed, showing an oval shape. From the fifth stadium to the seventh one, the sternal portion between the squamiform appendages is progressively reduced (Figs. 9-11). After each moult, these appendages appear bigger, closer, and more embedded in the sternite than in the previous stadia. The spiracles are no more present.

With the seventh and last moult, the males achieve the adult stadium (the eighth one). At this point the gonopods are finally formed (Figs. 12-17). They appear like complex and bulky structures oriented in posterior-anterior direction. These appendages consist in three principal parts: a basal gonocoxa, a medial praefemur and a distal telopodite. The gonocoxa is characterized by a cylindrical shape and it is basally connected to the sternite while two articles are inserted at the distal part: a big one, the praefemur, and a small one, the cannula. The cannula has a hook shape and is situated on the medial side of the gonopod: because of its small size and position, if the gonopod is not removed from the body it is not possible to see it. In resting position the apex of the cannula, fits into a small depression of the basis of the praefemur, the spermatic groove. The praefemur is entirely covered by setae and forms a knee joint between gonocoxa and telopodite. The telopodite is the distal article of the gonopod and is composed by a basal subconical part articulated to the praefemur and a complex distal part with four elongated elements: the solenomerite, the tibiotarsus, and the femoral and praefemoral processes. Femoral and praefemoral processes have similar length; the first one has a flattened end, while the other ends with two branches. The medial branch has the terminal part covered by small spines. The solenomerite is the most intricate element: the basal part is formed by a tubular portion partially rounded by a cuticular lamella, while the distal part is a flagellar structure partially inserted in the medial branch of the praefemoral process. The tibiotarsus, situated opposite to the solenomerite, is the shortest element and it has a hook shape.

Anatomy

The internal anatomy has been studied to investigate the degree of structural change associated to the modifications in the eighth pair of appendages. During the third stadium, no difference is found between the seventh ring and the others: the extrinsic muscles of the eighth pair of legs are in the typical arrangement as described by, e.g, Silvestri (1903) and Manton (1977). The internal arrangement of the ring is similar to the other ones. The spiracles open internally into a spacious atrium or tracheal pouch: this provides an apodeme for the attachment of the extrinsic muscles of the coxae.

In the fourth stadium, the extrinsic musculature of the eighth pair of legs is absent; in particular the coxal muscles (protractor and retractor), that in the previous stadium are inserted in the tracheal pouch, are not present. The squamiform appendages contain a group of tightly packed cells that seem to be an undifferentiated tissue (Fig. 18).

The fifth stadium shows the same internal arrangement of the fourth one (Fig. 19).

During the sixth and the seventh stadium, the undifferentiated tissue occupies the distal area of the appendages, while, in the proximal part, some tiny muscles are present (Figs 20, 21).

At the adult stadium, the internal anatomy sensibly changes, because of the presence of the gonopods. Each gonopod possesses apodeme (Fig. 25) that offers insertion points for a pair of antagonistic muscles (protractor and retractor) (Fig. 22). One single trasversal muscle (abductor) connects right and left gonocoxae (Fig. 23). The arrangement of these extrinsic muscles is summarized in Fig. 26. Each gonocoxa posses three muscles: extensor and flexor of prefemorite and an abductor of the cannula. The prefemorite and telopodite anatomy is less understood: no musculature are detected inside this articles and the first one seems to contain mainly connective tissues while in the second one is present only the epidermid (Fig. 24).

Pharate phase of the eighth stadium

The moulting process between the seventh and the eighth stadium lasted about 20 days in agreement with Causey (1943) data. During this quite long period is not easy to distinguish the beginning of the pharate phase (Fig. 27), also for the high individual diversity in the duration of this moult (from 12 to 29 days, N=19). To identify the major processes in gonopod formation, frequent observations of the specimens were required. Three steps can be distinguished:

a) the squamiform appendages are elongated in proximodistal direction and are formed by three parts, separeted by two constrictions; nothing is identifiable inside the appendages (Fig. 28).

b) a small increase in length accompanied by the formation of an elongated structure inside the appendage (Fig. 29).

c) the distal processes of the gonopod telopodite are recognizable inside the squamiform appendages (Fig. 30).

The histological investigations confirm the *in vivo* observations. During the first step, only an epidermic layer was identifiable (Fig. 31). In the second step, the entire appendage contains a mass of tightly packaged cells (probably undifferentiated tissue), that appears compact in the basal part while it is branched in two directions distally (Fig. 32). Finally, this tissue starts to form the gonopods and in the distal part are recognizable several branchings that probably correspond to the distal telopodite parts (Fig. 33).

CONCLUSIONS

The formation of gonopods of *Oxidus gracilis* occurs across five moult events starting from specimens in the third developmental stadium, which are provided with a normal eighth pair of legs. The loss of this pair of locomotory appendages represents the first visible step in this complex process. It is reasonable to think that this event is under hormonal control, like the loss of the larval legs in the Lepidoptera *Manduca sexta* (Weeks & Truman 1985) corresponding to an high level of ecdysteroids. Unfortunately there are no available information about the hormones involved in moulting process in diplopods. During the following three developmental stadia the squamiform appendages undergo an increase in size and the pair of tracheal openings coupled with the eighth pair of appendages disappears. In this stadium the cells contained in the squamiform appendages show a moderate proliferation activity. Most of the morphogenetic processes happen in a quite short period as confirmed by observations

effected during the pharate phase of last moult. Finally at the eighth stadium a couple of bulky and complex gonopods are completely formed. In other arthropods, several morphogenetic events occur also during the intermoult period: in *Lithobius forficatus*, for instance, new neuromeres are differentiating continuously during the intermoult (Minelli et al., 2006). At the contrary eighth pair of appendages of *O. gracilis* has no relevant modifications for the duration of each stadium; moreover in the period between the begin of the fourth and the end of seventh stadium the changes are minimal.

The association of a high level of morphogenesis with the moult to the adult stadium is obvious and not rare within Arthropods, but could not explain the reason why four "preliminary stages" with squamiform appendages are required. The fact that in all Helmintomorpha millipedes the gonopods formation is preceded by at least one stadium with squamiform appendages underlines their crucial role in this process. The cluster of cells responsible for gonopod formation was not identified in specimens of the first three developmental stadia: this cells appears for the first time with the squamiform appendages. It is realistic to assume that the stadium (or the stadia) with squamiform appendages represent a developmental constrain for the organization of the cluster of undifferentiated cells that will form gonopods in the adult. Also the presence of the eighth pair of legs during the third stadium could be explained using a developmental approach: probably all juvenile millipedes undergo an identical developmental module which includes the formation of locomotory appendages in the seventh ring, until the sexual differentiation that could be activated by the production of a male signal (hormone). This signal could turn on a molecular marker that identifies gonopod position (Drago et al., 2008) only in this moment of the millipede life: this would explain why the formation of the eighth pair of legs in the juvenile males is not blocked. This hypothesis is supported by the fact that all juvenile millipedes have locomotory appendages in the seventh ring at least during one developmental stadium; in addition, the most preserved peculiarity of gonopods among millipedes is their precise position in this trunk unit.

REFERENCES

Berns MW, 1968. The development of the copulatory organs (gonopods) of a spiroboloid milliped. Journal of Morphology 126:447-461.

Blower JG, 1974. Food consumption and growth in a laboratory population of *Ophyiulus pilosus* (Newport). Symposia of the Zoological Society of London 32:527-551.

Brölemann HW, 1923. Blaniulidae Myriapodes. Biospeologica - Archives de zoologie expérimentale et générale 61:99-453.

Causey NB, 1943. Studies on the life history and the ecology of the hothouse millipede, *Orthomorpha gracilis* (C.L. Koch 1847). The American Midland Naturalist 29:670-682.

Dhaenens M, VandenSpiegel D, 2006. Contribution to the study of the post-embryonic development and reproduction of the African millipede *Epibolus pulchripes* (Gerstäcker, 1873)(Diplopoda, Pachybolidae). Belgian Journal of Zoology 136:43-51.

Drago L, Fusco G, Minelli A, 2008. Non-systemic metamorphosis in male millipede appendages: long delayed, reversible effect of an early localized positional marker? Frontiers in Zoology 5:5.

Enghoff H, 1976. Parthenogenesis and bisexuality in the millipede, *Nemasoma varicorne* C. L. Koch, 1847 (Diplopoda: Blaniulidae). Morphological, ecological, and biogeographical aspects. Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening 139:21-59.

Enghoff H, 1985. The millipede family Nemasomatidae. With the description of a new genus, and a revision of *Orinisobates* (Diplopoda: Julida). Entomologica Scandinavica 16:27-67.

Enghoff H, Dohle W, Blower JG, 1993. Anamorphosis in millipedes (Diplopoda) - the present state of knowledge with some developmental and phylogenetic considerations. Zoological Journal of the Linnean Society 109:103-234.

Manton SM, 1977. The Arthropoda: habits, functional morphology and evolution. London: Clarendon Press.

Mesibov R, 2003. The millipede genus *Gasterogramma* (Diplopoda: Polydesmida: Dalodesmidae) in Tasmania, Australia, with descriptions of seven new species. Memoirs of Museum Victoria 60:207-219.

Minelli A, Brena C, Deflorian G, Maruzzo D, Fusco G, 2006. From embryo to adult-beyond the conventional periodization of arthropod development. Development Genes and Evolution 216:373-383.

Nation JL, 1983. A New Method Using Hexamethyldisilazane for Preparation of Soft Insect Tissue for Scanning Electron Microscopy. Stain Technology 58:347-351.

Nijhout HF, 1994. Insect hormones. Princeton, NJ: Princeton University Press.

Pantin CFA, 1964. Notes on Microscopical Technique for Zoologists. Cambridge: Cambridge University Press.

Petit G, 1973. Étude Morphologique et Expérimentale de la Métamorphose d'un Appendice Ambulatoire en Gonopode Chez le Diplopode *Polydesmus angustus* Latz. Annales D'Embryologie et de Morphogenése 6:137-149.

Petit G, 1976. Développements comparés des appendices copulateurs (gonopodes) chez *Polydesmus angustus* Latzel et *Brachydesmus superus* Latzel (Diplopodes: Polydesmidae). International Journal of Insect Morphology and Embryology 5:261-272.

Schubart O, 1947. Os Diplopoda da viagem do naturalista Antenor Leitão de Carvalho aos Rios Araguaia e Amazonas em 1939 e 1940. Boletim do Museu Nacional (Nova Serie) 82:1-74.

Sierwald P, Shear WA, Shelley RM, Bond JE, 2003. Millipede phylogeny revisited in the light of the enigmatic order Siphoniulida. Journal of Zoological Systematics and Evolutionary Research 41:87-99.

Simonsen Å, 1990. Phylogeny and Biogeography of the Millipede Order Polydesmida, with Special Emphasis on the Suborder Polydesmidea. Museum of Zoology, University of Bergen, Norway.

Silvestri F, 1903. Classis Diplopoda. Vol. 1. Anatome: Pars I, Segmenta, Tegumentum, Musculi. - Acari, Myriapoda et Scorpiones huscque in Italia reperta. Portici.

Weeks JC, Truman JW, 1985. Indipendents steroid control of the fates of motoneurons and their muscles during insect metamorphosis. The Journal of Neuroscience, 5:2290-2300.

Figure 2. Teloanamorphosis scheme in *Oxidus gracilis*.

Figure 3. Egg of *O. gracilis*. SEM.

Figure 4. First developmental stadium, lateral view. SEM.

Figure 5. Second developmental stadium, lateral view. SEM. Arrow indicates seventh trunk ring.

Figure 6. Third developmental stadium, ventral view. SEM. Arrow indicates seventh trunk ring.

Figure 7. Higher magnification of previous sample, ventral view. SEM. Arrow indicates seventh trunk ring.

Figure 8. Male, fourth developmental stadium. Ventral view of the seventh ring with squamiform appendages. SEM. sp indicates the spiracles associated to eighth pair of appendages.

Figure 9. Male fifth developmental stadium. Ventral view of the seventh ring with squamiform appendages. SEM.
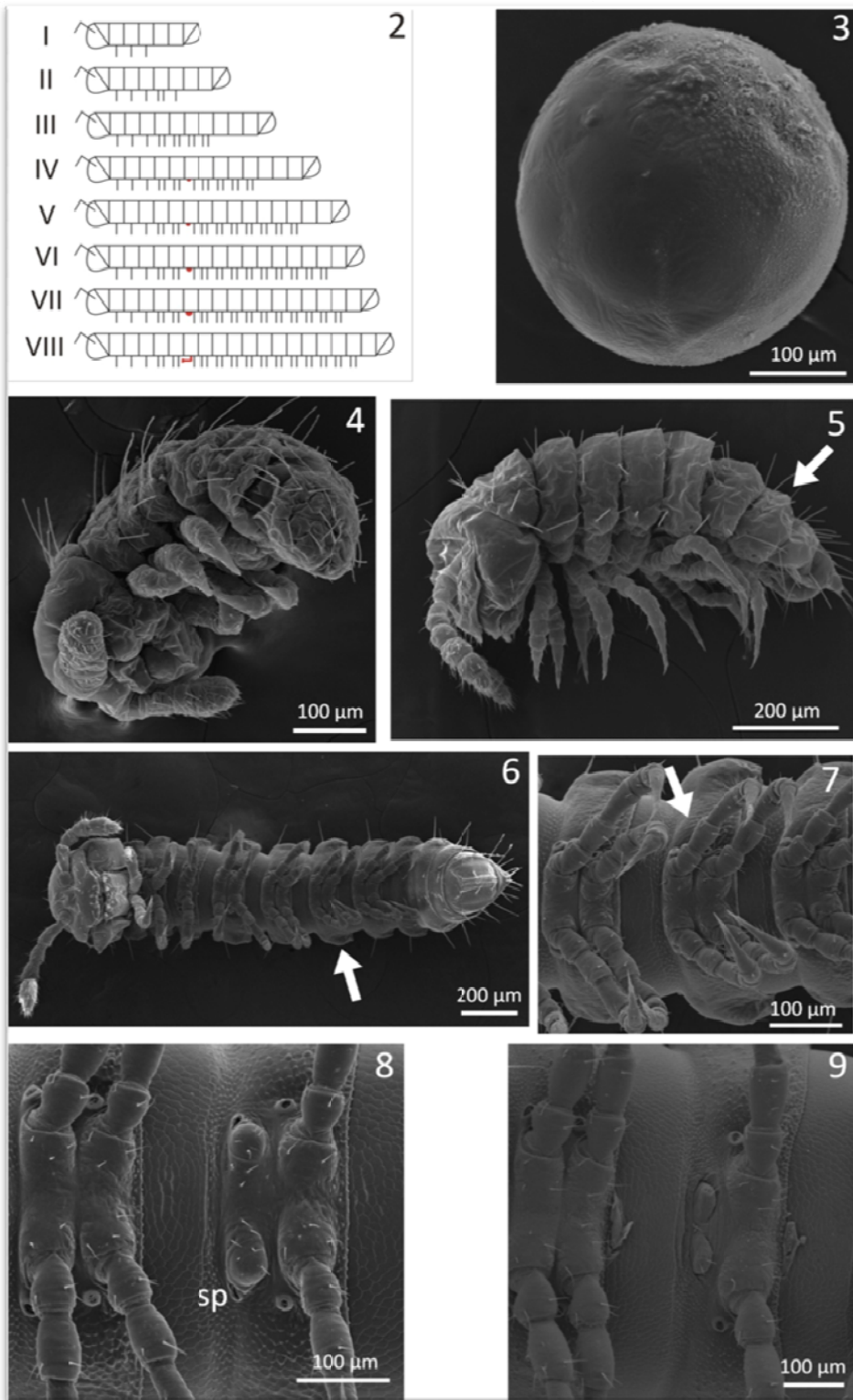
Figure 10. *O. gracilis* male, sixth developmental stadium. Ventral view of the seventh ring with squamiform appendages. SEM.

Figure 11. Seventh developmental stadium. Ventral view of the seventh ring with squamiform appendages. SEM.

Figure 12. Eighth developmental stadium. Ventral view of the seventh ring with gonopods. SEM.

Figure 13. *O. gracilis* right gonopod, lateral view of the external side. SEM.

Figure 14. Right gonopod, lateral view of the internal side. SEM.

Figure 15. Higher magnification of the cannula. The apex is fitted in the spermatic groove of the praefemorite. SEM.

Figure 16. *O. gracilis* left gonopod, lateral view of the internal side. Light microscope (fused EDF-stack).

Figure 17. Right gonopod, solenomerite inserted in the medial branch of the praefemoral process of the telopodite. SEM.

G = gonocoxa; P = praefemorite; T = telopodite; c = cannula; s = solenomerite;

tt = tibiotarsus; fp = femoral process; pp =praefemoral process
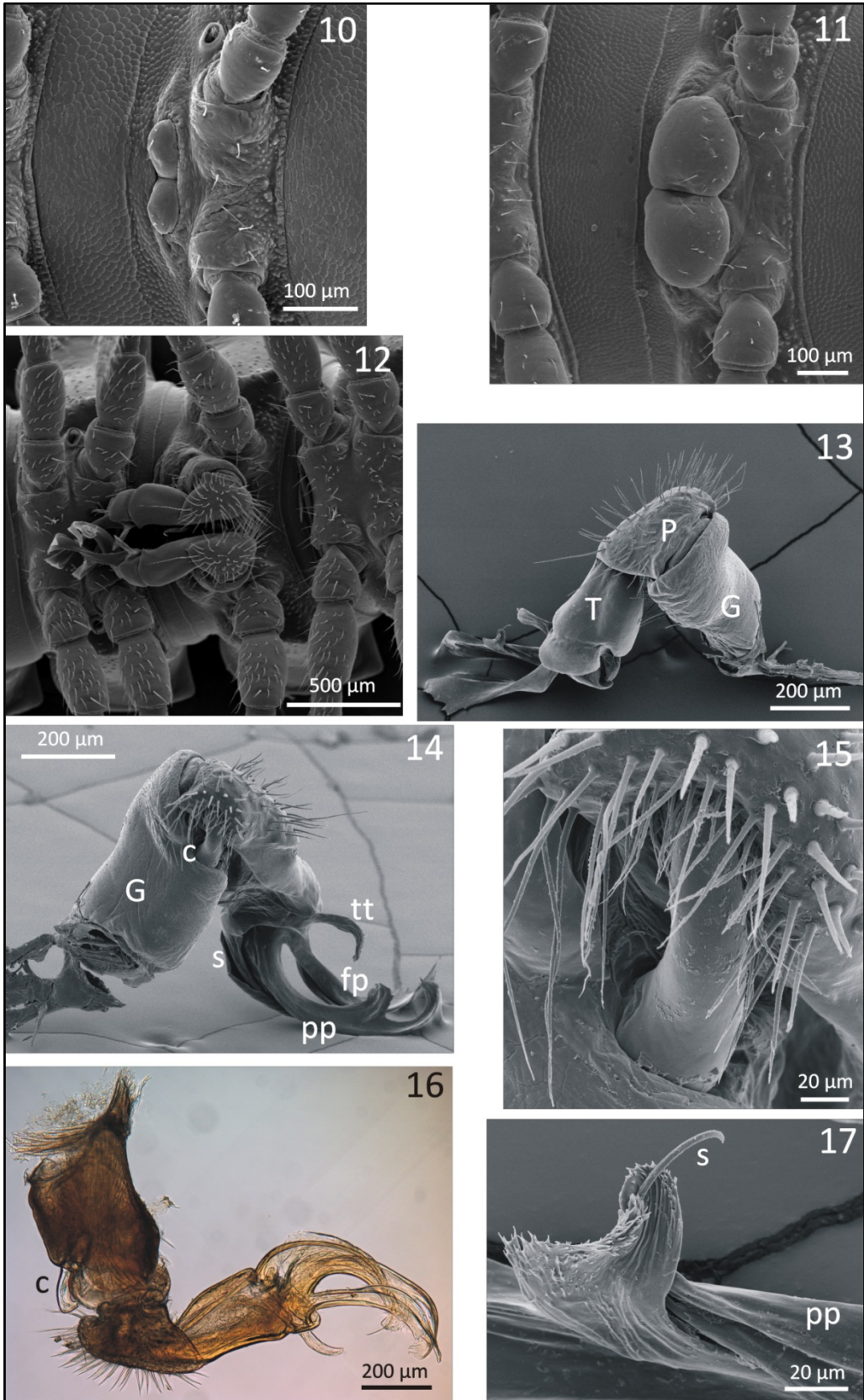
Figure 18. Para-midsagittal paraffin section of the squamiform appendage during the fourth stadium *O. gracilis* male. Mallory's triple stain. cc indicate the cluster of tightly packed cells.

Figure 19. Para-midsagittal paraffin section of the squamiform appendage during the fifth stadium. Mallory's triple stain. cc indicate the cluster of tightly packed cells.

Figure 20. Para-midsagittal paraffin section of the squamiform appendage during the sixth stadium. Mallory's triple stain. sm indicate the tiny muscle of squamiform appendages.

Figure 21. Para-midsagittal paraffin section of the squamiform appendage during the seventh stadium. Mallory's triple stain.

Figure 22. Frontal paraffin section of the seventh ring during the eighth stadium. Mallory's triple stain. gr = gonopod retractor muscle; gp = gonopod protractor muscle; gA =gonopod apodeme; vA = ventral apodeme.

Figure 23. Transverse paraffin section of seventh ring during the eight stadium. Mallory's triple stain. ga indicates the gonopod abductor muscle.

Figure 24. Para-midsagittal paraffin section of the gonopod. Mallory's triple stain.
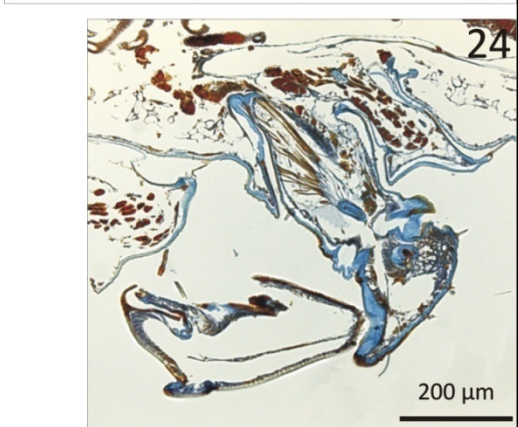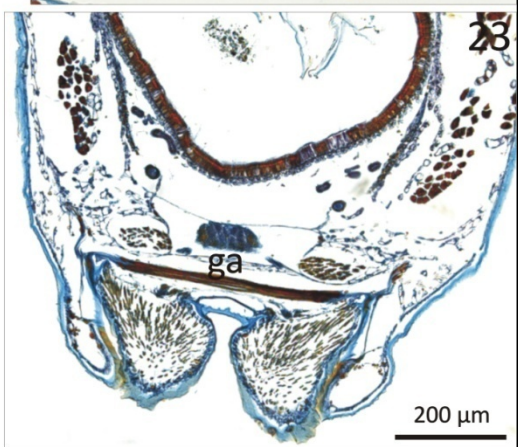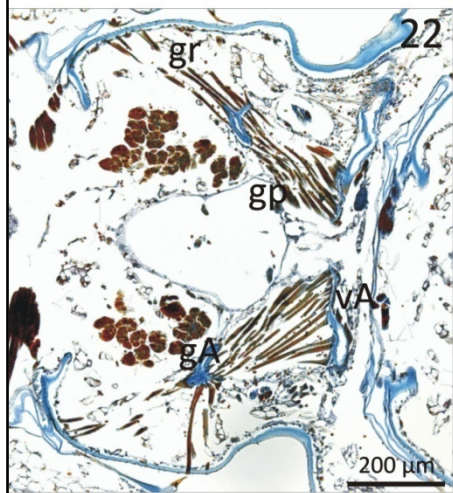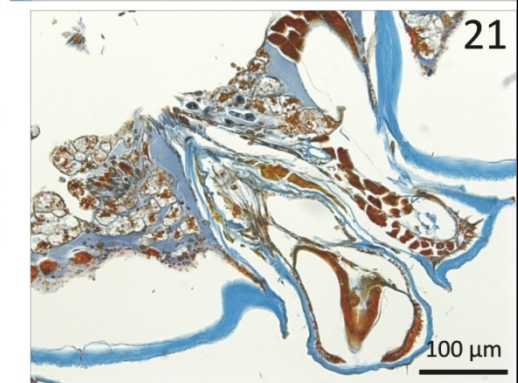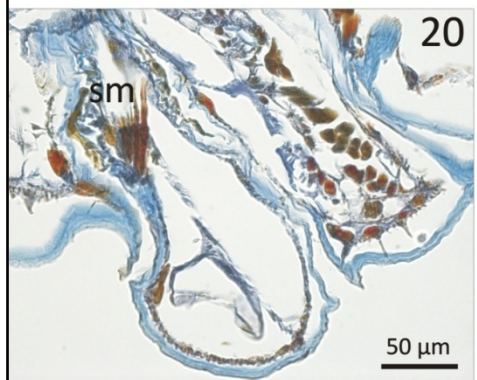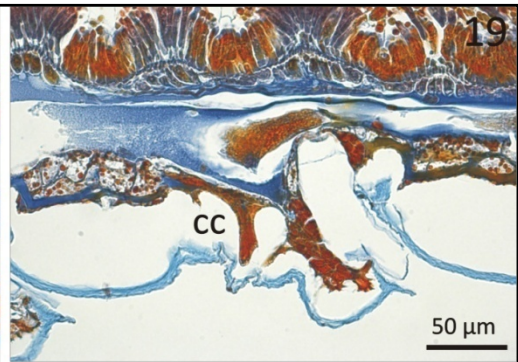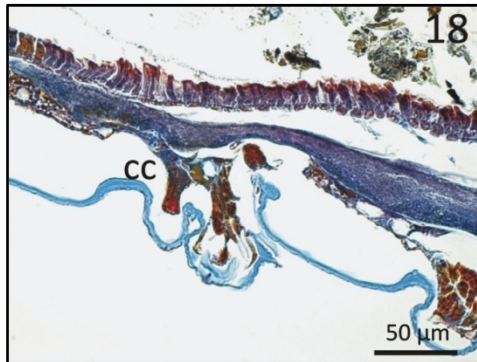
Figure 25. *O. gracilis* adult male. Endoskeleton of seventh trunk ring of adult. Anterior view. SEM after the digestion of the internal soft tissues. gA =gonopod apodeme; vA = ventral apodeme.

Figure 26. Scheme of extrinsic musculature of the gonopod. Same view of the previous picture. gp = gonopod protractor muscle; gr = gonopod retractor muscle; ga = gonopod abductor muscle.
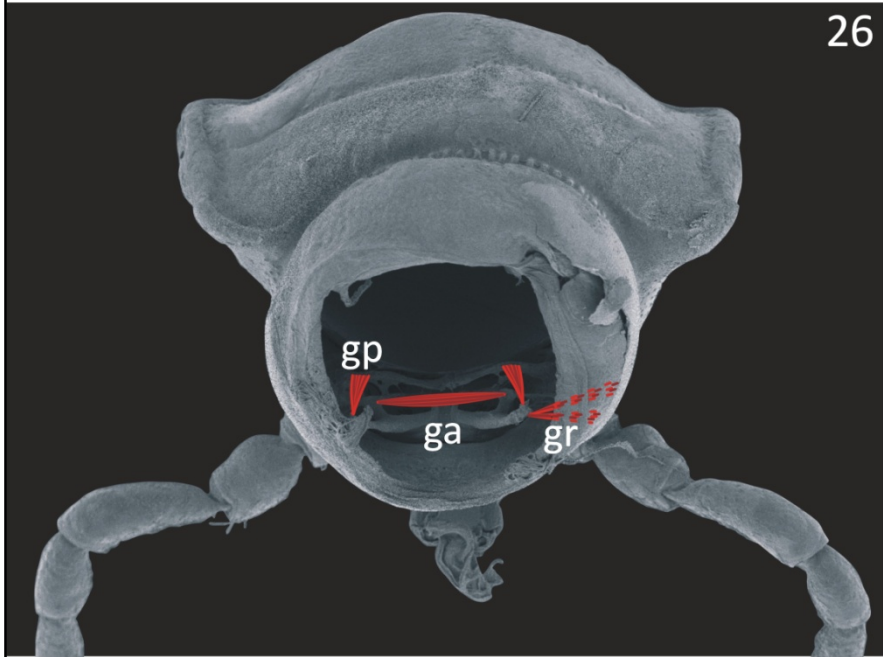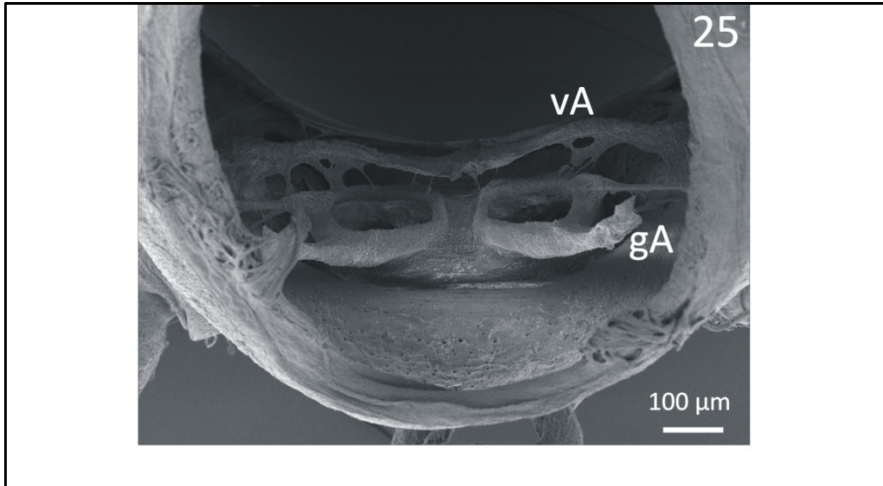
Figure 27. *O. gracilis* male during pharate phase of eighth stadium. The squamiform appendage is elongated in proximodistal direction sq = squamiform appendage. Lateral view.

Figure 28. Squamiform appendage during first step of pharate phase of eight stadium. The squamiform appendage is formed by three parts, separeted by two constrictions. Lateral view (fused EDF-stack).

Figure 29. Second step: an elongated structure is visible inside the appendage. Lateral view (fused EDF-stack).

Figure 30. Third step: the distal processes of the gonopod telopodite are recognizable inside the squamiform appendages. Lateral view (fused EDF-stack)

Figure 31. Para-midsagittal paraffin section of squamiform appendages during first step of pharate phase of eight stadium. Mallory's triple stain.

Figure 32. Second step: squamiform appendage contains a mass of tightly packaged cells. The mass of cells is branched in two directions in the distal part

Figure 33. Third step: several branches, corresponding to the distal telopodite parts, are present in the distal part of the appendages.
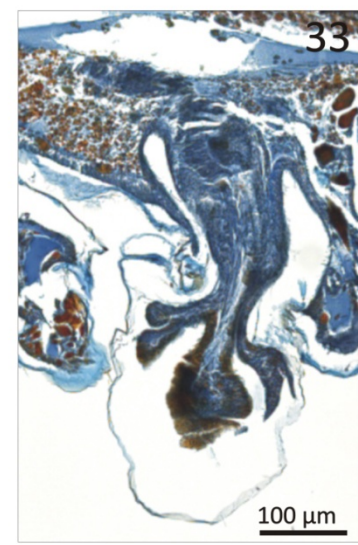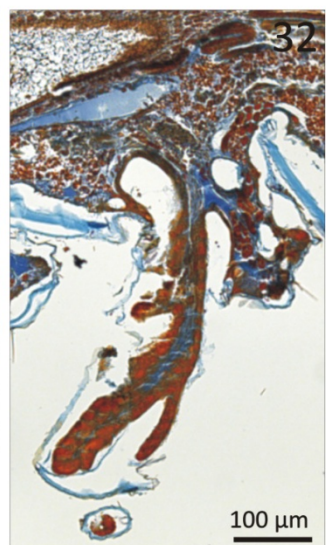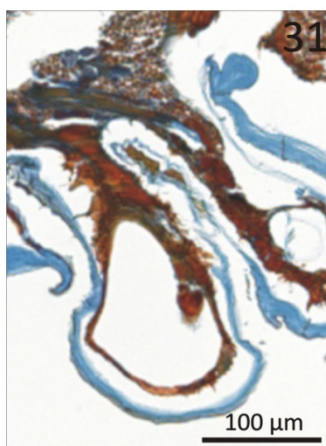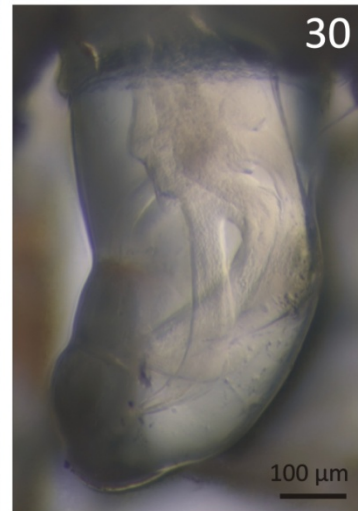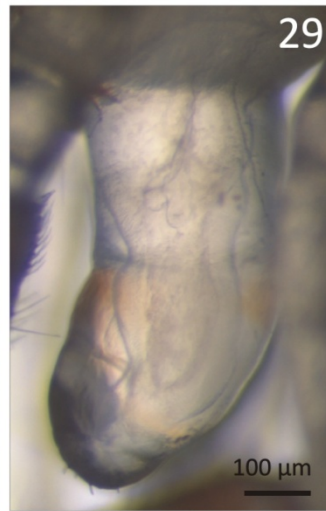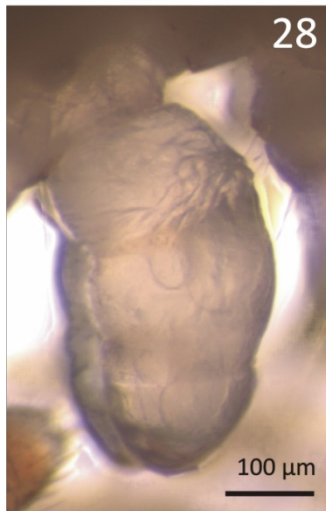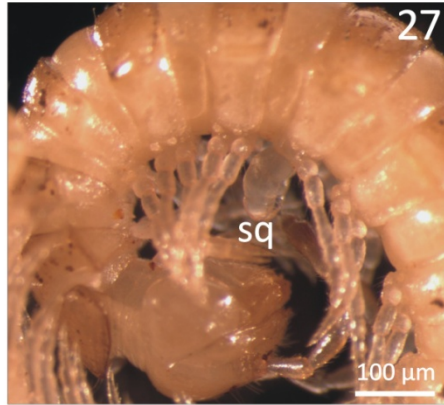
Consequences of non systemic metamorphosis in three julid millipedes: an anatomical point of view

ABSTRACT

The modifications of seventh trunk ring in three species of millipedes during subsequent developmental postembryonic stadia are described. Traditional techniques are used as well as a new technique based on the employ of confocal laser scanning microscope for the study of external morphology and internal anatomy. The analysis of two blaniulids and a nemasomatid reveals surprising remodeling of the seventh ring during gonopod formation.

INTRODUCTION

Arthropod postembryonic development is constrained by the presence of an hard exoskeleton encasing the whole animal body. The growth process, as consequence, is punctuated by a series of moulting events in that often involve conspicuous and relatively abrupt changes in the animal's structure (metamorphosis). The processes engaged during the metamorphosis are one of the themes that most fascinate developmental biologists. Current research has reached highly detailed understanding of mechanisms involved in metamorphosis, like genes expression pattern in adult butterfly leg formation (Tanaka and Truman, 2007) or motoneuron development (e.g., Consoulas et al., 2002) and fine gene regulation (e.g., Kayashima et al., 2005) in *Drosophila* development. But this level of knowledge is limited to a few model organisms, or to few developmental stadia, usually embryonic. At the contrary, for a lot of other species, the current status of understanding is limited to a general description of external morphology changes during the development, without any information about gene expression or even, at a morphological level, eventual modifications affecting internal anatomy. Diplopods offer an intriguing scenario for the study of metamorphosis. Among millipedes, the adult helmintomorph males possess gonopods on the seventh body ring. These specialized appendages differentiate in a very advanced phase of post-embryonic development, changing from normal walking legs, to squamiform appendages, to gonopods. This transformation is limited to the seventh trunk ring, while leaving unaffected the other – initially identical – rings, in a process described as non-systemic metamorphosis (Drago et al., 2008).

Millipede internal anatomy is poorly known: Silvestri (1903) and Manton (e.g., 1977) described muscular system and endoskeletal structure of several diplopods in a precise way, but did not provide information about gonopods. Wilson (2002) realized an excellent description of trunk and gonopod musculature in a spirobolid millipede, but in her work there are no information about the developmental stadia preceding the adult.

The current lacking of an adequate knowledge of the genes of these animals does not permit to start studying this topics from a molecular point of view. In addition, before considering the processes at a fine scale, it is essential to have a better knowledge of the anatomy of these animals, in order to understand the effects that metamorphosis may have on it.

Aim of this work is to describe the anatomical changes involved in the development of the seventh trunk ring and its appendages, comparing subsequent developmental stadia during postembryonic growth. In this paper I illustrate the data

collected working with *Nopoiulus kochii* (Gervais, 1847), *Blaniulus guttulatus* (Bosc, 1792), *Nemasoma varicorne* C.L. Koch, 1847, and I give a comparison between these three diplopod species.

I investigated external morphology using SEM, while a double approach has been required for the internal anatomy, combining the observation of histological serial sections with the study of data collected using a confocal laser scanning microscope.

This data can be the starting point for future investigations of the morphogenetic processes involved in the formation of the gonopods.

MATERIALS AND METHODS

<u>Animals</u>

Specimens of *Nopoiulus kochii* were collected in litter and in decaying wood in Sorio di Gambellara (Vicenza, Italy 45°27'14''N, 11°20'42''E). Breeding cultures were kept at 20°C in plastic Petri dishes with an approximately 5 mm layer of 2% agar (Blower, 1974; Enghoff, 1976). Original litter material, pieces of potatoes and decaying wood (*Populus, Salix*), was used as food. Males were reared until the desired stadium of development, then they were killed using ethyl acetate vapour at 4°C, in order to prevent rolling up, and immediately fixed in a mixture of 2,5% glutaraldehyde and 2% formaldehyde in PBS. For optimal fixation, the animals were cut in two or more pieces before putting them into the fixative where they were preserved for at least 48 hours at 4°C.

Specimens of *Blaniulus guttulatus* were collected in a garden in Hellerup (Copenhagen, Denmark 55°44'01''N, 12°33'01''E), while specimens of *Nemasoma varicorne* were collected under the bark of trees (usually *Fagus*) in Gentofte, Ermelunden (Copenhagen, Denmark 55°46'13''N, 12°32'11''E) and in Jægersborg Hegn (Copenhagen, Denmark 55°49'51''N, 12°34'00''E). Developmental stadia were determined counting the number of podous and apodous rings, and representative specimens were killed and fixed as described above. In addition some individuals from the collection of Zoological Museum in Copenhagen were studied to increase sample size. Material from collection was preserved in rthanol 70%, and was used only for SEM investigation.

In vivo observations were made using a Leica MZ125 stereomicroscope, fitted with Leica DFC 420 digital camera using Leica Application Suite software (version 2.6).

<u>Scanning electron microscopy</u>

Fixed specimens were prepared following the protocol described in Drago 2009a. Samples were briefly sonicated when necessary, to remove soil particles from the animals. The investigation was made with a Cambridge Stereoscan 260, or with a Jeol JSM-6490, or with a Jeol JSM-6335-F scanning electron microscope.

<u>Histology</u>

Internal anatomy was studied through serial paraffin sections. The protocol is the same used for *Oxidus gracilis* (Drago 2009a). Sagittal, frontal and transversal 7 μm sections were stained with Mallory's triple stain (Pantin, 1964).

Slides were observed with a Leica DM5000B microscope, fitted with a Leica DFC 300 FX camera.

Pictures were elaborated using CorelPHOTO-PAINT and CorelDRAW (version 11) software.

Confocal Laser Scanning Microscopy (CLSM)

Recently several works demonstrated that the external morphology of small arthropod appendages can be investigated using CLSM by taking advantage of the autofluorescence of the cuticle (Klaus et al., 2003; Klaus and Schawaroch, 2006; Michels, 2007; Zill et al., 2000). Using the same techniques, it was possible to study the complexity of the gonopod endoskeletal component *in situ*, avoiding the problems of accidental damage deriving from dissection.

Specimens were washed in PBS + 0.1% Triton®X 100 to remove the fixative. Internal soft tissues were digested with KOH 5% at 50°C for at least 48 hours. Immersion in KOH solution removes soft tissues while leaving the hard cuticular exoskeleton intact. The remaining cuticle was bleached with acetic acid for 5 minutes and washed with distilled water. This step makes the cuticle transparent and allows to observe the internal parts. Evans Blue staining (0,005% in water for 3 minutes) increases the fluorescence of the cuticle, improving resolution beyond the effect of autofluorescence alone. Several washes were made to remove excess stain. Thus the samples were mounted in buffered glycerol anti-fading (90% glycerol, 0,5% n-propyl gallate in PBS). In the mounting process care was paid to prevent deformation (compression or crushing) using spacers between the coverslip. Each sample was scanned several time under different orientation. Because increasing specimen thickness exacerbates aberrations in the optical setup (de Grauw et al., 2002, Diaspro et al., 2002), care was taken using just the amount of medium necessary to cover the specimens. The slides were studied using a Nikon Eclipse E600 microscope equipped with a Bio-Rad MRC 1024ES confocal laser scanning unit using a 543 nm helium/neon laser and a 570 nm long pass emission filter. CLSM data were analyzed with ImageJ (version 1.37) software.

Some digested specimens, eventually dissected and stained with Chlorazol black, were studied with a LeicaDM5000B microscope using bright field light or differential interference contrast (DIC). Picture were acquired with a Leica DFC 300 FX camera using Leica Application Suite software (version 2.6) and Helicon Focus software.

RESULTS

Non-systemic metamorphosis in *Nopoiulus kochii* (Gervais, 1847) (Diplopoda: Julida: Blaniulidae)

In specimens of the third and the fourth developmental stadium the eighth and ninth pairs of legs and the seventh trunk ring is identical to the other trunk units. These legs are replaced by two pairs of squamiform appendages during the moult to the fifth stadium and are later changed into two pairs of bulky and complex gonopods after a further moult (Fig. 70, Drago et al. 2008). This process is not so strictly fixed in time in respect to the animal's post-embryonic development: in some specimens the process starts one stadium later than in the others. In the studied population such exceptions have been recorded rarely (less than 5%), and these males have not been considered in this work. Several times males with completely formed gonopods in the sixth stadium could not to be considered mature (Kheirallah et al., 2000): in fact, mature functional males are morphologically distinct in the hooked form of the first pair of legs. When sixth stadium males lack this condition they are not able to copulate. From comparison between this males and the mature sixth stadium specimens no difference emerge in

gonopod morphology or in the anatomy of the seventh ring. Another peculiarity of *N. kochii* males is periodomorphosis: cyclically, mature males (with gonopods) moult into intercalary males, with reduced appendages comparable to the squamiform, and they resume the reproductive condition only following one or a few more moults. In this work I studied males until the first appearance of the gonopods.

The squamiform appendages are tiny structures located on the ventral side of the seventh ring (Figs. 34-36) There are an anterior pair (peltogonopod primordia) and a posterior one (gonopod primordia). The anterior pair can include an anterior sternite, two coxal processes primordia and two small peltogonopod telopodites, while the posterior pair are formed by a sternite (covered by the anterior squamiform appendages) and a couple of gonopod telopodite primordia. Brölemann (1923) described some morphological variations in these appendages in specimens of different stadia (intercalary males). He discussed also small changes within the same stadium, starting from sixth stadium males. My investigations reveal a further variation: in males of fifth stadium some short setae in the apical surface of the primordia of gonopod telopodite are not always present (Fig. 36). Histological investigation reveal that inside each squamiform appendage is present a cluster of tightly packed cells that seem to represent an undifferentiated tissue (Fig. 37). No muscles are detected in association with these appendages.

The general appearance of seventh ring appendages of adult males shows the characteristic peculiarity of blaniulid millipedes and has been adequately described by Brölemann (1923) (sub nomine *subtilis*) and Enghoff (1984). Here I move from their observations on, for a better understanding of general morphology including new data about the implications of the differentiation of these appendages on the internal anatomy of the seventh ring.

In this developmental stadium the shape of the seventh ring is changed: from SEM and CLSM investigations it emerges that a conventional sternite is no more present but it is modified in a thin layer of cuticle (cuticular membrane), which delimits the gonopodal sac. The most dramatic change is correlated to the impressive reduction of the ring volume: when the gonopods are in resting position, the gonopodal sac occupies more than half of the ring volume.

Anterior gonopods (peltogonopods) are composed by two long, clavate coxal processes, accompanied laterally by two short telopodites without setae (Figs. 38-40). The coxal processes are almost totally visible in the ventral side of the ring, and cannot to be retracted in the gonopodal sac. For the purpose of this paper, it is more interesting to look at the endoskeletal component of the anterior gonopods: this is a single U-shaped apodeme from which both coxal processes start. This structure was illustrated by Brölemann (1923) (sub nomine Breuili) who, however failed to provide information about muscles and their relationship with the trunk anatomy. The U-shaped apodeme is the prominent element inside the ring lumen (Figs. 40, 41 and Fig. 70F), and its anterior part reaches the posterior area of the sixth ring. The apodeme is divided in two symmetrical, left and right arms. Two bulky muscles (peltogonopod retractor, peltogonopod protractor) are inserted on each arm, along the dorsal margin. The peltogonopod retractor, which is inserted along the internal margin, is attached on the posterior ventral apodeme of the sixth ring. The peltogonopod protractor, which is inserted on the external margin of the apodeme, is attached to the border of the prophragma of seventh ring in dorso-lateral position (Figs. 42, 45). A third large unpaired muscle (peltogonopod abductor) is localized at the basis of the coxal processes (Figs. 43, 44) and is responsible for their abduction movements.

The posterior gonopod is composed by a single article, the telopodite, with two characteristic lamellae in the apical part: the lateral lamella and the mesal lamella. The

lateral lamella is surrounded by spines, while the mesal seems to protect the other (Figs. 39, 49). A third and smallest internal lamella can be seen in the interior side, after removing the gonopod from the trunk (Fig. 49). The telopodite is usually described as connected to the sternum, and for some authors it has the identity of a true article. SEM and CLSM investigations reveal that a conventional sternite is not present: the ventral part of the seventh ring is modified in a membranous cuticular layer, which delimits the gonopodal sac. Starting from this point, the posterior gonopod appears to be formed by two elements (Fig. 46), confirming the identity of a small article for the sternum which is basally articulated to the trunk, and forms a knee joint with the telopodite. No muscles have been found inside the telopodite. Two trunk muscles (gonopod protractor; gonopod retractor) find their insertion points in the sternum. The gonopod retractor muscle is inserted on the basis of the sternum, and is attached to the anterior part of the prophragma (Figs. 45, 47) of the eighth ring in dorso-lateral position; the gonopod protractor muscle is inserted on a long apodeme of the sternum (gonopod apodeme), and is attached on the anterior ventral apodeme of the eighth ring. The contraction of the protractor muscle causes the eversion of the gonopod from the gonopodal sac (Fig. 49), while with the contraction of the retractor muscle the gonopod comes back to the resting position (Fig. 39).

The presence of anterior and posterior voluminous gonopod apodemes and of their bulky muscles causes a further reduction in the volume available for the other organs. The ventral nerve cord and the digestive tract are the most influenced by these changes as they are displaced dorsally, comparing with immature stadia, with a consequent decrease in the gut lumen (Fig. 66).

Non-systemic metamorphosis in *Blaniulus guttulatus* (Bosc, 1792) (Diplopoda: Julida: Blaniulidae)

The development of the seventh ring in *B. guttulatus* is similar to the corresponding process in *N. kochii*, but it is shifted here to later stadia: the squamiform appendages appear after the seventh moult, that is, in the eighth stadium. These data are in agreement with the work of Brookes and Willoughby (1978) on a population from Wales.

The external morphology of squamiform appendages is shown in Figs. 50-52. The general organization is the same described for *N. kochii*. A difference is the presence of a seta at the apex of the primordia of peltogonopod telopodite. Also in this species several morphological modifications could be find in the squamiform appendages, in particular in the primordia of peltogonopod telopodite. Histological investigation does not show differences in the internal anatomy associated to these external modifications. The internal arrangement is very similar to *N. kochii*, with a cluster of tightly packed cells inside each squamiform appendage.

In adult males (ninth stadium) the general architecture of the seventh ring is the same as found in *N. kochii*, with a gonopodal sac that occupies more than a half of the ring volume.

The two peltogonopod coxal processes are fused in a single element, which possesses a short telopodite, with few apical setae, to each side (Fig. 53). Peltogonopods are associated to a U-shaped apodeme (Figs. 54,55) which offers insertion points for a pair of antagonistic muscles (protractor and retractor) in each arm (Fig. 56). The voluminous abductor muscle found in *N. kochii* at the base of coxal processes, is not present in *B. guttulatus*: actually, in this species, with the fusion of the peltogonopod coxal processes, any abduction movement is no more possible. The apodeme are less voluminous than in the previous species: in particular, the two arms are less divergent.

The posterior gonopods are composed by a telopodite connected to the sternum. Both these articles are thinner than in *N. kochii*. The terminal part of the telopodite is characterized by the presence of two small lamellae, one with long spines at the apex, while the other one possesses only a few short spines (Fig. 57). The trunk muscles taking insertion on the gonopods are organized like in *N. kochii*.

The metamorphosis from juvenile stadia to adult causes a considerable reduction in the internal volume of the seventh ring. The ventral nerve cord and digestive tract are displaced dorsally and the digestive lumen is reduced (Fig. 67) but this change is less conspicuous than in *N. kochii*.

<u>Non systemic metamorphosis in *Nemasoma varicorne* C.L. Koch, 1847 (Diplopoda: Julida: Nemasomatidae)</u>

In *N. varicorne* males the eighth and ninth pairs of legs are only present during the third developmental stadium. At this time the seventh trunk ring is identical to the other trunk units (Fig. 58).

Squamiform appendages (Fig. 59) appear after the following moult and possess two well-developed primordia of the peltogonopod telopodite, with a single seta in medial position. Posterior squamiform appendages are characterized by a cuticolar fold with a few short setae on the free margin. Histological sections reveal the presence of a cluster of cells in each squamiform appendage. These groups of cells are confined to the proximal part of the primordia and seem to be less tightly packed than the cells observed in the corresponding appendages of the two blaniulid species. In addition, there are two tiny extrinsic muscles in the basal part of the squamiform appendages (Fig. 60).

Adult males have the anterior gonopods similar to the peltogonopods of the Blaniulidae, while the posterior gonopods largely share a number of resemblance with the gonopods of the Julidae. Anterior gonopods (Fig. 61) are formed by two long coxal processes with hook shaped apex, and two long telopodites with a few setae. The telopodites are larger than the coxal processes. On a slender anterior gonopod apodeme (Fig. 64, 65) is inserted a protractor muscle which goes the ventro-lateral part of the seventh ring (Fig. 62). An abductor muscle is present at the base of coxal processes. Two muscle are responsible for telopodite movements; these muscles are inserted at the base of telopodites and are attached at the anterior ventral apodeme of the seventh ring. A flagellum connects the basis of anterior gonopod to the posterior gonopod. Posterior gonopods are formed by a large basal structure that ends with a thin telopodite, almost completely hidden in resting position (Fig. 61). Posterior gonopods possess an apodeme where a protractor muscle (attached to the anterior face of the prophragma of the eighth ring) (Fig. 63) and a retractor muscle, attached in the ventro-lateral part of the seventh ring, are inserted. A muscle is in the basal part of the posterior gonopod is probably responsible for the movements of the gonopod telopodite.

The gonopodal sac is smaller than in the two Blaniulidae and also the endoskeletal component of the gonopods are smaller. As a result, the influence of the presence of the gonopods on the ring volume is less important in this species (Fig. 68).

CONCLUSIONS

During the anamorphic post-embryonic development of male helmintomorph millipedes some remarkable changes affect a single segment of the trunk: in few moults

the fate of the seventh ring is modified from normal diplosegment bearing two pairs of legs to a very specialized ring bearing two pairs of gonopods. In this work I demonstrated that through this non-systemic metamorphosis (Drago et al., 2008) not only the external shape of the eighth and ninth pairs of appendages undergoes drastic modifications, but there are also dramatic changes in the internal anatomy of the seventh ring. Both the blaniulid species show a major remodelling of the architecture of this ring, mainly because of the reduction of the ring volume. Ventrally, the sternite is modified to a cuticular membrane which delimits the gonopodal sac, a kind of cavity where the gonopods can be retracted. In *Nemasoma varicorne* the gonopodal sac is smaller and the consequence for the ring are less evident. In addition, a conspicuous part of the remaining ring is occupied by the bulky apodeme and its musculature. Also in this case, the influence of this component is more conspicuous in the blaniulids than in the nemasomatid. In particular, in the blaniulids examined the changes caused by the presence of the gonopods deeply affect also the anatomy of the sixth and eight rings: in the sixth, there is a part of the peltogonopod apodeme, and the protractor muscles that are attached to the posterior ventral apodeme of this ring. This ventral apodeme is also peculiar for the coxal extrinsic muscle of the seventh pair of legs. The situation is similar for the anterior ventral apodeme of the eighth ring, where the protractor muscles of the posterior gonopods are attached (Fig. 45). The gonopod apodemes in the nemasomatid millipede are smaller and also the muscles inserted on these endoskeletal structures have smaller influence on the trunk anatomy. The result of this different organization is a different degree of dorsal displacement of ventral nerve corde and the digestive tract, which is most prominent in *N. kochii* and minimal in *N. varicorne* (summarized in Figs. 66-68).

The processes involved in the formation of the gonopods in the three species are broadly similar: in all cases gonopods are preceded by a stadium in which the seventh ring carries squamiform appendages. The study of the internal anatomy of these structure revealed the presence of undifferentiated clusters of cells in the three species. The same was seen in the squamiform appendages of the polydesmid millipede *Oxidus gracilis* (Drago 2009a): in this millipede squamiform appendages are presents during four subsequent developmental stadia, through which these rudimentary appendages increase size. The clusters of cells found in the polydesmid millipede during the fourth and fifth stadium show high similarity with the group of cells found in both blaniulids. At the contrary in sixth and seventh stadium *O. gracilis* squamiform appendages contain less tightly packed cells and small muscles in the basal part are recognisable: the same is seen in *N. varicorne* which seems to possess a small number of undifferentiated cells. Species with more complex gonopods (blaniulids) maintain all the cells inside the squamiform appendages undifferentiated for longer period, spending longer time to enlarge the cells number. Besides, the initial differentiation in the basal part of squamiform appendages found in *N. varicorne* and in *O. gracilis* is perhaps be correlated to the small endoskeletal component of their gonopods: the internal remodelling is not so conspicuous and the muscles inserted in the squamiform appendages could be a primordial version of some gonopod muscles. At the contrary in *N. kochii* and *B. guttulatus* it is not possible to anticipate the begin of differentiation because the bulky gonopod apodemes require a complete remodelling of the internal setting, similar to the changes an holometabolous insect undergoes during metamorphosis.

Independent from these differences and the complexity of the resulting organs, gonopods formation depends on the clusters of cells present in squamiform appendages: for some reasons, the apoptosis processes that cause the loss of the eighth and ninth pairs of legs and the proliferation processes that form the gonopods can not

go on at the same time. The stadium with squamiform appendages in the seventh ring is a developmental constraint, the basis of the non-systemic metamorphosis in millipedes.

REFERENCES

Blower JG, 1974. Food consumption and growth in a laboratory population of *Ophyiulus pilosus* (Newport). Symposia of the Zoological Society of London 32:527-551.

Brölemann HW, 1923. Blaniulidae Myriapodes. Biospeologica - Archives de zoologie expérimentale et générale 61:99-453.

Brookes CH, Willoughby J, 1978. An Investigation of the ecology and life history of the millipede *Blaniulus guttulatus* (BOSC) in a British woodland. In: Kraus O. editor. Myriapoda, III Internationaler Kongress für Myriapodologie. Hamburg; 105-114.

Consoulas C, Restifo LL, Levine RB, 2002. Dendritic remodeling and growth of motoneurons during metamorphosis of *Drosophila melanogaster*. The Journal of Neuroscience 22:4906-4917.

de Grauw CJ, Frederix PLTM, Gerritsen HC, 2002. Aberrations and penetration in in-depth confocal and two-photon microscopy. In: Diaspro A, editor. Confocal and two-photon microscopy: foundations, applications, and advances. New York: Wiley-Liss. p 153–69.

Diaspro A, Federici F, Robello M, 2002. Influence of refractiveindex mismatch in high-resolution three-dimensional confocal microscopy. Applied Optics 41:685–90.

Drago L, Fusco G, Minelli A, 2008. Non-systemic metamorphosis in male millipede appendages: long delayed, reversible effect of an early localized positional marker? Frontiers in Zoology 5:5.

Drago L, 2009a. Gonopod formation in the hothouse millipede *Oxidus gracilis* (C.L. Koch 1847). MS 1: pg. 33-51.

Enghoff H, 1976. Parthenogenesis and bisexuality in the millipede, *Nemasoma varicorne* C. L. Koch, 1847 (Diplopoda: Blaniulidae). Morphological, ecological, and biogeographical aspects. Videnskabelige Meddelelser fra Dansk Naturhistorisk Forening 139:21-59.

Enghoff H, 1984. A revision of the Nopoiulinae, with notes on the classification of blaniulid millipedes (Diplopoda, Julida: Blaniulidae). Senckenbergiana biologica 64:393-427.

Kayashima Y, Hirose S, Ueda H, 2005. Anterior epidermic-specific expression of the cuticle gene *EDG84A* is controlled by many cis-regulatory elements in *Drosophila melanogaster*. Development Genes and Evolution 215 (11):545-552.

Kheirallah A-M, Aly A-NH, Abdel-Wahed NY, 2000. Anamorphosis and life-history of the millipede *Nopoiulus kochii* (Gervais, 1847), new for Egypt. Zoology in the Middle East 21:159-168.

Klaus AV, Kulasekera VL, Schawaroch V, 2003. Three-dimensional visualization of insect morphology using confocal laser scanning microscopy. Journal of Microscopy 212:107-121.

Klaus AV, Schawaroch V, 2006. Novel methodology utilizing confocal laser scanning microscopy for systematic analysis in arthropods (Insecta). Integrative and Comparative Biology 46:207-214.

Manton SM, 1977. The Arthropoda: habits, functional morphology and evolution. London: Clarendon Press.

Michels J, 2007. Confocal laser scanning microscopy: using cuticolar autofluorescence for high resolution morphological imaging in small crustaceans. Journal of Microscopy 227:1-7.

Pantin CFA, 1964. Notes on Microscopical Technique for Zoologists. Cambridge: Cambridge University Press.

Silvestri F, 1903. Classis Diplopoda. Vol. 1. Anatome: Pars I, Segmenta, Tegumentum, Musculi. - Acari, Myriapoda et Scorpiones huscque in Italia reperta. Portici.

Tanaka K, Truman JW, 2007. Molecular patterning mechanism underlying methamorphosis of the thoracic leg in *Manduca sexta*. Developmental Biology 305:539-550.

Wilson HM, 2002. Muscular anatomy of the millipede *Phyllogonostreptus nigrolabiatus* (Diplopoda: Spirostreptida) and its bearing on the millipede "Thorax". Journal of Morphology 251:256-275.

Zill S, Faith Frazier S, Neff D, Quimby L, Carney M, Dicaprio R, Thuma J, Norton M, 2000. Three-dimensional graphic reconstruction of the insect exoskeleton through confocal imagining of endogenous fluorescence. Microscopy Research and Technique, 48:367-384.

Figures 34, 35, 36. Three *Nopoiulus kochii* males during the fifth developmental stadium. Ventral view of the squamiform appendages. Fig. 35 shows elongation of the primordia of coxal processes. Fig. 36 shows reduction of setae on the primordia of gonopod telopodites. SEM. st = sternite; cpp = coxal processes primordia; ptp = peltogonopod telopodite primordia; gtp = gonopod telopodite primordia.

Figure 37. Para-midsagittal paraffin section of the seventh ring with squamiform appendages during fifth developmental stadium. Mallory's triple stain. cc = cluster of packed cells.

Figure 38. Peltogonopods of *N. kochii* in situ. Sixth stadium male. Ventral view. SEM. pt = peltogonopod telopodite; cp = coxal process.

Figure 39. Lateral view of appendages of seventh trunk ring of sixth stadium male. SEM. pt = peltogonopod telopodite; cp = coxal process; ll = gonopod lateral lamella; ml = gonopod mesal lamella.

Figure 40. Dissected peltogonopods after digestion of soft tissues. Chlorazol black stain. Ventral view (fused EDF-stack). pt = peltogonopod telopodite; cp = coxal process; pA = peltogonopod apodeme.
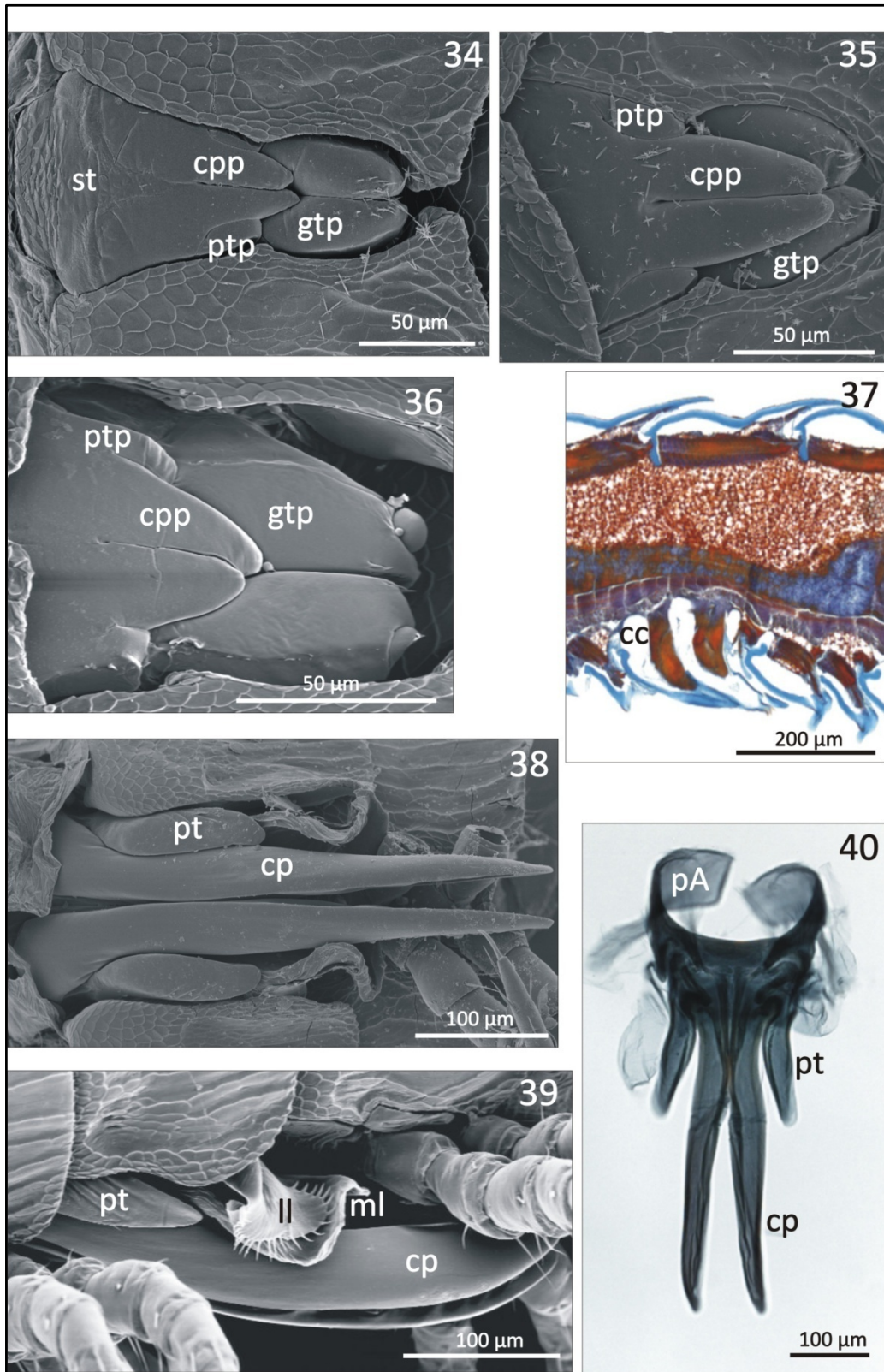
Figure 41. Seventh trunk ring of *Nopoiulus kochii* adult male, after digestion of internal soft tissues. Posterior view (fused EDF-stack)

Figure 42. Transverse paraffin section of seventh ring during the sixth stadium. Mallory's triple stain. pA = peltogonopod apodeme; pp = peltogonopod protractor muscle; pr = peltogonopod retractor muscle.

Figure 43. Transverse paraffin section of seventh ring during the sixth stadium. Mallory's triple stain. pa = peltogonopod abductor muscle

Figure 44. Frontal paraffin section of sixth and seventh ring during the sixth stadium. Mallory's triple stain. pa = peltogonopod abductor muscle; pt = peltogonopod telopodite; T = gonopod telopodite.

Figure 45. Scheme of extrinsic musculature related to the peltogonopods and gonopods. pA = peltogonopod apodeme; gA = gonopod apodeme; vA = ventral apodeme; pp = peltogonopod protractor muscle; pr = peltogonopod retractor muscle; gp = gonopod protractor muscle; gr = gonopod retractor muscle.
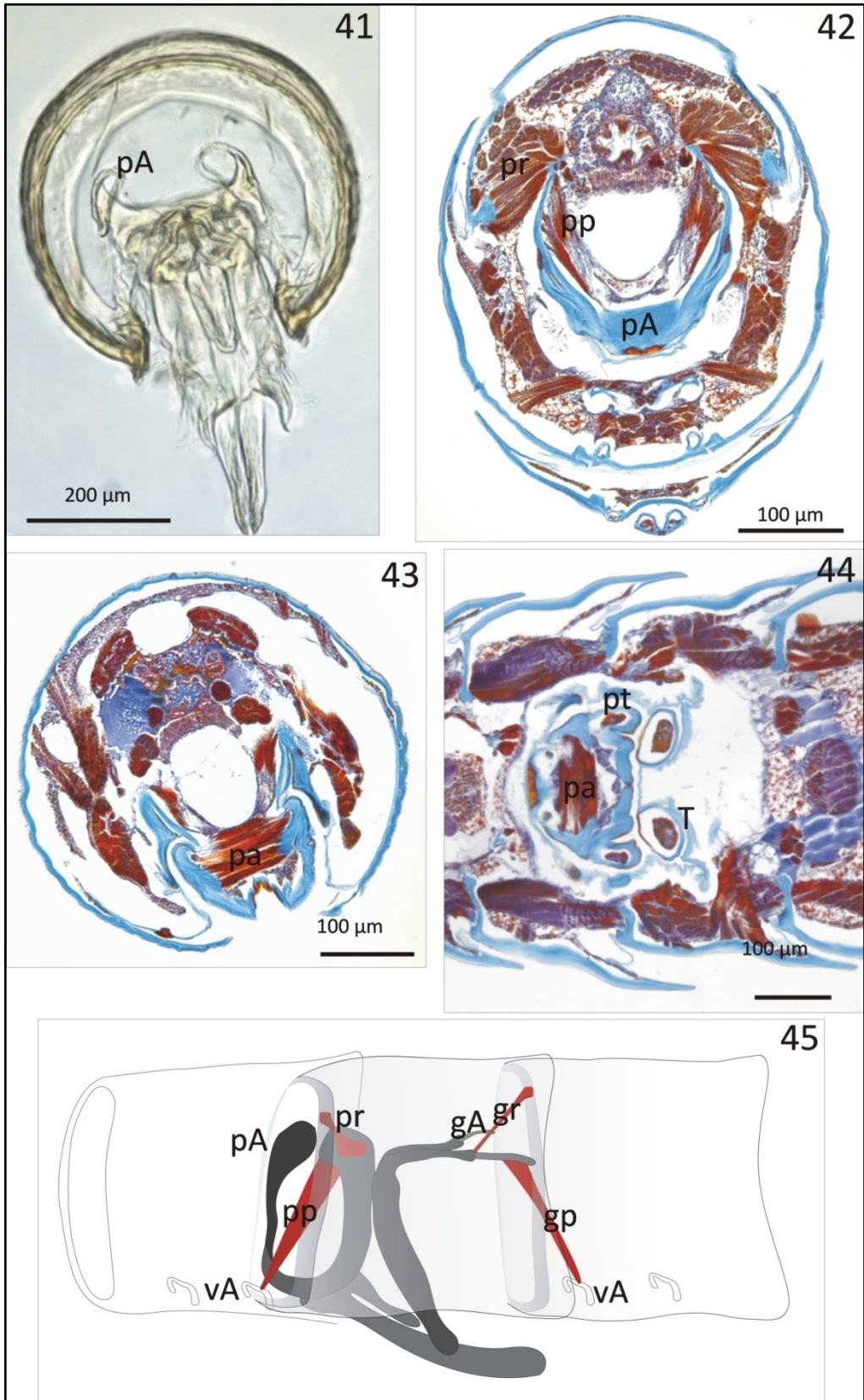
Figure 46. Gonopod of *Nopoiulus kochii* adult males. Maximum intensity projection of a stack of pictures obtained with CLSM. T = gonopod telopodite; S = gonopod sternum; gA = gonopod apodeme.

Figure 47. Frontal paraffin section of sixth and seventh ring during the sixth stadium. Mallory's triple stain. gr = gonopod retractor muscle.

Figure 48. Seventh ring of an adult males. Dorsal view. Maximum intensity projection of a stack of pictures obtained with CLSM. pA = peltogonopod apodeme; gA = gonopod apodeme.

Figure 49. Ventral view of the seventh ring of an adult male. The gonopods are everted outside the gonopodal sac. SEM. pt = peltogonopod telopodite; cp = coxal process; T= gonopod telopodite; ll = gonopod lateral lamella; ml = gonopod mesal lamella; il = gonopod internal lamella.
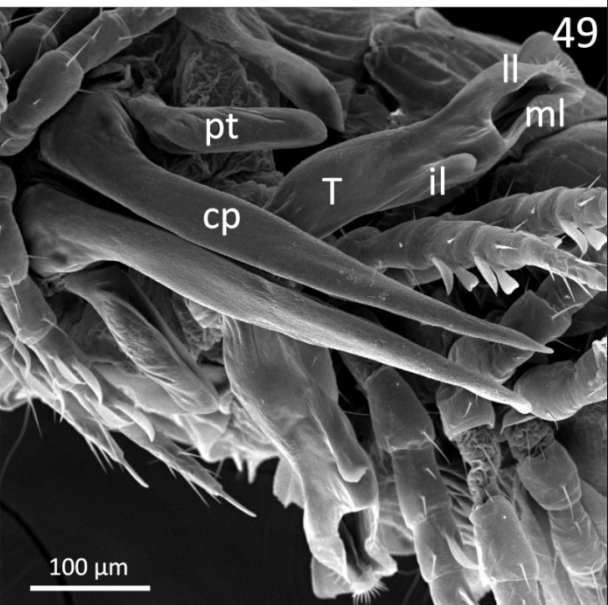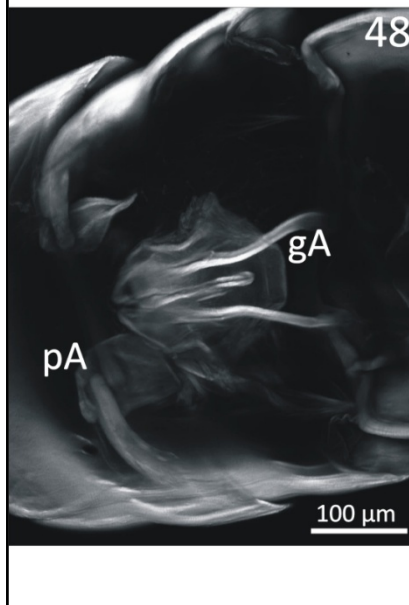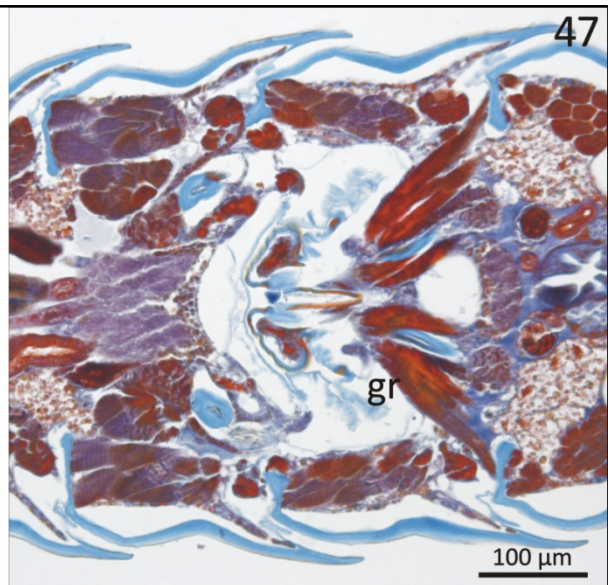
Figures 50, 51, 52. Squamiform appendages of *Blaniulus guttulatus.* Three males during eighth stadium with some morphological variation in the squamiform appendages. ventral view. Fig. 51 shows elongation of the primordia of coxal processes. Fig. 52 shows elongation of the primordia of peltogonopod telopodite. SEM. cpp = coxal processes primordia; ptp = peltogonopod telopodite primordia; gtp = gonopod telopodite primordia.
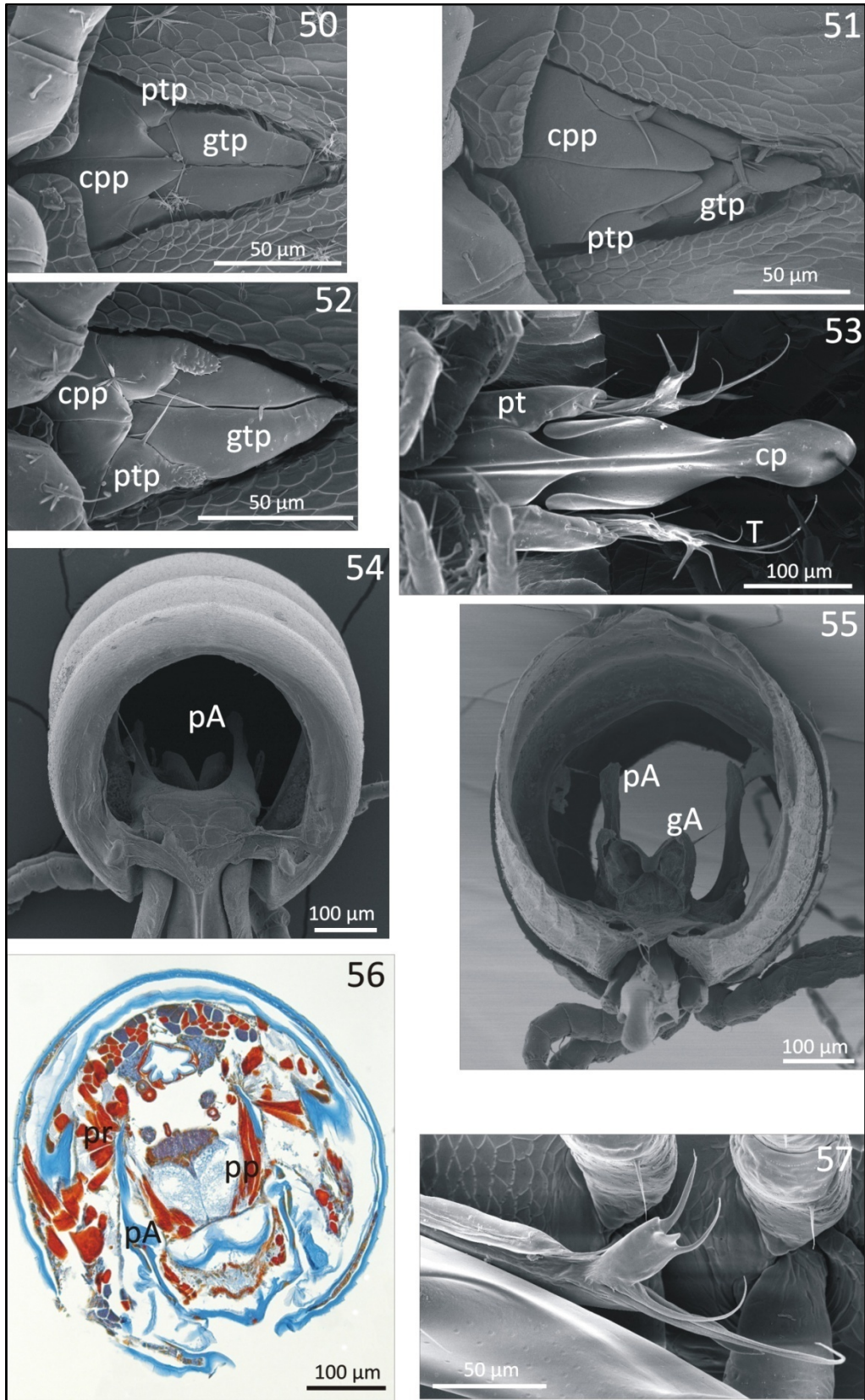
Figure 53. Appendages of seventh trunk ring of adult *B. guttulatus* male. Coxal processes of peltogonopods are fused medially. Ventral view. SEM. pt = peltogonopod telopodite; cp = coxal process; T = gonopod telopodite.

Figure 54. Endoskeleton of seventh trunk ring of adult male. Anterior view. SEM after the digestion of the internal soft tissues. pA =peltogonopod apodeme.
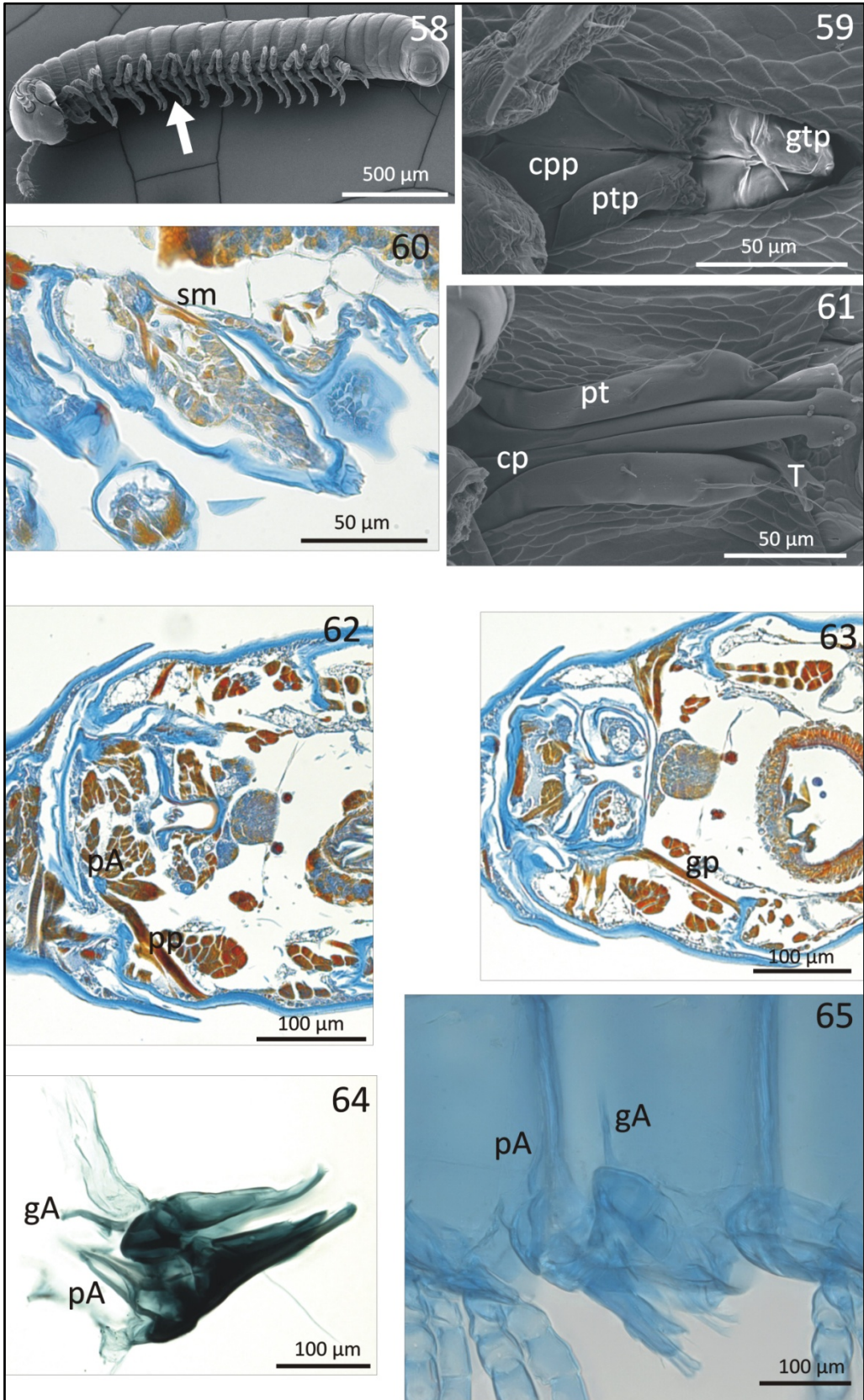
Figure 55. Endoskeleton of seventh trunk ring of adult male. Posterior view. SEM after the digestion of the internal soft tissues. pA =peltogonopod apodeme; gA = gonopod apodeme.

Figure 56. Transverse paraffin section of seventh ring during the ninth stadium. Mallory's triple stain. pA = peltogonopod apodeme; pp = peltogonopod protractor muscle; pr = peltogonopod retractor muscle.

Figure 57. Apex of gonopod telopodite.

Figure 58. *Nemasoma varicorne* third stadium. Seventh ring possess two pair of normal walking legs. Ventral view. SEM. Arrow indicate the seventh trunk ring.

Figure 59. *N. varicorne* squamiform appendages. Ventral view. SEM. cpp = coxal processes primordia; ptp = peltogonopod telopodite primordia; gtp = gonopod telopodite primordia.

Figure 60. Para-midsagittal paraffin section of the squamiform appendage during the fourth stadium. Mallory's triple stain. sm = tiny muscle of squamiform.

Figure 61. Appendages of seventh trunk ring of adult *N. varicorne* male. Ventral view. SEM. pt = peltogonopod telopodite; cp = coxal process; T = gonopod telopodite.

Figure 62. Frontal paraffin section of the seventh ring during the fifth stadium. Mallory's triple stain. pA = peltogonopod apodeme; pp = peltogonopod protractor muscle.

Figure 63. Frontal paraffin section of the seventh ring during the fifth stadium. Mallory's triple stain. gp = gonopod protractor muscle.

Figure 64. Seventh ring appendages of *N. varicorne* adult male, after digestion of internal soft tissues. Chlorazol black stain. lateral view (fused EDF-stack). pA = peltogonopod apodeme; gA = gonopod apodeme.

Figure 65. Seventh trunk ring of *N. varicorne* adult male, after digestion of internal soft tissues. Lateral view. Evans blue stain. (fused EDF-stack). pA = peltogonopod apodeme; gA = gonopod apodeme.

58

500 μm

59

cpp

ptp

gtp

50 μm

60

sm

50 μm

61

pt

cp

T

50 μm

62

pA

pp

100 μm

63

gp

100 μm

64

gA

pA

100 μm

65

pA

gA

100 μm

Figures 66, 67, 68. Transverse paraffin sections of seventh ring during the adult stadium. Mallory's triple stain. Fig. 66 = *Nopoiulus kochii*; Fig. 67 = *Blaniulus guttulatus*; Fig. 68 = *Nemasoma varicorne*. D = digestive tract; NC = nerve cord.
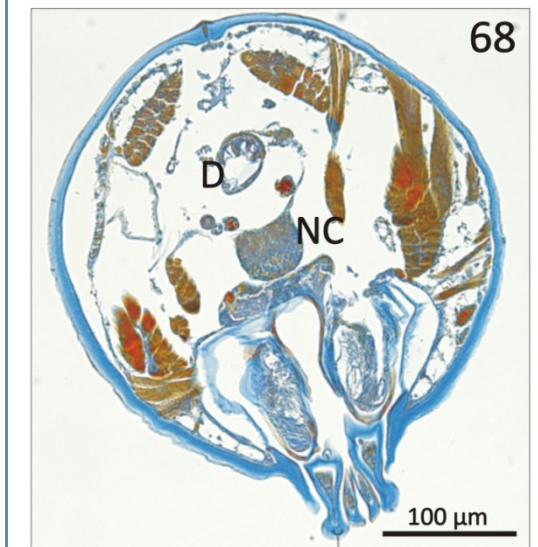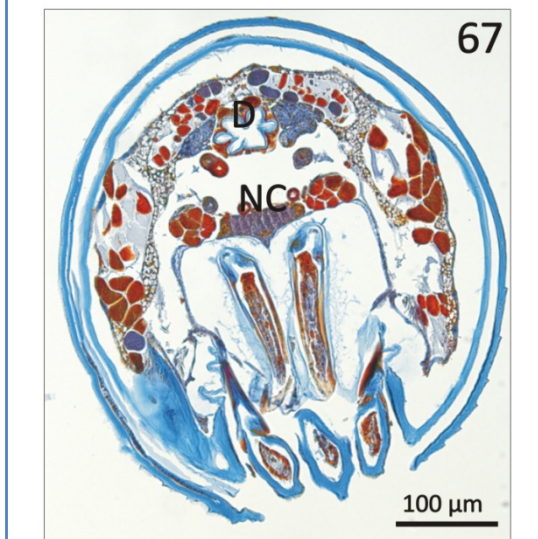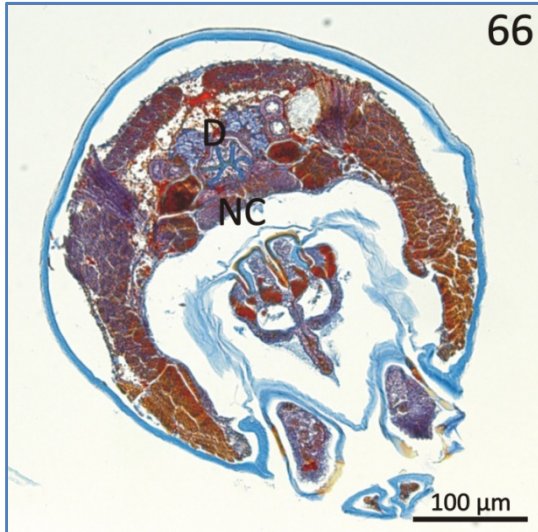
Non-systemic metamorphosis in male millipede appendages: long delayed, reversible effect of an early localized positional marker?[5]

ABSTRACT

Background: The development of specialized appendages involved in sperm transfer in the males of julid millipedes is an extreme case of specialized, complex structures differentiating in a very advanced phase of post-embryonic development. Here, a non-systemic metamorphosis affects the external morphology and the internal anatomy of a trunk double segment only.

Presentation of the hypothesis: We hypothesize that during early (possibly embryonic) development a segmental marker is produced that remains unexploited throughout late embryonic and early post-embryonic development, until, activated by a systemic signal, it finally determines the release of a segmentally localized but anatomically major change.

Testing the hypothesis: Key to testing the hypothesis are (1) the identification of both the putative segmental marker involved in the localization of the legs to be eventually metamorphosed into gonopods and the systemic signal activating it, (2) the identification of the cell population from which the gonopods are built, and (3) a longitudinal study of the marker's expression throughout late embryonic and, possibly, post-embryonic development.

Implications of the hypothesis: Proving the validity of this hypothesis would demonstrate the existence of a cryptic developmental module that will be activated only months, or years, after it has been first laid down during early development. This study also opens a window onto the very poorly explored domain of late expression of developmental genes and molecular control of late developmental events

BACKGROUND

Translation of positional information into the localized expression of anatomical structures is not limited to the embryonic phase of development, or to a metamorphosis systemically affecting the whole animal body. The development of specialized appendages (gonopods) involved in sperm transfer in the males of julid millipedes (Diplopoda, Julida) (Fig. 69) is an extreme case of specialized, complex and highly species-specific structures differentiating in a very advanced phase of post-embryonic development. This occurs through a metamorphosis that deeply affects external morphology and internal anatomy of the trunk diplosegment bearing the eighth and ninth pair of legs, while leaving unaffected the sections of the trunk that both precede and follow it. We propose to call this kind of dramatic post-embryonic transformations confined to a circumscribed body district *non-systemic metamorphosis*. Other examples of non-systemic metamorphosis are offered by the development of a new kind of appendages replacing the second and third pair of legs lost by some larval pycnogonids a few instars earlier (Dogier, 1913), and by the transformation of the forelegs of cicadas, from the robust digging tools of the nymph into the generalized walking legs of the adult. In the case of male Helminthomorpha (the large millipede clade that includes the julids), this metamorphosis is limited to one or very few non-terminal serially

homologous elements in an otherwise morphologically homogeneous series of trunk segments. In females, no special feature develops at any time at the corresponding location. In some julid species, this localized change is partially reversible, with the occurrence of the peculiar phenomenon called periodomorphosis (Enghoff et al., 1993; Sahli, 1990): cyclically, mature males with fully-developed gonopods moult into 'intercalary' males, with these limbs regressed to a condition similar to the small scale-like appendages of the last instar preceding sexual maturity, but resume the condition of reproductive adults with fully-developed gonopods within one or a few more moults.

PRESENTATION OF THE HYPOTHESIS

The precise localization of the metamorphic event eventually giving rise to the millipede male gonopods, with the accompanying changes in the overall arrangement of the internal organs in the corresponding segments (Fig. 70), must depend on the strict localization of a positional marker. We hypothesize that (1) a segmental marker is produced during early (possibly embryonic) development, although a later reinforcement of the signal through novel expression is probable; (2) this marker remains unexploited throughout late embryonic and early postembryonic development, until it finally determines the transcription of genes or the activation of gene products that release a segmentally localized but anatomically major change. That this position is marked since early post-embryonic development is suggested, in particular, by the conservation of the position across the Helminthomorpha, in spite of the diversity in post-embryonic segmentation schedules, both as number of segments already formed at hatching and, more conspicuously, as number of segments that are visibly added at each post-embryonic moult, a figure that in some lineages also varies intra-specifically. Thus, although, in principle, a previously unspecified position could subsequently acquire its distinctive identity through signals from sources localized elsewhere in the body, this seems unlikely to occur during millipede post-embryonic development. The positional mark may be provided by the sustained transcription of a key factor, perhaps assisted by trithorax-like proteins (Simon and Tamkun, 2002), or by the localized expression, determined by an earlier factor, of components of a signal transduction pathway, which make tissues competent to respond to a subsequent non-localized signal, as for instance a hormone.

TESTING THE HYPOTHESIS

It seems sensible to start the search for a positional marker by a candidate gene approach. The segmental position of gonopods would perhaps suggest that one of the posterior-group *Hox* genes is involved. At present, due to the incomplete expression data for posterior *Hox* genes in helminthomorph millipedes, the only term of comparison would be the pill millipede *Glomeris marginata* (Janssen and Damen, 2006), where the *Hox* complex respects standard collinearity. This is of limited interest,

however, as male pill millipedes do not possess gonopods, although they differentiate, at late post-embryonic instars, the terminal leg-pair as claspers (telopods). Position and nature of the specified organs might suggest to focus on *Abdominal-B* (*Abd-B*), as this gene is consistently involved in the specification of sexual structures in animals as different as spiders (Damen and Tautz, 1999), nematodes (Kagoshima et al., 1999) and mammals (Kondo et al., 1997). The expression pattern of *Abd-B* has not been studied in the only helminthomorph millipede, the polydesmoid *Oxidus gracilis*, for which data on *Hox* gene expression are available (Abzanhov et al., 1999); nevertheless, in this species the anterior border of expression domains of the other posterior-class *Hox* genes is known to occur, embryonically, at positions anterior to those of the gonopods. In parallel with establishing the molecular nature of the putative segmental marker involved in the localization of the legs to be eventually metamorphosed into gonopods, and identifying the signal that activates it (or releases its suppression), key to testing our hypothesis are (1) the identification of the cell population from which the gonopods are built, followed by (2) a longitudinal study of the marker's expression throughout late embryonic and, possibly, postembryonic development.

No study is available on the origin, localization, proliferation and differentiation of the cell population eventually giving rise to the gonopods in any millipede. The possible occurrence of small clusters of set-aside cells, comparable to insect histoblasts, should be considered. While their existence would reinforce our suggestion for an early positional specification, this would also open alternative scenarios as to the mechanism involved. To date, millipedes have been proved to be difficult subjects for the study of development. The few classic studies of their embryonic development (e.g., Plugfelder, 1932; Dohle, 1964) have been extended by gene expression work on pill millipedes (Damen and Tautz, 1999; Janssen et al., 2004) and by the already mentioned, very limited account on *Oxidus* (Abzanhov et al., 1999). Post-embryonic stages of helminthomorph millipedes are difficult to handle experimentally, because of the heavily calcified armour that covers their body, often completely, and also because of the disturbance to histochemical procedures caused by the diversity of repugnatory substances they produce.

IMPLICATIONS OF THE HYPOTHESIS

Proving the validity of this hypothesis would demonstrate the existence of a cryptic developmental module that will be activated only months, or years, after it has been first laid down during early development and also the existence of positional markers whose information can even be used repeatedly (periodomorphosis), following morphogenetic reversals of a magnitude and significance only comparable to the loss and regeneration of major body parts.

In evolutionary perspective, this change cannot be equated to the change from the thoracic legs of a caterpillar to the thoracic legs of a butterfly, because in the latter case it is the adult appendage which is phylogenetically conservative, while the larval leg is evolutionarily more derived. In the helminthomorph millipede, what is conserved is

the appendage found in the early developmental stages, followed by metamorphosis into a derived one. The evolution of gonopods is thus recapitulative, in so far as the development of the derived condition of the appendages is preceded by the full development of the primitive condition of the same structures. It is not easy to explain this costly developmental pathway in terms of adaptation. Generally, the legs that are to become gonopods are functionally lost (transformed into scale-like appendages) within one or two moults. On the other hand, an uninterrupted series of typical leg pairs, including those that will later metamorphose into gonopods, is observed since the first post-embryonic instar in a species (*Pachyjulus flavipes*) that hatches with more than twenty leg pairs, rather than the more usual three pairs only (Enghoff et al., 1993). Thus, gonopod evolution in millipedes possibly represent a case of developmental constraints, whose precise nature needs be investigated at molecular level.

This study can also open a window onto the very poorly explored domain of late expression of developmental genes and molecular control of late developmental events.

REFERENCES

Abzhanov A, Popadic A, Kaufman TC, 1999. Chelicerate *Hox* genes and the homology of arthropod segments. Evolution & Development 1:77–89.

Damen WGM, Tautz D, 1999. *Abdominal-B* expression in a spider suggests a general role for *Abdominal-B* in specifying the genital structures. Journal of experimental zoology 285:85-91.

Dogiel V, 1913. Embryologische Studien an Pantopoden. Zeitschrift für wissenschaftliche Zoologie 107:575-741.

Dohle W, 1964. Die Entwicklung von *Glomeris marginata* (Villers) im Vergleich zur Entwicklung anderer Diplopoden. Zoologische Jahrbücher, Abteilung für Anatomie und Ontogenie der Tiere 81:240-310.

Enghoff H, Dohle W, Blower JG, 1993. Anamorphosis in millipedes (Diplopoda). The present state of knowledge and phylogenetic considerations. Zoological journal of the Linnean Society 109:103–234.

Janssen R, Damen WG, 2006 The ten Hox genes of the millipede *Glomeris marginata*. Development Genes and Evolution 216:451-465.

Janssen R, Prpic NM, Damen WGM, 2004. Gene expression suggests decoupled dorsal and ventral segmentation in the millipede *Glomeris marginata* (Myriapoda: Diplopoda). Developmental Biology 268:89-104.

Kagoshima H, Cassata G, Bürglin TR, 1999. A *Caenorhabditis elegans* homeobox gene expressed in the male tail, a link between pattern formation and sexual dimorphism? Development Genes and Evolution 209:59-62.

Kondo T, Zakany J, Innis JW, Duboule D, 1997. Of fingers, toes and penises. Science 390:29.

Pflugfelder O, 1932. Über den Mechanismus der Segmentbildung bei der Embryonalentwicklung und Anamorphose von *Platyrrhacus amauros* Attems. Zeitschrift für wissenschaftliche Zoologie 140:650-723.

Sahli F, 1990. On post-adult moults in Julida (Myriapoda, Diplopoda). Why periodomorphosis and intercalaries occur in males? In Minelli A editor. Proceedings of the 7th International Congress of Myriapodology. Leiden: Brill EJ.

Simon JA, Tamkun JW, 2002. Programming off and on states in chromatin: mechanisms of Polycomb an trithorax group complexes. Current Opinion in Genetics & Development 12:210-218.

Figures 69. Simplified phylogeny of Diplopoda according to Enghoff et al. (1993), to illustrate the relationship between the taxa cited in the main text.

Figure 70. In the juliform millipede *Nopoiulus kochii* (Gervais, 1847) (Blaniulidae), the eighth and the ninth pair of trunk appendages are typical legs since their first appearance in the III postembryonic instar and so remain in the IV instar (A, D, G), and only turn into short, scale-like appendages with the moult to the V instar (B, E, H). With a further moult, the millipede, now in its VI post-embryonic instar, becomes an adult with two pairs of bulky, complex gonopods (C, F, I). (A-C) External morphology. SEM, scale bar 300 μm. (D-F) Esoskeletal and endoskeletal components. Projection at maximum intensity of serial pictures collected with Confocal Laser Scanning Microscope, soft tissues digested with KOH 10%, cuticle stained with Blue Evans 0.005%, scale bar 200 μm. (G-I) Internal anatomy. Para-midsagittal paraffin sections (7 μm), Mallory's triple stain, scale bar 200 μm. Arrow pairs point to the eighth and ninth pair of trunk appendages, as walking legs (A, D, G) or scale-like appendages (B, E, H).
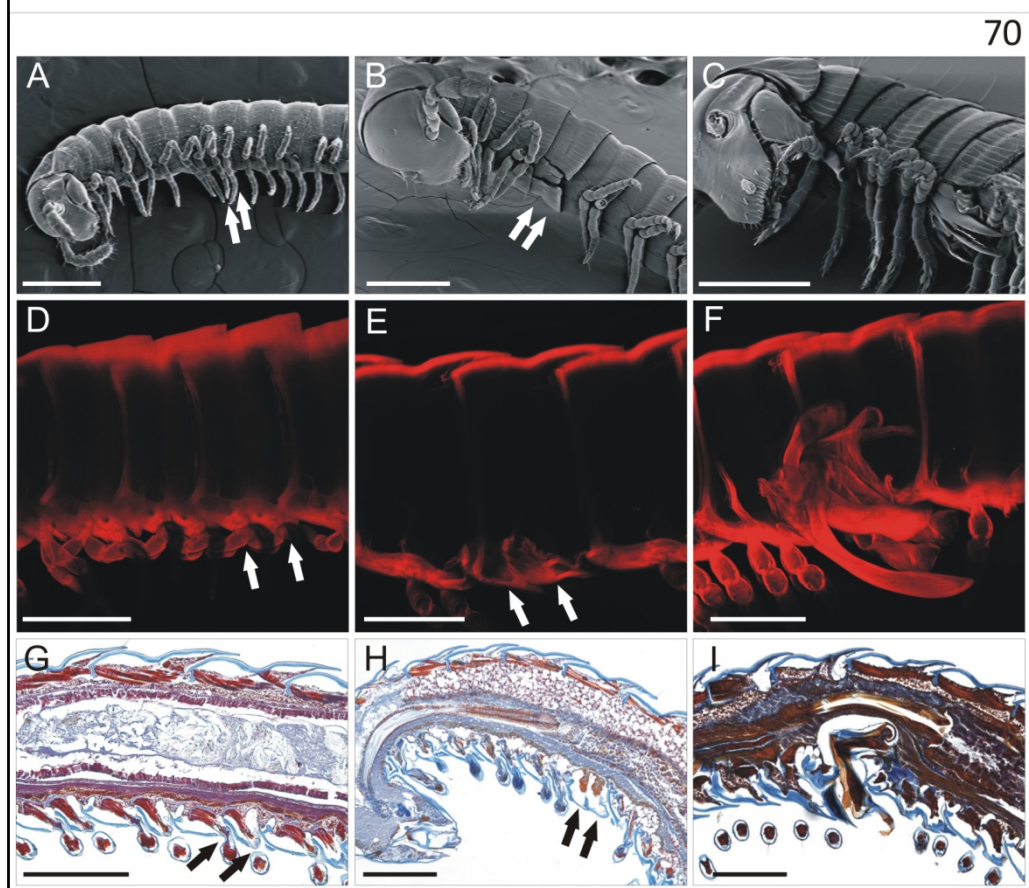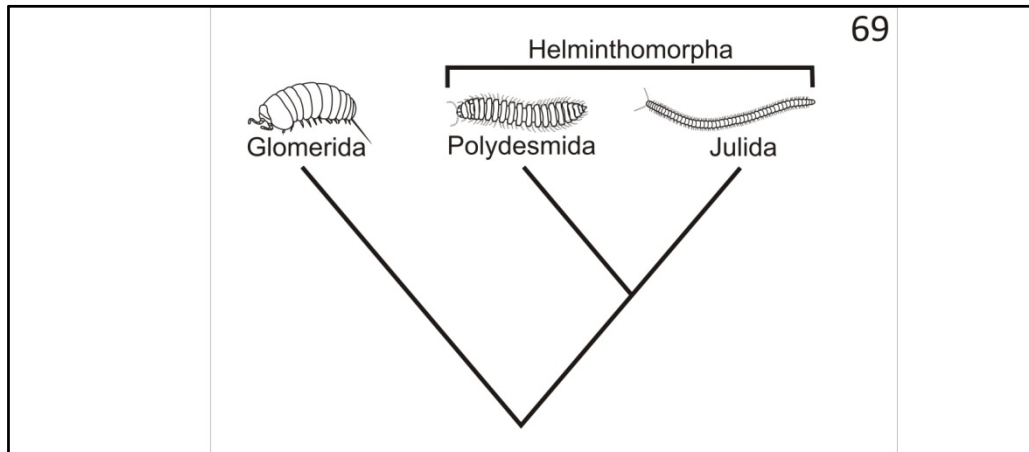
GENERAL CONCLUSIONS

The results obtained in this thesis demonstrate that gonopod formation in the helmintomorph millipedes represents a model for a particular kind of metamorphosis, the non-systemic metamorphosis. The seventh ring of the trunk undergoes a dramatic anatomical modification consequent to the differentiation of the very complex appendages used in copulation. The presence of at least one post-embryonic stage with squamiform appendages (Drago, 2009b) is widespread in helmintomorph species and probably is a developmental constraint in gonopod formation.

Gonopod formation affects trunk anatomy deeply, usually involving only the eighth and eventually the ninth pair of legs, but in some species of the order Chordeumatida up to five pairs of appendages are involved, from the seventh to the eleventh pair (Blower, 1985). In the latter group also the sixth and the eight ring are involved in non-systemic metamorphosis. What is most surprising is the strictly localization of the metamorphic event: gonopods are formed in the seventh ring independently from the kind of post-embryonic development (either hemi-, telo- or euanamorphosis) the male undergoes and independently from the number of segments added in the posterior part of the millipede body. Also in species which possess more than eight or nine pairs of legs already at hatching, as *Pachyjulus flavipes* with more than twenty legs pairs (Enghoff et al., 1993), the same localization of gonopod is confirmed. This fixation suggests the presence of a segmental marker, probably produced at a quite early embryonic stage, when the organization of the body plan is the same for all species of millipedes. This hypothesis implies that a cryptic developmental module will be activated months, or years, after its production during embryogenesis. This module could be used repeatedly in the species with periodomorphosis. The nature of the organs involved in the metamorphosis and the posterior position of the seventh diplosegment in the embryo suggest *Abd-B* as candidate marker. Unfortunately the expression pattern of *Abd-B* has not been studied in the only helminthomorph millipede, *Oxidus gracilis*, for which data on *Hox* gene expression are available (Abzhanov et al., 1999). Metazoans, generally, the future of the embryonic segments along the antero-posterior axis is determined by the expression pattern of *Hox* genes (e.g., Gilbert, 2006). In *Drosophila*, for example, through these markers, the position of the head, the thorax, the abdomen and the genitalia are differentiated. In adult millipedes the trunk and the abdomen are composed by a series of homonomous elements repeated several times. Gonopod development is the specialization of a single non-terminal unit among a long series of identical elements. Probably in the precocious millipede embryo the posterior part with the expression pattern border of *Abd-B* corresponds to the adult seventh ring. The seventh ring is then follows by a proliferation zone that represents a "sub-terminal" part of the embryo, which forms all other segments. Unfortunately, to date, millipedes have been proved to be difficult subjects for the study of development: post-embryonic stages are difficult to handle experimentally, because of the heavily calcified exoskeleton and also because of the disturbance to histochemical procedures caused by the diversity of repugnatory substances they produce. The study of gene expression

during embryonic development have been performed only on pill millipedes (Janssen et al., 2004; Damen and Tautz, 1999), which do not have gonopod.

To improve the study of gonopod formation in millipedes could be crucial for a better understanding of anamorphic growth in arthropods, revealing useful information for the study of developmental processes in this phylum.

REFERENCES

Abzhanov A, Popadic A, Kaufman TC, 1999. Chelicerate *Hox* genes and the homology of arthropod segments. Evolution & Development 1:77-89.

Blower JG, 1985. Millipedes Linnean Society Synopses of the British fauna(New Series). No. 35. London: E. J. Brill/Dr W. Bachuys.

Damen WGM, Tautz D, 1999. *Abdominal-B* expression in a spider suggests a general role for *Abdominal-B* in specifying the genital structures. Journal of experimental Zoology 285:85-91.

Drago L, 2009b. Consequences of non systemic metamorphosis in three julid millipedes: an anatomical point of view. MS 2. pg. 53-75.

Gilbert SF, 2006. Developmental Biology. Sinauer Associates Inc.

Enghoff H, Dohle W, Blower JG, 1993. Anamorphosis in millipedes (Diplopoda) - the present state of knowledge with some developmental and phylogenetic considerations. Zoological Journal of the Linnean Society 109:103-234.

Janssen R, Prpic N-M, Damen WGM, 2004. Gene expression suggests decoupled dorsal and ventral segmentation in the millipede *Glomeris marginata* (Myriapoda: Diplopoda). Developmental Biology 268:89-104.